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LIVER ENZYMES IN HEALTHY VOLUNTEERS: DATA DISTRIBUTION AND INTRAINDIVIDUAL VARIATION

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Interpretation of liver enzyme elevations in healthy volunteers participating in studies with investigative new drugs often cause difficulties. We investigated rank distributions of liver enzymes in a population of 431 healthy male volunteers (age 18 to 41 years) participating in phase-I-studies. Only pre-study evaluations have been included. Total number of study participation ranges from 1 to 25 with an average of 5.
We found no tendency towards more frequent and/or more elevated enzyme activities in volunteers with frequent study participation.
Determination of intraindividual variation over time has also been performed.
It is evident from our data that intraindividual variation of the investigated liver enzymes - even over years - is mostly very small compared to the interindividual variation in the group. Some subjects however do display a considerable intraindividual variation that is not due to single outliers but caused by multiple oscillations within a wide range.
Further investigations are planned to elucidate the nature of this phenomenon. We suggest the use of individual "reference ranges" as a first-in-line criterion for valuation of suspicious liver enzyme elevations in healthy subjects during studies with investigative new drugs.

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INFLUENCE OF MAGNESIUM- AND ALUMINIUMHYDROXIDE ON PHARMACOKINETIC DATA OF OFLOXACIN IN PATIENTS WITH A PROPHYLAXIS OF PEPTIC ULCER

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A recent study of Lode et al. (Rev Infect Dis 1988; 10 (suppl 1): S 138-S 139) has shown that a simultaneous administration of a single oral dose of ofloxacin (Tarivid®) and a single excessively high dose of 6000 mg magnesium- and 9000 mg aluminiumhydroxide leads to a significant reduction of ofloxacin serum concentrations in healthy volunteers. So far no data are available proving this result under steady-state conditions in patients with prophylaxis of peptic ulcer receiving a low dose of an antacid.
Therefore 13 patients (7 women, 6 men) with symptoms of an urinary tract infection were investigated in the following study. They received 400 mg ofloxacin (2 x 200 mg tablets) for six days and an antacid (Maalox 70®: 600 mg magnesium- and 900 mg aluminiumhydroxide) three times daily for three days. During the first three days the antacid was administered simultaneously with ofloxacin. During the last three days ofloxacin was given without an antacid. Blood samples were taken at day three and six to determine serum concentrations of ofloxacin by HPLC.

Results:

time after administration (h)	0	1	2	3	4	6	8	10
	serum concentrations ofloxacin (mg/ml)							
with Maalox 70®	0.45	0.836	1.197	1.079	1.052	0.791	0.742	0.752
without Maalox 70®	2.078*	2.533*	2.928*	2.582*	2.242*	2.219*	2.183*	1.888*

* t - test: $p < 0.05$

After concomitant intake of Maalox 70® serum concentrations of ofloxacin were significantly reduced. Furthermore the serum concentrations of ofloxacin were microbiologically inefficient almost four hours before the administration in the evening. As ofloxacin is mainly eliminated by the kidney sufficient concentrations are reached in the urinary tract. However other diseases may require higher serum concentrations to reach therapeutic tissue levels.
It is concluded that the concomitant administration of an antacid containing magnesium- or aluminiumhydroxide and ofloxacin leads to a significant decrease of serum concentrations of ofloxacin. For the prophylaxis of a peptic ulcer a H_2-receptor antagonist should be chosen to avoid pharmacokinetic interactions.

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Intra-individual variability of caffeine elimination in healthy volunteers

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The most widely used technique for assessing the activity of hepatic drug metabolising enzymes is to study the kinetics of model drug substrates in vivo. For a drug to be useful test substance, its clearance from plasma should be directly related to the activity of enzyme(s) responsible for its metabolism.
Caffeine is almost certainly the world's most popular test substance. A correct estimation of the relative magnitudes of *intra*-individual and *inter*-individual variations in caffeine elimination is significant for the use of the elimination parameter of caffeine to characterize the biotransformation capacity of a specific form of cytochrome P-450 (1AII) in vivo.
The purpose of this study is to demonstrate the magnitude of fluctuation of caffeine-clearance and half-life as well as *inter-* and *intra*-individual comparison in 12 healthy male subjects. Compared to the high reproducibility of caffeine decay curves in each healthy males, caffeine elimination varied more extensively between subjects. The distribution of variance amount to:
intra-individual 21.4 %
inter-individual 78.6 % .
The knowledge of variance provided precise evidence about the sample size, which are nessesary to prove previously defined differences.

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DIGITOXIN METABOLITES IN LIVER CIRRHOSIS.

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Although digitoxin has been used for many years the pharmacokinetic behaviour of this digitalis glycoside is still a matter of discussion, especially the results in patients with liver disease are divergent. Aim of the present study was to investigate the pharmacokinetics of digitoxin in patients with liver cirrhosis and to study the urinary excretion of its metabolites as digoxin, digitoxigenin, digitoxigenin-mono- and -bis-digitoxoside using a specific and sensitive combination of RIA after HPLC technique (1). Nine patients with biopsy confirmed liver cirrhosis (median antipyrine clearance 19.61 ± 3.08 ml/min; $\bar{X}$ ± SEM) and eight healthy volunteers received intravenous and p.o. administration of 1 mg digitoxin. The calculated and computed kinetic parameters derived from the digitoxin plasma concentration time curve and from urinary recovery including total clearance of unchanged digitoxin did not differ significantly between both groups investigated. Renal clearance of digitoxin (DGT) was 0.017 ± 0.005 ml/min/kg b.w. in the patient group and 0.011 ± 0.002 ml/min/kg b.w. in the volunteers (NS); it was 0.0034 ± 0.0005 ml/min/kg b.w. and 0.0022 ± 0.0004 ml/min/kg b.w., respectively (NS) for digitoxigenin-bis-digitoxoside (Bis DGTN), 0.00006 ± 0.00001 ml/min/kg b.w. and 0.00016 ± 0.00005 ml/min/kg b.w. ($p<0.05$) for digitoxigenin-mono-digitoxoside (Mono-DGTN), 0.0004 ± 0.0001 ml/min/kg b.w. and 0.0009 ± 0.0003 ml/min/kg b.w. ($p<0.05$) for digitoxigenin (DGTN), 0.0014 ± 0.0005 ml/min/kg b.w. and 0.0011 ± 0.0004 ml/min/kg b.w. (NS) for digoxin (DG). In conclusion, hydrolysis of digitoxin is altered in liver cirrhosis, whereby a significant reduction of renal clearance and urinary recovery for digitoxigenin-mono-digitoxoside and digitoxigenin was seen in the present study.

1) Santos SRCJ et al., J. Chromatogr. 1987; 419: 155-64.

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BIOAVAILABILITY OF TWO RETARDED NIFEDIPINE PREPARATIONS IN STEADY STATE

P. Banditt and F.P. Meyer

The relative bioavailability of Corinfar® retard dragees (test preparation T) was tested in 22 healthy volunteers in a cross-over design versus another retarded nifedipine (reference preparation R) under steady-state conditions (2 x 20 mg /d over 6 days). In preceding studies, the preparations were found to be bioequivalent after single application (Meyer and Banditt, Z. klin. Med. 46: 1141-1144, 1991). Blood was sampled just before (C_{6d}) and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 10, 12 and 24 h after application 12. Analysis: GLC as proposed by Jakobsen et al. (J. Chromatogr. 162: 81 - 87, 1979) and modified in our laboratories.

Nifedipine	T	R	Q
C_{6d} (ng/ml)	15 ± 8	17 ± 11	-
t_{max} (h)	2.5 ± 0.8	3.0 ± 1.1	1.0
C_{max} (ng/ml)	83 ± 21	74 ± 21	1.17
AUC (0-12) (h·ng/ml)	440 ± 154	460 ± 199	0.98
f	0.95	1.0	

Both preparations are considered to be bioequivalent.

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COMPARISON OF THE LIPID LOWERING EFFECT OF SITOSTEROL AND SITOSTANOL IN CHILDREN WITH SEVERE FAMILIAL HYPERCHOLESTEROLEMIA

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The current recommended therapy for management of severe familial hypercholesterolemia (FH) in childhood consists of dietary intervention and treatment with bile acid binding resins. However, acceptance and patient compliance have been poor in many children. Therefore, the current study was performed to compare the lipid lowering effect of two different plant sterols on their lipid lowering effects and their mechanism(s) of action in 9 children (age range 6.7 to 11.0 yrs) with severe FH (total- and LDL cholesterol averaged 367 mg/dl and 306 mg/dl, respectively). After 3-month dietary intervention, sitosterol pastils (3x2 g/d) were administered for 3 months, followed by a 3-months therapy with sitostanol (3x0.5 g/d). Serum lipoproteins, serum concentrations of campesterol and sitosterol were obtained in all, fecal excretion of neutral sterols were measured in 7 children at the end of the different therapeutic regimens. All children completed the study and no obvious side effects occured. During administration of sitosterol total- and LDL cholesterol were reduced by 17 % and 20 %. In contrast, sitostanol decreased total- and LDL cholesterol by 26 % and 33 % ($p<0.05$ compared to sitosterol). Whereas sitosterol did not alter serum concentrations of campesterol and sitosterol (3.04 mg/dl vs 2.84 mg/dl) a significant reduction (-47 %; $p<0.01$) occured during sitostanol therapy, indicating a reduction in intestinal sterol absorption. This was in line with a marked increase in fecal excretion of neutral sterols from 6.7 mg/kg/d^{-1} to 12.6 mg/kg/d^{-1} ($p<0.01$) during sitostanol administration. The data suggest that sitostanol, a non-absorbable plant sterol, reduces elevated serum cholesterol by inhibition of intestinal cholesterol absorption, and that the reduction in serum lipids is in the order of magnitude observed with HMG-CoA-reductase inhibitors.

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ABSENCE OF PARIETAL CELL cAMP STIMULATORY AUTOANTIBODIES IN ULCER PATIENTS WITH INADEQUATE RESPONSE TO RANITIDINE

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Approximately 10% of peptic ulcer remain unhealed after 4-8 weeks therapy with H_2-receptor antagonists. The hypothesis that parietal cell cAMP stimulatory autoantibodies to H_2-receptors are one reason of this inadequate response is conversely discussed [1,2]. In this study we investigated cAMP production induced by immunoglobulins (Ig) derived from ulcer patients with adequate and inadequate response to ranitidine.

We used human gastric tumor cells (HGT-1, kindly provided by Dr. C. Gespach, INSERM U55, Paris) expressing H_2-receptors as an in vitro model for measuring cAMP production. Ig-fractions from sera of adequate (AR; n = 16) and inadequate (IR; n = 20) responders were obtained by affinity chromatography on protein G sepharose (IgG) and precipitation by ammoniumsulfate (1.6 M) (Non-IgG). The HGT-1 cells were grown as monolayers and incubated with Ig-preparations for 10, 30, 180, 360 min in the presence of 1 mM phosphodiesterase inhibitor (IBMX). Standard IgG (Behring) was used as control and cAMP was measured by a radioimmunoassay (Amersham). All cAMP-values are given as pmol/mg protein (mean ±SD).

In all experiments (n = 38) the basal level of cAMP was 10.7 ± 1.7 and histamine (10^{-5} M) enhanced cAMP production after 10 min incubation to 80.4 ± 15.0. This stimulation could be blocked by ranitidine (IC_{50} = 34 nM) and cimetidine (IC_{50} = 400 nM) but not by triprolidine confirming the presence and specifity of H_2-receptors on these cells. Neither IgG (4 mg/ml) nor non-IgG (1 mg/ml) of both patient groups tested did affect cAMP production in HGT-1 cells (table).

Time (min)	0	10	30	180	360
Control	11.8 ± 0.5	12.2 ± 1.0	12.6 ± 1.3	12.8 ± 1.7	11.5 ± 1.0
AR: IgG	10.4 ± 1.7	10.7 ± 1.7	10.5 ± 2.2	12.0 ± 2.1	11.4 ± 2.2
Non-IgG	10.6 ± 1.2	13.0 ± 1.6	13.1 ± 1.3	11.9 ± 2.0	11.8 ± 2.5
IR: IgG	10.1 ± 1.7	10.5 ± 1.1	10.1 ± 1.8	11.8 ± 2.5	11.0 ± 1.9
Non-IgG	10.7 ± 0.9	12.7 ± 1.0	12.3 ± 1.6	11.7 ± 2.4	11.7 ± 2.0

In this in vitro system cAMP stimulating autoantibodies could not be detected in sera of adequate and inadequate responders to ranitidine confirming the results of Burman et al. [2]. However our study does not rule out an autoimmune disorder in subgroups of ulcer patients with this kind of drug resistence.

[1] De Lazzari et al. Gut 1988; 29: 94-100.
[2] Burman et al. Gut 1991; 2: 620-623.

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TOLERABILITY AND PHARMACODYNAMIC EFFECTS OF THE THROMBOXANE A_2 ANTAGONIST BAY U 3405 ADMINISTERED AS CONTROLLED RELEASE (CR) TABLETS TO HEALTHY VOLUNTEERS

H. Blume, W. Ritter, E. Perzborn*, and H. Weber

BAY u 3405, a cycloalkano-indolsulfonamide compound, exhibits specific thromboxane A_2 antagonistic properties in various pharmacological models. BAY u 3405 CR tablets were administered orally to healthy volunteers in single doses of 25 mg and 50 mg o.a.d. and multiple doses of 50 mg b.i.d and t.i.d. No specific side effects were observed; cardiovascular parameters like heart rate, blood pressure and ECG-time-intervals were not influenced. Clinical chemistry, haematology and urinalysis showed no difference between active and placebo treatment up to 7 days after multiple administration. Bleeding time was never increased above the upper limit of normal (4, 8, 12 and 24 h p.a.). U46619-induced platelet aggregation ex vivo was markedly inhibited (>80 %) in the volunteers up to 8 h after administration of 25 mg, and up to 12 h after 50 mg. About 100 % inhibition occurred at plasma levels of >10 µg/l. A correlation between platelet aggregation and bleeding time could not be found.

Conclusion: The BAY u 3405 CR formulation was well tolerated and seems to be appropriate to inhibit platelet aggregation continuously at a dose of 50 mg b.i.d.

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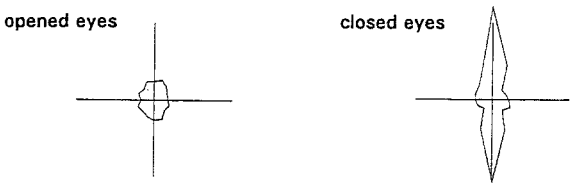
APPLICATION OF A MULTIFUNCTIONAL IMAGE ANALYSIS SYSTEM TO STATIC POSTUROGRAPHY IN CLINICAL PHARMACOLOGY

M. Böttcher, M. Beneke, H. Dietrich, R. Horstmann, W. Lüdtke, I. Seitz

Dizziness is a common side effect of several drugs. To quantify the intensity of symptoms, we introduced a multifunctional image analysis system for static posturography in clinical pharmacological studies. For evaluation of method applicability a study with 12 healthy volunteers was designed. Body swart of the volunteers was analysed with opened and closed eyes (Romberg).

In order to perform video recording of the motion pattern labels ("targets") are placed on the volunteers body. Up to 25 different targets have been identified in the video image by the system automatically. Parameters calculated were: target velocity, target direction and movement deviations.

Figure below demonstrates mean angle dependent deviations of the volunteers movement with opened and closed eyes:



Conlusions: Preliminary results indicate that image analysed static posturography is a reliable and less time consuming method.

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STEADY STATE PHARMACOKINETICS OF PROPAFENONE IN PATIENTS WITH RENAL FAILURE

S. Botsch, J. Evers*, E. Hardtmann+ and U. Kuhlmann+

Propafenone is an antiarrhythmic drug which undergoes extensive biotransformation to the active metabolites 5-hydroxypropafenone and N-desalkylpropafenone. Formation of 5-hydroxypropafenone cosegregates with spartein/debrisoquine polymorphism with about 90% of a population being extensive metabolizers (EM). Conjugates of both the parent compound and metabolites are excreted into urine summing up for a total of about 60% of a dose. No data are available regarding disposition of propafenone during chronic oral therapy in patients with renal failure. We therefore investigated interdose steady state plasma concentrations of propafenone, 5-hydroxypropafenone and N-desalkylpropafenone in 5 EM patients (4 hemodialysis, 1 CAPD; 3 male, 2 female, age 61.4 ± 6.1years, dose 150 mg tid) in comparison to five EM patients with intact renal function (creatinin clearance: 91 ± 15.5 ml/min). Both groups were matched for age, gender and weight. Results are expressed as mean interdose plasma concentrations (C_{ss}) of propafenone, 5-hydroxypropafenone and N-desalkylpropafenone.

C_{ss} (ng/ml)	patients	controls	P
Propafenone	428.9 ± 126.7	385 ± 325.4	n.s.
5-hydroxy-propafenone	216.9 ± 120.4	79.1 ± 29.8	< 0.03
N-desalkyl-propafenone	52.3 ± 28.9	190.1 ± 117.4	< 0.03

C_{ss} of propafenone were similar in patients and controls. Significant differences, however, were observed for C_{ss} of both metabolites.

We conclude while propafenone itself does not accumulate in extensive metabolizer patients with renal failure, disposition kinetics of the active metabolites are altered.

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Celiprolol's cardiovascular effects in man are unaffected by pretreatment with bisoprolol.

K. Breithaupt, C. de Mey, E. Appel and M. Herrmann.

Celiprolol (C) is a ß1-selective adrenoceptor-antagonist with ancillary vasodilatory properties. The present study describes and differentiates the cardiovascular effects of celiprolol in relation to the occupancy of ß1- and ß2-adrenoceptors, with and without preceding ß1-selective blockade by bisoprolol. 8 healthy male volunteers (mean age: 26 y.) were investigated twice (1 wk interval), receiving a single p.o. dose of 1200 mg C 3 h after either a p.o. dose of 20 mg bisoprolol (B) or placebo (P). Treatments BC and PC were randomly allocated in a double-blind cross-over design. Blood pressure, heart rate (HR, bpm), systolic time intervals, Heather Index (HI, $\Omega.s^{-2}$), cardiac output (by impedance cardiography) and total peripheral resistance (TPR, $dyn.s.cm^{-5}$) were investigated in recumbent resting condition, plus BP and HR responses to supine bicycle ergometry (4 min at constant Watt load aiming for HR 130 bpm). Mean results are detailed below (time 0 : baseline before P or B, time 3 : 3h later, immediately before C, time 6 and 22 : 6 and 22 h after P or B, i.e. 3 and 19 h after C) :

	Resting						Exercise	
Time	HR		TPR		HI		HR	
Tr:	PC	BC	PC	BC	PC	BC	PC	BC
0	56.0	55.4	1017	992	11.4	12.3		
3	55.4	51.5[a]	1026	1013	11.3	10.7[a]	141	111[c]
6	66.7[b]	67.0	852[b]	786	14.0[b]	13.8	125[b]	124
22	67.1[b]	60.8	787[b]	820	14.9[b]	15.1	124[b]	122

a : statistically significant effects of B (time 3 vs 0), b : statistically significant effects of C (treatment PC only, contrasts vs time 3); c : statistically significant difference between BC and PC (i.e. true B effects at time 3, and BxC interaction after time 3 h).

Thus, C's effects were not affected by pretreatment with B. B's effects on exercise HR were weakened by subsequent C. C and B yielded about similarly high ß1-adrenoceptor occupancies (ca. 90%), whereas ß2-adrenoceptor occupancy was detectable only for C (ca. 35%).

It is concluded that celiprolol caused net vasodilatory, positive chronotropic and cardiac performance enhancing effects in man independent of its ß1-antagonism. This might relate to (partial) ß2-agonism.

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INFLUENCE OF CIMETIDINE AND RANITIDINE ON THE PHARMACOKINETICS OF CONTROLLED-RELEASE FORMULATIONS OF THE TWO CALCIUM-ANTAGONISTS NIFEDIPINE AND NISOLDIPINE

E. Brendel, R. Heinig, G. Ahr and N. Wetzelsberger*

Studies formerly conducted with the immediate release tablets of both Nifedipine (Nif) and Nisoldipine (Nis) had shown an interaction between Cimetidine (C) and a lack of interaction between Ranitidine (R) and both drugs. To investigate this potential interaction also for the newly developed controlled-release forms (CR) of both drugs, two studies were performed in healthy male volunteers. In each study, twelve subjects were randomly allocated to 3 different treatments in a crossover fashion: 5 days treatment with either C (400 mg bid), R (150 mg bid) or placebo and an additional administration of Nif CR (60 mg) and Nis CR (20 mg), respectively, as single doses. Plasma concentrations of Nif and Nis were determined by GC-ECD. Geometric means of pharmacokinetic parameters :

Parameter	Nifedipine			Nisoldipine		
	+Plac.	+C	+R	+Plac.	+C	+R
Cmax [µg/l]	73.8	127.7	76.4	1.01	1.47	0.84
tmax [h]	3.2	2.9	2.5	7.5	3.4	7.3
AUC(0-oo) [µg*h/l]	742.8	1193.8	715.7	14.37*	19.02*	12.20*

*AUC(0-tn)

The results clearly indicate an interaction between C and both Nif and Nis, whereas R does not show any influence on the pharmacokinetics of either drug. The increase in Cmax (Nif: 73.0 %,Nis: 45.5 %) and AUC (Nif: 60.7 %, Nis: 32.4 %) has no influence on pharmacodynamics (heart rate and blood pressure) and is considered to be of no clinical relevance.

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METHYLENE BLUE POTENTIATES ACETYLCHOLINE-MEDIATED VASODILATATION IN THE VASCULAR BED OF THE HUMAN FOREARM

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5-Hydroxytryptamine (5-HT) and acetylcholine (ACh) have been reported to induce vasodilatation via the 'nitric-oxide pathway'. The 5-HT-induced transient and persistent vasodilatation in the forearm is abolished by the 5-HT_3 and 5-HT_4 receptor antagonist ICS 205-930 (Blauw *et al*, 1989).

To further elucidate the 5-HT-mediated vasodilatation in the vascular bed of the human forearm of normotensive volunteers (n=6-12), we infused 5-HT (1 ng/kg/min), sodium nitroprusside (SNP; 5 ng/kg/min), or ACh (100 ng/kg/min) in the presence of saline, the 5-HT_3 receptor antagonists ondansetron (OND; 0.3 and 1 µg/kg/min) and granisetron (GRAN; 0.3 and 1 µg/kg/min), or methylene blue (MB; 1 µg/kg/min), using automated R-wave triggered venous occlusion plethysmography.

OND, GRAN, and MB did not affect the transient or the persistent vasodilator responses to 5-HT. ACh-induced vasodilatation was markedly potentiated by concomitant infusion of MB, from +89% to +757% ($p<0.01$), but also by OND (without MB; 1 µg/kg/min) to +210% ($p<0.05$) of the basal value, respectively. MB as such did not influence SNP-induced vasodilatation.

Unlike ICS 205-930, OND or GRAN did not antagonize the transient or the persistent vasodilator responses to 5-HT. This finding may suggest the involvement of a 5-HT_4 receptor subtype. The mechanism by which MB enhances the vasodilator response to ACh requires further investigation.

Blauw GJ, van Brummelen P, and van Zwieten PA (1989) The 5-HT-induced biphasic vasodilatation in the human forearm is dose-dependently antagonized by ICS 205-930. Br J Clin Pharmacol 27:663P-664P

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14

DOSE DEPENDENCY OF FLECAINIDE PHARMACOKINETICS

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Plasma concentrations of the antiarrhythmic flecainide above the therapeutic range (300 to 750 ng/ml) can elicit serious proarrhythmic effects. One factor contributing to interindividual variability in flecainide plasma concentrations is the cosegregation of flecainide metabolism with that of sparteine/debrisoquine. At the same dose poor metabolisers (PM) have on average 2 to 3 times higher plasma concentrations of the drug than extensive metabolisers (EM). Since up to 80% of a dose is excreted unchanged in urine in PMs renal secretion of flecainide may be saturable at higher plasma concentrations and hence dose dependency of the pharmacokinetics could result.

Pharmacokinetic characteristics considering both polymorphic oxidation and saturable renal secretion of flecainide have not been described so far. We therefore studied the dose dependency of flecainide pharmacokinetics after administration of 50, 100 and 200 mg on healthy volunteers (5 EM and 5 PM of sparteine). Blood samples were obtained and urine was collected from 0 to 120 hours.

In the EM subset we observed a dose dependent decrease in apparent oral clearance (50 mg: 643 ± 65; 100 mg: 614 ± 76; 200 mg: 481 ± 51 ml/min; $P < 0.01$). This decrease was due to reduced non-renal clearance (50 mg: 423 ± 73; 100 mg: 365 ± 111; 200 mg: 237 ± 66 ml/min; $P = 0.01$) while renal clearance remained unchanged (50 mg: 219 ± 83; 100 mg: 249 ± 61; 200 mg: 244 ± 58 ml/min; P = ns). In contrast, the PM subset showed linear pharmacokinetics (apparent oral clearance: 50 mg: 306 ± 60; 100 mg: 296 ± 37; 200 mg: 287 ± 62 ml/min; P = ns). Both renal (50 mg: 173 ± 30; 100 mg: 219 ± 19; 200 mg: 210 ± 24 ml/min; P = ns) and non-renal clearances (50 mg: 132 ± 66; 100 mg: 90 ± 39; 200 mg: 77 ± 39 ml/min; P = ns) were not affected by the increase of dosages.

In summary, flecainide shows nonlinear pharmacokinetics in the extensive metaboliser subset. Therefore, increase of dosages can lead to a more than proportional rise in plasma concentrations bearing the risk of proarrhythmic side effects. We have no evidence for saturation of renal clearance in either subset.

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EFFECT OF IBOPAMINE AND CAPTOPRIL ON CARDIAC AND RENAL FUNCTION IN PATIENTS WITH CONGESTIVE HEART FAILURE

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In some countries for several years ibopamine, an orally effective dopamine agonist, is used for treatment of congestive heart failure. Its influence on cardiac and renal function was studied in two groups of patients with congestive heart failure NYHA stage II in comparison with captopril. 10 patients had a mean creatinine clearance of 91.3 ± 3.4 ml/min ($\tilde{x}$ ± SEM), 10 patients had an impaired renal function (creatinine clearance 36.4 ± 3.9 ml/min). All patients were treated with placebo t.i.d. for one week and afterwards with ibopamine 100 mg t.i.d. again for seven days. In the third week the two groups of patients first got placebo t.i.d. again and afterwards captopril 12.5 mg t.i.d. for one week. 7 day treatment with ibopamine led to a significant increase of stroke volume and cardiac output 45 and 90 minutes after the morning dose, whereas captopril did not cause any significant changes.

Table 1: Parameters of renal function, *$p<0.05$ compared with placebo

Pts.	Treatment	Urine Volume (ml/24h)	PAH-Clear. (ml/min)	Inuline-Clear. (ml/min
normal	placebo	1440 ± 211	555 ± 18	93 ± 4.6
renal	ibopamine	1946 ± 243*	574 ± 32	93 ± 3.6
funct.	captopril	1510 ± 207	536 ± 17	87 ± 3.1
imp.	placebo	1504 ± 307	239 ± 45	40 ± 6.7
renal	ibopamine	2010 ± 307*	269 ± 50*	44 ± 8.0
funct.	captopril	1538 ± 253	251 ± 44	39 ± 7.2

In conclusion ibopamine but not captopril significantly increased diuresis and renal perfusion in patients with congestive heart failure and different degrees of renal function.

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ANALYSIS OF PHENPROCOUMON IN HUMAN BREAST MILK BY HPLC AFTER SOLID PHASE EXTRACTION

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R. von Kries[2]

Many drugs administered to lactating women are detectable in breast milk; however, no data are available about the conc. and excretion of phenprocoumon (PH) in human breast milk from women on long-term anticoagulant therapy; this is crucial for the assessment of possible risks for infants on breast feeding. An assay was developed for the analysis of PH in breast milk. *METHOD*: Breast milk samples from a woman on PH treatment were collected at time intervals; she had recently born a premature child. Samples were cleaned up by successive precipitations and centrifugations using 30% perchloric acid, methanol, 5 *N* sodium hydroxide and glacial acetic acid; a final purification step with octadecyl columns was used before reversed phase HPLC analysis. *RESULTS*: PH calibration curves were linear from 20 - 100 ng/ml (r^2 = 0.9994); good precision (cv = 8%), sensitivity (5 ng/ml) and recoveries (92%) were found. PH conc. were in the range 26 - 76 ng/ml; many drugs did not interfere. *DISCUSSION*: The purification steps previously used for the assay of PH in other biological fluids were modified for the ana-lysis of human milk due to the presence of pro-teins and lipids; the assay is fast and requires only small sample volumes. *CONCLUSION*: This pro-cedure allows the determination of PH conc. in human breast milk, and will be used in further excretion surveys.

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EVALUATION OF DRUG EFFECTS ON CNS BY DYNAMIC LIGHT EVOKED PUPILLOMETRY

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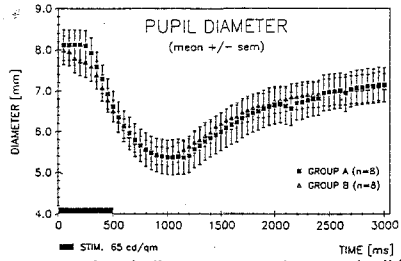
Investigations of drug effects on CNS play an important role in clinical pharmacological studies. Sympathic and parasympathic nervous system are frequently affected by CNS active drugs. Dynamic light evoked pupillometry is a non invasive method, which acts by calculating the pupil diameter.

To investigate reliability and validity of this method, two studies were designed with 8 healthy volunteers each (group A,B). Pupil diameters were measured over a period of 3 sec with a sampling rate of 20 Hz. Intensity of light stimulus was 65 Cd/qm, duration 0.5 sec. Variables recorded were pupil diameter (start,end,minimum) and time elapsed from begin of stimulation to achieving minimum diameter. For estimation of reproducibility repeated measurements were performed. The figure shows the time course of pupil diameter after light stimulus:



Conlusions: The results indicate a good reproducibility of the method. Dynamic light evoked pupillometry is a reliable method for clinical pharmacological investigations.

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BIOTRANSFORMATION OF CAFFEINE AND THEOPHYLLINE IN GENETICALLY ENGINEERED CYTOCHROME P450 EXPRESSING V79 CELLS

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Primary steps in the metabolism of caffeine and theophylline are cleavage of methyl groups and/or hydroxylation at position 8, mediated by cytochromes P450.

V79 Chinese hamster cells genetically engineered for stable expression of rat cytochromes P450IA1, IA2, IIB1, and human cytochrome P450IA2 were applied in metabolism studies in order to identify the metabolites formed and to identify the metabolically competent cytochrome P450 isoform.

V79 derived cells were exposed to culture medium containing caffeine and/or theophylline. Medium was checked three days later for metabolites formed by HPLC.

Caffeine was metabolized by human and rat P450IA2 to four primary demethylated and hydroxylated metabolites. However, a species specific difference was observed. The human IA2 predominantly mediated 3-demethylation of caffeine, whereas the rat IA2 mediated both 3-, and 1-demethylation of caffeine to the same extent.

Theophylline was metabolized in all cell lines via 8-hydroxylation.

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DETERMINATION OF GENETIC VARIATION IN HUMAN ARYLAMINE N-ACETYLTRANSFERASE (NAT2) LOCUS

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Human arylamine N-acetyltransferase (NAT-2) plays an important role in metabolization of drugs and other xenobiotics. This enzyme exibits genetic polymorphism. Among the European population 50% are phenotypically slow acetylators. We have investigated the relationship between phenotype and genotype. Phenotypisation was performed by the caffeine-method. For genotype determination specific oligonucleotide primers were synthesized according to published polymorphic NAT-2 sequence to produce a gene-probe by polymerase chain reaction (PCR) using genomic DNA as starting material [Grant et al (1989) Nucl Acid Res 17: 3978]. The NAT-2 restriction fragment length polymorphism (RFLP) was determined after hybridization of KpnI digested DNA by Southern-blot in 34 patients. NAT-2 gene-probe hybridization revealed the following RFLPs: 15/5.2 kb (a), 19/15/5.2 kb (b), 15/5.2/4.6 kb (c), single 19 kb band (d), 15/4.6 kb (e), and 19/15/4.6 kb (f). Combination (a) represents the homozygous fast acetylator, (d) one and (e) another homozygous mutation causing impaired acetylation. The remaining combinations represent the heterozygous status of either fast (b, c) or slow acetylators (f) [Drakoulis et al (1991) Naunyn-Schmiedeberg's Arch Pharmacol (Suppl) 344: R88]. Allele specific PCR was applied to determine the mutations causing slow acetylation [Blum et al (1991) Proc Natl Acad Sci USA 88: 5237]. The RFLPs were then correlated with the PCR results. 39% of the allels had the 481C→T mutation corresponding to the single 19 kb band in RFLP, 28% to the 282C→T mutation corresponding in the 15/4.6 bands and 33% of the allels were wild type and revealed the 15/5.6 kb bands. The 857G→A mutation which appeared only in the heterozygous status could not be determined by RFLP after KpnI digest. The allele frequency of 2% for this mutation was determined by PCR.

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DISTRIBUTION OF QUINOLONES AND ß-LACTAM-ANTIBIOTICS INTO NECROTIC AREAS IN SEVERE ACUTE PANCREATITIS

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Bacterial infections of necrotic regions are the most common cause of mortality in severe acute pancreatitis. To prevent these infections effective levels of antibiotics are necessary in inflamed tissue and necrotic areas. Following investigations concerning the penetration of antibiotics into the healthy pancreas (Chirurg 62 (1991) 317), the aim of this study was, whether systematically administered antibiotics are able to enter necrotic areas in severe necrotizing pancreatitis.

We studied the distribution of cefotiam, ceftazidim, ciprofloxacin and ofloxacin between serum and necrotic regions in patients with necrotizing pancreatitis in comparison to healthy rats and rats after induction of acute pancreatitis.

We found the highest necrotic pancreatic tissue/serum partitition coefficient after treatment of patients with ofloxacin (0.43-3.14; mean: 1.25). The mean tissue/serum ratios of the other antibiotics studied in humans were: ciprofloxacin = 0.54 (range: 0.2-0.78), ceftazidim = 0.45 (range: 0.17-0.9) and cefotiam = 0.17 (range: 0.11-0.24). In rats with necrotizing pancreatitis a considerable elevation of pancreatic tissue/serum level ratios in comparison to the control group was observed after application of ciprofloxacin and ceftazidim.

CONCLUSION: Antibiotics are able to penetrate into necrotic pancreatic tissue. The levels of several antibacterial drugs show therapeutic values abow MICs of relevant pathogens.

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PHARMACODYNAMICS, TOLERABILITY AND PHARMACOKINETICS OF DKAH 3 CL, A NEW "SINUS NODE INHIBITOR"

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In a range finding study the dose-response relation of DKAH 3 CL, a compound which pharmacologically shows a long and pronounced reduction of heart rate (HR) without impairing contractility and coronary perfusion, was assessed in healthy volunteers regarding pharmacodynamics, tolerability and pharmacokinetics. DKAH 3 CL was administered orally in increasing doses (5, 7.5, 10 and 15 mg) as a single dose. The doses were divided over 4 treatment groups, each consisting of 4 volunteers. Of each group, 1 subject received placebo and 3 subjects received DKAH 3 CL in a randomised double-blind design. The effect on HR and blood pressure was measured at rest as well as during exercise conditions (bicycle ergometry).

Results

At rest: 15 mg DKAH 3 CL reduced HR markedly between 4 h and 24 h p.a.
During exercise: A pronounced decrease of HR was observed 4 h p.a. with 5, 7.5, 10 and 15 mg. When compared to placebo, the effect was still present 48 h p.a. with 10 and 15 mg.

Heart rate reduction compared to pre-drug values

	placebo	5 mg	7.5 mg	10 mg	15 mg
during exercise, 4 h p.a.	+ 4.8 %	- 2.0 %	- 4.4 %	- 3.9 %	- 12.6 %
during exercise, 6 h p.a.	+ 2.7 %	- 5.3 %	- 7.5 %	- 6.4 %	- 14.4 %
during exercise, 48 h p.a.	- 1.4 %	- 4.6 %	- 5.7 %	- 3.9 %	- 10.8 %

DKAH 3 CL is relatively rapidly absorbed. t_{max}: 1 h (median), range 0.5 - 2 h ;
MRT (mean residence time) median 4.5 h, range 3.2 - 8.8 h.
Due to the short MRT of 4,5 h no accumulation can be expected with a once daily administration.
The subjective tolerability was good up to the dose of 15 mg.

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CONJUNCTIVAL CAPILLAROSCOPY (CC) AS A METHOD FOR THE INVESTIGATION OF DRUGS WITH VASODILATIVE PROPERTIES

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A study was performed to investigate the effect of two vasodilating compounds, the calciumantagonist Nifedipine (NIF) and the ACE-inhibitor Captopril (CAP), on conjunctival vessel diameter, cardiac output (CO), total peripheral resistance (TPR) and heart rate (HR).
Following a single blind design with intraindividual comparison 12 healthy male volunteers were given placebo, 20mg NIF and 25mg CAP. HR, CO and TPR were recorded predose and six times between 40 and 440 min. postdose using impedance cardiography. The measurements were done with the volunteers laying in supine position after 15 min. rest. Following each impedance measurement, a photograph from the left lateral eyeball of the volunteer was taken to depict the conjunctival vessels. For the exact measurement of the vessel diameter the pictures were magnified via microscope. Both, NIF and CAP increased the vessel diameter by a maximum of 14% and 12%, compared to the corresponding placebo values. The maximum effect was reached 80 min. after dosing of NIF and 120 min. after dosing of CAP. TPR was decreased by 10% and 12%, respectively. The correlation between CC and TPR was -0,89 for NIF and -0,46 for CAP. HR was slightly increased (8% NIF and 4% CAP). Following the administration of NIF CO was increased by 11% 80 to 120 min. post dose, CAP caused only an initial increase in CO by 5%.
20mg NIF and 25mg CAP increased the conjunctival vessel diameter and decreased TPR in a comparable manner, while the effect on HR and CO was more different. From the good inverse correlation between increase in conjunctival vessel diameter and decrease in TPR we conclude that the dilatation of the conjunctival vessels is predictive for a systemic peripheral vasodilation. CC, a method easily to perform, may be a useful tool to directly measure vasodilative properties of drugs and to get pharmacodynamic profiles of these compounds in man.

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PHASE I CLINICAL TRIAL OF R-VERAPAMIL AS A MODIFIER OF MULTIDRUG RESISTANCE IN CANCER CHEMOTHERAPY

M. Eichelbaum, G. Engel, E. Ladda*, K. Schumacher*, A.Weimer*

Verapamil has been shown both in vitro and in vivo to reverse multidrug resistance (MDR). However, at the large doses required to reverse MDR significant cardiovascular toxicity occurs. Clinically used verapamil is a racemate, i.e. a 50:50 mixture of R- and S-verapamil. R-verapamil which has only 10 to 20% of the cardiovascular activity of the S-enantiomer is equipotent in vitro to racemic verapamil and the S-enantiomer to reverse MDR. Thus R-verpamil could be superior to racemic verapamil to reverse MDR because of its lesser cardiovascular activity larger doses can be administered.
We have evaluated in 12 patients with advanced breast cancer the maximum tolerated R-verapamil dose, the dose limiting cardiovascular and noncardiovascular toxicity and the drug plasma concentrations achieved. The patients were treated with oral R-verapamil for 5 days starting with a dose of 200 mg q.i.d. in combination with infusion of adriamycin (70 mg/m^2). Combined treatment was repeated 3 weekly for a total of 4 treatment cycles with an option to escalate the R-verapamil dose by an additional 50mg q.i.d. at the each new treatment cycle up to 350 mg q.i.d.
4 patients completed all 4 treatment cycles. The maximal tolerated R-verapamil doses ranged from 400 to 1400 mg/day.
The dose limiting side effect was arterial hypotension with a systolic blood pressure < 80 mm Hg which occured in 4 patients. First degree av-block developed in 9, 2nd degree av-block in 1 patient. The maximum plasma verapamil concentrations ranged from 667 to 1646 ng/ml.
R-verapamil administered in doses between 400 to 1400 mg daily for 5 days caused less cardiovascular toxicity in combination with chemotherapy than it has been reported for i.v. racemic verapamil. Therefore R-verapamil might be better suited than the racemate to reverse MDR.

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RELATION BETWEEN ARTERIAL AND VENOUS PLASMA CONCENTRATION OF EXOGENOUS CATECHOLAMINES

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The site of sampling to determine the catecholamine (CAT) plasma concentration has been a matter of controversy. Arterial sampling was recommended(1). However, this site is not devoid of risk for the patient or volunteer studied. In a study on the haemodynamic and metabolic effects of exogenous CAT in volunteers, we investigated the relationship between arterial and peripheral venous concentration during infusion of noradrenaline (NOR) or adrenaline (ADR).
After approval by the ethics committee cumulative dose-response curves were performed in volunteers. NOR (n=8) or ADR (n=8) were administered using 5 different infusion rates (IR) at 0.01-0.2 μg/kg min, each infusion lasting 30 min. Arterial and venous blood samples were simultaneously taken from radial artery and forearm venous cannulas. The CAT plasma concentrations (ng/l) were determined by HPLC/ECD. Linear regression analyses were calculated for the IR and the CAT plasma concentration. Means $\pm$ SD are given.
Arterial and venous NOR plasma concentrations increased in linear correlation to their infusion rates from 199$\pm$75 to 7475$\pm$1071 pg/ml and from 192$\pm$58 to 5388$\pm$1291 pg/ml. Arterial and venous adrenaline plasma concentrations increased from 53$\pm$44 to 4349$\pm$818 pg/ml and from 51$\pm$47 to 3521$\pm$754 pg/ml. The linear regression equations are:
NOR_{ven} = 0.757 * NOR_{art} - 131 pg/ml, r = 0.96; ven = venous
ADR_{ven} = 0.733 * ADR_{art} + 12 pg/ml, r = 0.92; art = arterial
both intercepts not significantly different from 0.
Our results show a linear relationship between venous and arterial plasma concentrations of NOR and ADR.

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PHARMACOKINETIC COMPARISON OF TWO ORAL FORMULATIONS OF REPIRINAST AFTER SINGLE DOSE ADMINISTRATION

C.Ewald, D.Beermann, H.G.Schaefer, H.Blume, and R.Süverkrüp*

Repirinast(R) is a new antiallergic agent, which is marketed for asthma treatment in Japan as tablet.
To investigate the influence of the galenic preparation of R on relative bioavailability of the active metabolite(M) six healthy male caucasian volunteers were treated with 300 mg R as suspension and as tablet in a randomized crossover trial. Plasma, urine and faeces samples were collected until 96 hours after administration and analysed for R and M using HPLC/UV methods.
R could not be detected in plasma and urine.
Pharmacokinetic parameters (geom. means:/ and ar. means: ±):

	suspension	tablet	units
AUC(0-12)(M)	0.80/ 1.42	0.72/ 1.33	[mg*h/L]
Cmax (M)	0.28/ 1.53	0.22/ 1.39	[mg/L]
Ae urine (M)	11.5 ± 2.9	11.1 ± 2.9	[% of dose]
Ae faeces(M)	56.5 ±14.0	45.1 ± 9.2	[% of dose]
Ae faeces(R)	24.4 ±16.7	17.0 ± 6.3	[% of dose]

Based on plasma AUC relative bioavailability of M was 10 % lower after administration of the tablet as compared to the suspension. Total recovery of R and M in urine and faeces accounted for 92% and 73% of the dose for tablet and suspension, resp. However, this difference was not statistically significant. - In conclusion, both formulations of R were pharmacokinetically equivalent.

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The impact of liver cirrhosis on the stereoselective disposition of *R/S*- nimodipine

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Liver cirrhosis has a profound impact on the first-pass metabolism of drugs. Due to a decrease in functional liver cell mass and extra- and intrahepatic shunting of portal blood supply a substantial proportion of an oral dose will bypass the liver thereby escaping first-pass metabolism. In the case of racemic drugs which are subjected to stereoselective first-pass metabolism the stereoselectivity of this process can be altered in liver cirrhosis.
The first-pass metabolism of nimodipine is known to occur in a stereoselective manner in healthy volunteers (Naunyn-Schmiedeberg's Arch. Pharmacol. **343**, Suppl. 1991: A496). Since no data on the impact of liver cirrhosis on the stereoselectivity of the first-pass metabolism of nimodipine are available we studied the absolute bioavailability of racemic nimodipine in 8 patients with liver cirrhosis (2 mg of the stable isotope labelled racemic analogue i.v. simultaneously administered with 30 mg orally).
The following pharmacokinetic data were obtained:

	volunteers		p	patients		p	p	
	S	*R*	*R/S*	*S*	*R*	*R/S*	*S/S*	*R/R*
F %	3.6±1.6	20.3±5.6	*	42.1±19.0	67.3±28.2	#	*	*
Cl l/min	66.8±27.6	9.7±4.1	*	3.6±2.0	2.0±0.8	#	*	*
$t_{1/2}$ hr	10.5±1.5	10.5±1.5	#	16.9±7.9	16.2±7.7	#	#	#

* $p < 0.05$; # $p > 0.05$

These results revealed a dramatic increase of the bioavailability in patients with liver cirrhosis according to the reduced liver function. As a consequence of more than 10 fold increase in the bioavailability of the cardiovascular more potent *S*-enantiomer a significant decrease in arterial blood pressure was observed. F_R increases only by factor 3. In healthy volunteers a remarkable enantioselectivity was observed expressed as F_R / F_S ratio = 5.6. In contrast the patients exhibit only a modest enantioselective first-pass metabolism with a F_R / F_S ratio = 1.6. The clearances of the stereoisomers are significantly reduced and consequently the AUCs significantly greater when compared to the subjects of the control group. The terminal half-lives were prolonged when compared to healthy subjects.
Conclusion: In case of racemic drugs with extensive stereoselective first-pass metabolism the aspect of altered stereoselective disposition has to be considered.

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GRAPEFRUIT JUICE INHIBITS CYTOCHROME P450 1A2 DEPENDENT METABOLISM OF CAFFEINE IN MAN

U. Fuhr and K. Klittich

Grapefruit juice increases the bioavailability of dihydropyridines in man (1) which may be caused by the inhibition of cytochrome P450 3A4 through the grapefruit component naringenin (2). Inhibition of benzo(a)pyren hydroxylation in vitro (via the P450 1A subfamily) by naringenin (3) indicates that other P450 isoforms also may be involved.
The objective of this study was to investigate the effect of grapefruit juice on cytochrome P450 1A2 in man. Caffeine metabolism was used as a specific probe for P450 1A2 activity, both in vitro and in vivo.
In incubations with human liver microsomes, naringenin was a competitive inhibitor of caffeine 3-demethylation, with K_I values for naringenin one order of magnitude lower than K_M values for caffeine.
In vivo, a randomized cross over design was applied to 12 healthy subjects (5 m, 7 f, 5 smokers, 7 nonsmokers, age 22 - 56 years, weight 39 - 98 kg). 167 mg of caffeine (single dose) was given in both study periods. Additionally, the subjects drank 300 ml of either grapefruit juice (freshly prepared) or water 1/2 h before caffeine application and every 6 h during the 36 h sampling period. Concomitant intake of grapefruit juice prolonged caffeine elimination significantly (see tab.). These changes correlated (r=0.761, p<0.01) to the relative grapefruit juice dose given as ml/min/kg.

Tab.: Effect of grapefruit juice on caffeine kinetics in man

caffeine parameter in plasma (mean±SD)	+ water (absol.)	+ grapefruit (% of control)	Wilcoxon test
$AUC_{0-\infty}$ (umol*h/l)	142±75	134±38 %	p<0.05
Clearance (ml/min)	128±63	81±24 %	p<0.05
$t_{1/2el}$ (h)	4.7±2.1	127±16 %	p<0.005
V_d (l/kg)	0.6±0.1	100±15 %	n.s.

The marked inhibition of caffeine metabolism in vitro identifies naringenin as one agent responsible for the interaction in vivo, although it is unknown whether further components of grapefruit contribute to this effect. The extent of P450 1A2 inhibition, reflected by an increase in AUC of caffeine occasionally exceeding 50 %, may have a relevant influence on the effect of drugs which are metabolized completely or in part by this cytochrome P450 isoform.
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EFFECTS OF DIHYDROSPIRORENONE/ETHINYLESTRADIOL ON RAA-SYSTEM AND SALT-WATER BALANCE IN OVULATION INHIBITION DOSE

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The synthesized progestogens which are usually found in oral contraceptives (OC) failed to show an anti-mineralcorticoid effect as is seen in natural progesterone. An unopposed estrogen effect may cause weight gain, water retention and hypertension in susceptible women. Dihydrospirorenone (DHSP) is proven as a potent progestogen and a competitive aldosterone antagonist in rat and man. The effect of two fixed combinations of 30 µg Ethinylestradiol (EE) and 2 mg and 4 mg DHSP respectively on salt-water balance was investigated under standardized conditions.

Twenty-seven healthy young women received a calorie balanced diet with 100 mmol sodium and 60 - 70 mmol potassium from day 1 - 24. Nine women each took 2 mg DHSP/30 µg EE/d, 4 mg DHSP/30µg EE/d or 0.150 mg levonorgestrel (LNG)/30 µg EE/d (control treament) from day 1 - 21.

Urinary sodium excretion in volunteers treated with DHSP revealed a distinct and dose dependent increase compared with control treatment within the first week of administration. Urinary potassium excretion showed little reduction in the same period. In the subsequent two weeks there was no difference between the treatment groups in urinary salt excretion. Initially, weight loss was slightly greater after DHSP than after control treatment. In the resting period body weight remained constant. Plasma renin increased minimally. Plasma aldosterone and angiotensin II increased significantly during the whole DHSP medication period. Correspondingly a significant increase was demonstrated in urinary aldosterone-18-glucuronide, tetrahydroaldosterone, 18-hydroxy-corticosterone and free aldosterone. No considerable changes were observed in systolic and diastolic blood pressure after administration of DHSP/EE. LNG/EE exerted no aldosterone antagonistic reaction. These three combined dosages suppressed ovulation and progesterone increase.

It was concluded that DHSP/EE produces anti-aldosterone effects in ovulation inhibition dose. The initially increased sodium excretion was largely compensated for, by an endogenous counter-regulation. The progesterone-like pharmacological profile of DHSP may be useful in oral contraception as well as in special gynecological indications e.g. premenstrual syndrome.

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NON-INVASIVE MEASUREMENTS OF PHARMACOLOGICAL EFFECTS ON HUMAN SKIN.

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The assessment of skin function and reactivity with bioengineering tools is now possible through the availability of measuring devices. Thus, skin color, dermal capillary blood flow and water content in the horny layer can be precisely quantified with the Minolta Chromameter, the Periflux laser-Doppler and the Nova DPM among others, respectively. Duration and intensity of the reactions can be recorded, areas under the curves can be calculated and the availability of metrical data allows statistical comparisons to be made.

The following examples will illustrate the measuring techniques and their possibilities:

1. Application of nicotinic acid methyl ester on the skin results in a dose-dependent erythema which can be quantified with the measurement of the skin color and of the capillary blood flow. Pretreatment of the application area with a non-steroidal antiinflammatory drug such as diclofenac durably inhibits the development of the erythema.
2. The vasoconstriction noticed after the application of a glucocorticoid (the known "blanching" effect) similarly can be quantified with the measurement of the skin color. Occlusive treatment with a dermatological base containing urea leads to an enhanced vasoconstriction and also to an increase of the water content of the horny layer as compared to the same base without urea.

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THE INFLUENCE OF GALANTHAMINE HYDROBROMICUM ON REM SLEEP REGULATION

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Animal experiments have demonstrated, that the triggering and maintenance of REM sleep is mainly under the control of cholinergic neurons in the brain stem. In human studies, it has been shown that cholinergic agonists or cholinesterase inhibitors like physostigmine, arecholine or RS 86 administered during or prior to sleep lead to an earlier onset of REM sleep. The effect of cholinergic drugs on REM latency (i.e. the time between sleep onset and the first REM period is even more pronounced in patients with a major depression, thus supporting the hypothesis of a central nervous cholinergic/aminergic transmitter imbalance in depressed patients. In the present study we are investigating the influence of Galanthamine hydrobromicum, a reversible cholinesterase blocker on REM sleep regulation in healthy volunteers. Up to now we have studied 8 subjects (6 females, 2 males) with a mean age of 39.0 ± 7.5 years in the sleep laboratory. After an adaptation night the subjects are given two doses of Galanthamine hydrobromicum (10 mg and 15 mg) in a randomized double blind design compared to placebo at 10 p.m. prior to sleep. Galanthamine 10 mg led the shortening of mean REM latency from 80.6 ± 27.5 min. to 54.4 ± 31.4 min. A dose of 15 mg led to a shortening of REM latency from 74.3 ± 31.5 to 54.1 ± 27.2 min. With both doses, 6 of the 8 investigated subjects showed a shortening of REM latency. The preliminary data suggest, that Galanthamine had the expected influence on REM sleep regulation which proves the central nervous efficacy of the compound.

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THE ABSORPTION OF GRISEOFULVIN FROM DIFFERENT REGIONS OF THE HUMAN SMALL INTESTINE

Th. Gramatté

Steady state intestinal perfusion with three lumen tubes was used to evaluate site specific intestinal absorption of the antifungal drug griseofulvin along the human small intestine. This substance was chosen as a weak acid with extremely low watersolubility (14 mg/l) and moderate lipidsolubility. Preliminary results (3 healthy volunteers) reported here show no distinct intraindividual differences of the mean amount absorbed per unit time (µg/cm x hr): duodeno-jejunal junction 47. 57. 39 : jejunum 30, 62 and ileum 46. Griseofulvin absorption was neither dependent on water movement nor bile acid concentration within the intestinal segment under study.

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POSSIBLE ROLE OF PROTEIN BINDING RATES IN DISCREPANT PLASMA LEVEL-RESPONSE-RELATIONSHIP

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There are numerous attempts to correlate pharmacodynamic effects to (total) plasma levels of drugs. Total plasma levels are routinely measured, however, free plasma levels are supposed to be interrelated with the effect. A linear relationship between free and total plasma levels as suggested by giving the percentage of free drug vs total drug is not necessarily given especially when the plasma protein is saturated or when time did not allow to obtain equilibrium between free and bound portion of the drug. If the exchange between the bound and free pool of the drug is slow, considerable discrepancies between free and total drug especially when starting or stopping e.g. intravenous therapy will be imaginable.

Unfortunately, routinely employed equilibrium dialysis to determine plasma protein binding does not allow to evaluate appropiate association and dissociation rates of protein binding due to the very slow methodology.

Urapidil, an α_1-blocking antihypertensive agent, is chosen as an example, where binding rates to and from protein might explain discrepancies between total plasma levels and effect.

Application of estimated binding rate constants representative of slow protein binding in a model, that treats bound and free drug as separate compartments leads to a plasma level-effect relationship, that follows closely what is published on experimental results with the drug.

Rate constants of plasma protein binding in the case of Urapidil remain to be determined experimentally to either support or discard this hypothesis.

Although difficult to obtain by standard determination procedures for protein binding it appears worthwhile to consider protein binding rates when plasma level and effect do not seem to correlate.

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ENANTIOSPECIFIC FLUORESCENCE ASSAY FOR NOREPHEDRINE (PHENYLPROPANOLAMINE) AND ITS APPLICABILITY IN PHARMACOKINETIC STUDIES

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The racemic erythro form of phenylpropanolamine (rac-norephedrine) is clinically used as a nasal decongestant and an anorectic. The pharmacokinetics of the two enantiomers of norephedrine have not yet been studied in man. In order to measure low concentrations of norephedrine enantiomers [levo-norephedrine, (1R,2S)-(-)-phenylpropanolamine; dextro-norephedrine, (1S,2R)-(+)-phenylpropanolamine] in plasma after administration of racemic drug dosage forms, chiral fluorescent derivatisation and HPLC was employed. The internal standard levo-norpseudoephedrine [(1R,2R)-(-)-phenylpropanolamine] and borate buffer pH 11 were added to a sample aliquot and a liquid-liquid extraction was performed using diethyl ether containing 1.5% ethanol. After solvent evaporation the remaining residue was derivatized with o-phthaldialdehyde and the chiral mercaptan N-acetyl-L-cysteine. The HPLC resolution of diastereomeric derivatives was possible on an achiral octadecylsilane column. The mobile phase consisted of sodium phosphate buffer pH 6.5, methanol and acetonitrile (100:40:30, v/v). The fluorescence of the eluate was monitored at 344/442 nm. Norephedrine enantiomers and the internal standard were well separated. The capacity factors for levo-norephedrine, dextro-norephedrine and levo-norpseudoephedrine derivatives were 7.7, 9.5 and 14.4, respectively. The separation and resolution factors for the norephedrine enantiomers were calculated to 1.22 and 4.22, respectively. The intraday coefficients of variation were less than 10% at concentrations of 5 ng/ml or more of each enantiomer. The detection limits are less than 2 ng/ml (signal to noise ratio 5:1). The specificity of the assay was tested for some endogenous compounds and drugs structurally related to norephedrine but none of them did interfere. In a pharmacokinetic study in 10 healthy volunteers with dosage of 50 mg rac-norephedrine as oral solution, virtually no differences were found between the pharmacokinetic parameters of the two enantiomers.

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INFLUENCE OF CIMETIDINE ON THE STEREOPHARMACOKINETICS OF CARVEDILOL AFTER ORAL DOSAGE

W. Henke, D. Henke, and H. Spahn-Langguth

The ß- and α_1-adrenoceptor antagonist carvedilol exhibits distinct differences between the clearances of its two enantiomers including a highly stereoselective first-pass effect after peroral administration with preferential extraction of the S-(-)-enantiomer. As the compound is mainly eliminated via oxidative metabolism it was hypothesized that inhibitors of this elimination route may affect the extent and the stereoselectivity of clearance and first-pass elimination.

Thus, the pharmacokinetics of carvedilol enantiomers and the respective conjugates were studied in six healthy volunteers after a single oral dose of 100 mg rac-carvedilol in a control period and under concomitant cimetidine treatment, respectively. Enantiomer concentrations were assayed with an enantiospecific analytical method that is based on chiral derivatization with R-phenylethyl isocyanate, normal-phase HPLC and subsequent fluorescent detection (1).

Interestingly, only minor changes due to cimetidine were detected for parent carvedilol enantiomers. The stereoselectivity of the pharmacokinetic parameters was not significantly affected by cimetidine. Average C_{max} values increased from 29 to 45 ng/ml for S- and from 63 to 94 ng/ml for R-carvedilol when cimetidine was coadministered, while the apparent oral clearance decreased from 5.3 to 4.3 l/min for S- and from 3.2 to 2.1 l/min for R-carvedilol. Only a small fraction of the dose (0.5%) was excreted unchanged into urine. Virtually no effect of cimetidine on the renal clearance of the parent enantiomers was detected (R/S-ratio, appr. 1.5). The plasma concentrations of carvedilol conjugates exceeded those of parent drug. The fraction of the dose excreted into urine as conjugates was <5%. Under cimetidine treatment the plasma concentrations of the conjugates were enhanced, while the renal clearances of the conjugates were reduced.

(1) W. Henke, H. Spahn, and E. Mutschler, ISSX 2nd European Meeting on Foreign Compound Metabolism, Frankfurt/Main, F.R.G., March/April 1987.

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CARDIOVASCULAR EFFECTS OF ESMOLOL IN MAN - INFLUENCE OF RATE OF INFUSION, PLASMA CONCENTRATION AND OCCUPANCY OF ß1-ADRENOCEPTORS

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Esmolol (E), a rapidly metabolized and therefore ultrashort acting ß1-selective adrenoceptor antagonist, was infused over 75 min in 8 healthy male volunteers at rates of 100(A) , 250(B) and 500(C) $\mu g \cdot kg^{-1} \cdot min^{-1}$ on three different days in a randomized manner. Bicycle ergometry was carried out before, 60 min after beginning and 45 min after termination of infusion. Parallel to in vivo measurements blood samples were drawn during and 45 min after infusion and conditioned with tricresyl phosphate (TCP) in order to prevent rapid esterolysis of E. In plasma samples occupancy of β_1-adrenoceptors was quantified by a radioreceptorassay (RRA); concentrations of E as well as of its acid metabolite were determined by a HPLC method.

During infusion of E maximal exercise heart rates were reduced dependent on the rate of infusion: from 146 (control) to 131 (A), 120 (B) and 117 (C) bpm (mean values). Systolic blood pressure was reduced from 191 to 157 (A), 143 (B) and 131 (C) mm Hg , respectively . These results corresponded well to the ß1-adrenoceptor occupancies of 61 (A), 77 (B) and 85 % (C). The antagonistic equivalents in plasma as determined by the RRA were directly correlated to the plasma concentrations of E obtained by HPLC under steady state conditions (0.46/0.67 (A), 1.08/1.238 (B) and 2.42/1.99 (C) µg/ml - HPLC/RRA). Thus, the acid metabolite of E, despite high plasma concentrations (max. 77 µg/ml), is obviously without any antagonistic effect; these results are supported by the identical cardiovascular effects measured during exercise before and 45 min after termination of infusion.

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PHARMACOKINETICS AND PHARMACODYNAMICS OF GALLOPAMIL IN PATIENTS WITH LIVER CIRRHOSIS

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Gallopamil (D 600) is a calcium antagonist with phenylalkylamine structure which is a methoxy derivative of verapamil. Although the drug is completely absorbed after oral administration its bioavailability is only 9.5 to 25.4 % due to an extensive first pass metabolism. The total plasma clearance is on average 8.2 l/min, the terminal half life 3 -6 hours. 1 % of the dose is recovered in urine as unchanged drug. Due to a decrease in functional liver cell mass and shunting of the portal blood in patients with liver cirrhosis first pass metabolism can be reduced substantially. So far no data on the pharmacokinetics and bioavailability of gallopamil in liver cirrhosis are known. Therefore we studied the pharmacokinetics of gallopamil in six patients with liver cirrhosis (Child B) and in six matched healthy volunteers after a single oral dose of 25 mg and 50 mg gallopamil, respectively. Blood pressure, heart rate and electrocardiographic parameters were monitored prior to and for twelve hours following gallopamil administration.

In patients with liver cirrhosis a dramatic reduction in clearance to 1.82 (0.7 - 3.8) l/min was observed. As a consequence of the reduced clearance absolute bioavailability increased to 60.0 % (26.2 - 95.6 %), the terminal half life was prolonged to 11.6 (5.1 - 20.2) hours.

These changes in pharmacokinetics were accompanied by distinct cardiovascular effects. In contrast to the healthy subjects where no changes in blood pressure were observed, gallopamil reduced systolic and diastolic blood pressure by 19.2 ± 12.5 and 10.1 ± 3.2 mm Hg respectively as maximum effects in patients with liver cirrhosis. The intensity and duration of the negative dromotropic effect on the atrioventricular conduction was greater in patients with liver cirrhosis.

Conclusion: Based on pharmacokinetic and pharmacodynamic data in patients with liver cirrhosis with Child B the oral dose of gallopamil should be reduced to 25 to 50 % of normal dose.

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A.Gross et al., Eur.J.Pharmacol.183(5),1651-1652(1990)

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PHARMACOKINETICS OF A NEW DEVELOPED NICOTINE TTS

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Tobacco use has resulted in major health related problems worldwide. Nicotine replacement is one of the most promizing strategy in smoking cessation. The pharmacokinetic properties of a new developed transdermal nicotine patch (two different sizes: 16 cm^2 and 24 cm^2) was investigated in three separate studies.
Mean daily nicotine delivery of the two patches were 14.3 ± 2.4 mg (16 cm^2) and 24.6 ± 1.8 mg (24 cm^2) nicotin, where the release followed a first-oder kinetic. A linear relationship between individual dose and AUC- and c_{max}-values of both patches were obtained. As a result of single and multiple dose kinetic, no significant accumulation of nicotine were observed. $T_{1/2el}$ (3.5 h - 4.5 h) of nicotine after removal of the patches was longer than reported values in the literature after i.v. administration (40-120 min). Detection of a longer elimination half life may be due to more sensetive analytical methods used in this study.

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ANALYSIS OF CYTOCHROME P-450*2D6* GENOTYPE AND PHENOTYPE IN 390 CAUCASIANS

R.Kerb and J. Brockmöller

Genetic characterization of patients deficient in cytochrome P-450*2D6* is of great value in individualization of dosage with certain drugs and in studies on diseases associated with toxic environmental compounds. Data on frequency of the different mutant alleles and the related enzyme activities are an important prerequisite.

After phenotyping with debrisoquine, a partially preselected group of 390 Caucasians, 326 extensive metabolizers (EMs) and 64 poor metabolizers (PMs), were analyzed for their restriction fragment length patterns (RFLPs) using the restriction enzyme *Xba*I and by polymerase chain reaction (PCR) (Heim & Meyer, 1990, Lancet 336: 529). Two different active alleles (associated with EM phenotype) and eight different mutant alleles (inactive, PM phenotype) were found.

wt	44E	29B	44B	29A	11.5	29C	10	16+9	32+9	Allele
181	8	76	26	6	10	*	4	1	3	wt
	1	*	6	-	3	-	-	1	-	44E
		9	13	6	5	4	-	2	1	29B
			10	1	4	3	1	1	-	44B
				-	-	1	-	-	-	29A
					2	1	-	-	-	11.5
						-	-	-	-	29C
							-	-	-	10
								-	-	16+9
									-	32+9

The numbers of individuals with the respective allelic conformations are provided; * cannot be diagnosed

190 of the EMs with two active alleles (wt or 44E) had a mean metabolic ratio (MR, ratio of debrisoquine to OH-debrisoquine in urine) of 0.4, ranging from 0.1 to 8. Interestingly, presence of an 44E allele was always associated with very high (MR < 0.4) metabolic activity, as observed in 9 cases. All 64 persons with two mutant alleles showed a MR > 12. Persons carrying a mutant allele (independent of the type) combined with an active allele (n = 136) had a mean MR of 1.0, ranging from 0.1 to 12. Thus, these heterozygotes had a significantly lower metabolic activity than carriers of two active alleles, a fact, which may also be true for other drugs metabolized by cytochrome P-450*2D6* to a quantitatively relevant degree.

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PHARMACOKINETICS OF THE MONOAMINE OXIDASE-A INHIBITOR BROFAROMINE IN YOUNG HEALTHY VOLUNTEERS AND IN PATIENTS WITH CHRONIC RENAL FAILURE

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Brofaromine is a new reversible and selective inhibitor of the MAO-A isozyme. Several studies revealed good tolerability and high therapeutic safety. It has been shown to be an effective antidepressant in patients with major depression. The compound is extensively metabolized by the liver. The primary metabolite is pharmacologically inactive, its glucuronide and sulfate conjugates are excreted in urine. To compare the results in renal disease with those obtained in healthy volunteers, brofaromine was given in open studies to 12 young healthy subjects [A: 7 females (f), 5 males (m); 20 - 35 yr] and to 19 patients with chronic renal failure [B: 6 patients with creatinine clearence from 15 to 25 ml/min; 4 f, 2 m; 35 - 65 yr; C: 13 patients on hemodialysis; 9 f, 4 m; 25 - 65 yr]. Each subject received a single oral dose of 50 mg brofaromine. Blood samples were obtained during 48 h and plasma concentrations of brofaromine were measured by GC-EC.
Results: (mean ± SD)

Study	C_{max}/dose [nmol/(L*mg)]	AUC_{0-48}/dose [(nmol*h)/(L*mg)]	$t_{1/2}$ [h]
A	15.3 ± 6.1	236 ± 125	13.3 ± 3.1
B	22.3 ± 5.5	368 ± 122	12.6 ± 3.5
C	32.7 ± 12.8	585 ± 328	17.5 ± 7.1

Patients had higher C_{max} and increased AUC values ($p \leq 0.05$). Half-lives were not significantly different from values obtained in young healthy volunteers. Possible explanations for the differences are age, liver function, drug distribution and comedication.

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INFLUENCE OF SMOKING ON THE CORRELATION OF IN VITRO AND IN VIVO PARAMETERS OF BIOTRANSFORMATION IN PATIENTS WITH CHRONIC LIVER DISEASE

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7-ethoxycoumarin O-deethylase (ECOD) activity was measured in liver biopsy samples from 30 patients with liver disease (13x fatty liver, 10x chronic-active hepatitis, 7x cirrhosis) to estimate oxidative drug metabolism in vitro. Caffeine and metamizol elimination as in vivo parameter of cytochrome P450-dependent biotransformation were determined in the same subjects within 3 days after biopsy.
ECOD-activity was significantly correlated ($p<0.05$) with the pharmacokinetics of metamizol and with the amount of metabolites excreted in the urine. In contrast, no correlation was found between ECOD-activity and all parameters of caffeine elimination in serum.
Caffeine elimination was elevated in smokers if smokers (n=15) were compared to non-smokers (n=15). However, ECOD-activity and metamizol elimination were not different in both groups. In smokers a correlation was found only between ECOD-activity and different parameters of metamizol elimination ($t_{½}$, MRT and metabolites excreted). In non-smokers an additional correlation was observed between in vitro biotransformation and CL of caffeine as well as metamizol.
Results confirm that smoking differently influences cytochrome P450-dependent drug metabolism caused by different isozymes measured. Consequently, smoking modifies correlations calculated between different in vitro and in vivo measurements of drug metabolism, too. Therefore, smoking must generally be taken into consideration in correlation studies.

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A modified Marples Kligman Test for the evaluation of antibacterial effects of topical drugs

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Several modifications of the Marples Kligman Test (MKT) have been used to improve the performance with respect to easier or faster conductance. After having proven the back to be a convenient localisation for MKT, we have been looking for an appropriate time schedule for the expanded flora test. 24 hours occlusion and treatment under occlusion for another 24 hours gave the best results as to growth of bacteria and measurable antibacterial effects.

In a randomized double-blind study on the back of 18 healthy volunteers, two different models were compared in a half-side method. The original occlusion test of Marples and Kligman (treatment 48 hrs. under occlusion) was compared to a modified expanded flora test (preocclusion 24 hrs., treatment under occlusion for 24 hrs.). Test areas one on each side were treated with 6 α-methylprednisolone aceponate 0.1 %/ clorquinaldol 1%, 6 α-methylprednisoloneaceponate 0.1 %, dexamethasone 0.04%/nandrolone-decanoate 0.04%/chlorhexidine 1%, dexamethasone 0.035%/diphenhydramine-hydrochloride 0.5%/dequaliniumchloride 0.1%, flupredniden-21-acetate 0.1%/chlorquinaldol 1%, betamethasone-17-valerate 0.1%/neomycinesulfate 0.5%, betamethasone-17-valerate 0.1%/gentamycinesulfate 0.1%. An occluded test area on each side without treatment served as control. Bacterial scrubs, dilution, cultures and calculations were made according to Marples and Kligman.

On both sides the antibacterial effects of all substances tested were better than the control. The lowest values were found under topical treatment with antibiotic combinations. The combination corticosteroid/diphenhydramine-hydrocloride/dequaliniumchloride was almost as effective as the combinations with antibiotics. The same tendencies were seen on both sides. In contrast to the original expanded flora test (48 hrs./48 hrs.) the modified expanded flora test (24hrs./24hrs.) gave better discrimination of single drug effects due to a larger range of bacterial numbers. 24 hrs. of preocclusion did not lead to maximum numbers of bacteria (no ceiling-effect).

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EVIDENCE FOR GENETICALLY AND ENVIRONMENTALLY DETERMINED METABOLISM OF METHADONE

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Methadone ((*S*/*R*)-6-dimethylamino-4,4-diphenylheptan-3-one) is frequently used as a treatment drug for maintainance therapy of opiate addicts. The drug undergoes extensive and variable metabolism in man which may be a limiting factor in achieving plasma concentrations necessary for successful maintainance therapy. The enzymes involved in methadone biotransformation have not been identified so far. We therefore investigated methadone metabolism and disposition both in vitro (microsomal fraction of human liver) and in vivo (20 opiate addicts during chronic methadone therapy).

In vitro experiments showed both the CYP2D6 catalyzed 5-hydroxylation and the CYP3A4 and CYP1A2 catalyzed N-dealkylation of propafenone to be inhibited by methadone in a competitive manner (K_i for 5-hydroxylation and N-Dealkylation 395 μM and 230 μM, respectively), thereby indicating affinity of methadone to these enzymes. The major metabolite formed in vitro was the N-demethylated product (2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine). The V_{max} for the formation of this metabolite correlated with the content of CYP3A3/4 ($r = 0.94$; $P < 0.01$) and CYP1A2 ($r = 0.92$; $P < 0.01$) as estimated by Western blotting in five different liver preparations.

The average dose corrected steady state plasma concentrations of methadone during chronic treatment revealed a wide interindividual variability (6.9 ± 3.2 ng/ml/mg; range 2.3-16.9 ng/ml/mg). Phenotypisation of patients with sparteine during methadone treatment showed an increased metabolic ratio compared to a non treated population indicating partial inhibition of CYP2D6 mediated sparteine metabolism in vivo.

We conclude that CYP3A4, CYP1A2 and CYP2D6 can modulate methadone disposition. Therefore, genetic (CYP2D6) and/or environmental (CYP3A4) factors may contribute to the metabolic profile and interaction potential of methadone.

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MONITORING OF MICROPROTEINURIA IN PHASE I STUDIES

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Monitoring of renal integrity by serum creatinine, creatinine clearance or proteinuria in test strips is too insensitive to detect early renal impairment. Therefore, determination of urinary albumin (Alb), α-1 microglobulin (a-1MG) and N-acetyl-ß-D-glucosaminidase (NAG) excretion was carried out to detect and discriminate glomerular and tubular alterations. Alb and a1-MG were determined immunologically by radial diffusion, NAG was determined enzymatically in substrate excess. Comparing creatinine adjusted excretion of these proteins by healthy volunteers in collection urine (0-12h, 12-24h) and 1st and 2nd morning urine in a prospective study revealed that levels were highest in the 2nd morning urine, which should be chosen for routine determinations. Day to day variability was lowest with NAG (7-25 %) and highest with Alb (>300 %). Upper "normal limit" was not exceeded with a1-MG, but in 2 % with Alb and NAG. Historical data of renal tubular enzyme excretion in the 2nd morning urine of more than 100 volunteers in pre-examinations and placebo periods of phase I studies were evaluated to get further information on biological variability and to compare values of young male volunteers with established "norm values". Depending on methods used for the measurement of enzyme activity, the corresponding indicated "upper limits" were exceeded in 3-6 % with NAG.

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PHARMACOKINETIC DETERMINATION OF DOSE EQUIVALENCY BETWEEN INTRAVENOUS AND ORAL DOSES OF CIPROFLOXACIN IN HEALTHY VOLUNTEERS

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In order to get information on the equivalence between oral and IV ciprofloxacin dosages the drug was administered to 12 healthy volunteers at doses of 300 mg and 400 mg intravenously (infusion time 60 minutes) and 500 mg and 750 mg orally in a randomized, double-blind, single-dose, four-period crossover study. On each treatment day, each subject received both oral and intravenous formulations, one of which was placebo. Blood and urine samples were obtained through 24 hours post-dose. The following pharmacokinetic parameter were calculated using non-compartmental methods. (geom. means, n=11).

	300IV	400IV	500PO	750PO
AUC(0-24)[mg*h/l]	8.21	10.98	10.36	15.80
AUC(0-24)/D[h/l]	0.0274	0.0275	0.0207	0.021
Cmax[mg/l]	3.23	3.94	2.67	3.63
Cmax/D[1/l]	0.0108	0.0099	0.0053	0.005
t1/2[h]	5.47	4.65	4.57	4.55

Within each dosing route, the pharmacokinetic profiles were dose-proportional. The 400 mg IV dose was equivalent to 500 mg oral dose with respect to AUC and equivalent to 750 mg oral with respect to Cmax. The absolute bioavailability after oral administration was about 78%. No significant adverse events were associated with either route of administration.

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THE METABOLIC DISPOSITION OF ^{14}C-CARBOCYSTEINE IN HUMANS

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The metabolism of carbocysteine (CMC) has been reported to proceed predominantly *via* sulphoxidation. The so called sulphoxidation index (ratio between drug-related thioethers and sulphoxide metabolites excreted in the urine during 0-8h) shows polymodal distribution. Based on this index the population can be divided into extensive and poor sulphoxidisers. The polymorphic sulphoxidation of CMC has been linked to certain diseases and adverse drug reactions [S. C. Mitchell and R H. Waring, *Pharmacol. Ther.* **43**, 237 (1989)]. However, recent studies using HPLC, GC-MS and ^{13}C NMR techniques could not confirm that sulphoxidation of CMC is a major biotrans-formation pathway [C. O. Meese et al., *Lancet* **336**, 693 (1990)]. In view of these discrepancies we have studied the disposition and metabolism of the drug following oral administration of $^{14(13)}$C-CMC to two subjects. Recovery of ^{14}C-activity in breath and urine corresponded to 7% and 16%, respectively, and 88% and 80%, respectively (totally 95-96% within 168 hrs). A combination of radio-tlc, ^{13}C NMR and GC-MS was used to identify and quantitate CMC and its metabolites in urine. The ^{14}C-activity in urine could be identified as CMC (20%) thiodiglycolic acid (TDGA, 20%), thiodiglycolic acid sulphoxide (TDGA-SO, 32%), *S*-(carboxymethylthio)-L-cysteine (CMTC, 4%), (3-carboxymethyl-thio)lactic acid (TLA, 2%). Sulphoxide metabolites were excreted only in trace amounts accounting for ≤ 2% of the dose. During the 0-8 hr urine collection period which is used to determine the sulphoxidation index only 30% of the dose was excreted. This urine fraction consisted mainly of CMC (65% of radioactivity), the remainder being TDGA, TDGA-SO and TLA. Less than 0.5% of the dose was excreted during this time period as sulphoxide metabolites and CMTC. The findings of this study confirm our previous data in 34 volunteers, where a similar metabolic pattern was observed. CMTC was the only metabolite which showed bimodality in its urinary excretion [C. O. Meese et al., *Biochem. Pharmacol.* **42**, R13 (1991)].

Conclusion: More than 90% of the urinary metabolites of CMC have been identified. Excretion of the parent drug and formation of TDGA, TDGA-SO, CMTC and TLA are the major routes of elimination. Thus it can be ruled out that cysteinyl sulphoxide formation constitutes a major pathway of CMC biotransformation.

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PHARMACODYNAMIC CONSEQUENCES OF THE CODEINE O-DEMETHYLATION POLYMORPHISM IN AN ANIMAL MODEL

G. Mikus, J.F. Cleary, N.C. Kerry, A.A. Somogyi, and F. Bochner

The analgesic action of the weak opioid codeine is thought to be mediated by its O-demethylated metabolite morphine. This reaction has been shown to be under the genetic control of cytochrome P-450IID6 (man) and P-450IID1 (rat). The female Dark Agouti (DA) rat shows decreased morphine formation as compared to the female Sprague Dawley (SD) rat due to a deficiency in cytochrome P-450IID1. The aim of this study was to test the involvement of codeine O-demethylation to morphine in the analgesic action of codeine. The enzyme kinetics of O- and N-demethylation of codeine were investigated *in vitro* using liver microsomes from both rat strains. In addition the nociceptive tail-flick reflex was used to test the analgesic action of codeine. Analgesia was obtained when the tail-flick latency time was greater than 150% of the baseline latency and dose-response curves were constructed using the number of animals analgesic.

There were no strain differences in codeine N-demethylation to norcodeine, however a 10-fold diminished intrinsic clearance of morphine formation was observed in DA liver microsomes. Quinine 10 µM, a potent selective inhibitor of P-450IID1, diminished the clearance to morphine in SD rats to 6% of that observed without quinine, no change occurred in codeine N-demethylation. To account for potential inter-strain differences in the rat tail-flick model, morphine was given as a positive control and a 3-fold difference in ED_{50} was observed between the two strains (SD: 10.4 vs DA: 31.0 µmol/kg). After s.c. administration of codeine a dose-response curve was obtained only in SD rats with the ED_{50}=76 µmol/kg. However, even after 400 µmol/kg codeine no analgesia was observed. When quinine (60 mg/kg i.p.) was administered to SD rats 1 hour before codeine (100 µmol/kg) in order to inhibit morphine formation, a reduction in analgesia from 87.5% to 37.5% was observed. Codeine analgesia can be abolished if either the metabolising enzyme (cytochrome P-450IID1) is inhibited by other drugs (phenocopying) or the enzyme itself is lacking. In conclusion, codeine elicits its analgesic effect via the formation of morphine.

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LIVER FUNCTION TESTS IN PHARMACOKINETIC STUDIES ON PATIENTS WITH IMPAIRED LIVER FUNCTION: AMINOPYRIN-BREATHTEST VERSUS CAFFEINECLEARANCE

K.-H. Molz, G. Haug und D. Grune

Pharmacokinetic data generated on healthy volunteers cannot be applied to patients without certain limitations. This applies particularly to patients with a limited elimination capacity due to an impaired renal or liver function. Hence, pharmacokinetic studies on these patients are gaining increasing importance during the development of a drug. The renal function can be measured with the creatinine clearance as a simple, well tolerated and commonly accepted method. Due to the complexity of its function, an analogous test has not been established for the liver.

Amongst a number of widely exercised methods the aminopyrine breath test as well as coffeine clearance were prefered as screening methods in several pharmacokinetic studies. The primary reason was their simplified and reliable feasibility for gaining quantitative information on hepatic microsomal activity and their good correlation with the clinical symptomatology. [1,2,3]. Since both techniques assess functionally related microsomal isoenzyme systems (cytochrome P-450 and P-448), the results were compared and the correlation between calculated. A regression analysis for n = 38 data pairs, which were collected from 20 patients with impaired liver function, revealed a strong correlation between both tests (correlation coefficient = 0.811). A power function (multiplicative model) in form of $c = c(a) = pa^q$ fits best to reflect the functional relationship; a stands for aminopyrine breath test [% dose*kg body weight/2 mmol CO_2] and c for caffeine clearance [ml/min*kg body weight]; p = 2.241 and q = 0.886.

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[2] A. Holstege et al, 1989, Klin. Wochenschr, 67: pp. 6-15
[3] D.A. Henry et al, 1985, Dig Dis Sci, 30, No.9: pp. 813-818

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IN VITRO INVESTIGATION OF THE TRAMADOL-METABOLISM - EVIDENCE FOR STEREOSELECTIVITY

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Tramadol [rac-1(e)-(m-methoxyphenyl)-2(e)-(dimethylaminomethyl)cyclohexan-1(a)-ol hydrochloride] (T) is a widely used centrally acting analgesic. Therapeutically the drug is administered as a racemate. T is metabolized via O-demethylation to M1 and via N-demethylation to M2. Further demethylation of the primary metabolites results in the formation of three other metabolites (M3 - M5). A sensitive HPLC-method for the simultaneous quantification of T and the major metabolites in biological samples has been developed. T and its metabolites were extracted by dichloromethane after alkalization using ammonia solution. Extraction efficiencies of T and its metabolites from microsomal incubation mixtures ranged from 78 - 93 %. The isocratic HPLC system employed a C_{18} reversed phase column (10 µm; precolumn 12,5 cm x 3 mm i.d.; column 30 cm x 4 mm i.d.). Sensitivity of the assay was 0.5 µg/ml and 0.2 µg/ml for T and its metabolites respectively. Precision (C.V.) of the assay was 3.6, 5.2 and 4.4 % for T, M1 and M2 respectively.

Microsomes from human livers were incubated with (+/-)-T,(-)-T or (+)-T in the presence of NADPH. M1 formation (O-demethylation) was considerably faster when incubating microsomes with (-)-T in comparison to (+)-T or the racemate. In contrast N-demethylation (M2 formation) was found to be faster after incubation with the (+)-enantiomer as compared to (-)-T or the racemate.

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INVESTIGATIONS ON KINETICS AND DYNAMICS OF 4'-EPIDOXORUBICIN ON ITS ONFLOATING SPEED TO THE LIVER CELL *R.Preiss, H.Kelm and U.Ulrich

In preliminary investigations on tumor patients we have found a linear pharmacokinetics of doxorubicin in the tested dose range of 30 to 70mg/m^2,whereas both the elimination of its main metabolite doxorubicinol and the formation rate of the aglycone metabolites were capacity limited (R.Preiss et al. 1989: Int.J.Clin.Pharmacol.Ther.Toxicol. 27(4), 156-64). In the present investigations on 12 tumor patients the kinetics of 4'-epidoxorubicin (4'-ed), a stereoisomere of doxorubicin, and its main metabolite 4'-epidoxorubicinol (4'-ed-OH) in the systemic circulation, the renal elimination of 4'-ed-glucuronides, 4'-ed and 4'-ed-OH, the toxic side effects and the therapeutic effect of 4'-ed were investigated in dependence on the onfloating speed of 4'-ed to the liver cells. 4'-ed was administered into the Arteria hepatica in form of a bolus injection and a 3-h infusion, respectively. In comparison to the bolus injection the 3-h infusion led to 30-fold and to more than 10-fold lower plasma Cmax values for 4'-ed and 4'-ed-OH, respectively. Both the AUC of 4'-ed and 4'-ed-OH were also significantly lower in the systemic circulation after the 3-h infusion than after the bolus injection. Some heavily metastasized patients show a saturated hepatic elimination also under the conditions of the 3-h infusion. The formation rate of 4'-ed-OH was high. The renal elimination pattern of the 4'-ed glucuronides was also dependent on the onfloating speed of 4'-ed to the liver cells. A good correlation was found between the liver-leaving amounts of 4'-ed and 4'-ed-OH and the tumorstatic side effects. Hemodepression, nausea and vomiting were smaller after the 3-h infusion of 4'-ed than after its bolus injection. From the kinetic and therapeutic point of view a 3-h infusion of 4'-ed should be preferred to a bolus injection in patients such as those underlying this study.

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PIROXIMONE I.V. (MDL 19.205) IN AIDING WEANING FROM CARDIOPULMONARY BYPASS FOLLOWING CARDIAC SURGERY: FIRST EXPERIENCES

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Piroximone is a new non-glycoside, non-catecholamine imidazolone derivative with both positive inotropic and vasodilator properties. Patients may often be pre-selected prior to cardiac surgery (CS) as being at risk of requiring inotropic assistance in weaning from cardiopulmonary bypass (CPB) or developing acute low-output-states refractory to ß-adrenergic stimulation.
In an open study 15 pre-selected patients (11 male, 4 female), mean age 62.1 ± 4.8 years (range 54 - 70) with a mean CI of 2.2 l*min^{-1}*m^{-2} and a mean PCWP of 15 mmHg received a single piroximone bolus-dose of 0.5 mg/kg bodyweight. Operation types were 3-vessel coronary-aorto-bypass-graft in 13 cases and mitral valve replacement in two cases. CPB and cross clamp duration were 104 ± 21 min (76 - 140) and 51 ± 8 min (37 - 55), respectively. Three patients additionally received i.v. nitrates and one patient Ca^{2+}-agonist-therapy. Haemodynamic parameters were measured using Swan-Ganz-catheter prior to surgery, before piroximone bolus and after 15, 30, 50 and 60 min. In most cases the maximum effect were seen after 15 min.

	UNIT	pre OP t_{pre}=-153min	after OP t_0=0min	after OP t_{15}=15min
CI	l*min^{-1}*m^{-2}	2.2 ± 0.2	2.1 ± 0.2	3.1 ± 0.8 * °
PCWP	mmHg	15 ± 6	16 ± 4	13 ± 4
HR	min^{-1}	69 ± 11	85 ± 11	90 ± 17 °
RAP	mmHg	13 ± 4	15 ± 3	13 ± 4
AP_m	mmHg	93 ± 13	76 ± 8	79 ± 15 °
SVR	dyn*sec*cm^{-5}	1643 ± 277	1308 ± 168	1021 ± 271 * °
PAP_m	mmHg	26 ± 6	24 ± 3	21 ± 2
PVR	dyn*sec*cm^{-5}	221 ± 96	175 ± 73	123 ± 53 * °

Signif.: paired t-Test * $p < 0.01$ t_{15} vs t_0; ° $p < 0.01$ t_{15} vs t_{pre}

In one patient prolonged ECC and IABP combined with catecholamine-therapy were necessary because of hypotonia. Another patient had an episode of moderate tachycardia but recovered without counteractive intervention. No adverse events on laboratory parameters or life-threatening arrhythmias occurred.
It is concluded that a single dose of 0.5 mg/kg piroximone in addition to conventional therapy is safe and effective for use in weaning patients from CPB. Further comparative studies are indicated.

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METHODOLOGICAL STUDIES ON THE KINETICS OF CYCLOSPORINE UPTAKE INTO ERYTHROCYTES

Ch. Reichel and M. v. Falkenhausen

More than 70 % of cyclosporine (CyA) are bound to erythrocytes at whole blood concentrations of 50 - 1000 ng/ml (1). Up to now kinetics of CyA uptake could not be determined sufficiently, because it was not possible to inhibit this uptake at a certain point of time. We describe a method to measure the kinetic parameters of this uptake based on rapid cooling of the erythrocyte suspension.
After centrifugation and filtration erythrocytes were resuspended in RPMI medium (1 % BSA) to a hematocrit of 10 %. CyA was added in ethanolic solution, final concentration being 5 µg/ml. At fixed times, 6 ml of the suspension were pipetted into 30 ml of normal saline kept at 0°C in order to lower rapidly the temperature. After centrifugation and twofold washing of the erythrocytes in normal saline at 0°C, CyA was determined by HPLC.

After 5 minutes CyA uptake amounted to 43 * 10^{-5} nmol/10^6 cells and to 4 * 10^{-5} nmol/10^6 cells at 22°C and 0°C respectively. This demonstrates that cooling effectively inhibits CyA uptake into erythrocytes.
The cooling to 0°C results in prompt inhibition of CyA uptake. This allows further investigations of the kinetics of CyA uptake e.g. with respect to temperature dependency or inhibitory effects of drugs.

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INCREASED CYP1A2 ACTIVITY AFTER OMEPRAZOLE AS SHOWN BY THE ^{13}C-CAFFEINE BREATH TEST AND THE URINARY PARAXANTHINE/CAFFEINE RATIO IN MAN

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The gastric H^+-K^+-ATPase inhibitor omeprazole is rapidly and completely metabolized by the liver. Formation of the hydroxymetabolite cosegregates with the *S*-mephenytoin hydroxylation polymorphism. Moreover, omeprazole was shown to induce CYP1A1 and CYP1A2 activity in hepatocytes and liver microsomes (Diaz et al., Gastroenterology 1990; 99; 737). In this study the inducing property of omeprazole is evidenced in vivo by the ^{13}C-[*N3*-methyl]-caffeine breath test and urinary metabolic ratios of caffeine.

13 volunteers (18-82 y; 6 f, 7 m) were treated with 40 mg omeprazole for 7 days. After administration of 3 mg/kg ^{13}C-[*N3*-methyl]-caffeine p.o. an 8h breath test and 24h collection of urine were performed before, at the 7th day of treatment, and after 7-day washout. The ^{13}C-content of the breath samples was measured by isotope ratio mass spectrometry, specifically indicating the *N3*-demethylation activity. The corresponding urinary paraxanthine/caffeine ratio was established by rp-HPLC gradient elution.

Treatment with omeprazole lead to a mean increase of 23.4 ± 16.8 % (median = 16.1 %, 95 % confidence: 10.1 - 36.7 %, range -10 to +63 %) in the ^{13}C-[*N3*-methyl]-caffeine breath test. This increase was statistically significant, $p = 0.002$. The paraxanthine/caffeine urinary ratios before and after omeprazole treatment were correlated with the results of the ^{13}C-[*N3*-methyl]-caffeine breath test ($p = 0.05$). The increase of 23.9 % was of the same magnitude as observed in the breath test. The results suggest that omeprazole induces the *N3*-demethylation of caffeine (CYP1A2 activity) in vivo. Furthermore, evidence is presented that the urinary paraxanthine/caffeine ratio may serve as a convenient indicator of CYP1A2 activity as compared to the more laborious ^{13}C-[*N3*-methyl]-caffeine breath test.

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DOSE-RESPONSE RELATIONSHIP OF INTRAVENOUSLY ADMINISTERED QUINAPRILAT IN MAN USING ANGIOTENSIN I - CHALLENGE

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Following oral administration of the non-sulfhydryl ACE-inhibitor quinapril approximately 38 % of the dose are absorbed and converted to the active compound quinaprilat. Information on the extent and the time course of the hemodynamic effects of i.v. quinaprilat in comparison with orally administerd quinapril is lacking. Thus, we investigated in a double-blind, cross-over study in two parallel groups of 12 healthy volunteers the dose-response relationship between rising doses of i.v. quinaprilat (0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 mg) or placebo by means of diastolic blood pressure response after repeated infusions of angiotensin I at a dose which produced an increase in diastolic blood pressure of 25 mmHg under standard conditions. The individual dose of angiotensin I was determined in each subject during an initial challenge. The effect of quinaprilat per dose was characterized by the decrease in the area (AUC) under the hemodynamic index (mean blood pressure/heart rate) versus time curve within the period of observation. A clear dose-response curve was obtained for this hemodynamic index following the different doses of quinaprilat: AUCs were 4.64 ± 1.39 (Placebo), 4.28 ± 0.85 (0.1), 2.89 ± 0.41 (0.5), 2.56 ± 0.54 (1), 1.73 ± 0.55 (2.5), 1.70 ± 0.76 (5), and 0.85 ± 0.03 (10 mg quinaprilat), respectively.
Thus, approximately 50 % inhibition is obtained with a quinaprilat dose of 1.5 mg; 90 % inhibition is reached with a dose of 10 mg quinaprilat i.v. These doses correspond to oral doses of 2.5 and 20 mg quinapril.

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SAFETY AND PHARMACOKINETICS OF MURINE MONOCLONAL ANTIBODY TO HUMAN TUMOR NECROSIS FACTOR (TNF-MAB)

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Tumor Necrosis Factor (TNF) is a significant mediator of the clinical manifestations of sepsis. A murine monoclonal antibody against recombinant human TNF (TNF-MAb) with a high TNF neutralizing activity was developed and selected for a phase I trial. This study evaluated the safety and pharmacokinetics of TNF-MAb in 20 noninfected patients at high risk for developing sepsis and 16 infected patients with the sepsis syndrome. For the first study a dose escalation was administered to 5 groups each with 4 patients: 1, 3, 7.5, 15 and 15 mg/kg x 2. For the second study a single dose of 15 mg/kg was administered to all 16 patients enrolled. There were no infusion related reactions and overall tolerance was good. Transient liver function test alterations were seen in 4 and 6 patients, respectively, all attributed to underlying illnesses. Platelet rises occurred in 7 patients and 4 patients, respectively. No thrombotic or ischemic events were noted. A linear dose response was noted with $t_{\frac{1}{2}}$=50.5 hours and 49 hours, respectively. Approximately 2/3 of the patients developed a human anti mouse antibody response and a smaller proportion an anti-idiotypic response. The mortality rate of the septic patients was 4 of 16 at 28 days. It is concluded that TNF-MAb administration is safe and efficacy will be evaluated in a large trial with septic patients.

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TOLERABILITY AND PHARMACOKINETICS OF MELOXICAM AS A SINGLE I.M. INJECTION COMPARED TO AN I.V. INJECTION IN HEALTHY VOLUNTEERS

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Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) from the structural class of oxicams. Due to the potential utility of i.m. administration as a convenient alternative to i.v. dosing, tolerability and pharmacokinetics of the i.m. mode of administration were assessed in comparison to i.v. in twelve healthy male volunteers. The study was designed as an open randomised two way cross over study. The plasma concentration-time curve after i.v. administration demonstrated a rapid distribution followed by a dominant elimination phase with an elimination half-life of about 16 hours. The Meloxicam plasma profile after i.m. administration demonstrated a rapid (maximum concentration was reached after 1 hour) and complete absorption of the drug.
The following pharmacokinetic parameters (mean ± SD) were obtained from fitting using the TOPFIT programm:

	units	i.v.	i.m.
$AUC_{(0\text{-infin.})}$	mcg x h/ml	29.8 ± 7.8	30.1 ± 6.8
* $t_{1/2}$	h	15.6 ± 3.0	
$V_{ss/f}$	l	11.2 ± 1.7	11.2 ± 1.4

* elimination half life was fitted simultaneously for i.v. and i.m.

Meloxicam was well tolerated. CK-MM isoenzymes decreased after both treatments.

	CK-MM [U/l], median, n=12		% change compared to 0 h	
Time point	i.m.	i.v.	i.m.	i.v.
0 h	48.2	50.2		
24 h	38.4	40.3	- 11.0	- 22.3
96 h	39.9	35.5	- 7.3	-22.4

The favorable data on tolerance and the similarity of pharmacokinetics between both routes of administration suggest that i.m. administration is an useful alternative for attaining therapeutically effective plasma concentrations of Meloxicam.

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PEDIATRIC PRESCRIPTIONS OF CHEMOTHERAPEUTIC AGENTS

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Chemotherapeutic agents are widely used in pediatric therapy. The present study was undertaken to analyse this pharmacotherapy in the pediatric division of an outpatient department in an East German large town. We studied 13176 prescriptions written out for 6415 patients (3223 girls, 3192 boys) in 1988. Furthermore, 11905 prescriptions of 5371 patients could be compared with appropriate medical records. Data were obtained in a standardized manner and analysed by means of a computer program. Following portions of different groups of drugs had been prescribed: oral antibiotics 30.8 %, local antibiotics 22.5 %, oral sulfonamides 19.7 %, local sulfonamides 3.8 %, local antimycotics 4.3 %, other chemotherapeutic agents 18.8 %. Most prescriptions concerned oral phenoxymethyl penicillin (4059) and oral trimethoprim plus sulfamerazine (2181). In medical records 88.6 % of prescriptions were documented, but only 5.1 % completely. Side effects of prescribed chemotherapeutic agents were recorded only in 0.8 % of all prescriptions. Most drugs were used for treatment of infections of the respiratory tract (53.9 %), eye (11.8 %) and ear (10.6 %). Compared to chemotherapeutic guidelines accepted generally, 71.4 % of analysed prescriptions were indicated, 14.5 % were not indicated and 14.1 % could not be assessed.

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PHARMACOKINETICS OF RECOMBINANT FVIII IN COMPARISON TO PLASMA-DERIVED FVIII(KOATE-HS®)

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As part of a world wide multicenter clinical trial pharmacokinetics of pdFVIII and rFVIII were investigated in 17 patients with severe or moderate hemophilia A. After a therapy free period of 72 hours each patient received a single iv dose of 50IU/kg BW pdFVIII and started two weeks later a treatment of three times weekly rFVIII prophylaxis. Pharmacokinetic profiles of rFVIII were obtained on weeks 1, 13 and 25. The following parameters were calculated (geom. means, 1s-range).

	pdFVIII	rFVIII week 1	rFVIII week 13	rFVIII week 25
AUCnorm[IU-h/l]	0.32	0.42	0.40	0.46
	(0.25-0.41)	(0.30-0.57)	(0.29-0.57)	(0.34-0.62)
CL[ml/h/kg]	3.12	2.41	2.48	2.18
	(2.4-4.0)	(1.76-3.3)	(1.76-3.5)	(1.61-2.96)
MRT[h]	17.9	21.1	19.1	22.5
	(14.5-22.0)	(16.5-27.0)	(14.7-24.7)	(18.8-26.8)
t1/2[h]	13.3	15.7	13.9	16.3
	(10.6-16.6)	(12.5-19.6)	(10.7-18.1)	(13.7-19.5)
Vss[l]	4.2	3.8	3.6	3.9
	(3.5-5.0)	(3.0-4.8)	(2.9-4.3)	(2.9-5.1)

The shape of the individual curves for pdFVIII- and rFVIII-infusions were nearly identical. AUCnorm-values for rFVIII were about 23-34% higher compared to pdFVIII, but were not changed during repeated administration. CL of rFVIII was smaller than that of pdFVIII due to a lower Vss for rFVIII, since t1/2 and MRT showed no difference. In general, pharmacokinetic behaviour of rFVIII and pdFVIII was comparable.

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EFFECT OF (-)-NIGULDIPINE (B8059-035) ON THE PHARMACOKINETICS OF DAUNORUBICIN (DNR) IN PLASMA, PERIPHERAL BLASTS (MB) AND THEIR NUCLEI IN PATIENTS (PTS) WITH ACUTE MYELOGENOUS LEUKEMIA (AML).

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The occurrence of multidrug resistance (MDR) may be one of the major obstacles to an effective chemotherapy of pts with AML. Its association with the overexpression of a membrane glycoprotein (Gp-170) acting as an energy-dependent efflux pump for anthracyclines and other xenobiotics. Serveral drugs including calcium antagonists have been proven to inhibit Gp-170 in vitro. However, effective concentrations mostly cannot be obtained in plasma without severe side effects. In contrast, B8059-035 may be a nontoxic modulator of MDR. Pts with AML in relapse were sequentially treated with hAD (2x1,000 mg/m²/d ara-C i.v., d 2-5, 60 mg/m²/d DNR i.v., d 1-3) and hAD & B8059-035 (1,250 mg/d p.o., d (-2)-7), respectively. Plasma kinetics of DNR and kinetics of cellular and nuclear uptake of DNR were determined in MB isolated from peripheral blood (PB) dependent on B8059-035 either by FACS-analysis or by HPLC with fluorescence detection. Up to now, there was no significant difference between hAD and hAD & B589-035 with respect to response as well as toxicity. Plasma kinetics of DNR was not significantly influenced by B8059-035. In vivo cellular uptake of DNR in MB was about 25% higher when B589-35 was added. In vitro investigations of MB from PB or bone marrow concomitantly performed showed that the efflux of rhodamine 123 was significantly higher from CD 34-positive subpopulations and could be more effectively inhibited by B589-35 than by verapamil (VER). However, MB expressing Gp-170 as determined by MRK 16 immunefluorescence were rare and could be only identified in 3/20 pts. In conclusion, B589-35 seems to be a well tolerated substance to more effectively reverse multidrug resistance in MB than VER.

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EARLY INTRAVASCULAR DISTRIBUTION AND ELIMINATION OF DRUG SIMULATED BY REPETETIVE CONVOLUTION WITH A CIRCULATORY TRANSPORT FUNCTION

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The aim of this study was to develop a widely applicable model for circulatory indicator dispersion in order to describe simultaneously the pharmacokinetics of early drug distribution and drug elimination after i.v. injection.
The indicator dilution curve measured in the aorta is the result of repetitive circulation of the indicator through the body. The concentration time course of the indicator after the first recirculation, I_1, can be described by convolution of the concentration of the first aortic pass, I_0, with the circulatory transport function g: $I_1 = I_0 * g$. I_n is the concentration time course after the n^{th} recirculation pass of the initial bolus. The resulting final concentration time course (R) after n recirculations is:

$$R = \sum_{n=1}^{\infty} I_{n-1} * (k\ g)$$

The asterisk denotes the convolution operation.

The circulatory transport function g can be represented by a left skewed distribution function like a lagged normal density[1] or a lognormal distribution[2]. Elimination of the indicator is represented by a recovery factor k, which indicates the fraction of the indicator appearing after recirculation. Simulation for intravascular indicators such as indocyanine green yield concentration time courses, which are similar to in vivo measured time courses for the early distribution phase as well as for the elimination phase.
Repetive convolution with a left skewed circulatory transport function is a practical algorithm serving as a model for the drug distribution and elimination after i.v. injection.

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2 J.C. Böck, A. Hoeft, G. Hellige (1990): Biomed Instr Tech 24, 42-49.

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THE PHARMACOKINETICS OF THE β_1-SELECTIVE ANTAGONIST TALINOLOL AFTER MULTIPLE DOSING IN HEALTHY VOLUNTEERS

B.Terhaag, U. Möbus, R. Oertel und K. Richter

The pharmacokinetics and the steady state serum level of talinolol were investigated after oral administration of 50 mg talinolol with a dosing intervall (τ) of 12 hours for 7 days in 12 (2 female, 10 male) healthy subjects. Blood was drawn in each trough before the next dosing and also during the first (1^{st} day) and last (8^{th} day) in order to describe the kinetics.
The t_{max} values are in the same range at the 1^{st} and 8^{th} day (2.8 h and 2.5 h, resp.). C_{max} is increased (mean ± SD) from 94 ± 47 ng/ml (1^{st} day) to 128 ± 38 ng/ml at the 8^{th} day. The terminal half life is 9.2 ± 4.2 h (range: 4.3 - 19.6 h) and not different to the first day. The AUCo-∞ is increased by a factor of 1.6 (range 0.5 - 3.7) from 605 ng/ml.h (range: 72 - 987 ng/ml.h) to 814 ng/ml.h ($AUC_{168-180}$, range: 267 - 1203 ng/ml.h).
C_{min} is increased by factor of 1.8 (range: 1.0 - 2.9). According to c_{min} steady state is reached after third dosing. The peak-trough difference in steady state is 2.4 according too the pharmacokinetic simulation.
In this dosage regimen the cumulation factor is about two .

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THE EFFECT OF BILLROTH II-GASTRECTOMY ON THE PHARMACOKINETIC OF A SOLID OR LIQUID ORAL SULFAMERAZINE PREPARATION IN MAN M. Trausch, B. Trausch, G. le Petit

Billroth II-gastrectomy changes the physiology of the upper gastrointestinal tract (pH, transit time) and affects the pharmacokinetic of drugs. Sulfamerazine was used as a model drug, because of it's physicochemical properties: acid (pk_a: 6,9) and pH-dependent octanol:water partition coefficient (pH ≤5: 1,5; pH 7: 0,65; ph 7,4: 0,35) and water solubility (pH ≤5: 0,29 mg/ml; pH 7: 0,7 mg, pH 7,4: 1,22 mg/ml). Sulfamerazine concentrations were determined by spectrofluometry in serum of 5 male patients (group I: 37 - 63 years, 65 - 85 kg body weight, >2 years after gastrectomy) and 5 healthy male volunteers (group II, 24 - 32 years, 64 - 84 kg body weight) after oral administration of sulfamerazine as a solid form and a solution (pH: 8 - 8,5) in a cross over design. A dose of 25 mg/kg body weight were applicated.

	group I		group II	
preparation	**solid**	**liquid**	**solid**	**liquid**
pharmacikinetical parameters (mean ± standard deviation)				
c_{max} (µg/ml)	58±26*	186±20	91±39	147±40
t_{max} (min)	252±94*	15±4	240±85	81±100
AUC $^{0-10h}$ (µg.ml^{-1}.h)	453±213	974±145	730±318	915±396

paired t-Test (p <0,05): * group I solid - group I liquid

Conclusion: Billroth II-gastrectomy has a non-significant effect on the bioavailability of different sulfamerazine preparations. For drugs with comparable physicochemical properties such kind of changes in pharmacokinetics are possible.

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HEMODYNAMIC EFFECTS OF GLYCERYL TRINITRATE FOLLOWING INTERMITTENT OR CONTINUOUS TRANSDERMAL ADMINISTRATION D. Trenk, A. Wiegand, R. Bonn

Continuous administration of glyceryl trinitrate via transdermal delivery systems (TDS) with constant release characteristics provokes hemodynamic tolerance to glyceryl trinitrate within a few days of treatment. Thus, we investigated in a double-blind, placebo-controlled, 3-way-cross-over design in 11 healthy male volunteers, if the development of tolerance can be circumvented or diminished by intermittent administration (12 hours verum-TDS/ 12 hours placebo-TDS) of glyceryl trinitrate-TDS (Deponit 10^{R}, Schwarz Pharma, Monheim) in comparison to continuous administration (verum/verum) over a treatment period of 4 days. In addition, we focused our interest on the possible involvement of counter-regulatory mechanisms in the development of hemodynamic tolerance to glyceryl trinitrate. Hemodynamic effects were characterized by a/b-ratio of the digital pulse curve, and blood pressure/heart rate following orthostatic challenge. Continuous administration of glyceryl trinitrate-TDS results in a marked decrease of the effects of glyceryl trinitrate on the a/b-ratio: The mean integrated effect as characterized by the area under the a/b-ratio within a dosing interval was attenuated by 14 % (day 2), 43 % (day 3) and 53 % (day 4), while it was preserved following intermittent dosing. The same results were obtained with respect to systolic blood pressure and heart rate during orthostatic challenge. Epinephrine and norepinephrine plasma concentrations and plasma renin activity increased in parallel to the hemodynamic effects. The latter increases were also blunted in the state of tolerance to glyceryl trinitrate. In contrast, we observed a significant ($p<0.05$) decrease of the hematocrit (-2.5 %) during continuous administration of glyceryl trinitrate-TDS, indicating that extra- to intravascular volume shifts might be involved in the development of hemodynamic tolerance to glyceryl trinitrate.

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HEMODYNAMIC EFFECTS OF NEBIVOLOL AND ATENOLOL

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Nebivolol (N) is a selective beta-1 adrenoceptor antagonist with an ancillary property. In a double-blind placebo (P) controlled cross-over study, cardiac function was measured invasively (dye dilution) in 9 patients (8 males, 1 female; 30-60 y) with essential hypertension after 4 weeks of treatment with N 5 mg once daily. In a 2nd study with a similar P controlled cross-over design, cardiac function of 30 hypertensive patients (20 males, 10 females; 33-68 y) was measured non-invasively with echo Doppler. In this study effects of N were compared with P and atenolol (A) 50 mg once daily.

	MAP	HR	CO	SV	TPR
	mmHg	b/min	l/min	ml	kPa.s.l^{-1}
Dye dilution					
P	122±4	70±4	6.3±0.4	91±6	119±7
N	111±3*	59±3*	5.7±0.2	97±4	118±5
Echo Doppler					
P	118±2	73±2	5.4±0.2	78±2	136±6
N	110±2*	65±2*	4.9±0.2*	81±2	139±5
A	109±2*	62±2*#	4.5±0.2*	78±2	149±5*#

*p<0.05 vs. P; #p<0.05 vs. N.

The results of the non-invasive study were comparable to those of the invasive study. The antihypertensive effect (MAP) of N 5 mg once daily and A 50 mg once daily was similar. HR decreased more during A than during N. CO decreased during A and N but the decrease during N tended to be smaller than during A. In contrast to A, in both studies N tended to increase SV and did not change TPR. These studies show that for a similar antihypertensive effect N does not increase TPR, which might result in a smaller afterload than during A.

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DOSE-RESPONSE RELATIONSHIP OF BM 06.022, A NOVEL RECOMBINANT PLASMINOGEN ACTIVATOR, IN HEALTHY VOLUNTEERS

E.v. Möllendorff, W. Akpan, G. Neugebauer

BM 06.022 is a recombinant plasminogen activator (rPA) expressed by E coli cells, containing active centers of human tPA. The tolerability, hemostatic effects, and pharmacokinetics of rPA were investigated in healthy volunteers. Single ascending i.v.-doses from 0.1125 to 5.5 megaunits rPA were administered each dose to 3 volunteers of n=18. Plasma samples were taken in order to measure the following parameters: activity-concentration of rPA, clotting times, fibrinogen, plasminogen, α_2-antiplasmin, and fibrin-D-dimers.
With increasing doses the maximum concentrations (c_{max}) and the activity concentration time curves (AUC) of rPA increased dose dependently and linearly. Clotting timss and fibrinogen were not influenced, plasminogen only at higher doses. α_2-antiplasmin decreased, fibrin D-dimers increased dose-dependently. After the highest dose residual plasminogen was 87±3 %, α_2-antiplasmin 79±3 % and fibrin D-dimers 1147±380 ng/ml. r-PA was well tolerated. The doses tested in these healthy humans showed a beginning fibrinolytic action.

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METABOLISM OF EUGENOL IN MAN AFTER ORAL APPLICATION OF EUGENOL AND EUGENOL-D_3

G.E. von Unruh, I.U. Fischer, J. Reinhardt, and A. Schwalbe

Eugenol, 4-hydroxy-3-methoxy-allylbenzene (I), is the main component of clove oil; (I) is a component of spices and herbal medicines. Similar allylbenzenes, safrole and estragole, are hepatotoxins and carcinogens for man and animal. (I) is generally recognized as safe in human food. We ingest about ½ mg per day.
Recently, the metabolism of eugenol has been studied for the first time in humans[1]. Orally applied (I) was metabolized rapidly. Some of the suspected metabolites of (I) found in the urine of the volunteers were similar to or identical with components of food or their metabolites. Therefore, confirmation of the precursor - metabolite relationship was desired.
According to Rice et al.[2] eugenol-D_3 was synthesized, the three deuteriums being in the metabolicaly stable methoxy group. In the urine of 3 male volunteers, dosed with a 1 : 1 mixture of (I) and (I)-D_3, 200 mg each, conjugates and all major metabolites were identified by their typical twin peaks as well as by co-injection of the appropriate reference compounds. In addition to the previously described metabolites cis- and trans-coniferyl alcohol were shown to be metabolites of (I).

References: 1) I.U. Fischer, G.E. von Unruh and H.J. Dengler, Xenobiotica 20, 209-222 (1990).
2) J.E. Rice, N. Hussain and E.J. LaVoie, Journal of Labelled Compounds, 24, 1043-1049 (1987).

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BLOOD TO PLASMA CONCENTRATION RATIO OF CYCLOSPORINE AND ITS PRIMARY METABOLITES 1 AND 17

W. Weber, M. Looby, M. Nitz, and P. Neuhaus*

The kinetic time course of Cyclosporine (CyA), and its primary metabolites 17 and 1 were measured using HPLC in plasma and whole blood of 11 transplant patients following iv administration of CyA. Plasma was seperated immediately after sampling at 37°C. The blood to plasma concentration ratio of these 3 substances was subsequently determined. This ratio was found to be concentration dependent for both metabolites and independent for CyA. However, the nonlinear effect was only apparent below concentrations of 30 ng/ml.
Since almost all CyA plasma concentrations were above 25 ng/ml, a concentration dependency for CyA may still exist, but was not determinable as the therapeutic concentrations were too high. In contrast M1 concentrations were almost all below this value and M17 concentrations were evenly distributed between 10 and 100 ng/ml.
The most likely explanation for the observed concentration dependency of the metabolites is saturation of binding to blood cells.
These observations have consequences for the interpretation of blood and plasma concentration data. To date there have been much disagreement about which biophase is the better for kinetic evaluation. Our data clearly show that either biophase is suitable equivalent at concentrations above 30 ng/ml. However, below this value only whole blood concentrations provide reliable estimate of the amount of metabolite in circulation.

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EFFECTS OF THE PUTATIVE DOPAMINE AUTORECEPTOR AGONIST ROXINDOLE IN PATIENTS WITH DEPRESSION

K. WIEDEMANN, M. KELLNER, J.-C. KRIEG and F. HOLSBOER

In several in vitro and in vivo studies Roxindole (EMD 49980, 5-hydroxy-3-(4-phenyl-1,2,3,6-tetrahydropyridil-(1)-butyl)-indol) proved to be a potent presynaptic dopamine autoreceptor agonist. In addition, Roxindole inhibits serotonin (5-HT) uptake. In binding studies the dopamine D2 receptor affinity was confirmed in the low nanomolar range, however, effects were also observed at 5-HT-1 A serotonin binding sites with a dissociation constant of about 1 nM. Therefore the compound might dispose of antipsychotic as well as antidepressive properties. In recent investigations applying Roxindole to patients suffering from schizophrenia with negative symptoms an activating and antidepressive effect could be observed. We therefore conducted another pilot study applying Roxindole to patients suffering from a major depressive syndrome especially with psychomotor retardation. Roxindole was applied for 28 days in an open pilot study. The maximum dosage applied was 7.5 to 12.5 mg/day. Up to now 10 patients were investigated. One patient showed a marked psychomotor activation which prompted the termination of the trial at day 4. Seven patients responded favorably with a reduction of the HDRS (Hamilton depression rating scale) score by 40 % or more, 2 patients remained without clearcut effects upon depressive symptomatology. Adverse effects were not observed throughout the treatment period of 28 days. It remains unclear to which specific pharmacological receptor interaction the clinically observed effects of Roxindole can be attached. Possibly Roxindole is able to act on both, dopamine autoreceptors and supersensitive postsynaptic dopamine receptors. From clinical and pharmacological observations an additional effect of the compound upon 5-HT receptors has to be also taken into consideration.

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SUBCELLULAR DISTRIBUTION AND ACTIVITY OF GLUCOSE TRANSPORTER ISOFORMS GLUT1 AND GLUT4 TRANSIENTLY EXPRESSED IN COS-CELLS

A. Schürmann, I. Monden, and K. Keller

Glucose transporters (GLUT) represent a family of homologous membrane proteins exhibiting a remarkable tissue specificity of expression. Insulin-sensitive cells predominantly express the isoform GLUT4. In the these cells, GLUT4 is present in an intracellular, vesicular compartment (low-density microsomes) from which it is translocated to the plasma membrane in response to insulin. In order to test the hypothesis that insulin sensitivity is conferred by the presence of the GLUT4 and its preferential targeting to intracellular vesicles, we transiently expressed GLUT4 and the erythrocyte/brain-type glucose transporter GLUT1 in COS-cells. Full-length cDNA was ligated into an expression vector driven by the cytomegalovirus promoter (pCMV), and introduced into COS cells by the DEAE-dextran method. After 48 h, cells were homogenized and fractionated by differential centrifugation to yield plasma membranes and a Golgi-enriched fraction of intracellular microsomes (low-density microsomes). In these membrane fractions, the abundance of glucose transporters was assessed by immunoblotting with specific antibodies against GLUT1 and GLUT4, and their transport activity was assayed after solubilization and reconstitution into lecithin liposomes. In parallel samples, cells were incubated in the presence or absence of insulin, and 2-deoxyglucose uptake rates were assayed. Uptake rates of 2-deoxyglucose were higher (maximal effect 2-fold) in cells expressing GLUT1 or GLUT4 as compared with control cells (transfection of pCMV without transporter cDNA); insulin failed to alter the uptake rates. Reconstituted glucose transport activity in plasma membranes was about 5-fold higher after expression of GLUT1 and GLUT4 as compared with control cells. The relative amount of GLUT4 in the low-density microsomes as detected by reconstitution exceeded that of the GLUT1, but was much lower than that observed in typical insulin-sensitive cells, e.g. rat fat cells or 3T3-L1 adipocytes. These data indicate that COS cells transfected with glucose transporter cDNA express the active transporter protein and can thus be used for further studies on these proteins. Furthermore, the data indicate that the GLUT4 isoform, when expressed in an insulin-insensitive cell line, is inserted into the intracellular pool only to a small extent. Thus, its cellular targeting does not only depend on the sequence of GLUT4, but also on yet to be defined components of the cellular machinery.

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BINDING DOMAINS OF INHIBITORY LIGANDS IN GLUCOSE TRANSPORTER GLUT4 AS LOCALIZED BY PHOTOLABELING, PROTEOLYTIC CLEAVAGE AND SITE-SPECIFIC ANTISERUM
B. Hellwig, F.M. Brown, M.F. Shanahan, and H.G. Joost

Glucose transporters (GLUT) bind several ligands with high affinity, namely cytochalasin B, forskolin, and a number of methylxanthines. These agents label a domain of the transporter involved in its function, since binding of the ligands results in inhibition of transport and is competitively inhibited by glucose. The different isoforms of the transporters, GLUT1, GLUT2, and GLUT4, exhibit striking differences in the affinity to these ligands (Hellwig and Joost, Mol. Pharmacol. 40:383-389, 1991). In the present study, we further defined the binding region of the ligand forskolin in GLUT4 with the aid of the photoreactive derivative, ^{125}IAPS-forskolin (3-[^{125}I]iodo-4-azidophenethylamido-7-O-succinyldeacetylforskolin). Plasma membranes from insulin-treated rat adipocytes containing predominantly the GLUT4 isoform were irradiated with UV light in the presence of ^{125}IAPS-forskolin. The covalently labeled glucose transporters were solubilized, immunoprecipitated with specific antiserum, and partially digested with trypsin and elastase. The fragments were separated by gel electrophoresis, transferred onto nitrocellulose membranes, and identified by immunoassay with antiserum against a peptide sequence corresponding to the C-terminus of GLUT4. Labeling of the fragments was assessed by direct autoradiography. After digestion with a high-purity grade trypsin, two photolabeled fragments with apparent molecular weights of 21 and 16 kDa were detected, both containing the intact C-terminus of GLUT4. In contrast, after digestion with elastase only a single photolabeled fragment at 21 kDa was detected, although the enzyme had generated two fragments with intact C-terminus at 21 and 15 kDa. A less pure trypsin preparation generated three fragments with intact C-terminus (21, 17, and 15 kDa), and two labeled fragments at 21 and 17 kDa. Based on the sequence of GLUT4, the tryptic cleavage site generating the 16 kDa fragment can be localized at Arg 346, 349 or 350. Since the size of the smallest labeled fragment containing the intact C-terminus was 16 kDa, and that of the largest unlabeled fragment was only slightly lower (15 kDa), binding of the photolabel appears to occur in close proximity to this tryptic cleavage site.

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STABLE EXPRESSION OF THE CALCIUM CHANNEL SUBUNITS IN SOMATIC CELL LINES
A. Welling, R. Bottlender, E. Bosse, F. Hofmann

Chinese hamster ovary cells (CHO^--cells) were stably transfected with complementary DNA encoding the calcium channel α_1 subunit from smooth muscle ($\alpha 1_{SM}$) and a combination of $\alpha 1_{SM}$ and skeletal muscle ß. Specific high affinity binding sites for calcium channel blockers (ccb) were present in all selected cell clones indicating the functional expression of the $\alpha 1_{SM}$. Expression of ß was demonstrated by Western blot analysis. The apparent KD for the ccb isradipine was 0.1 to 0.3 nM and the specific binding capacities of cell homogenates were 10 (CHO^-), 143 (α1, CHOCa9) and 364 fmol/mg protein ($\alpha 1+$ß, CHOCa9ß3). Transfection of $\alpha 1_{SM}$ alone led to the expression of functional calcium channels which are sensitive to ccb and BayK8644. Currents were measured with the whole-cell version of the patch-clamp method in a solution containing 30 mM $BaCl_2$. Coexpression of $\alpha 1_{SM}+$ß enhanced I_{Ba} 4-fold from -11.3 (α1) to -45.0 $\mu A/cm^2$ ($\alpha 1+$ß). The cotransfection of the ß-subunit has accelarating effects on both the current activation (ttp90%: 9.1 msec (α1), 6.1 msec ($\alpha 1+$ß)) and inactivation kinetics (dec_{100}: 84.8% (α1), 37.4% ($\alpha 1+$ß)). The half maximal steady state inactivation and activation were shifted significantly to more negative potentials. Our results demonstrate that the skeletal muscle ß-subunit has a modulatory effect on the current evoked by smooth muscle α1. Coexpression of $\alpha 1+$ß enhances the expression of calcium channels and fastens the current kinetics.

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MEMBRANE DEPOLARIZATION AND CALCIUM INFLUX ACTIVATES GENE TRANSCRIPTION IN THE ENDOCRINE PANCREAS
M. Schwaninger, R. Blume, G. Lux, and W. Knepel

The islet cells of the endocrine pancreas are electrically excitable. Action potentials occur spontaneously and get more frequent in the presence of secretagogues. Depolarization of the plasma membrane leads to influx of calcium through voltage dependent calcium channels and thus to a stimulation of hormone secretion. The role of elevated intracellular calcium levels in the regulation of pancreatic hormone gene transcription is unknown. Therefore, the coding region of a reporter enzyme gene was placed under the transcriptional control of 350 base pairs of the rat glucagon gene promoter and enhancer. This fusion gene was transiently transfected into HIT cells, a well characterized pancreatic islet cell line. Depolarization of cell membrane by high potassium activated gene expression irrespective of the reporter enzyme used (firefly luciferase or chloramphenicolacetyltransferase). The increase in gene expression was related to the potassium concentration in the medium. In contrast, reporter enzyme activity was not enhanced by high potassium concentration when the reporter gene was placed under the control of the herpes simplex virus thymidine kinase promoter, suggesting that potassium-induced membrane depolarization does not enhance protein or mRNA stability but stimulates gene transcription driven by the rat glucagon gene promoter and enhancer. The effect of membrane depolarization on glucagon gene transcription is mediated by calcium influx into the cells, since nifedipine, a blocker of L-type voltage dependent calcium channels, inhibited the response. The molecular mechanism of calcium-induced glucagon gene transcription seems to differ from that of cAMP-induced transcription, because the effects induced by membrane depolarization and 8-Br-cAMP, an analogue of cAMP, were additive. How the calcium signal is transmitted into the nucleus, as well as the transcription factors and cis-acting DNA control elements involved remain to be defined.

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REGULATION OF THE AGONIST-EVOKED CALCIUM RESPONSE IN HUMAN PLATELETS BY PROSTAGLANDINS, ENDOTHELIAL FACTORS AND SELECTIVE ACTIVATORS OF THE cAMP-DEPENDENT PROTEIN KINASE
J.Geiger and U.Walter

Human platelet activation is inhibited by both cAMP- and cGMP-elevating agents. Certain prostaglandins [PG-E_1, PG-I_2 and its stable analog iloprost (ZK 36374)] and forskolin are potent activators of the platelet adenylyl cyclase and strong inhibitors of the agonist-induced platelet calcium response. In this study with human platelets, we investigated the regulation of the ADP- and thrombin-evoked calcium response (analyzed by fluorescence spectrometry and stopped-flow fluorometry [Geiger J, Nolte C, Butt E, Sage SO, Walter U, (1992) Proc. Natl. Acad. Sci. USA,in press]) by PG-E_1, iloprost, endothelial-derived factors and the selective cAMP-protein kinase activator Sp-5,6-dichloro-1-ß-D-ribofuranosyl-benzimidazole-3',5'monophosphorothioate.
Human endothelial cells were untreated or preincubated with either hemoglobin or indomethacin. In all cases, the agonist evoked Ca^{2+}-response was partially or strongly inhibited in the presence or absence of extracellular calcium, respectively, while the ADP-receptor-activated cation channel was not significantly affected. The results suggest that cAMP-elevating prostaglandins including the endothelial-cell-derived prostacyclin primarily inhibit the agonist-evoked Ca^{2+}-discharge from intracellular platelet stores, an effect mediated by the cAMP-dependent protein kinase.

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ENDOGENOUS ADP-RIBOSYLATION OF ACTIN I.Just, G.Koch, and K.Aktories

Various bacterial toxins such as *C. botulinum* C2 toxin and *C. perfringens* iota toxin ADP-ribosylate regulatory proteins. There is some evidence that bacterial ADP-ribosyltransferases mimic endogenous cell regulation mechanisms. Here we report on the endogenous ADP-ribosylation of actin. The incubation of the lysate of mononuclear cells (MNC) in the presence of [^{32}P]NAD resulted in the labelling of several proteins. One labelled protein with the MW of 42 000 (p^{42}) was precipitated by DNAse-Sepharose which is known to bind selectively actin with high affinity. Formation of F-actin by phalloidin treatment inhibited ADP-ribosylation. The DNAse-precipitated p^{42} was digested by snake venom phosphodiesterase and the product was identified as AMP. ADP-ribosylated p^{42} and actin ADP-ribosylated by iota toxin showed the same isoelectric point in 2-D gel electrophoresis. Furthermore, the proteolytic peptide maps of p^{42} and of actin both ADP-ribosylated were similar. In contrast to toxin-induced ADP-ribosylation of actin which is at arginine-177 the modification of p^{42} was sensitive to mercury ions indicating an ADP-ribose-cysteine bond. Thymidine did not effect the ADP-ribosylation of p^{42} whereas ATP, ADP and ADP-ribose were inhibitory. In the presence of ADP-ribose p^{42} was ADP-ribosylated much faster and to a greater extend than in the presence of NAD.
The data indicate that actin is endogenously mono-ADP-ribosylated in mononuclear cell lysate and that ADP-ribosylation occurs also by a non-enzymic process. Since ADP-ribosylation of actin catalyzed by clostridial toxins causes dramatic effects on the cytoskeleton the endogenous ADP-ribosylation of actin might be involved in the regulation of cytoskeletal organization.

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BIOCHEMICAL CHANGES IN MALIGNANT HYPERTHERMIA: CONTENT OF cAMP AND INOSITOL PHOSPHATES IN PORCINE SKELETAL AND CARDIAC MUSCLE
J. Scholz, U. Troll, M. Patten*, J. Schulte am Esch

The ryanodine receptor seems to be involved in the development of malignant hyperthermia (MH). Recently, it was presumed that altered second messengers may lead to alteration of the ryanodine receptor. Consequently, we have determined the basal content of cyclic AMP and inositol phosphates in skeletal and cardiac muscle of MH susceptible (MHS) and healthy control (MHN) swine.
MHS (n=8) and MHN (n=6) Pietrain swine were used and tested by halothane challenge for MH susceptibility. In addition, an in-vitro skeletal muscle contracture test according to the protocol of the European MH Group was done to confirm MHS or MHN status. Skeletal and cardiac muscles were prepared and frozen in liquid nitrogen. For determination of inositol phosphates a non-radiometric HPLC-metal dye detection technique (Mayr, Biochem J, 254: 585, 1988) was used. cAMP was measured by radioimmunoassay.
The following IP isomers could be detected: 1,3,4-IP3, 1,4,5-IP3, 1,5,6-IP3, 4,5,6-IP3, 1,3,4,6-IP4, 1,3,4,5-IP4, 1,2,5,6-IP4, 2,4,5,6-IP4, 1,4,5,6-IP4, 1,2,3,4,5-IP5, 1,2,4,5,6-IP5, 1,3,4,5,6-IP5 and IP6. Almost all inositol phosphates measured had a higher concentration in MHS than MHN in skeletal as well as heart muscles. There was no difference in cAMP content of cardiac muscle whereas there was a higher content of cAMP in MHS than MHN skeletal muscle. In conclusion, firstly, besides involvement of the skeletal muscles a primary myocardial abnormality in MHS is possible, and secondly, the idea that the inositol phosphate metabolism could be involved in the development of MH is additionally supported.

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LOCALIZATION OF THE CYTOTOXIN GENE IN *PSEUDOMONAS AERUGINOSA* AS A CONTRIBUTION TO STUDY THE BACTERIAL VIRULENCE.

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The cytotoxin gene demonstrated in a temperate phage of 35.5 kb [1] was investigated for its possible role in virulence of *P.aeruginosa* strain PA158. We present evidence that the phage is integrated in the host DNA as a prophage and that the phage genome cohesive ends are associated covalently during phage gene integration. The restriction profiles of phage DNA digested with *Eco*RI, *Hin*dIII, and *Bam*HI have been identified. The cohesive ends (*cos*) and the cytotoxin gene are located close to each other within the circular form in the 4 kb-*Eco*RI B fragment, 8.5 kb-*Bam*HI B fragment and 11 kb-*Hin*dIII A fragment. The phage attachment site (*attB*) is present in the same 4 kb-*Eco*RI B fragment with the cytotoxin gene and *cos* ends. Replication of the vegetative form of the phage was found to be higher at 37°C than at 30°C, while the production of the cytotoxin was higher at 30°C than at 37°C. Preparations of the phage showed at least 7 proteins as detected by SDS-PAGE. However, cytotoxin could not be found by Western blotting. It suggests that the cytotoxin gene integrated in the cellular DNA is responsible for toxin production.

[1] Hayashi, T., Baba, T., Matsumoto, H. and Terawaki, Y. (1990) Mol. Microbiol. 4:1703-1709.

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EXPRESSION OF A NOVEL PENICILLIN-BINDING PROTEIN IN BETA-LACTAMASE PRODUCING STAPHYLOCOCCUS AUREUS
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The beta-lactamase dependent penicillin resistance of S.aureus can be greatly reduced by polidocanol (PDO), a dodecyl polyethyleneoxid ether (Bruns et al. 1985, Antimicrob. Agents Chemother. 27, 632-639). PDO does not affect the activity of beta-lactamase, but the induced synthesis of this enzyme is inhibited by PDO-concentrations which are not inhibitory for bacterial cell growth.
We studied the consequences of this effect on the expression of penicillin-binding proteins (PBPs) with three resistant strains (S 108, 8325, 5814 S). Staphylococcal cells grown with and without PDO were incubated with various concentrations of ^{3}H-benzylpenicillin. The cytoplasmic membranes were isolated and subjected to SDS-PAGE and fluorography.

By growth in the presence of PDO (30 mg/l) an additional PBP was expressed. This concentration-dependent effect was present in all three strains tested. The additional PBP runs between PBP2 and 3 and was named PBP2α. It possesses a high affinity for penicillin similar to that of the PBPs from susceptible staphylococci. The mol.wt. is 71,000. In the PDO-free control cells PBP2α was always absent; this was also true when PDO was added to non-growing cells. Furthermore, in the penicillin-susceptible S.aureus H PBP2α could not be expressed by PDO under any experimental conditions tested.
In spite of the fact that it is still unclear in which way PBP2α works, one can assume that it is involved in the regulation of beta-lactamase synthesis. In plasmid curing experiments it could be shown that PBP2α is encoded by chromosomal DNA and does not represent a plasmid-mediated protein.

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DIFFERENTIATION OF [^{3}H]-LIDOCAINE BINDING IN ISOLATED MYOCARDIAL CELLS OF RAT

W. Voigt and R. Mannhold

The molecular mechanisms of antiarrhythmic drug action are not yet completely understood. Electrophysiological findings support the existence of voltage dependent binding sites for class I antiarrhythmics in myocardial and neuronal tissues; however, a high affinity plasmalemmal binding site has not been detected so far. In former investigations (*Voigt* and *Mannhold*, Eur.J.Pharmacol. 169, 103, 1989) showing a lidocaine binding site in crude membranes of bovine heart, the association of this binding site to the plasmalemma could not be demonstrated. In contrast, we now found lidocaine binding sites (lido-BS) in myocardial plasmalemma. Myocardial cells were desaggregated with collagenase. Binding with [^{3}H]-lidocaine was performed in intact cells and in membranes derived from intact cells. After disruption of cells, membranes were purified by centrifugation using a linear sucrose gradient. ß-Adrenoceptors and Ca^{2+}-channels of plasmalemmal membranes were identified with (-)-[^{125}J]pindolol and (+)-[^{3}H]isradipine, respectively. Lido-BS cosedimented with both plasmalemmal markers. In addition, the density of binding sites (BS) for lidocaine (220 fmol/mg of protein) was in the same range as for (-)-[^{125}J]pindolol and (+)-[^{3}H]isradipine. These data correspond well with densities of Na^+-channels extrapolated from electrophysiological findings. Competition binding experiments yielded a K_D for lidocaine of 80-200 nM for plasmalemmal membranes showing a 500 fold higher affinity than estimated from the allosteric inhibition of [^{3}H]batrachotoxinin A-20-α-benzoate binding by lidocaine. These results further support that lidocaine binds to a plasmalemmal BS, probably located on the sodium channel.

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INFLUENCE OF LOCAL ANESTHETICS ON CALCIUM FLUXES IN HUMAN PLATELETS.

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Local anesthetics (LA) influence vascular tone via sympathetic blockade which leads to vasodilatation and by direct action on vascular smooth muscle resulting in either vasodilatation or vasoconstriction depending upon the local anesthetic, its concentration, the site of injection, the type of blood vessel, and pre-existing vascular tone. Since the mechanism(s) of direct vascular action is not well understood the influence of two LA, different in their physico-chemical properties, were studied on intracellular calcium fluxes of human platelets using the calcium indicator fura-2. The results show that bupivacaine and lidocaine suppressed dose dependently the thrombin and epinephrine induced intracellular calcium release and calcium influx from the extracellular space. Bupivacain was more potent in affecting the hormone-sensitive calcium pool than lidocain.

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LOCALIZATION BY *IN SITU* HYBRIDIZATION OF Na,K-ATPase α1 SUBUNIT mRNA IN *XENOPUS* OOCYTES USING A PCR-GENERATED PROBE

S. Friehs and G. Schmalzing

In oocytes of *Xenopus laevis* several maternal mRNAs have been identifed that are spatially localized and inherited by particular blastomers after fertilization. Spatial localization of mRNAs could provide a mechanism whereby proteins are synthesized close to their site of plasma membrane insertion and function. In support of this view, we have previously found that proteins synthesized in *Xenopus* oocytes from synthetic cRNAs for various Na,K-ATPase subunits are confined to that part of the plasma membrane which is close to the site of cRNA injection.

In the present study we have examined in *Xenopus* oocytes the spatial distribution of the endogenous mRNA encoding the Na,K-ATPase α1 subunit. Stage VI oocytes of an albino line were fixed with formaldehyde, dehydrated with ethanol, and embedded in paraffin. Sections cut at 5 μm were hybridized with cDNA probes generated and internally labeled with digoxigenin or biotin by PCR (polymerase chain reaction). Biotin or digoxigenin in mRNA-DNA hybrids were detected with the fluorescent dye Cy 3 coupled to avidin or with an antibody against digoxigenin coupled to alkaline phosphatase combined with an appropriate indicator reaction, respectively. Negative controls included omission of cDNA probes, injection of an antisense oligodeoxynucleotide complementary to 54 bases of the *Xenopus* Na,K-ATPase α1 subunit mRNA, and RNase treatment of the sections prior to hybridization. Retention of cytoplasmic RNA within the sections during the hybridization steps was confirmed by acridine orange staining. We found that the mRNA for the α1 subunit of the *Xenopus* Na,K-ATPase is more abundant in the cytoplasm of the animal than of the vegetal hemisphere. In addition, the α1 subunit mRNA is concentrated in distinct cytoplasmic regions including the perinuclear zone.

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Molecular mechanisms of vasopressin receptor sensitization using the oocyte system

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Arginine-vasopressin (AVP) injected intracerebroventricularly and into distinct brain areas increases mean arterial blood pressure (MAP), heart rate (HR) and splanchnic nerve activity via central V_1-AVP receptors. The central AVP system is unique in that the cardiovascular and sympathetic responses can be enhanced by repeated central applications of AVP. We have previously shown that this phenomenon is linked to V_1-AVP receptor stimulated phosphoinositol turnover. In order to investigate its molecular mechanisms, AVP receptors were expressed in Xenopus laevis oocytes by microinjection of poly(A+)RNA from liver containing V1-AVP receptors and were analysed using the voltage clamp technique.

Oocytes (n=9) responded to 1 μM AVP at a holding potential of -60 mV with currents between 5 and 220 nA (mean 102 ± 25,2 nA) and a latency between 18 and 54 sec (mean 36,7 ± 4,2 sec). Non-injected oocytes or H_20-injected oocytes did not respond to AVP. Repeated applications of 1 μM AVP induced an enhancement (50 %) of the inward currents (mean 151 ± 28 nA) without influencing latency (mean 30,2 ± 4,3 sec). To test for the specificity of this effect, substance P receptors were expressed in the oocyte by microinjection of Poly(A+)RNA from rat brain. In contrast to AVP, repeated applications of SP (1 μM) induced the well known tachyphylaxis, i.e a loss of the membrane current initially recorded.

These results show for the first time a molecular mechanism underlying the short term sensitization of AVP V_1-receptors. This AVP receptor sensitization may be a critical regulatory mechanism for AVP not only in central blood pressure regulation and hypertension but also in memory processes.

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DIFFERENT CONTENT OF CALBINDIN IN BRAIN AREAS OF TWO SUBLINES OF WISTAR RATS

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The intraneuronal buffering capacity for free calcium-ions is partly determined by the content of calcium-binding proteins such as calbindin and parvalbumin. It has been shown that neurons that contain these proteins are more resistant to functional states where a massive influx or an increased release of calcium-ions occurs (hypoxia, seizures).
Using a competitive ELISA for calbindin D-28K, the content of this protein was determined in total cytoplasmic proteins obtained from hippocampus, frontal cortex , striatum and cerebellum of Wistar rats (Crl: (WI) BR [Charles River] and Shoe: Wist/I [Schönwalde]). We confirmed the about ten-fold higher content of this protein in cerebellum compared to the other brain regions studied. Charles-River rats exhibited a lower hippocampal content of calbindin compared to Schönwalde rats whereas the content of calbindin in the striatum and cerebellum of Charles-River rats was higher than in Schönwalde rats. The protein content in cytoplasmic proteins obtained from the brain areas of both sublines of rats was identical. Further investigations are required to detect possible histological differences in the distribution of calbindin in the brain of these rats.

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INJURY-INDUCED INCREASE IN EXPRESSION OF THE PREPROCHOLECYSTOKININ GENE IN RAT BRAIN CORTEX: IS IT AFFECTED BY NMDA-RECEPTOR ANTAGONISTS?

C. Olenik, A. Uhl and D.K. Meyer

The preprocholecystokinin gene is expressed in rat cortex in interneurons which contain γ-aminobutyric acid as a cotransmitter. Injuries to rat cortex as induced by a needle prick or knife cut cause a transient threefold increase in the concentration of the preprocholecystokinin-mRNA in the whole ipsilateral cortex. This phenomenon is observed 48 and 72 hours after the injury. The "immediate early genes" c-fos- and c-jun may be involved by producing the transcription factor AP-1 which can bind to the promoter area of the preprocholecystokinin gene (Olenik et al., Mol. Brain Res. 10: 259-265 (1991)).
All these changes can be prevented, when 4mg/kg MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) are injected i.m. 2 hours before the operation-induced injury. MK-801 blocks NMDA-receptors and in a dose-dependent manner also nicotine-receptors as well as the re-uptake of biogenic amines. Therefore it was tested, whether other blockers of nicotine receptors or uptake also diminished the injury-induced increase in preprocholecystokinin-mRNA.
All drugs were injected i.m. 30 min. prior to the cortex injury which was caused by a knife cut in pentobarbital-anaesthetized rats. Neither the competitive NMDA-receptor antagonist CPP (25 mg/kg; 3-((±)2-carboxypiperazin-4-yl)propyl-1-phosphonic acid) nor the nicotine receptor antagonist pempidine (8 mg/kg) affected the injury-induced increase in gene expression. However, blockers of noradrenaline re-uptake, such as (+)oxaprotiline (2.5mg/kg), amitriptyline (7.5mg/kg) or nomifensine (4mg/kg), prevented the injury-induced increase in preprocholecystokinin-mRNA. Agents which preferentially block re-uptake of serotonin, such as citalopram (2mg/kg) and fluvoxamine (2mg/kg), were without effect.
It is concluded that accumulation of noradrenaline in the synaptic cleft and the ensueing strong stimulation of adrenoceptors can prevent the injury-induced changes in neuropeptide gene expression in rat cortex.

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EXPRESSION OF RECEPTORS FOR NEUROTROPHINS IN NERVOUS AND IMMUNE SYSTEMS

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Nerve growth factor (NGF) is the best characterized protein in a family of related neurotrophic factors (neurotrophins) including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). The actions of these neurotrophins are mediated by the interactions with specific membrane receptors on target cells. Recent findings indicate that the high affinity NGF receptor is associated with the tyrosine kinase encoded by the trk proto-oncogene, whereas the trk B protein kinase is a component of the BDNF receptor.
We investigated the developmental expression of trk and trk B in rat peripheral sympathetic and sensory ganglia as well as in thymus and spleen by reverse transcription combined with polymerase chain reaction. Our results show that NGF- and BDNF-receptors are developmentally regulated in a tissue-specific manner. In addition, trk expression was found to be induced on immune cells such as cyclic AMP-stimulated human monocytes. Analysis of the expression of trk or related receptors in nerve and immune cells should help define the biological role of neurotrophins in neuronal development and regeneration and in immunomodulation.

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ROLE OF THE PITUITARY SPECIFIC TRANSCRIPTION FACTOR Pit-1 IN TRH AND cAMP STIMULATION OF HUMAN THYROTROPIN ß-SUBUNIT (hTSHß) GENE EXPRESSION

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We have recently shown that the expression of hTSHßCAT fusion genes in pituitary GH_3 cells is stimulated up to 4fold by TRH and up to 10fold by cAMP. DNA elements mediating this induction were located in the region between -128 and -61 bp of the hTSHß 5'flanking DNA. This area of the hTSHß gene contains 3 regions with a 75-90% homology to the consensus recognition sequence of the pituitary specific transcription factor Pit-1. Two footprints were observed in this area when DNaseI digestion was performed in the presence of a mouse thyrotropic tumor nuclear extract. Both footprints were suppressed by a 100fold molar excess of an oligonucleotide with the sequence of a high affinity Pit-1 binding site from the rat growth hormone gene. A mutant of this oligonucleotide with no binding affinity for Pit-1 did not suppress the footprints. DNA binding (ABCD) assays revealed that oligonucleotides identical to each of the 3 potential Pit-1 recognition sequences in the hTSHß 5'flanking DNA bound significantly more ^{35}S labeled *in vitro* synthesized Pit-1 than DNA fragments with no homology to the Pit-1 recognition sequence from either the hTSHß or the rGH genes. The involvement of Pit-1 in regulated expression of hTSHßCAT plasmids was further tested in cotransfection experiments. Cotransfection of a Pit-1 expression vector in a human kidney tumor cell line (293) induced a cAMP responsiveness of the expression of transfected hTSHßCAT plasmids in these cells that was comparable to that observed in GH_3 cells.

In conclusion, functional and structural data support the hypothesis that the pituitary transcription factor Pit-1 is involved in hormonal stimulation of hTSHß gene expression. Stimulation is mediated via sequences located between -128 and -61 bp of the hTSHß 5'flanking region.

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INHIBITION OF MICROSOMAL LIVER ENZYMES BY N-(2-CHLOROETHYL)-N-ETHYL-2-BROMOBENZYLAMINE (DSP-4) IN VITRO

F.J.Hock & F.E.Beyhl

The action of the centrally active, norepinephrine-depleting agent, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), on a series of hepatic mitochondrial and microsomal enzymes was studied in vitro.

DSP-4 inhibits microsomal drug-metabolizing mixed-function oxidases such as ketamine and aminopyrine N-demethylases, papaverine, 4-methoxybiphenyl, and 4-methylayapanine O-demethylases, and 7-ethoxycoumarin O-deethylase but not 4-methylaminoantipyrine N-demethylase and biphenyl 4- and coumarin 7-hydroxylases. It does not affect microsomal hydroperoxide formation and has no effect on microsomal NADPH-specific cytochrome c and neotetrazolium reductases.

DSP-4 does not act on glucose-6-phosphatase, another microsomal enzyme, and on succinate dehydrogenase, a mitochondrial marker enzyme.

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CELLULAR KINETICS AND EFFECTS OF PREDNIMUSTINE COMPARED TO ITS COMPONENTS CHLORAMBUCIL AND PREDNISOLONE

Musch E., M. Malek, H. Egge and J. Peter-Katalinic

The cellular uptake of prednimustine (PM), the 21-prednisolone (P) ester of chlorambucil (CLB) was investigated in comparison to that of a mixture of CLB and P in four different tumor cell lines, namely mammary carcinoma MCF-7, melanoma MEWO, acute lymphoblastic leukemia CEM and colon carcinoma HT-29 cells. The cellular pharmakokinetics analysis using High Performance Liquid Chromatography (HPLC) and Fast Atom Bombardment Mass Spectrometry (FAB-MS) was performed on cell extracts obtained after incubation of cells with the cytotoxice agent, washing and lysis. FAB MS data provided direct evidence for the intracellular uptake of intact PM as well as for the existence of a hihherto unknown metabolic oxidation product showing molecular and fragment ions (MH^+/MNa^+ at m/z= 646 / 648; 668 / 670 and m/z =642 / 644; 664 / 666 respectively). No ß-oxidation products were detected. Experimental tumor cell lines as well as lymphocytes separated from patients with (CLL) cells exposed to PM exibited higher and longer-lasting concentrations of PM and CLB as compared to cells incubated with CLB + P. The alkylating potency (= concentration-time integrals of all alkylating agents) did not differ significantly in both incubation series (ΣPM + CLB = 1087 nmol/ml·min versus ΣCLB+phenylacetic acid mustard = 878 nmol/ml·min). Lymphocytes from healthy volunteers and from lymphoma patients showed enhanced cellular uptake of PM compared to CLB. Thus, a facilitated cellular uptake of the hormone conjugated PM seems to be confirmed.

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CHANGES OF LIDOCAIN IN RAT SERUM AND TISSUE LEVELS UNDER STRESS AND HYPERLIPIDAEMIC DIETS

C.Tesseromatis,C.Tsopanakis,M.Loukissa,E.Sgourakis, and G.Symeonoglou

Hyperlipidaemic diet increases serum lipids.Furthermore stress interferes in lipid metabolism.Many drugs (alkaline) modidy the pharmacokinetic behaviour in presence of lipoproteins.In the present study Wistar rats were used and were treated with stress (cold water swimming),various lipid diets (olive oil,butter,sun flower oil)and lidocain-C^{14} (04 mg/kg wt).Free fatty acids (FFA),triglycerides (TG),total cholesterol (TC) and high density lipoproteins (HDL) levels were estimated in serum and tissues. The rats under stress and lipid diets showed increased serum levels in FFA and TG. The animals under stress showed a serum TC decrease compared to controls.HDL levels showed a relationship to the quality of diet lipid (increased with sunflower decreased with butter). The levels of lidocain were increased in all lipid diet groups,probably due to drug displacement from its binding with serum and tissue proteins.Stress enhanced furthermore lidocain levels possibly due to the further FFA levels increase which potentiate the displacement process.

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TRANSPLACENTAL PHARMACOKINETICS OF A SYNTHETIC RETINOID WHICH IS NOT BOUND TO EMBRYONIC CYTOSOLIC RETINOIC ACID BINDING PROTEIN

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Teratogenicity is an important side effect of retinoids which are widely used in dermatology and oncology. The binding of retinoids to cytosolic retinoic acid binding protein (CRABP) has been suggested to be important for the mechanism of retinoid embryopathy. Here data are presented on the transplacental pharmacokinetics of CD394 *(4-[3-(1-adamantyl)-4-methoxybenzamido] benzoic acid*) which is not bound to CRABP but has a teratogenic potential in whole rat embryo culture similar to that of retinoic acid (Hensby et al., Pharmacol Skin. Basel, Karger, 1989, vol 3, pp 153-156).
On day 11 of pregnancy NMRI mice received intragastrically 10 mg/kg CD394 in an aqueous suspension with 25 % (w/w) Cremophor EL. Maternal blood, placentas and embryos were collected after decapitation of the dams at various time points between 0 and 48 hours after administration. Radioligand experiments demonstrated the lack of binding of CD394 to embryonic CRABP. Analysis of plasma and of homogenized embryos and placentas was conducted with reversed phase-HPLC on C18-columns with an acetonitrile/water gradient system. Prior to HPLC solid phase sample enrichment on C18- sorbent material was applied.
C_{max} values reached after 1 to 2 hours were: 1370 ng/ml for plasma, 200 ng/g for embryo and 860 ng/g for placenta.
AUC values (0-48 hours) were: 4430 ng·h/ml for plasma, 870 ng·h/g for embryo and 3130 ng·h/g for placenta.
Our results indicate that CD394 reaches the embryo in vivo without binding to CRABP although embryonic concentrations stayed well below plasma levels. Thus binding to CRABP is not a prerequisite for the placental transfer and the teratogenicity of retinoids.

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INFLUENCE OF ALBUMIN ON THE ACTIVITY OF ANTIFUNGAL AGENTS WITH WIDELY VARYING PROTEIN BINDING
M. Schäfer-Korting, W. Rittler and H.C. Korting

Intense plasma protein binding is considered to reduce drug activity. The inhibition of C. albicans by itraconazole (f_b > 0.99) levels as obtained in humans, however, is not reduced by albumin (Schäfer-Korting M. et al., Antimicrob. Ag. Chemother., in press). To investigate the influence of albumin on drug activity in more detail, we have determined the minimum inhibitory concentrations (MIC's) for 8 antifungal agents in 40 clinical isolates of C. albicans in a protein free incubation medium and in the presence of 4% human albumin (HSA). Above that, MIC values for itra- (I) and ketoconazole (K, f_b: 0.99) as well as griseofulvin have been evaluated in 10 strains of T. rubrum. The increase in the ratio of the MIC's by HSA is compared to the expected inactivation due to drug protein binding.

HSA reduces the antifungal effects of fluconazole, miconazole, amphotericin B, and griseofulvin (f_b: 0.11, 0.92, 0.95 and 0.81 resp.) as expected. The inhibition of I and K activity, however, is considerably less pronounced. The MIC's are raised no more than 2fold with I and only 4- and 5fold with K (C. albicans and T. rubrum) whereas 500- and 100-fold increases are expected. Moreover, HSA does not inhibit the anti-Candida effect of terbinafine despite strong protein binding. Thus, the results suggest that not only itraconazole activity is not reduced by protein binding but also the activity of other antifungal agents.

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PERCUTANEOUS ABSORPTION OF ISOSORBIDE DINITRATE IN THE ISOLATED PERFUSED BOVINE UDDER.
M. Kietzmann, B. Wenzel[1], D. Arens, B. Blume[1]

Using a novel isolated perfused bovine udder model, the percutaneous absorption of isosorbide dinitrate (ISDN) was studied in vitro. Within 30 minutes after cattle slaughtering, udder perfusion with gassed tyrode solution was started. Thereafter, ISDN (60 mg/400 cm^2) was administered as spray (TD Spray Iso Mack^R) and as ointment (Isoket^R). In addition, a transdermal delivery system containing a microemulsion of ISDN (120 mg/30 cm^2) was used. Over a period of up to 8 hours, perfusate fractions were sampled. By capillary column gas-liquid chromatography with electron-capture detection after solid-phase extraction, the concentration of ISDN, isosorbide-2-mononitrate and isosorbide-5-mononitrate was measured in the perfusate fractions and also in skin. The amount of ISDN absorbed within the perfusion period was calculated from analyzed concentrations considering the perfusate flux. After treatment with the various formulations, ISDN was well absorbed via the skin. Demonstrating the viability of the udder skin, both metabolites could be detected in perfusate as well as in skin samples.
In conclusion, the isolated perfused bovine udder is a novel useful in vitro model which maintains a viable skin. It is possible to compare the dermal penetration and absorption rate after topical treatment with different drug formulations, including transdermal delivery systems.

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UPTAKE AND EFFECTS OF THIOPENTAL IN ISOLATED HEART PREPARATIONS OF RATS UNDER THE INFLUENCE OF VOLATILE ANESTHETICS
J.Ch. Isenberg, and J. Baldauf

Binding of thiopental in tissue homogenate of rat hearts was increased to a factor of 1.4 in the presence of halothane and to a factor of 1.2 in that of isoflurane (Büch et al, Drug Res **41**, 696, 1991). Question arose whether this binding increase was reflected in the thiopental uptake of an isolated, spontaneously beating heart preparation (Langendorff) when halothane or isoflurane were present. Krebs-Henseleit solution gassed with carbogen (pH 7.4) was used as perfusion fluid containing thiopental (0.13-0.19 $mmol \cdot l^{-1}$) without a volatile anesthetic and in the presence of 0.8, 1.5 and 2.0 vol% halothane or 1, 2 and 3 vol% isoflurane, respectively. Thiopental concentration in heart tissue was measured UV-photometrically (305 nm) after extraction with benzene and TLC. The thiopental concentration ratio (tissue-/Krebs-Henseleit-solution), if steady state was reached, was about 3.5 independent of the concentration used. In the presence of halothane (0.8 vol%) a small (+12%), yet significant, increase of the thiopental uptake in the heart tissue was observed for the whole concentration range used; higher halothane concentrations (1.5-2 Vol%) enlarged this difference maximally up to +43%. Isoflurane which was applied simultaneously with thiopental in the Langendorff preparation under the same conditions did not change the thiopental uptake in heart tissue. Using right and left atria of rat hearts the negative chronotropic and inotropic action of thiopental without and in the presence of halothane or isoflurane, respectively, was studied.

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STUDIES ON THE METABOLISM OF PLANT INGREDIENTS BY ISOLATED PERFUSED RAT LIVER
U. Vahlensieck, H.G. Gumbinger, C. Egen-Schwind, F.H. Kemper

A variety of phenolic acids - known to be ingredients of Lamiaceae and Boraginaceae, e.g. Melissa officinalis - shows a distinct antihormonal activity in vivo. The metabolism of some of these phenolic compounds, phenylpropanoids like caffeic acid, rosmarinic acid, etc. was investigated - using isolated rat liver perfusion and HPLC-analysis - to find out, if these phenolic acids pass the liver mainly unchanged or underlie a distinct first-pass-effect.

Following a standard operation procedure, the rat liver was perfused with a Krebs-Henseleit-buffer containing glucose and 0,5% bovine serum albumin. As vitality parameters of the liver, O_2-consumption and K^+-release as well as the enzymes GOT and GPT were determined.
After liver passage the single phenolic acids and their metabolites were examined in the perfusate, in the bile fluid and in the liver tissue by HPLC and DAD-detection.

Concerning caffeic acid, metabolites like ferulic acid, esculetin and different oxidation products of caffeic acid were found, which represent different metabolic pathways of the liver. In contrast to the unoxidized mother substance these oxidation products showed a distinct biological activity in diverse in vitro systems.

Thus studies on the metabolism of a substance using the isolated perfused rat liver should be a prerequisite especially for in-vitro but also for in vivo screeening procedures. Also the number of whole-animal experiments can be reduced.

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DEVELOPMENT OF A FORMULATION OF KAVA-KAVA EXTRACT THROUGH PHARMACOKINETIC EXPERIMENTS IN ANIMALS

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The active constituents of Kava-Kava rhizome (Piper methysticum, G. Forster) are the lactones dihydrokavain (DHK), Yangonin (Y), Kavain (K), Dihydromethysticin (DHM) and Methysticin (M). This presentation describes the results of some pharmacokinetic studies conducted to develop a new special Kava-formulation based on extracts of the rhizome. Plasma and brain lactone levels were determined after oral administration of 100 mg/kg of different extracts or formulations to male mice and rats, and of 6 and 10 mg/kg to dogs. The Kava lactones were quantified by HPLC (UV-detection). All the lactones except Kavain could be unequivocally analyzed.
Preliminary experiments in mice with 100 mg/kg extract per os suspended in 0.2 % agar revealed maximum plasma levels of the individual lactones (except Yangonin) ranging between 300 and 900 ng/ml already after 5 minutes. Elimination half-life was determined as approx. 30 minutes.
In further experiments with mice and rats treated orally (100 mg/kg) with a formulation of the extract bioavailability clearly increased, and maximum plasma levels of 1.7-2.5 µg/ml of DHK, K, DHM, M, and of 0.3 µg/ml of Y were attained 0.5 hours after administration. However, in rats two absorption peaks of the lactones at 0.25 and approx. 2 hours were observed. Surprisingly the lactone levels in brain showed peak concentrations (1.1-2 µg/g brain) at the same time point as in plasma, in mice, however, the brain levels decreased faster than in rats. Elimination half-lives of the lactones in mouse plasma and brain were approx. 1 hour, and even longer in the rat.
In contrast to the results obtained from rodents, dogs administered an extract formulation or a pure blend of the individual lactones showed quite obvious variations in tmax (0.7-4 hours). In this species DHK and Y could only be detected qualitatively. Maximum plasma levels of K, M, and DHM ranged from 100 to > 500 ng/ml, and elimination half-lives varied from approx. 90 minutes to several hours. The plasma lactone levels after administration of the blend of individual lactones were lower than those after the extract formulation.
In summary, bioavailability of Kava-extract could be increased up to 10-fold by improvement of galenical formulations after administration of a new special formulation to mice and rats.

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DIRECT DETERMINATION OF PROPOFOL IN BIOLOGICAL FLUIDS BY HPLC P. Altmayer, U. Büch, and R. Larsen

Methods for quantitative analysis of propofol (P) described are based on time consuming and difficult extraction procedures. A new HPLC method was developed allowing direct, rapid and sensitive analysis of very low P concentrations in biological fluids. The HPLC apparatus consisted of two pumps (A and B), an extraction-column and an analytical column (both filled with RP-18), a loop-type injector, a 6-way valve and a fluorescence detector. An aliquot of the biological fluid (serum, other protein containing samples, urine, etc.) was injected without any sample preparation directly onto the extraction column in a polar mobile phase (pump B, 0.01 M phosphate buffer pH 7.8). Under these conditions the polar constituents of the biological material such as proteins, amino acids, electrolytes etc. passed through and were eluted to waste, whereas the highly lipophilic P was retained on the extraction column. After 2 min the valve was switched automatically, whereby the analytical mobile phase (pump A, CH_3CN:0.01 M phosphate buffer pH 7.8) backflushed the loaded content (P and other apolar constituents of the biological sample) from the extraction column on the analytical column, where P was separated and detected by fluorescence (excitation 276 nm, emission 310 nm). The recovery rate for P in this precolumn extraction system was > 98%. With the method presented P can be determined directly in biological fluids without sample workup in about 6 minutes with a limit of detection of 2 ng/ml. The HPLC procedure provided linearity in the range from 5 ng/ml to 5 µg/ml with an analytical variability of less then 2 %.

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ESTER CLEAVAGE AND PHASE II METABOLISM OF MONOCYCLIC XENOBIOTICS IN SKIN OF RABBIT EARS

B.M. Henrikus and H.G. Kampffmeyer

The xenobiotics (procaine, 2-chloroprocaine, ethyl-aminobenzoate and methylsalicylate) were applied dermally or arterially at various concentrations to single pass perfused (protein-free) rabbit ears, or to purified liver esterase, or to supernatant of rabbit skin homogenate.
The respective Michaelis-Menten kinetics were calculated from steady-state product formation rates in the efflux by computer assisted non-linear adaptation. An only crude correlation was observed for the three enzyme containing systems.
In addition to free 4-aminobenzoic acid, acetamidobenzoic acid was found in quantities of about 10 % of the free 4-aminobenzoic acid during arterial or dermal application of procaine. Using ethyl-aminobenzoate as substrate, this product was not observed, but most likely , a conjugate of 4-aminobenzoic acid. In order to qualify this metabolite, further experiments with 4-amino-(14C ring)-benzoic acid will be carried out.
The capacity of 4-nitrophenol conjugation was 10 times lager than O-demethylation of 4-nitroanisole.

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Hypothermia elevates atracurium plasma levels and induces an increase of neuromuscular blockade in patients with cardiopulmonary bypass. E.Koundourakis*, M.Theisohn*, C.Diefenbach** and M.Abel **.

Flynn et al.(Br.J.Anaesth. 56:967,1984) reported that during hypothermia the infusion velocity of the short acting, curare-like muscle relaxant atracurium (A) had to be reduced to maintain a medium degree of muscle relaxation. In this investigation the extend of the neuromuscular blockade (NMB) and the concentrations (CONC) of (A) and its metabolite laudanosine (L) (HPLC method; modified according to Varin et al., J. Chromatography 529,319,1990) were determined in 20 patients (12 m,8 f, age:48-70 y,body weight 50-87 kg) undergoing cardiac surgery with cardiopulmonary bypass and hypothermia after giving informed consent. Anaesthesia was induced by an iv-bolus of midazolam (10-15 mg) and fentanyl (0.75 -1.5 mg). Neuromuscular monitoring was done at the m.adductor poll. stimulating the n.ulnaris over the wrist with supramaximal voltage (train-of-four) . Muscle relaxation was started with iv-bolus of (A) (0.46 mg/kg). After NMB had declined to 70 %, infusion of (A) (3.7+/-1.3 ug/kg*min) was started to establish a NMB of 52 % (+/-11). During hypothermia (28+/-2°C) NMB increased considerably (90+/-11%) parelled by an elevation of (A) from 0.46+/-0.16 mg/l to 0.61+/-0.20 (2p<0.01) whereas (L) decreased from 0.14+/-0.04 to 0.10+/-0.03 (2p<0.02). According to Olkkola et al.(Acta Anaesthesi-ol.Scand. 35:420-423,1991) the plasma CONCs of (A) were fitted to a 2 compartment model. NMB could be fitted to the equation $E(t)=E_{max} * y(t)^H / [IC_{50} + y(t)^H]$. As there was a time-lag between (A) in plasma and the NMB of about 10 min the effect compartment was appended to the 2nd compartment. (A) CONCs in this compartment and the NMB was significantly correlated exhibiting a S-shaped CONC effect curve (E_{max}=106%,IC_{50}=3.2 mg/l and H=3.5).
Hence, the increase of the neuromuscular blockade during hypothermia is mainly caused by the elevation of (A) induced by a decreased metabolic elimination during hypothermia.

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S-CARBOXYMETHYLCYSTEINE DISPOSITION IN HUMANS: METABOLITE RECOVERY IN URINE AFTER ORAL AND INTRAVENOUS APPLICATION B. Staffeldt

Different studies on the proposed genetic polymorphism of carbocysteine (CMC) *S*-oxidation at different places (Specht D et al (1990) Fresenius J Anal Chem 337: 13; Brockmöller J et al (1991) Eur J Clin Pharmacol 40: 387) failed to confirm the data described by R. Waring (1980, Eur J Drug Metab Pharmacokinet 5: 49). However, most of our experiments evaluated the urinary metabolite recovery only over a period of the first eight hours after application, in which the total recovery was between 20-50%.

Now, elimination of CMC and its metabolites in urine was investigated in 12 healthy volunteers (5 female, 7 male, age between 22 and 34 yrs) over a period of 60 hours. Furthermore, kinetics of CMC was studied in a single volunteer after intravenous infusion. CMC and its metabolites were analysed by HPLC using precolumn derivatization with 9-fluorenylmethylchloroformate (FMOC) and 1-pyrenyldiazomethane (Staffeldt B et al (1991) J Chrom 571: 133).

Following a single oral dose of 1.125 g, CMC was eliminated in urine between 1 and 12 h, with a maximum at 4 h (total: 13 - 28 % of the dose). The primary metabolites (R)- and (S)-CMC sulfoxide were detectable in urine fractions between 4 and 8 h (total: 0.1 - 1.0 % of dose, sum of both enantiomers). The secondary metabolite 2,2'-thiodiacetic acid was eliminated between 4 and 36 h (total: 4 - 25 % of dose). The sulfoxide of thiodiacetic acid was detected between 5 and 48 h (total: 1 - 12 % of dose), with a maximum 12 h after application.

Bioavailability of a neutral carbocysteine solution was 70 % and drug/metabolite ratios did not significantly differ in dependence on oral or intravenous dosage. The recently discovered mixed disulfide metabolite (Meese CO et al (1991) Biochem Pharmacol 42: R13) S-(carboxymethylthio)-L-cysteine, could also be quantified as a fluorescent FMOC derivative with a maximum amount excreted in urine samples collected between 8 and 24 hours after administration. With this qualitatively and quantitatively revised metabolic pattern, previous studies on the sulfoxidation polymorphism and even the disputed therapeutic efficacy of CMC now appear in a completely new light.

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INFLUENCE OF CHEMICAL MODIFICATION OF CYTOCHROME b5 WITH TETRANITROMETHANE ON THE DIVERSE ELECTRON TRANSFER PATHWAYS AND 7-ETHOXYCOUMARIN DEETHYLATION. **I. Golly and P. Hlavica.**

Highly purified cytochrome b5 (b5) from hepatic microsomes of untreated male Chinchilla rabbits was chemically modified with a 50-fold molar excess of tetranitromethane (TNM) over b5 at pH 7.5 such as to produce 1.4 nmol 3-nitrotyrosine (3-NT)/nmol b5. Tyrosine modification was also verified by means of second-derivative absorption spectroscopy. Selectivity of tyrosine derivatization was proven by the presence of unmodified tryptophan residues as well as by the unimpaired spectral properties of b5 after nitration.

TNM-modification of b5 resulted in a decreased extent of complex formation (70%) with cytochrome P-450LM2 (P450IIB4) associated with an 11-fold increase in affinity for the electron mediator. Anaerobic stopped-flow measurements of the NADH-dependent transfer of the 1. electron to P-450LM2 in a reconstituted system consisting of P-450LM2, NADH-cytochrome b5 reductase (Fp1), benzphetamine and native or nitrated b5, revealed a drastic inhibition (98.5%) of the rate of P-450LM2 reduction. TNM-induced changes in the redox potential of b5 could be ruled out to account for this phenomenon, because the reduction of b5 was neither perturbed when the electron flow was allowed to proceed via the NADH-Fp1 segment nor via the NADPH-cytochrome P-450 reductase (Fp2) chain. Kinetic experiments in which cytochrome c substituted for P-450LM2 served to elucidate the specificity of the b5/P-450LM2 interaction. Ethoxycoumarin deethylation, a b5-dependent reaction, as investigated in a reconstituted system containing NADPH, Fp2, P-450LM2 and either native or modified b5, was used to study the functional role of the modified site(s) in the b5 molecule in substrate turnover. Experiments are underway to precisely localize the derivatized tyrosine(s).

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CHANGES OF CARBONYL REDUCTASE CONTENT AND ACTIVITY IN CULTURED HEPATOCYTES OF RATS.

K. Hupe-Sodmann and E. Maser

Carbonyl reduction of xenobiotic aldehydes and ketones is a significant step in the metabolism and elimination of these compounds. The enzymes involved constitute the family of carbonyl reductases which share similar features such as monomeric structure (30-40 kDa), NADPH dependence, cytosolic subcellular localization and broad substrate specificity.

In previous investigations we purified and characterized a membrane-bound carbonyl reductase (34 kDa) from mouse liver microsomes using metyrapone (MPON) as substrate and a sensitive HPLC method for metabolite determination. In addition, the occurrence of carbonyl reduction was demonstrated in continuous cell lines thus emphasizing the significance of this metabolic pathway.

In this study the content and activity of carbonyl reductase was investigated in microsomes of rat liver (solid organ) and in hepatocytes of the same species cultured for 9 days. In addition, the occurrence of immunoreactive enzymes within the respective fractions was tested by using antibodies against the microsomal mouse liver MPON reductase.

We found high MPON reducing specific activity in microsomes of freshly isolated rat hepatocytes (22.7 nmol/mg/30min) to be very similar to that in microsomes derived from the solid organ (16 nmol/mg/30min). The enzyme activity increases about 2.3-fold (52.9 nmol/mg/30min) 24 h after preparation and decreases from day 1 to day 6 to about 2.3 nmol/mg/30min then remaining constant until day 9.

Immunoreactivity in Western blot analysis always parallels enzyme activity, even in day 1-hepatocytes, where the degree of immunological crossreaction also proved to be stronger than in day 0-hepatocytes.

In microsomes of day 6-hepatocytes and thereafter no immunological crossreaction occurred, although enzyme activity could be detected by HPLC analysis.

Because the time related changes in activity are reproducible, primary cultured hepatocytes may thus be used as an alternative to whole animal experiments to study various aspects of xenobiotic carbonyl compound metabolism.

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IN VITRO INVESTIGATIONS OF METABOLIC CHIRAL INVERSION OF 2-ARYLPROPIONATES IN RAT H4IIE AND HUMAN HEP G2 HEPATOMA CELLS

S. Menzel-Soglowek, G. Geisslinger and K. Brune

An unusual feature of the metabolism of 2-arylpropionic acids is the unidirectional inversion of configuration at the chiral centre which can be observed in man and various animal species.

The inversion of 2-arylpropionic acids has been investigated in vitro using rat H4IIE and human Hep G2 hepatoma cells in continuous culture. The effect of substrate concentration (15-150 μg/ml), cell density (1.5-12x10^6 cells/dish) and serum content of the culture medium (0-20%) on inversion was examined in rat H4IIE hepatoma cells using R-ibuprofen as model compound. Increasing R-ibuprofen concentrations and decreasing serum content of the medium resulted in increased inversion whereas variation of cell density had no effect. Furthermore rat H4IIE and human Hep G2 hepatoma cells were incubated with the individual enantiomers of ibuprofen, ketoprofen and flurbiprofen under optimized culture conditions (serum-free culture medium). The elimination rate constants (k_{el}) and fractions inverted (Fi) were determined. Although inversion occurred slowly in the tumor cells and thus long incubation periods (120h) were required, the hepatoma cells were nevertheless able to mimick qualitatively the species and substance specifity of inversion of 2-arylpropionic acids as observed in vivo.

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INVESTIGATIONS INTO THE STRUCTURE-RELATIONSHIP OF ACETYLENIC STEROIDS TO INHIBIT HUMAN LIVER CYTOCHROME P-450 IN VITRO

R. Böcker, U.Richter, M.Eichhorn, and B.Kleingeist

It has recently been demonstrated that acetylenic steroids inhibit *in vitro* the human liver cytochrome P-450 3A enzymes [Guengerich FP (1990) Chem.Res.Toxicol.3:363; Böcker R and Lepper H (1991) Naunyn-Schmiedeberg's Arch.Pharmacol.343: R13]. To investigate a structure deteminant which addtionally to the acatylenic moiety at position 17 of the steroid basic structure may trigger the mechanism based inhibition of cytochrome P-450 3A enzymes 7 progestogenic compounds derived from norethisterone (including levonorgestrel,gestodene,3-ketodesogestrel, and some others) were compared in an *in vitro* test system on human liver microsomes. The synthetic steroids were kindly provided by Organon Int., Oss, The Netherlands, and Schering AG, Berlin, Germany. The experimental setup was as described in the above mentioned literature. Human liver microsomes were preincubated in presence of various concentrations of the steroids and an NADPH-generating system. After a tenfold dilution of the preincubation mixture the test assays for the determination of 3 P-450 activities (3A, 2E1, 2D6) were started. Gestodene was the most potent inhibitor of the 8 compounds with a mean IC_{50} of 4.8 µM. Levonorgestrel, 3-ketodesogestrel, and the other compounds were about 6 -10 fold weaker in their inhibitory potency. Only theP-450 3A activity was affected.

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DETERMINATION OF EROD AND MROD ACTIVITY IN HEPATIC MICROSOMES OF TCDD-TREATED RATS AND MARMOSETS

T. Schulz-Schalge, E. Koch

The inducibility of ethoxyresorufin O-deethylase (**EROD**) in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**TCDD**)-treated rats and marmosets (*Callithrix jacchus*) has been used as an indicator for *Ah*-receptor mediated reactions, especially for the induction of P4501A1. Recently we confirmed findings of Namkung et al. (Mol. Pharmacol. **34**: 628-637, 1988) that methoxyresorufin O-demethylase (**MROD**) is a very specific assay for P4501A2-mediated reactions. Investigations by Sesardic et al. (Carcinogenesis **11**: 1183-1188, 1990) have demonstrated that in the human liver only P4501A2 was inducible. In order to investigate these enzyme activities, we have performed studies in rats and marmoset monkeys.
The animals received a single subcutaneous injection of different doses of TCDD, the control animals received 100 µl/kg body wt of the vehicle (dimethylsulfoxide/toluene 2+1) only. The enzymatic activities were investigated in hepatic microsomes and the following results were obtained:

	Rat		Marmoset	
Dose[1)]	MROD[2)]	EROD[2)]	MROD[2)]	EROD[2)]
control	34 ± 2	24 ± 8	98 ± 44	97 ± 18
3	44 ± 9	24 ± 2	n.d.	n.d.
11	47 ± 5	28 ± 7	n.d.	n.d.
32	87 ± 27	149 ± 63	n.d.	n.d.
107	251 ± 124	1270 ± 404	n.d.	n.d.
167	n.d.	n.d.	433 ± 239	499 ± 232
300	n.d.	n.d.	791 ± 170	1065 ± 128
644	616 ± 156	5210 ± 985	n.d.	n.d.
1000	n.d.	n.d.	579 ± 131	757 ± 206

1) doses in ng TCDD x kg body wt^{-1}; n.d. = not determined
2) values in pmoles resorufin x mg $prot^{-1}$ x min^{-1} (mean of -at least- three animals)

In **rat** liver there is a higher inducibility of MROD as compared to EROD, using low doses. MROD activity in the highest dose group was only 18-fold in comparison to a 220-fold increase of EROD activity. The inducibility of both enzyme activities is in the same range in **marmoset** liver microsomes. Since it is feasible that EROD is catalysed by both P4501A1 and 1A2, it has to be elucidated, whether P4501A2 is the only TCDD-inducible P450 in the liver of marmosets.

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INDUCTION OF CYTOCHROMES P450IIB1 AND P450IA BY METYRAPONE (MP) IN PRIMARY RAT HEPATOCYTE CULTURE

J.Aubrecht, M.W.Höhne, and G.F.Kahl

Rat hepatocytes in primary culture are an useful model for the investigation of the metabolism of xenobiotics. However, long term culturing of hepatocytes generally leads to a rapid loss of differentiated functions and P450 enzyme activities. To prevent this dedifferentiation serum-free culture conditions are necessary and the addition of MP was recommended (Pain AJ(1990) Chem.Biol.Interact.74:1). We investigated the induction of P450IA1/IA2 and P450IIB1/IIB2 in serum-free culture of rat hepatocytes under the influence of the inductor phenobarbital (PB) and of MP in MX-83 medium. After 4 days in culture PROD and EROD enzyme activities were determined and the transcription of the P450's measured by Northern blot analysis with gene specific c-DNA probes or oligonucleotides. PROD activities were more than 4fold higher under PB or MP induction in comparison to freshly isolated hepatocytes whereas without inductor no activities were measurable after 3 days in culture. EROD activity was 3fold (PB) or 9fold (MP) higher as in controls. PB induced the transcription of only P450IIB1, whereas MP induced P450IIB1 and P450IA1. From these results it is evident that MP can induce the transcription of specific genes of P450IA and P450IIB gene families and that stabilization of P450 activities may be explained by transcriptional induction.

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DISSOCIATION OF A NEW GLUTATHIONE S-TRANSFERASE ACTIVITY IN HUMAN ERYTHROCYTES

K. R. Schröder, E. Hallier, H. M. Bolt

Previous investigations on the metabolism of monohalogenated methanes in erythrocytes have shown that the conversion was restricted to man. Two populations were found: conjugators (70%) are able to conjugate monohalogenated methanes to glutathione in erythrocytes and non-conjugators (30%) lack this ability. These findings suggest that this phenomenon is due to an enzyme polymorphism. Furthermore it was demonstrated that this polymorphism also applied to ethylene oxide and methylene chloride as substrates.

Since glutathione S-transferases are known to conjugate electrophiles to glutathione and methyl iodide was formerly used to classify the classes of transferases, we purified glutathione transferases from erythrocytes from individual donors („conjugators") using affinity chromatography. The eluted transferase fraction contained both known transferases from human erythrocytes, σ and ρ. The eluate did not show any activity directed to methyl bromide. The effluent of the affinity column contained almost the complete activity. As the glutathione S-transferase activity against 1-chloro-2,4-dinitrobenzene could be separated from the activity against methyl bromide in purification steps not involving affinity chromatography, this indicates that the hitherto identified transferases from human erythrocytes cannot be responsible for the observed enzyme polymorphism.

Altogether, the results show the existence of a glutathione S-transferase activity in erythrocytes involving different C_1- and C_2-substrates. This activity has not yet been characterized probably due to unusual substrate spectrum.

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STRUCTURAL REQUIREMENTS OF SUBSTRATES SELECTIVE FOR THE MURINE P450Coh (Cyp2a-5)

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Earlier contributions report systematic variations of substrates being selective for the murine hepatic coumarin 7-hydroxylase (Coh). Chemical variations include the following three points of the substrate molecule: 1. The length of the side chain (C_1-C_6; Mangoura et al, Naunyn-Schmiedeberg's Arch Pharmacol 341 Suppl:R7, 1990). 2. The aromatic ring system (coumarin, quinoline, naphthalene; Feil et al, NSAP 341 Suppl:R6, 1990). 3. The linkage of the side chain to the ring system (ether, amine; Tegtmeier et al, NSAP 343 Suppl:R14, 1991).

In this study according to Erlenmeyer's rule the ether oxygen was replaced by -NH- leading to secondary amines which were checked for their suitability as substrates of the Coh. Two 7-(mono)-alkylamino-4-methyl-coumarins (methyl or ethyl-substituted) were incubated at final conc. of 10^{-4}M with microsomes of differently pretreated male mice (37°C, up to 10 min). The pretreatments consisted of two i.p. administrations of either phenobarbital (PB), 3-methylcholanthrene (MC) or pyrazole (PL). As the fluorimetric quantification reveals, only after MC and PL the N-dealkylation to the free amine is enhanced 2-fold or 1.5-fold, respectively. Furthermore these reactions were studied after HPLC separation analyzing the absorbances at 360 nm.

In conclusion, three structural requirements are essential for substrates of the Coh: ① An oxygen linkage to the side chain (ether) or at least a secondary amine. Ternary aminocoumarins behave as ethylmorphine predominantly indicating a PB-type of induction. ② An ethyl side chain. ③ An electron rich region in the bimembered ring: as a lactone of coumarin (substrate) or of coumaranone (inhibitor) or as a nitrogen of the quinoline; naphthalenes do not serve at all as substrates of the Coh.

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DIFFERENTIAL INDUCIBILITY OF THE HEPATIC AND RENAL COUMARIN 7-HYDROXYLASE ACTIVITY BY TIN IN MICE

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Recently tin was reported to provoke a drastic and selective induction of the coumarin 7-hydroxylase (Coh, Cyp2a-5) after subcutaneous administration of 50 mg of tin(II)chloride/kg b. wt. daily for two days (Emde et al, EUROTOX 1991, Maastricht, p 86). This parallels the inductive action of other heavy metals such as cobalt, indium and cerium. The selectivity of the Coh-induction is furthermore observed for several N-containing heteroaromatic compounds such as aminotriazole, pyrazole and pyrazine, which in general decrease the overall content of microsomal cytochrome P450.

This study compares the inducibility of the Coh in kidney with that in liver. Tin was administered as a $SnCl_2$-citrate-complex as mentioned above. Controls were injected with either isotonic saline or isotonic citrate buffer, pH 7.4. Umbelliferone was quantified fluorimetrically using final substrate concentrations of 0.1 mM for coumarin and 7-ethoxycoumarin. Due to very low concentrations renal cytochrome P450 could not be quantified spectrophotometrically. However, the hydroxylation and deethylation was totally blocked by carbon monoxide.

Tin increased the hepatic Coh up to 20-fold, that of the kidney up to 48-fold compared to controls. This suggests that after tin renal cytochromes P450 contain a higher fraction of the $P450_{Coh}$ than the hepatic. As metyrapone (MP) is known as a preferential inhibitor of the Coh higher specific Coh activities should be correlated with stronger inhibition. Indeed, this is true for kidneys: controls are inhibited to 52% only, tin pretreated microsomes to 2% ($3x10^{-4}$M MP). Data of liver samples indicate a greater fraction (25%) which is not affected by this concentration of MP.

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STRUCTURE DETERMINANTS OF STEROIDS INVOLVED IN THE MECHANISM-BASED INHIBITION OF CYTOCHROME P-450 OF PRIMARY RAT HEPATOCYTES

H. Lepper and G. Schmitz

Earlier investigations with freshly isolated rat hepatocytes have shown that 17α-ethinylated progestogenic and estrogenic steroids like norethisterone, ethisterone and ethinylestradiol have marked inhibitory effects on the nifedipin-oxidase activity of these cells while the structure analogous steroids without the 17α-ethinyl function have only very low or even no inhibitory effects on this cytochrome P-450 isoenzyme (Lepper H et al, Naunyn-Schmiedeberg's Arch Pharmacol 1991; 344 : R62, Nr. 115). To clearify whether the 17α- ethinyl moiety of the steroids is the only structurally relevant determinant involved in the mechanism-based inhibition of the nifedipine-oxidase activity we tested synthetic steroids with different side chains in 17α-position: 17α-*methyl*-testosteron, 17α-*allyl*-estrenol, 17α-*propinyl*-estradiol and Dienogest, a progestogen with a 17α-*cyanomethyl* group. Furthermore, we tested the interaction of the MAO B-inhibitor R-(-)-Deprenyl, which has no steroid structure but also possesses an ethinyl-moiety.

All studies were carried out with freshly prepared hepatocytes from male Wistar rats. As substrate for the nifedipine-oxidase we used a nifedipine analogous compound not being substituted at the aryl moiety. This compound has no Ca^{++} antagonistic effects and does not exhibit cytotoxic effects at the concentrations used in the experiments. All substances were tested under preincubation and coincubation conditions over 30 min. in concentrations ranging from 0μM-50μM.

The 17α-propinyl and the 17α-allyl compounds exhibited a marked inhibitory effect on the nifedipine-oxidase activity of the cells similar to the inhibitory effect of the 17α-ethinyl compounds ethinylestradiol and norethisterone. Dienogest,17α-methyltestosterone and R-(-)-Deprenyl as the non-ethinylated steroids testosterone and 19-nortestosterone had only very low influence on the nifedipine oxidation.

It can be concluded that beside the 17α -ethinyl function also other π-bond systems at the 17α-position of the steroids can cause a mechanism-based inhibition of the nifedipine-oxidase activity. The mechanism of the interaction may be similar to that described for the 17α-ethinylated steroids.

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GLUCURONIDATION OF ALIPHATIC ALCOHOLS BY SPRAGUE DAWLEY RATS

S. Iwersen, A. Schmoldt

As previously reported aliphatic alcohols (C3-C7) can be glucuronidated by liver microsomes of Wistar rats (Iwersen et al. this journal 344:241,1991). The UDP-glucuronyltransferases (GT) mainly involved in this reaction are the steroid GT's. In order to study wether the glucuronidation rates of aliphatic alcohols can be enhanced by pretreatment with ethanol we chose Sprague Dawley instead of Wistar rats because it is known that Wistar rats do not respond to ethanol. However, Sprague Dawley rats show higher glucuronidation rates e.g. Acetaminophen and Phenolphtalein after ethanol pretreatment. Therefore we tested the alcohol-glucuronidation in rats which were pretreated with ethanol given as 10% of the drinking water for 14 days and controls.

Results:1. Also in SD rats aliphatic alcohols were glucuronidated by 17β-OH-steroid and 3α-OH-steroid- GT. 2.Compaired to Wistar rats there were considerable differences respect of the affinities and V_{max} values for different alcohols. 3.Ethanol pretreatment caused an increase of alcohol conjugation rates by a factor of 1,4-1,6 which corresponded to that of the steroid conjugation rates.

It may be concluded that the alcohols conjugating steroid GT's of Sprague Dawley rats can be induced by pretreatment of ethanol.

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HIGH AFFINITY PARACETAMOL UDP-GLUCURONOSYLTRANSFERASE (UGT) ACTIVITY IS CATALYZED BY HUMAN AND RAT PHENOL UGT (UGT1A1)

A. Forster and S. Fournel-Gigleux[a]

Paracetamol glucuronidation has been found to be increased in heavy smokers (Bock et al., Eur. J. Clin. Pharmacol. 31, 677-683, 1987). However, little is known about the UGT isozymes responsible for paracetamol glucuronidation and their regulation. Therefore we investigated paracetamol UGT in human and rat liver and kidney and in V79 cells (derived from hamster fibroblasts) expressing human or rat UGT1A1.
Kinetic analysis of human liver paracetamol UGT activity revealed biphasic kinetics suggesting high and low affinity enzymes with apparent k_M values of 3 and 20 mM, respectively. With human UGT1A1 expressed in V79 cells an apparent k_M of 2.5 mM was determined suggesting that UGT1A1 represents the high affinity UGT activity.
With rat liver microsomes no biphasic kinetics were found. Apparent k_M values of 12.5, 7.5 and 3 mM were determined with liver microsomes from untreated rats, 3-methylcholanthrene(MC)-treated rats and with microsomes from V79 cells expressing rat UGT1A1, respectively. Together with a 2-fold increase of v_{max} with liver microsomes of MC-treated rats the kinetic analysis suggests that high affinity paracetamol UGT activity is catalyzed by rat UGT1A1.
UGT1A1 is widely distributed in tissues. In support of extrahepatic expression paracetamol UGT activity could be clearly detected in human and rat kidney. It was inducible in rat kidney and liver by the MC-type inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin.
The results suggest that UGT1A1 is responsible for high affinity paracetamol UGT activity in rats and man.

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METHYLCHOLANTHRENE-INDUCIBLE GLUCURONIDATION OF CHRYSENE AND BENZO(a)PYRENE MONO- AND DIPHENOLS

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It has been postulated that some phenolic metabolites of polycyclic aromatic hydrocarbons such as 3-OH-benzo(a)pyrene (BP) and 3,6-dihydroxy-BP are more selective substrates for a 3-methylcholanthrene (MC)-inducible UDP-glucuronosyltransferase (UGT1A1) than standard substrates such as 1-naphthol. The postulate was based on induction factors of UGT activity determined in liver microsomes after treatment of male Wistar rats with MC (40 mg/kg). Induction factors were 4-fold with 1-naphthol, 10-fold with 3-OH-BP, and 10- and 40-fold with BP-3,6-quinol for mono- and diglucuronide formation, respectively. These studies have been extended to 6-OH-chrysene and 3,6-hydroxychrysene which are easier to synthesize, more stable and safer to use. Conjugates were separated by HPLC, identified and quantified after biosynthesis with UDP-(^{14}C)glucuronic acid, and by fluorescence spectroscopy. It was found that glucuronidation of 6-OH-chrysene was stimulated 10-fold by MC-treatment. With 3,6-dihydroxychrysene 6-OH-monoglucuronide and diglucuronide formation were increased 24- and 310-fold, respectively. The results show that induction factors obtained for monoglucuronide formation corresponded to those found with the BP phenols and with increases of mRNA for UGT1A1 (9-fold). Additional factors may be responsible for the much higher induction of diglucuronide formation. Diglucuronide formation of 3,6-dihydroxy-BP was clearly detectable with V79-expressed UGT1A1 and with the conventionally purified enzyme. Hence, no additional enzyme may be required for diglucuronide formation. It is therefore conceivable that the strong induction of diglucuronide formation is due to both the induction of UGT1A1 subunits and their facilitated interaction.

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EXPRESSION OF PHENOL UDP-GLUCURONOSYLTRANSFERASE (UGT1A1) IN HEPATIC AND EXTRAHEPATIC RAT TISSUES

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Previous studies have shown that UDP-glucuronosyltransferase (UGT) represents a family of isozymes with differential inducibility by xenobiotics and overlapping substrate specificity. One of these isozymes, phenol UGT (UGT1A1), shares exons 2 - 5 with other UGTs of the phenol/bilirubin UGT gene complex. UGT1A1 appears to be expressed in a wide variety of rat tissues. Therefore tissue-specific regulation of this isozyme by endogenous factors (e.g., sex-linked factors) and by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was investigated. Male and female Wistar rats (200 g) were treated with TCDD (10 μg/kg, dissolved in corn oil, s.c.) and organs were isolated after 7 days. Levels of UGT1A1 mRNA were measured using a selective DNA probe together with UGT activities toward 1-naphthol and 6-hydroxychrysene. Constitutive UGT1A1 expression in kidney appeared to be higher than in liver, in line with UGT activity toward 6-hydroxychrysene but in contrast to the UGT activity toward 1-naphthol. Moreover, constitutive expression appeared to be higher in kidney and lung from female rats in comparison with males. TCDD induction of hepatic and renal 7-ethoxyresorufin O-deethylase activity (catalyzed by P4501A1), of hepatic UGT1A1 mRNA and of hepatic UGT activity toward 6-hydroxychrysene were clearly detectable. In contrast, renal UGT1A1 mRNA was not increased following TCDD treatment. However, renal UGT activity toward 6-hydroxychrysene was slightly induced in males and markedly increased in female rats, suggesting posttranscriptional mechanisms. The results emphasize the need to study tissue-specific regulation using selective functional and molecular probes of different UGT isozymes.

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N-ACETYLTRANSFERASE ACTIVITIES IN HAMSTER CELL LINES RESISTANT OR SENSITIVE TO 1,6-DINITROPYRENE

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Permanent cell lines differ widely in their sensitivity to the cytotoxic and genotoxic effects of nitroaromatic compounds such as 1,6-dinitropyrene (1,6-DNP). Since acetylation may be a limiting step in the process of 1,6-DNP activation, we have investigated acetyltransferase activities in various 1,6-DNP-sensitive and 1,6-DNP-resistant hamster cell lines using isoniazid (ISO) and p-aminobenzoic acid (PABA) as marker substrates. N-Acetyltransferase (NAT) activitities toward these substrates were determined by high pressure liquid chromatography. In addition, we studied the appearance of N-acetylated 1,6-DNP products formed by intact cells.

The results showed that the expression of NATs considerably varied not only between the hamster cell lines examined but also some of their sub-strains. Thus a high diversity was observed for strains of V79 cells which had been obtained from several laboratories throughout Europe and the US. The majority of these strains contained neither ISO-NAT nor PABA-NAT. Two strains expressed significant activities of PABA-NAT but not ISO-NAT, and only one contained both NAT activities. Toxicity of 1,6-DNP and appearance of N-acetylated 1,6-DNP products correlated with the expression of ISO-NAT. Similar observations were made on V79 variants which had been selected for their resistance against 1,6-DNP as well as on single strains of hamster CHO and BHK cells.

The results suggest that the acetyltransferase form involved in 1,6-DNP activation is similar, if not identical, to ISO-NAT in the hamster cells examined. The results, furthermore, point out the need for thorougly ascertaining the metabolic capabilities of cell lines to be used for toxicity testing.

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XENOBIOTIC METABOLIZING ENZYME ACTIVITIES IN IMMORTALIZED RAT LIVER PARENCHYMAL CELLS

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Primary rat liver parenchymal cells (PC) dedifferentiate rather rapidly and do not show permanent cell proliferation *in vitro*. Permanent lines of different cell types, on the other hand, have a low xenobiotic metabolizing capacity. To overcome this discrepancy, PC have been immortalized by fusion with a proliferating hepatoma cell line.

To characterize the resulting hybrid cell lines, several phase I and -II metabolizing enzyme activities were determined and quantitatively compared to the activities in the two parental cell types. Cytochrome P-450-dependent enzyme activities and phenol sulfotransferase activity of the hybrids were low or even below detection limit. Calculated on a "per cell basis", the hybrids however expressed about 10 % of microsomal and cytosolic epoxide hydrolase, as well as of glutathione transferase activities measured in freshly isolated PC. The α-naphthol UDP-glucuronosyl transferase activity of the hybrid cells was as high as in PC. Total metabolic conversion of benzo[a]pyrene in the hybrids was about 50% of that found in PC. From the presented data it is however not clear if this was P-450-mediated.

Thus, the differentiated status of PC was not conserved as a whole in the hybrids. Particular xenobiotic metabolizing enzyme activities where however stabilized. For that reason these cells might be a useful system for *in vitro* induction or inhibition studies of xenobiotics.

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EFFECT OF METHANOL OR GLUTATHIONE-DEPLETING AGENTS ON THE METABOLISM OF DICHLOROMETHANE TO CARBON MONOXIDE IN RATS

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Cytochrome P-450 IIE1 (CYP 2E1) is the major, if not the principal, catalyst involved in the oxidative metabolism of dichloromethane (DCM) to carbon monoxide (CO). The second metabolic pathway of DCM is dependent upon glutathione (GSH) and GSH-S-transferase and yields carbon dioxide. The purpose of this study was to evaluate the effect of methanol (MET) on the carboxyhemoglobin (COHb) formation resulting from DCM gavage. Male Wistar rats received a single dose of water (10 ml/kg p.o.) or MET, 98.8 mmol/kg p.o. as a 40 % (v/v) solution in water. At various intervals after MET or water gavage, DCM (6.2 mmol/kg p.o. as a 10 % (v/v) solution in Oleum pedum tauri) was administered. The COHb concentration in the blood was measured 6 hours after the administration. A stimulation of the COHb formation was observed, if DCM was administered 12 h - 48 h after MET. The MET administration produced a significant decrease in hepatic GSH content. Therefore, the effect of depletion of GSH by diethylmaleate, phorone or buthionine sulfoximine was studied on the metabolism of DCM to CO. The COHb formation was not enhanced following these pretreatments.
On the basis of these results it can be concluded that hepatic GSH depletion per se does not promote the DCM metabolism to CO. It seems that MET pretreatment produced an induction of CYP 2E1. The COHb formation was not significantly affected following a single simultaneous administration of both MET and DCM, and showed a slight but significant decrease when higher doses of MET (148 and 198 mmol/kg p.o.) were used. This demonstrates competition between DCM and MET on CYP 2E1.

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THE BIOTRANSFORMATION OF METHALLYLCHLORIDE BY RAT LIVER MICROSOMES

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The metabolism of methallylchloride (MAC) in microsomal incubations was investigated using headspace gas chromatography (HS-GC). Microsomes were isolated from male F-344 rats, pretreated with phenobarbital (PB), acetone, methylcholanthrene (MC), diethylhexylphthalate (DEHP) or saline. The metabolism of MAC was found to be NADPH dependent and was inhibited by CO, indicating the involvement of cytochrome P-450 dependent monooxygenases in MAC metabolism. One metabolite was detected in the headspace and was identified as methacroleine (MET) by gas chromatography/mass spectrometry. No other metabolite was detected using HS-GC. The rate of MAC metabolism and the rate of MET formation were measured and the specific activity (nmol/min*mg protein) and the turnover number (nmol/min*nmol cytochrome P-450) were calculated for the individual microsomal preparations. The specific activity in microsomes from acetone and PB pretreated rats was 8.0 and 8.3, respectively, compared to 2.1 and 1.8 nmol MAC metabolized/(min*mg protein) in microsomes from saline treated rats. The turnover numbers for these microsomal preparations were 10.8, 6.0, 3.2 and 2.8 nmol MAC metabolized/(min*nmol P-450), respectively. In contrast the specific activity and turnover number were slightly decreased in microsomes from DEHP and MC treated rats compared to microsomes from saline treated rats. These results suggest a possible role of the acetone inducible isozyme(s) and the PB inducible isozyme(s) of the cytochrome P-450 enzyme family in the metabolism of MAC.
The amount of MET formed in the incubation mixture was a fifth to a tenth compared to the amount of MAC metabolized. This might be explained by binding of MET to components present in the incubation mixture or by the formation of other metabolites, which were not detected by HS-GC. The rate of MET binding and the formation of other metabolites, especially the possible formation of the epoxide, are currently investigated.

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METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE IN ISOLATED PERFUSED RAT LUNG

H. Foth

4-Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nitrosamine found in tobacco products and smoke, is a strong carcinogen producing mainly tumors in the lung. NNK is extensively metabolized by cytochrome P-450 isoenzymes in a variety of organs including tissues from the respiratory and alimentary tract. Few data, however, are available concerning the degradation and metabolic activation, respectively, of NNK by the lung. We investigated the metabolism of NNK by intact isolated perfused rat lung.

In lungs from untreated rats, NNK (2 μM) has been extensively metabolized within 3 h with 9.9 ± 3.1 % (n=6) of the total radioactivity (RA) still eluting as NNK. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the product of NNK reduction, contributed 6 ± 1 % of total radioactivity. The largest peak of NNK-metabolites coeluted with NNK-N-oxide and contributed 49 ± 2 % of total RA. Additional metabolites identified by coelution with authentic reference compounds were NNAL-N-oxide with 6 ± 1 %, 4-hydroxy-1-(3-pyridyl)-butanone (HPB, ketoalcohol) with 14 ± 2 %, 4-keto-4-(3-pyridyl)-butyric acid (keto acid) with 15 ± 2 % and 4-hydroxy-4-(3-pyridyl)-butyric acid (hydroxy acid) with 2 ± 0.3 of total radioactivity. During the first 2 h of perfusion time NNK disappearance was paralleled by an almost linear appearance of NNK-metabolites derived by the three principal reactions, α-hydroxylation (ketoalcohol, keto acid and hydroxy acid), N-oxidation (NNK-N-oxide and NNAL-N-oxide) and reduction (NNAL). N-Oxidation was by for the most prominent pathway of NNK metabolism in rat lung. Metabolites that are formally derivatives of NNAL appeared with some delay compared to NNAL indicating that reduction of NNK to NNAL is a prerequisite for further metabolism toward NNK-N-oxide and hydroxy acid.

Starvation of rats for two days, which is known to increase intestinal metabolism of NNK and to decrease the acitivity of microsomes from nasal mucosa, had no visible effect on NNK elimination and metabolite formation. Also, the addition of nicotine, the main alkaloid of tabacco smoke, did not alter the time course of NNK elimination and the pattern of NNK metabolism, irrespective whether the addition was performed concomitantly with NNK or at later points of perfusion time. Further experiments should clarify if inhibitors of N-oxidation and α-hydroxylation in vitro exert any effects upon NNK metabolism in intact lungs.

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SPECIES SPECIFIC METABOLISM OF NNK IN ISOLATED PERFUSED INTESTINAL SEGMENTS

Richter, E.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine derived nitrosamine, is suspected to be the major agent in tobacco smoke to induce lung cancer. Its carcinogenic properties toward lung and pancreas have been demonstrated in vivo; however this activity has not yet been linked to a specific metabolic pathway. NNK metabolism has drawn considerable attention, and its liver metabolism has been thoroughly investigated. The predominant pathway formed in rat liver microsomes is reduction to NNAl (4-methylnitrosamino-1-(3-pyridyl)-1-butanol). However, in lung and intestinal segments of rats, N-oxidation and α-hydroxylation also contribute considerable amounts. We therefore investigated the intestinal metabolism of NNK in mouse, rat and hamster.

In all three species NNK (1 μmol/l in perfusion fluid) underwent extensive metabolic activation, with rat being the least active species. Overall metabolism in mice was 3 fold, in hamster 10 fold higher. The relative contribution of the three major activation pathways, i.e. α-hydroxylation, N-oxidation and keto-reduction, displayed different kinds of behavior. Keto-reduction was found most extensively in hamster, whereas in mouse NNAl-formation was only a minor metabolite (hamster > rat > mouse). α-Hydroxylation on the other hand, was most extensive in mice and even exceeded the amount found in hamster (mouse > hamster > rat). N-oxidation was the major pathway in hamster and rat, but in mouse was less than α-hydroxylation (hamster > mouse > rat).

NNK-metabolism was inducible by starvation or acetone treatment in all three species. In intestinal absorbates NNK-metabolism was increased up 5fold in rats to 95% metabolites (of total radioactivity). However in the perfusion fluid only NNK could be detected. In mouse and hamster metabolism in uninduced animals was higher in absorbates (50% and 95% resp.). After acetone pretreatment the amount of metabolites found in the perfusion fluid increased also about 5fold in these species.

We conclude that different cytochrome P450-content in mouse, rat and hamster intestines give rise to both qualitative and quantitative different pattern of presystemic NNK-metabolism in these species.

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EXCRETION OF NNK-METABOLITES IN RAT BILE: NNAL-GLUCURONIDE

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine, has strong carcinogenic activities toward lung and pancreas in animal experiments. The organospecificity toward the pancreas can be enhanced by intragastral application. Therefore metabolism and excretion of NNK as model compound has attracted considerable attention.

Earlier research has shown different metabolic activation pathways in liver vs. intestine and lung, with the latter organs showing higher degrees of α-hydroxylation, whereas the liver predominantly reduces NNK to NNAl (4-methylnitrosamino-1-(3-pyridyl)-1-butanol). To elucidate the metabolite pattern we collected bile from rats dosed with 0.7 or 240 umol/kg NNK. The bile was analyzed by HPLC and time course and total excretion of total radioactivity and NNK-metabolites determined. The main metabolite detected at both low and high concentrations of NNK was NNAl-glucuronide, contributing up to 80% of the NNK metabolites in rat bile. Other metabolites detected were hydroxy acid and keto acid, products of α-hydroxylation. Products of N-oxidation, the predominant metabolites in lung metabolism, could be detected only in the first two hours after NNK-application. At longer time periods NNAl-glucuronide was the only metabolite detected.

Excretion of both total radioactivity and NNK-metabolites followed a time course consistent with a one-compartment model. At the low NNK dose (0.7 umol/kg bw) half life for NNK was 37 min, in agreement with values reported earlier. The $t_{½}$ values for hydroxy- and keto acid, NNAl and NNAl-glucuronide were larger, ranging from 50 to 110 min. At high concentrations (240 umol/kg bw) $t_{½}$ values were larger, ranging from 150 to 300 min.

NNK as well as NNAl are known pancreatic carcinogens in animal experiments. The finding of large amounts of NNAl-glucuronide as products of NNK-metabolism in rat liver supports the hypothesis that pancreatic carcinogens are excreted in bile, and enter the pancreas. Thus after cleaving adducts by pancreas specific enzymes a locally high concentration of carcinogens would lead to organ specificity.

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N-GLUCURONIDATION OF 1- AND 2-NAPHTHYLAMINE IN ISOLATED RAT HEPATOCYTES

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Aromatic amines such as 2-naphthylamine were among the first chemicals recognized to induce bladder cancer in humans. Interestingly, no carcinogenic risk has been observed after exposure to the isomeric 1-naphthylamine. To further investigate the underlying mechanisms of metabolic activation and deactivation we used freshly isolated hepatocytes from untreated and 3-methylcholanthrene(MC)-treated Wistar rats as an intact biological system to compare the metabolism of 1- and 2-naphthylamine. Incubations were carried out at a substrate concentration of 50 μM for 1 hour at 37°C. Metabolites were quantified by direct analysis of the concentrated supernatant by reversed phase HPLC and were identified by various spectroscopic methods. With hepatocytes from untreated animals incubated with (^{3}H)-2-naphthylamine 9 metabolites could be separated. 2-Naphthylamine-N-glucuronide (40% of total metabolites), 2-amino-1-naphthylsulfate (21%) and N-acetyl-2-naphthylamine (20%) represented the major metabolites, whereas N-hydroxylation occured at about 2-4 %. Pretreatment of rats with MC led to a 2-fold increase of total metabolism, a 2- to 3-fold increase of 2-amino-1-naphthylsulfate (40-50% of total metabolites), a relative decrease of 2-naphthylamine-N-glucuronide (20-30%) and N-acetyl-2-naphthylamine (1-3%) and the appearance of about 6% 2-amino-1-naphthylglucuronide. In contrast, with 1-naphthylamine oxidative metabolism leading to 1-amino-2-naphthylsulfate occured only to a minor extend in hepatocytes from untreated and MC-treated rats, whereas extensive N-glucuronidation was observed (60-70%). N-Hydroxylated compounds were not detectable in incubations with 1-naphthylamine. In conclusion, N-glucuronidation represents a major metabolic pathway of 1- and 2-naphthylamine in rat hepatocytes. Our results suggest that efficient N-glucuronidation together with the lack of N-hydroxylation is the reason for the inability of 1-naphthylamine to induce cancer.

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BISDIHYDRODIOL EPOXIDES ARE IMPORTANT GENOTOXIC METABOLITES OF TUMORIGENIC DIBENZ[A,H]ANTHRACENE

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Most tumorigenic polycyclic aromatic hydrocarbons (PAH) possess the structural element of a bay-region. The metabolic activation of tumorigenic PAH by sequential enzymatic attack of cytochrome P-450 dependent monooxygenase(s) and microsomal epoxide hydrolase results in the formation of genotoxic bay-region dihydrodiol epoxides as the biologically most important ultimate mutagens and carcinogens. In the case of tumorigenic dibenz[a,h]anthracene (DBA), however, the formation of bay-region dihydrodiol epoxides, i.e. DBA-3,4-dihydrodiol-1,2-oxides, constitutes only a minor metabolic pathway. Investigations concerned with the microsomal metabolism of DBA-(3R,4R)-dihydrodiol, the main metabolite of DBA and the potential precursor of vicinal bay-region dihydrodiol epoxides, revealed that the monooxygenase does not preferentially attack the vicinal olefinic double bond (1,2-position) but aromatic carbon-carbon bonds at distant sites of the DBA molecule finally leading to DBA-3,4:8,9-, -3,4:10,11- and -3,4:12,13-bisdihydrodiols. Among these bisdihydrodiols DBA-3,4:10,11-bisdihydrodiol is formed to the highest extent and exhibits the strongest bacterial mutagenicity after metabolic activation. Thus it can be concluded that DBA-3,4:10,11-bisdihydrodiol is the proximate and DBA-3,4:10,11-bisdihydrodiol-8,9-epoxide may be the ultimate genotoxic metabolite of DBA-3,4-dihydrodiol and consequently of DBA with the greatest biological significance. This conclusion is further supported by recent observations showing that the major DNA adducts of DBA and of DBA-3,4-dihydrodiol in vitro and in vivo do not originate from vicinal bay-region dihydrodiol epoxides but from more polar metabolite(s), presumably bisdihydrodiol epoxide(s).

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PROSTAGLANDIN H SYNTHASE MEDIATED TOXICITY OF XENOBIOTICS: STUDIES IN SEMV CELL CULTURES

G.H. Degen and J. Foth

Several xenobiotics and carcinogens are oxidized in vitro by prostaglandin-H-synthase (PHS). This has been suggested to contribute to their metabolic activation, particularly in tissues low in monooxygenase (MFO) activity. In vivo systems impose certain limitations on studies of the involvement of PHS in mediating adverse effects of chemicals but cell cultures which express PHS but no MFO-activity can serve as useful models for investigating PHS-catalyzed bioactivation and its role in the toxicity of certain xenobiotics.
Such a cell line has been derived from ram seminal vesicles in our lab[1] for studies on the metabolism of the carcinogenic estrogen diethylstilbestrol (DES) and its effect on an endpoint for genotoxicity. Recently, PHS-catalyzed oxidation of DES has been demonstrated in these cells[2] which lack constitutively expressed MFO-activity, but contain PHS. DES-oxidation is modulated by compounds known to affect PHS-activity: it is inhibited by indomethacin and increased upon addition of arachidonic acid. Now, DES has been found to induce micronuclei at concentrations which are not acutely toxic for SEMV cells[3]; the induction of micronuclei by DES is dose-dependently inhibited by indomethacin.
The data support the hypothesis that PHS-dependent oxidation of DES plays a role in its genotoxicity. Moreover, the results show that SEMV cells are a useful tool for studies on the PHS-dependent bioactivation of xenobiotics and its toxicological consequences.

1 *Freyberger A et al (1987) Molecular Toxicology 1: 503-512*
2 *Foth J, Degen GH (1991) Arch Toxicol. 65: 344-437*
3 *Foth J et al Toxicol in Vitro (submitted)*

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INHALATION PHARMACOKINETICS OF N-HEPTANE IN RAT AND MAN

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The aliphatic hydrocarbons n-hexane (HEX) and n-heptane (HEP) are widely used industrial solvents. HEX caused polyneuropathy in experimental animals and in occupationally exposed humans. These symptoms are related to its metabolite 2,5-hexanedione, a γ-diketone which binds to neurofilamental proteins. HEP might possibly have neurotoxic properties, too, since the equivalent γ-diketone 2,5-heptanedione has also been found in urine of exposed rats and humans. Whereas metabolism and pharmacokinetics of HEX have been investigated extensively, little is known about HEP. Therefore, we investigated the pharmacokinetics of inhaled HEP in Sprague-Dawley rats and healthy volunteers.
Exposures were carried out using closed exposure chambers. These chambers were an all-glass device for rats and a modified spirometer for humans (Filser, 1991, Arch. Toxicol. in press). HEP was administered as single doses into the atmosphere of the chambers resulting in various initial atmospheric concentrations of 158, 364, 2800, and 10300 ppm in the chambers containing rats and of 100 ppm in the spirometer system used for humans. Gas samples were drawn periodically and analyzed by gas chromatography. The resulting concentration-time courses were pharmacokinetically analyzed by a two-compartment model (Filser, 1991).
Thermodynamic partition coefficient organism/air which is mainly determined by the solubility of HEP in fatty tissues was 30 in rat and 47 in man according to his higher fat content. Below an atmospheric concentration of HEP of about 40 ppm metabolism in rats followed first-order kinetics. At higher concentrations metabolism showed saturation kinetics, V_{max} being about 110 µmol/h/kg body weight and atmospheric concentration at $V_{max}/2$ being about 70 ppm. Besides this metabolic pathway another one was found following first-order kinetics. A similar metabolic behavior had been observed for n-pentane and n-hexane. In man, a metabolic elimination according to first-order kinetics was found for the concentration range investigated. At low exposure concentrations of HEP metabolism in both species is limited not by enzyme capacity, but by transport to the metabolizing enzymes.
The pharmacokinetic data acquired will be used in the estimation of the neurotoxic risk related to HEP exposures.

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DETERMINATION OF URINARY 2,5-HEXANEDIONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) WITH POST COLUMN DERIVATISATION TO 3-METHYL-2-CYCLOPENTENONE

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n-Hexane causes peripheral neuropathies which are attributed to the ultimate neurotoxic metabolite 2,5-hexanedione (HDO). In order to determine urinary HDO after exposure to n-hexane a HPLC method was developed for biological monitoring.
2.5 mL of urine were mixed with 0.15 mL of a solution of HCl (32 %) and hydrolyzed at 95 °C for 45 min. 0.75 g NaCl were added and the mixture was extracted once with 1 mL of dichloromethane. After extracting with silica solidphase columns the HDO containing fraction was eluted using 1 mL of the mobile phase of HPLC. 20 µL samples were chromatographed on a column (250*4.6 mm) packed with Nucleosil 120-5C18 at a flow rate of 1 mL/min; the mobile phase was composed of 1 mol/L ethanol and 5 mmol/L phosphate buffer (pH 7.2). HDO was converted to 3-methyl-2-cyclopentenone by continuously adding of a solution of NaOH (6.25 mol/L; 0.2 mL/min) and heating in a reactor (165 °C, PEEK-capillary 4.2 m*0.5 mm). UV absorption was measured at 235 nm.
A linear relationship between concentration and absorption was obtained in the range between 0.5 - 200 mg HDO/L. Urine samples of non-exposed humans were analyzed. No interference was detectable. 6 mg/L HDO were added to two urine samples which were analysed 5 and 6 times, respectively: Recovery was 97 ± 5 % and 92 ± 6 %. The detection limit, defined as threefold background noise, was less than 0.7 mg/L urine. One chromatographic run lastet 35 minutes, purging of the column with 6 mol/L ethanol included.
By the post column derivatisation the absorption coefficient increased about 300fold. Therefore interferences of UV-absorbing material in the urine matrix became negligible small. This new method is a useful tool for the determination of urinary HDO after exposure to n-hexane.

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METABOLISM OF 1,3-BUTADIENE IN MICROSOMES FROM LIVERS OF MOUSE, RAT, AND MAN

J.G. Filser, B. Altthaler, H.F. Welter*, C. Baur**, and G. Johanson***

1,3-Butadiene (BU) was carcinogenic in mice and rats. This effect is assumed to be based on biotransformation to the epoxides 1,2-epoxybutene-3 (BO) and 1,2,3,4-diepoxybutane. The former is formed in the first metabolic step via cytochrome P-450 dependent monooxygenases. In mice, carcinogenic potency of BU was considerably higher than in rats. One reason for these findings could be a different target dose of BO resulting from differences in the rate of its formation and elimination. Therefore, a basis for a risk estimate for BU is information on kinetics of its biotransformation to BO and of its further metabolism. In previous studies we established kinetic parameters of BU and BO in vivo in mice and rats (Kreiling et al., Arch. Toxicol. 58: 235, 1986; 61: 7, 1987). Since, for ethical reasons, kinetic data of both compounds in humans cannot be obtained in vivo, we determined kinetic parameters of BO in vitro using liver microsomes (LM) of humans and, comparatively, of mice and rats (Kreuzer et al., Arch. Toxicol. 65: 59, 1991). In this communication we report on enzyme specific kinetics of BU in LM.
LM were prepared from livers of mice and rats and from a specimen of one healthy human liver obtained during organ transplantation. Suspensions of LM containing a NADPH-regenerating system were exposed at 37 °C to gaseous BU by means of an in vitro headspace apparatus (Kreuzer et al. 1991). The headspaces of incubation vials contained initial BU concentrations of 30, 300, 1.000, and 10.000 ppm. BU in headspace was analyzed by gas chromatography. Control experiments were done without NADPH and with boiled LM. Kinetic parameters were calculated using a two compartment model (Filser, Arch. Toxicol. 1991, in press). BU accumulated barely in aqueous systems: At 37°C partition coefficient water/air was 0.06. NADPH dependent metabolism was found in LM of all three species, V_{max} (nmol BU/min/mg protein) being 3.22 (mouse), 2.17 (rat), and 2.56 (man). The apparent Michaelis-Menten constant, K_{mapp} (mmol BU/L), was related to the liquid phase. The values obtained were: 0.024 (mouse), 0.035 (rat), 0.023 (man). The similarity of these values might originate from the low solubility of BU. V_{max} was used to calculate maximum rate of BU metabolism in vivo: Assuming liver and body weights of 1 and 24 g (mouse), 10 and 250 g (rat), and 1.5 and 70 kg (man) and a protein content of 30 mg/g liver, maximum rates (µmol/h/kg body weight) were: 243 (mouse), 157 (rat) 99 (man). The estimates for mice and rats were 61% and 71%, respectively, of actual values determined experimentally in vivo (Kreiling et al., 1986). With regard to the resemblance between estimated and measured values in the animal species one should expect the same being also true in humans.
The data are a basis for the development of a physiological pharmacokinetic model, describing the burden of organs and tissues in mouse, rat and man with BU and BO.

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STEREOMETABOLISM OF ETHYLBENZENE IN MAN: URINARY EXCRETION OF MANDELIC ACID AND 1-PHENYLETHANOL ENANTIOMERS

M. Korn, I. Wodarz, and W. Gfrörer

Inhaled ethylbenzene (EB) vapors are known health hazards to workers in manifold areas of industry and craft. The major metabolites of EB are mandelic acid and phenylglyoxylic acid. Different views of the main pathway, especially the importance of the intermediate 1-phenylethanol, are discussed in the literature. Biological monitoring in man is recommended either by determination of the inhaled solvent vapor equivalent in blood or alveolar air or by determination of the metabolites mandelic acid or phenylglyoxylic acid in urine samples.
In the present study we analysed 50 urine samples from workers with respect to mandelic acid enantiomers, phenylglyoxylic acid, and 1-phenylethanol enantiomers. Urine samples were taken at the end of the shift. We estimated the concentration of these metabolites by gas chromatographic procedures using a glass capillary column coated with the chiral stationary phase Chirasil-L-Val. Details in sample preparation have been published elsewere (Korn et al., J. High Res. Chromatogr. & Chromatogr. Communications 11:313, 1988). Average excretion of phenylglyoxylic acid is 53.4 mg/l urine. Total excretion of mandelic acids is 153.6 mg/l, which is approximately 3-fold over the excretion of phenylglyoxylic acid. The average amount of 137.4 mg/l for R-mandelic acid and 9.4 mg/l for S-mandelic acid results in a S/R ratio of about 1 : 18. R-mandelic acid and phenylglyoxylic acid are preferably formed and excreted in humans, S-mandelic acid seems to be a minor metabolite in man. The average amount in free and conjugated form of 8.5 mg/l for R-phenylethanol and 2.7 mg/l for S-phenylethanol results in a S/R ratio of about 1 : 3. R-phenylethanol is preferably formed and excreted in humans. They seem to be minor metabolites in man.
According to our results we suggest a metabolic scheme for the biotransformation of EB as follows: The initial step of the biotransformation of ethylbenzene is an alpha-oxidation to S- and R-1-phenylethanol. Optical activity is thus introduced into the detoxication pattern by the formation of S-(-)- and R-(+)-phenylethanol. The subsequent enzymatical oxidation of S-1-phenylethanol by the alcohol dehydrogenase to R-phenylethylene glycol as well as further oxidative conversions via R-phenylglycol aldehyde to R-mandelic acid accordingly proceed stereospecifically. The theory of Sullivan et al. (Xenobiotica, 6:49, 1976), that the optical activ center of methylphenylcarbinol (1-phenylethanol) is destroyed and subsequently reformed during oxidation steps to mandelic acid, seems to be unlikely.

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INVOLVEMENT OF CYTOCHROME P-450IIE1 IN THE NADPH-DEPENDENT MICROSOMAL OXIDATION OF ALDEHYDES

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An NADPH-dependent oxidation of aldehydes by rat liver microsomes was demonstrated recently and suggested to be catalyzed by the P-450 isoenzyme IIE1 based on the sensitivity of carboxylic acid formation to inhibition by indazole and the marked enhancement of this reaction by pretreatment of rats with isopropanol (Gans & Werringloer, Naunyn-Schmiedeberg's Arch. Pharmacol. 341:R15, 1990). In order to verify the function of P-450IIE1 in the oxidation of aldehydes and to estimate its contribution to the microsomal formation of carboxylic acids studies were carried out with the purified enzyme system incorporated into phospholipid vesicles and with anti-(P-450IIE1)-IgG, respectively. The following results were obtained using reversed-phase HPLC for the separation and UV/Vis detection for the analysis of the products formed: 1. In the reconstituted enzyme system 4-nitrobenzaldehyde was converted to the corresponding carboxy compound with a specific activity of 12 nmol 4-nitrobenzoic acid formed nmol P-450IIE1^{-1} min^{-1} at 37 °C. The velocity of product formation was found to be dependent on the concentration of P-450IIE1, thus, providing conclusive evidence for the function of P-450IIE1 in the oxidation of aldehydes. 2. In liver microsomes benzphetamine is N-dealkylated primarily by isoenzymes of the P-450IIB family yielding either formaldehyde or benzaldehyde which are formed at a ratio of appr. 4:1, the latter being metabolized further to benzoic acid. Comparative studies with liver microsomes of control, phenobarbital- or isopropanol-pretreated animals revealed a marked susceptibitlity of this reaction to inhibition by anti-(P-450IIE1)-IgG as well as indazole (K_i < 1 µM) ranging from 60% in controls to appr. 80 % after induction of P-450 IIE1 by isopropanol. It is concluded, therefore, that P-450 IIE1 plays a major role in the NADPH-dependent microsomal oxidation of aldehydes and that indazole functions as a highly specific inhibitor of this particular enzyme.

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TRANSPORT AND DEACETYLATION OF ACETYLSALICYLIC ACID IN ISOLATED GASTRIC MUCOSAL CELLS.

M. Schwenk and I. Radziejowsky

Introduction: It is generally assumed, that the ulcerogenic effect of acetylsalicylic acid (ASA) is caused by a combination of cyclooxygenase-inhibition and direct cytotoxic effects after accumulation of the protonated drug from the acidic lumen into epithelial cells. However, only insufficient data about the possible effect of ASA deacetylation to salicylic acid (SA) on the overall kinetics in the gastric epithelium are presently available.

Methods: Cells were isolated from guinea pig gastric mucosal scrapings by treatment with collagenase and pronase. Cell suspensions were incubated at 37° C with various concentrations of ^{14}C-ASA. Cells were separated at various times by silicone filtration, and intra- and extracellular ASA- and SA-levels were assessed by thin layer chromatography.

Results: Cellular uptake rates of ASA were strongly pH dependent: The relative uptake rates at pH 7, 6, 5 and 4 amounted to 1, 3, 10 and 79. There was no saturation between 1 µM and 1 mM, and no Na^+- or energy-dependency. ASA was very rapidly deacetylated within cells to SA. The rate of deacetylation was 12 times faster at pH 4 than at pH 7. At steady state (20 min), the intracellular/extracellular concentration ratios of ASA (SA) were at pH 7 = 0.2 (2.5) and at pH 4 = 30 (290). Enriched mucous-, chief- and parietal cells all exhibited similar deacetylation rates.

Conclusions: Acidic luminal pH strongly favors uptake and accumulation of ASS. Epithelial cells protect themselves by deacetylating ASA to the less toxic and less cyclooxygenase-inhibiting SA.

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CYTOTOXICITY OF ALLYL ALCOHOL AND SEVERAL GLYCOLS TO BALB/c 3T3 CELL CULTURES AND COCULTURES OF 3T3 CELLS WITH MICROCARRIER-ATTACHED RAT HEPATOCYTES

J.-U. Voß, U. Jürgens & S. Mörchel

The cytotoxicity of allyl alcohol and several glycols (ethylene glycol, 2-methoxyethanol, propanediol-1,2, propanediol-1,3, and dioxane) was studied in cultures of 3T3 cells and in cocultures of 3T3 cells with rat hepatocytes. Hepatocytes isolated from male rats were inoculated with collagen-coated microcarriers. 24 hours after initiating both cultures, microcarrier-attached hepatocytes were transferred to plastic inserts covered by a 80 µm nylon mesh. These inserts were placed into the wells of 6-well cluster dishes with 3T3 cell cultures. 3T3 cells were exposed to various concentrations of the chemicals for 48 hours with or without cocultivation with rat hepatocytes for 24 hours. Growth of the 3T3 cell cultures was assessed by determination of cellular protein. Effects on hepatocyte viability (intracellular LDH-activity) and on the activity of the 7-ethoxycoumarin O-deethylase were determined at the end of the cocultivation period. The chemicals exhibited different effects on both cell types. Allyl alcohol was more toxic to hepatocytes than to 3T3 cells cultured separately, whereas the opposite was found for the other substances. The cytotoxicity of allyl alcohol, ethylene glycol and propanediol-1,3 to 3T3 cells was markedly enhanced by cocultivation with hepatocytes. Addition of 500 µM pyrazole to the cocultures reduced the toxicity of ethylene glycol and propanediol-1,3 to 3T3 cells and of allyl alcohol to both cell types. The results indicate that a) the cytotoxicity of allyl alcohol, ethylene glycol and propanediol-1,3 depends on the formation of toxic metabolites and b) the described coculture system represents a suitable approach to detect effects of biotransformation on toxic actions of xenobiotics.

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INFLUENCE OF CALCIUM ANTAGONISTS ON THE HEXACHLOROPHENE INDUCED CYTOTOXIC BRAIN OEDEMA, ETHANOL TOLERANCE AND WITHDRAWAL SEIZURE THRESHOLD K. Andreas

The hexachlorophene-induced cytotoxic brain oedema had been valued in former studies as a suitable model to test cerebroprotective substances. Its importance with respect to clinical concern is based on the occurence of oedema in connection with different kinds of brain injuries e.g. hypoxia, ischemia, trauma, and intoxications. The primary target of hexachlorophene is the neuronal cell membrane. Secondary effects are disruption of myelin lamellae and vacuolation of the white matter. Besides alteration of water and electrolyte content the coordinative motor responses is disturbed. In rats this motor disturbance is diminished by calcium antagonists. Nifedipin (0.3 mg . kg^{-1}. d^{-1}) as well as the calcium influencing nootropic substances flunaricine (20 mg . kg^{-1}. d^{-1}) and cinnarizin (30 mg . kg^{-1} . d^{-1}) abbreviate the recovery time after cessation of hexachlorophene treatment.
Calcium antagonist also decrease ethanol effects. The substances mentioned above and verapamil (10 or 30 mg . kg^{-1}) diminish or suppress the within session tolerance and withdrawal seizure threshold decrease. The results demonstrate the importance of calcium ion movement with respect to the mechanism of action of brain-injuring substances.

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STRAIN DIFFERENCES IN ACETAMINOPHEN NEPHROTOXICITY IN THE RAT - FACT OR ARTEFACT? G. Birner, M. Koob, P. Hauser and W. Dekant.

Acetaminophen has been described to be nephrotoxic in some strains of rodents and major differences in the nephrotoxic response have been reported. Fischer F344 rats were considered to be more susceptible to acetaminophen induced renal cortical necrosis than Wistar or Sprague-Dawley rats. Selective deacetylation of acetaminophen to p-aminophenol in Fischer rats was suggested to be responsible for nephrotoxicity.
The experiments reported here were designed to further characterize these strain differences. However, we were not able to confirm the previous findings. Acetaminophen (900 mg/kg oral) did not induce renal cortical necrosis in Fischer and Wistar rats and did also not induce changes in urinary parameters indicative for nephrotoxicity. Moreover, no differences in the structure and concentrations of biliary and urinary metabolites were observed. Acetaminophen-glucuronide, -sulfate and 3-(glutathion-*S*-yl)acetaminophen, resp. 3-(*N*-acetyl-cysteine-*S*-yl)acetaminophen were the major metabolites. *In vitro*, acetaminophen (up to 5mM) was not toxic to cells isolated from both Wistar and Fischer F344 rats and formation of p-aminophenol could not be demonstrated in kidney cytosol of both rat strains.

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CELL CULTURE IN NEPHROTOXICITY TESTING
H. L'Eplattenier, J. Zhao, F. Pfannkuch, G. Scholtysik* and A. Wüthrich*

The need to develop alternative methods has become obvious in industrial toxicology where there is an increasing concern as to refining the tests performed on animals. Cell culture is one of many in vitro methods to reach this aim. The present study describes a cell culture model for nephrotoxicity testing. Experiments were performed in order to evaluate the suitability of such a model.
Two different types of cells were used: proximal tubular epithelial (PTE) cells and fibroblasts. The PTE cells were from the pig kidney cell line LLC-PK_1 because of the advantages of using a cell line and because these cells still possess many of the morphological and functional characteristics that are present in vivo. The fibroblasts were from the Baby Hamster Kidney (BHK-21) cell line. Four substances were tested: three known nephrotoxicants gentamicin, $HgCl_2$, the carbapenem antibiotic CGP 31 608 [(5R,6S)-2-Aminomethyl-6- ((1R)-hydroxyethyl) -2- penem -3-carboxylic acid] and one general toxicant benzalkonium chloride.
The first step of the investigation consisted in assessing the general cytotoxicity of the test compounds by performing the neutral red assay [Borenfreund E and Puerner JA (1985) Toxicol. Letters 24:119] on the fibroblasts. In the second step compounds were tested on the tubular cells in order to assess specific nephrotoxicity by determination of (1) The activity of different enzymes in the cells (lactate dehydrogonase, alkaline phosphatase, N-acetyl-β-D-glucosaminidase and glutamate dehydrogenase); (2) The release of these enzymes into the culture medium; (3) DNA-synthesis.
It was an interesting result that the comparison between cytotoxicity in vitro in the neutral red assay and oral LD_{50} in the rat showed a close correlation ($r = 0.94$). Gentamicin and CGP 31 608 as expected produced changes in the enzyme parameters in the cells as well as in the culture medium. $HgCl_2$ surprisingly did not alter the activities of any of the enzymes considered for unknown reasons. Also benzalkonium chloride, being a non-specific toxicant, did not alter these parameters. Gentamicin caused a slight stimulation of cell growth during exposure whereas to other test substances did not.
In conclusion the cell culture approach and particularly the neutral red assay in fibroblasts may be suitable for comparing the toxicity of compounds and can be predictive for in vivo acute toxicity. Detection in cell culture of selective nephrotoxicity needs further investigation.

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NUMBER OF SPERMATIDES IN TESTES AND TESTICULAR MORPHOLOGICAL EVALUATION AFTER PRENATAL GANCICLOVIR EXPOSURE IN RATS. J. Hartmann[1] and G. Rune[2]

Ganciclovir is used in therapy of cytomegaly virus infections in AIDS patients. The present study was aimed at finding out whether effects on testes development in the offspring may be induced by prenatal treatment. Dams were treated (s.c.) on day 10 of pregnancy with three injections of 100 mg ganciclovir/kg body wt. Since postnatal (pn) day 50 is the start of spermatogenesis in rats and the ultimate function is reached on day 100, the testes (morphology and spermatid count) were investigated on days 50, 75, 100, 130 and 180 postnatally. The number of spermatides in the testes of exposed offspring was reduced on all days evaluated when compared to controls. On days 50 and 75 the difference was not statistically significant. The table shows the number of spermatids (in Mio) per testes, the values are given as M ± S.D.; testes weights were significantly reduced ($p < 0.05$), data not shown.

(pn) day	n	Ganciclovir	n	Controls
50	10	45 ± 35	16	60 ± 20
75	10	85 ± 23	13	101 ± 43
100	20	64 ± 17*	14	164 ± 32
130	20	97 ± 47*	20	147 ± 31
180	20	76 ± 41*	20	148 ± 49

* t-test; $p < 0.05$; n = number of testes examined

Morphometric evaluation of semi-thin sections of testis tissue revealed a significant decrease of the tubular diameter, which in turn corresponded to the fact that spermatogenesis is obviously not initiated in all tubules. Summarizing, ganciclovir affects morphological as well as functional development of testes in rat offspring exposed prenatally on day 10 of pregnancy to the substance.

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POSTNATAL EVALUATION AFTER PRENATAL GANCICLOVIR EXPOSURE IN RATS. I. Chahoud, M. Mayer, S. Ebel

When the embryotoxicity of substances are studied, the postnatal manifestation of prenatally induced alterations or lesions in organs must also be investigated. Ganciclovir is used in therapy against Herpes viruses and cytomegaly virus. Testis atrophy and Azoospermia, as well as neurotoxicity, are described as side-effects of ganciclovir in man. We performed a study aimed at answering the questions: Do substances such as ganciclovir, which harm many organs in adults, affect these organs if they are exposed prenatally? Do the effects manifest themselves postnatally? Pregnant rats were injected (s.c.) with 100 mg ganciclovir/kg body wt three times on day 10 of pregnancy. The dams were allowed to deliver and the postnatal development of the offspring was studied. In the offspring exposed prenatally to ganciclovir, the absolute and relative weight of the testes was significantly reduced ($p < 0.05$, see table) when compared to the controls. In some offspring macroscopical alterations were also found in the brain, kidney and liver.

Testes weight (g), M ± S.D.

Age (day)	n	Ganciclovir	Controls
50	10	0.66 ± 0.25	0.87 ± 0.12
75	10	0.97 ± 0.36	1.40 ± 0.15
100	10	1.09 ± 0.20	1.56 ± 0.16
130	10	1.13 ± 0.29	1.54 ± 0.08
180	10	1.24 ± 0.46	1.56 ± 0.14

These results and the results presented by J. Hartmann at this meeting indicate that ganciclovir, which harms testes in adult humans, also affects the testes in prenatally exposed offspring.

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INDUCTION OF APOPTOSIS IN CULTURED HEPATOCYTES AND IN THE REGRESSING LIVER BY TRANSFORMING GROWTH FACTOR-ß1 F. Oberhammer, M. Pavelka*, A. Purchio§, and R.Schulte-Hermann

In previous studies in vivo apoptotic liver cells were found to be stronger positive for an epitope of the pro-region than for an epitope of mature transforming growth factor-ß1 (TGF-ß1). Therefore we compared the potency of both forms to induce apoptosis in hepatocyte cultures. TGF-ß1 induces rounding up and fragmentation of the cells into multiple vesicles. As revealed by the DNA specific stain H33258 the chromatin of these cells condensed and segregated into masses at the nuclear membrane; this was obviously followed by fragmentation of the nucleus. Ultrastructurally the cytoplasm was well preserved as demonstrated by the presence of intact cell organelles. These features strongly suggest the occurrence of apoptosis. Quantification of nuclei with condensed chromatin and fragmented nuclei revealed that mature TGF-ß1 was found 30 fold more effective than the TGF-ß1 latency associated protein complex (LAP). Finally we administered TGF-ß1 in vivo using an experimental model in which regression of the liver was initiated by a short preceeding treatment with the hepatomitogen cyproterone acetate (CPA). Two doses of 1nM TGF-ß1/kg each augmented the incidence of apoptotic hepatocytes 5 fold. An equimolar dose of TGF-ß1 LAP was ineffective. These studies suggest that TGF-ß1 is involved in the initiation of apoptosis in the liver and that the mature form of TGF-ß1 is the active principle.

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DETERMINATION OF NEUROTRANSMITTER CONTENTS IN THE STRIATUM OF RATS AND MARMOSETS (CALLITHRIX JACCHUS) ON DIFFERENT POSTNATAL DAYS. R. Schwabe, X. Haun, U. Rahm

To obtain information about the developmental stage of the brain in animals who have the same biological age but belong to different species, determination of the neurotransmitter dopamine and the corresponding metabolites was carried out. Since the marmoset can only raise two babies from triplets, we used only the third baby of a litter for our study. The determination of the neurotransmitter and the corresponding metabolites was performed by HPLC with electrochemical detection after preparation and homogenisation of the brain areas on day 1 (rat, marmoset) and day 6 (marmoset) and day 7 (rat). The results (preliminary results for marmoset day 6) are shown in the table.

Table 1: Neurotransmitter contents(mean ± S.D.) in striatum of rat and marmoset on different postnatal days.

Striatum (ng/g wet tissue)				
	Rat		**Marmoset**	
	Day 1 (n=3)	**Day 7** (n=4)	**Day 1** (n=9)	**Day 6** (n=2)
DA	884±166	2681±405	2923±1350	3584±756
Dopac	359±179	488±256	352±271	363±24
HVA	419±375	321±102	1225±566	1952±17

DA = 3-Hydroxytyramine, Dopac=3,4-Dihydroxyphenylacetic acid, HVA=Homovanillic acid,

A great difference was found for dopamine levels in striatum between rat and marmoset. In the rat 33% of the content found on postnatal day 7 is found on day 1. In the marmoset on day 1, 82% of the content determined on day 6 was found. The ratio of DA/HVA increases from 2.11 (day 1) to 8.35 (day 7) in the rat, whereas it decreases from 2.39 (day 1) to 1.84 (day 6) in the marmoset. This may be a clue that at this stage of development there is a difference in the dopamine metabolism.

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DOSE-RESPONSE RELATIONSHIP OF TERATOGENICITY OF ETHYL CARBAMATE IN MICE

Thomas Platzek*, Gerd Bochert, Blanka Pauli

Ethyl carbamate (urethane) is a contaminant of various foodstuffs, especially in certain alcoholic beverages it is found in appreciable amounts. It is well known as a strong carcinogen in various laboratory animal species. Additionally, ethyl carbamate is teratogenic in rats, mice, and hamsters. We performed a dose-response study in NMRI mice. The animals were treated p.o. on day 11 of pregnancy. Four doses from 300 to 1000 mg/kg were applied. Sectio was performed on day 18 of pregnancy. Following the highest dose fetomortality was not changed compared to controls, fetal weight was decreased and the number of weight reduced fetuses was increased (8.3%). The predominant skeletal abnormalities were lateral-ventral fusion of thoracic and lumbal vertebrae. Using probit analysis dose-response data were established.

Table: Dose-response data, per-fetus approach, values mg ethyl carbamate per kg bw

	ED-10	ED-50	ED-90
all anomalies:			
	420 ±21	862 ±32	(1768 ±149)
ventral-lateral fusion of vertebrals:			
	621 ±21	941 ±24	(1426 ±78)

Studies are in progress to estimate the prenatal-toxic risk of ethyl carbamate based on several approaches including dosimetry.

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INFLUENCE OF HEAVY METALS AND METABOLIC INHIBITORS ON THE TRANSEPITHELIAL ELECTRICAL RESISTANCE IN MDCK CELLS

H. Mückter, B. Liebl, E. Doklea, B. Fichtl

The transepithelial electrical resistance (TER) is a sensitive parameter for the functional assessment of epithelial integrity. Madin-Darby canine kidney (MDCK) cells have been widely used for those studies. We have studied the effect of various heavy metals and metabolic inhibitors on the time course of TER in MDCK cells.

Wild-type MDCK cells were grown on permeable supports in DMEM/F12 medium at 37°C in moist atmosphere containing 5 % CO_2. The cells developed TER values above 2000 Ωcm^2 within 2 - 3 days after reaching confluency.

When various heavy metals were added to the cells, a sharp drop in TER within the first few hours of exposure was noted with some substances, e.g. MeHgCl, $CdCl_2$, but not with others, e.g. Tl_2SO_4. Various arsenicals that inhibit cellular energy metabolism also decreased TER values in MDCK cells. For example, an exposure time of 15 min to oxophenylarsine (PhAsO), a potent inhibitor of pyruvate dehydrogenase, irreversibly damaged the monolayer, while the cellular ATP values were still within the normal range. On the other hand, phenylarsonic acid, a less toxic analogue of PhAsO affected neither TER nor ATP.

Uncouplers of oxidative phosphorylation, e.g. 2,4-dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone, rapidly abolished TER in MDCK cells. Since these substances do not irreversibly bind to cellular structures, the time course of TER recovery could be studied after removal of the inhibitors. Whereas other parameters of cellular viability, e.g. ATP content, were completely normal within a few hours, recovery of TER rarely occurred before 24 h.

Our data suggest that TER measurements can complement the current bundle of in-vitro toxicity screening methods.

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BIOCHEMICAL CHARACTERIZATION OF TILORONE-INDUCED LYSOSOMAL ACCUMULATION OF SULFATED GLYCOSAMINO-GLYCANS IN CULTURED BOVINE FIBROBLASTS.

J. Fischer

The di-cationic amphiphilic compound tilorone induces lysosomal accumulation of sulfated glycosaminoglycans (GAG) in cultured corneal fibroblasts from several species (Lüllmann-Rauch and Ziegenhagen, Virchows Arch. B Cell Pathol. 60:99, 1991).

Aim of the present study was to characterize the accumulated GAG. Cultured bovine corneal fibroblasts were exposed to 5 µM tilorone for 96h without changing the culture medium, control cells were kept under identical conditions but without tilorone. The cells were harvested by trypsinization. Since the intracellular compartment is known to contain a mixture of proteoglycans and free GAG-chains (H. Kresse and J. Gössel, Adv. Enzymol. 60:217, 1987), the s-GAG-chains were liberated from core proteins by a ß-elimination reaction. Isolation of free GAG-chains was achieved by ion-exchange chromatography (DEAE-Trisacryl, 50 mM Bistris, pH 6.4, 0.1% Triton x 100, elution with rising concentrations of NaCl) followed by analytical electrophoresis and enzymatic degradation with chondroitin lyases AC/ABC. After staining with alcian blue, the relative amounts of individual GAGs were estimated by scanning densitometry. In untreated cells, dermatan sulfate (DS) was found to be the major compound accompanied by smaller amounts of heparan sulfate (HS) and chondroitin sulfate (CS). Treatment with 5 µM tilorone induced marked elevation of DS. When the tilorone-concentration was increased to 20 µM, not only DS but also HS was markedly augmented. This resulted in an elevated relative contribution of HS towards total GAG when compared with the GAG-storage induced by 5 µM tilorone. This concentration-dependent change in the GAG-pattern was accompanied by qualitative changes concerning the cytological lesions. At 5 µM tilorone, the cells showed GAG-containing lysosomes; at 20 µM tilorone, the cells additionally displayed clear vacuoles and polymorphic inclusions which suggested unspecific perturbation of the general digestive functions of the lysosomes.

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CHARACTERIZATION OF CULTURED URINARY BLADDER EPITHELIAL CELLS

Christine Guhe, Hartmut Hentschel#, Wolfram Föllmann

Epithelial cells of the urinary bladder of pigs, rat and humans were isolated by a mechanic preparation combined with an enzymatic collagenase digestion. The method yields dividing cells that can be cultured for several weeks under serum-free conditions without evident changes in cell morphology. After this period the cells become senescent and die.

Material from pigs is probably better suited for an extrapolation of toxicological data from cell culture experiments than rats, which have mainly been used in the past. The experience gained from the use of pig bladder cells may provide a basis for the development of a test system with human material, which is far less readily available.

Ultrastructural investigations demonstrate that the cultured cells develop a monolayer with a morphologic polarity resembling the epithelium in vivo. Chromosome analysis shows no changes in the chromosome set during the culture period. Growth curves indicate a doubling-time of 24 hours in the log-phase during the first 4 days when the cells are routinely seeded in a concentration of $1x10^6$ cells per 75 cm^3 flask. After seven days the cells build up a monolayer and reach a lag-phase caused by contact inhibition which stipulate a passage.

Following this characterization, the cultures were used for in vitro genotoxicity tests. Investigation of sister chromatid exchange (SCE) under the influence of an alkylating agent (N-Methyl-N´-nitro-N-nitrosoguanidine (MNNG)) shows an increase of SCE´s in dependence of the MNNG concentration. DNA repair of the cultured cells is measured autoradiographically by unscheduled DNA synthesis.

The final aim of this project is to obtain an in vitro model with human urinary bladder epithelial cells with characterized properties which can be used as a target for agents inducing bladder cancer to clarify the sensitivity to such agents in man on the level of the target organ.

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RESULTS OF THE GERMAN VALIDATION PROJECT OF ALTERNATIVES TO THE DRAIZE EYE TEST

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Since 1988 ZEBET has coordinated a national German interlaboratory study on the validation of two alternative methods to the Draize rabbit's eye test, the neutral red/kenacid blue (NR/KB) cytotoxicity assay and the hen's egg chorioallantoic membrane (HET-CAM) test.

During the first two years the two methods were established in twelve laboratories to ensure intra- and interlaboratory reproducibility. Testing 32 chemicals from a variety of chemical classes indicated a better correlation between data from the HET/CAM test and both, human and Draize test data than between cytotoxicity and the *in vivo* data.

During the final experimental stage of "data base development" (Balls et al., ATLA 18, 313-337,1990) 165 chemicals were tested in both test systems to provide information whether and to what extent the in vitro tests might be able to replace the Draize rabbit's eye test. The test chemicals selected are representing a broad spectrum of both chemical classes and in vivo eye irritating properties.

The evaluation of a total of 200 chemicals tested during the validation study indicates a sufficient positive predictive value of the HET-CAM test and a poor correlation between cytotoxocity and *in vivo* data.

It will be discussed with industry and governement agencies to which extent the validated *in vitro* methods can replace the mandatory Draize test in international guidelines of the CEC and OECD.

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EFFECTS OF 4-NITROSOPHENETOL ON THE ENDOGENOUS METABOLISM OF HUMAN RED CELLS

D. GALLEMANN AND P. EYER

The phenacetin metabolite 4-nitrosophenetol (NOPt) has been shown to induce methemoglobinemia and to exert toxic, mutagenic and probably carcinogenic effects. Since the disposition of NOPt is assumed to be markedly influenced by red cells, we studied the endogenous metabolic responses of human erythrocytes. As discovered by Kiese (M. Kiese, Methemoglobinemia: A comprehensive treatise, CRC Press, Clevland 1974), nitrosoarenes are reduced in red cells to the corresponding hydroxylamines by a NADPH-dependent diaphorase. The hydroxylamine is cooxidized with oxyHb, yielding ferriHb and regenerating the nitrosoarene, until it is eliminated by side reactions, especially with GSH (H. Klehr, P. Eyer, (1987) Naunyn-Schmiedeberg's Arch. Pharmacol., Suppl. 335, R12). The NADPH-supply for this deleterious cycle is maintained by the pentose phosphate pathway.

In suspensions of human red cells (15 g Hb/100 ml), NOPt (1.0 µmol/ml) rapidly produced ferriHb (27 % within 10 min). The flux through the pentose phosphate pathway was drastically increased, yielding 31 nmol/ml·min $^{14}CO_2$ from u-^{14}C-glucose (control value: 1.1 nmol/ml·min). During the first 10 min after NOPt, the G6P steady-state concentration was in the range of 2 nmol/ml (22 nmol/ml in controls), indicating a limiting hexokinase reaction. During this period, the formation rate of the main tricarbon metabolites of glycolysis (lactate, pyruvate and 2.3-DPG) steadily increased from 13 nmol/ml·min in controls to 23 nmol/ml·min, yielding 5 nmol/ml·min net NADH to allow ferriHb reduction. NOPt itself rapidly decreased to 0.07 µmol/ml within 10 min. Correspondingly, the GSH status was heavily disturbed: 5 min after NOPt, GSSG peaked at 0.28 µmol/ml, while GSH decreased from 1.00 to 0.20 µmol/ml, indicating a loss of 0.25 µmol/ml total GSH equivalents.

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ANTIESTROGEN INDUCED CELL DEATH IN CULTURED MAMMARY CARCINOMA (MCF-7) CELLS, H. Kienzl and W. Bursch

Many studies have demonstrated that antiestrogens block cell proliferation in estrogen dependent cell systems. Relatively little attention has been paid to the occurrence of cell death under antiestrogen treatment. Recent in vivo investigations showed that apoptosis ("programmed cell death") is involved in the regression of an estrogen dependent kidney tumor (H-301). Characteristic signs of apoptotic cell death are condensation of nucleus and cytoplasm and in some cell types the activation of an endogenous endonuclease which cuts the DNA into (oligo-) mononucleosomes.

We have tested whether the antiestrogens Tamoxifen (4-OH-Tamoxifen) and ICI 164.384 (N-n-butyl-11-(3,17ß-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-N-methylundecanamide) are able to induce cell death of cultured estrogen dependent human mammary carcinoma cells (MCF-7).

Our results show:

-inhibition of cell proliferation after antiestrogens, as demonstrated by others

-increase of number of cells with morphological changes such as nucleus condensation and fragmentation

-increase of unsedimentable DNA after antiestrogen treatment, indicating DNA degradation

-induction cell death and inhibition of cell proliferation by antiestrogens are dose dependent

-cell death can be inhibited by estradiol in a dose dependent way

Conclusion:

Tamoxifen and ICI 164.384 are able to induce cell death in MCF-7 cultures, some of its features suggest the occurrence of apoptosis.

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CELL DEATH BY APOPTOSIS IN LYMPHOID CELLS M.Cejna, D.Printz and G.Fritsch

We investigate the in vitro tumoricidal mechanism of antileukemic chemotherapeutic agents, like glucocorticoids, etoposide, cis-Platin on murine and human lymphoid cells such as CCRF-CEM, S.49 and primary rat thymocytes. Induction of apoptosis (= "Programmed Cell Death") appears to be - in addition to inhibition of proliferation - an important mechanism of these drugs. A prerequisite for screening on the significance of apoptosis in the therapy of lymphoid malignancies is its rapid and specific detection.

We investigated whether Flow Cytometry (= FCM) can be used to identify the characteristic nuclear changes of condensation of chromatin and fragmentation of DNA into (oligo-) mononucleosomes in apoptotic cells. We found the formation of a hypodiploid DNA population in the drug treated cells which did not appear in control samples. Sorting of this population with consecutive histological and biochemical characterization confirmed this population to be truely apoptotic. The incidence of apoptotic cells in the other sorted population (G_0, S, G_2/M) was below 5%. Our results suggest that FCM analysis can rapidly quantitate apoptotic cells in small sample volumes. Therefore detection of nuclear changes typical of apoptotic cell death may be a usefull endpoint for therapy monitoring and development of new cytotoxic assays.

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INFLUENCE OF THE EXPERIMENTAL DESIGN ON THE OUTCOME OF IN VITRO CYTOTOXICITY TESTS WITH PROLIFERATING CELLS

M. Gülden, M. Kolossa and H. Seibert

In vitro cytotoxicity tests with proliferating cells have attained considerable popularity. One common practice is to expose growing cultures for 24 hr to various concentrations of a test compound and to characterize the cytotoxicity by an EC_{50}-value referring to the reduction of an indirect measure for the final number of (viable) cells. The present study shows that this approach may result in an underestimation of the general cytotoxicity of some substances. Balb/c 3T3 cells were plated at a density of $6x10^3$ cells per well into 96-well culture plates and exposed after 48 hr to various concentrations of test compounds, either selected for different mechanisms of action or taken from the MEIC-list (Cell Biol. Toxicol. 5: 331-347, 1989). At the beginning and at the end of a 24 hr exposure period the cellular protein content and the amount of lysosomal neutral red (NR) accumulation were measured. The activity of the test compounds was characterized by EC_{50}-values a) for the inhibition of cell growth determined from the 24 hr increase of protein content and NR accumulation, respectively, and b) for the reduction of both measures for the final cell number. Some chemicals revealed remarkably lower EC_{50}-values for growth inhibition than for the reduction of the final cell number indicating a specific inhibition of cell proliferation at subcytolethal concentrations. Other compounds exhibited only sligthly different EC_{50}-values indicating growth inhibition by cytolethal action. We conclude from these results that in in vitro cytotoxicity tests using proliferating cells either the increase of cell number during the exposure period should be determined or exposure times markedly longer than the population doubling time should be employed to ensure the assessment of the cell growth inhibiting activity.

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Lymphocyte subset analysis in peripheral blood of Wistar rats after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

M. Korte, U. Jacob-Müller and R. Stahlmann

Thymus atrophy and immunosuppression are well known effects after exposure to high doses of TCDD in many species. In marmoset monkeys changes in the lymphocyte subpopulations were found to be a sensitive parameter in reflecting biological effects of low doses of TCDD. We investigated whether similar effects on lymphocyte subpopulations could be observed in the Wistar rat. The animals were treated subcutaneously with either a loading-dose of 3000 ng TCDD/kg body wt and weekly maintenance doses of 600 ng/kg body wt or with the vehicle only (toluene/DMSO; 1+2). Blood was taken from a tail vein one, five and eleven weeks after initial treatment. Lymphocytes were isolated using a Ficoll separation procedure and were stained with monoclonal antibodies. Analysis of the surface markers was performed using a Becton Dickinson FACScan. Results are shown in the table (mean ± S.D.).

Group time after treatment	Lymphocytes (% of leucocytes)	$CD4^+$ (%)	$CD8^+$ (%)	B-Cells (%)
Vehicle (n=10)				
1 week	82.0 ± 4.1	68.2 ± 2.0	26.0 ± 2.4	14.7 ± 5.2
5 weeks	84.8 ± 3.2	68.0 ± 3.1	24.7 ± 3.7	14.0 ± 3.4
11 weeks	84.7 ± 5.6	67.2 ± 4.8	23.2 ± 3.2	13.7 ± 4.3
TCDD (n=10)				
1 week	79.6 ± 7.9	66.4 ± 4.7	27.8 ± 4.7	12.7 ± 3.8
5 weeks	83.9 ± 4.3	68.5 ± 3.9	29.7 ± 3.3*	12.0 ± 2.0
11 weeks	86.1 ± 3.8	66.7 ± 3.0	28.1 ± 3.8*	12.2 ± 2.5

* statistically significant compared to controls (t-test; $p < 0.05$)

Although we observed a difference in the $CD8^+$ cells in the two groups we do not consider this as a TCDD-induced effect since the change occurs in the control group. 12 weeks after initial treatment, body and organ weights were determined. The thymus weight was significantly reduced in the TCDD treated group (Control: 230 mg ± 60 mg; TCDD: 150 mg ± 30 mg).

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COMPARISON OF THE INDUCTIVE POTENCY AND TISSUE CONCENTRATION OF 2,3,7,8-Cl4DD WITH A DEFINED PCDD-MIXTURE IN RATS.

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A defined mixture of polychlorinated dibenzo-*p*-dioxins (PCDDs) was administered subcutaneously 16 times (every third day) to male Wistar rats. The applied dose was calculated to contain 57 ng I-TE (international TCDD toxic equivalencies)/kg body wt. The mixture contained the following 2,3,7,8-substituted congeners: 61% Cl5DD, 32% Cl6DD, 6.5% Cl7DD and 0.5% Cl8DD (calculated as % of the total ng I-TE/kg body wt), as well as several non-2,3,7,8-substituted congeners. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Cl4DD) was not a component of the mixture. Concentrations in hepatic and adipose tissue were determined during the treatment period (one day after the 3rd, 8th and 16th injection) as well as 13 and 34 days after the last treatment and correlated with the corresponding **EROD** (ethoxyresorufin O-deethylase) activities in liver microsomes.
Tissue concentrations of the mixture in the liver were between 1.1 and 13.6 ng I-TE/g. A good linear relationship (R^2=87.1%) was found (when using a double-log plot) between the hepatic concentration of the mixture (ng I-TE/g tissue) and the EROD activity. A similar relationship (R^2=95.6%) was observed in rats treated with 2,3,7,8-Cl4DD alone (concentration range in liver tissue 0.2 to 9.7 ng/g). The concentration-response curves for both the PCDD-mixture and 2,3,7,8-Cl4DD run parallel. However, the inductive potency of 2,3,7,8-Cl4DD was approximately 4-fold compared with the mixture using I-TE's (factor for 1,2,3,7,8-Cl5DD : 0.5). When applying the original TE-factor for 1,2,3,7,8-Cl5DD (namely 0.1) the data for the mixture become identical with the data for 2,3,7,8-Cl4DD. There is no difference whether the non-2,3,7,8-substituted congeners are considered (according to UBA/BGA) or not.

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PCB-BINDING TO NUCLEAR COMPONENTS OF LIVER CELLS

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Polychlorinated biphenyls (PCB) are known to cause a great variety of disturbances in cell metabolism including tumor-promoting activity. Most of these effects emanate from reactions within the nucleus. We have therefore investigated the uptake and distribution of the PCB-congener 2,2',4,4',5,5'-hexachlorobiphenyl (HCB) in rat liver nuclei.

Nuclei were incubated with HCB at a concentration of 8.6 nM. In order to prevent dissociation of interacting HCB by subsequent treatment with organic solvents the ligand was covalently linked to nuclear macromolecules by photofixation. This procedure allows for analysis of HCB binding to highly purified nuclear components as checked by electron microscopy and gel electrophoresis.

We found most of the HCB (43 %) was retained in the nuclear envelope. Chromatin bound 34 %, whereas only 7 % were found in the nucleoplasm, bound to soluble proteins. Considering the vital importance of chromatin in the cell we investigated several subfractions of chromatin. A minor percentage (2 %) only of total HCB in the nucleus bound to histones, specifically to the core histones, whereas no HCB could be detected on histone H1. The major part bound to non-histone proteins, namely acidic chromosomal proteins and high mobility group proteins which are both supposed to play a specific role in transcription regulation. Binding of HCB to DNA has been proven to a minor extent of 0,01 %.

From our experiments we conclude that the uneven distribution of HCB in the nucleus hints at its potential impact on cell functions.

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ENDOGENEOUS INTESTINAL METALLOTHIONEIN POSSIBLY CONTRIBUTES TO THE RENAL ACCUMULATION OF DIETARY CADMIUM

B. Elsenhans, K. Kolb and K.H. Flaig

The major target organ of the chronic toxicity of cadmium (Cd) is the kidney. Generally one supposes that after enteral absorption of Cd, the metal first accumulates in the liver inducing hepatic metallothionein (MT) and then redistributes to the kidneys. However, small amounts of dietary $CdCl_2$ seem to accumulate preferentially in the kidneys rather than being accumulated in the liver as shown by feeding $CdCl_2$ at low dietary concentrations (0.3, 1, and 3 mg Cd/kg diet) to female Sprague-Dawley rats for 10, 20, and 30 days. Enterally administered Cd induces MT in the small-intestinal mucosa in a concentration-dependent manner and thus, may directly contribute to the renal accumulation of oral Cd. However, this requires some kind of serosal release of mucosal Cd-MT. To test this hypothesis, we used the Fisher-Parsons method of perfusion of isolated rat small intestine to investigate the serosal release of intestinal MT induced by dietary Cd. Substantial amounts of small-intestinal MT were induced in female Sprague-Dawley rats by feeding dietary $CdCl_2$ (33 and 112 mg Cd/kg diet, group Cd-I and Cd-II, respectively) for 2 wk. In jejunal segments mucosal MT levels were 5±1, 71±15 and 112±13 µg MT/g wet wt. in the control, Cd-I and Cd-II group, respectively; the corresponding levels in the ileum were 4±1, 43±5 and 181±35 µg MT/g wet wt. (n=6, M±SD). The rate of the appearance of MT on the serosal side of the tissue preparation, i.e., the rate of the release of MT into the absorbate, was on average 8, 39 and 46 ng MT/cm/h in jejunal segments of the control, Cd-I and Cd-II group, respectively; corresponding rates in ileal segments were 8, 42 and 162 ng MT/cm/h. These results demonstrate a concentration-dependent release of intestinal MT to the serosal side. When ^{109}Cd-$CdCl_2$ was present in the perfusion medium, ^{109}Cd mainly appeared in the MT fraction of the absorbate. The present findings indicate that endogeneous intestinal MT may deliver Cd-MT to the organism which may contribute to a direct renal accumulation of orally ingested Cd.

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THE INFLUENCE OF OCCUPATIONAL MERCURY EXPOSURE ON THE HUMAN IMMUNE SYSTEM.

Dagmar Lühmann, O. Strubelt* and H. Kirchner

There is some experimental evidence that mercury can depress humoral as well as cell-mediated immunity (Dieter et al., Toxicol. appl. Pharmacol. 68, 218;1983). In order to elucidate the practical relevance of these observations we investigated blood mercury levels and a variety of immunological parameters in 8 male workers from a mercury recycling plant. These data were compared 1. with those of 20 age-matched males and 2. with a group of 20 non-selected blood donors. Mercury blood levels were determined by AAS, serum immunoglobulin (IgG, IgA, IgM) by nephelometry, lymphocyte subpopulations (T, B, T4, T8, NK-cells) by flow cytometry after labelling with monoclonal antibodies and cytokine production with cultured cells in a whole blood assay using phytohaemagglutinin (PHA) to stimulate interleukin-2 (IL-2), interleukin-6 (IL-6) and interferon-gamma (IFN-gamma) production and Newcastle Disease Virus to induce interferon-α_2 (IFN-α_2). Mercury blood levels amounted to 10.5 ± 1.77 µg/l in the plant workers and 1.74 ± 0.34 µg/l and 1.63 ± 0.28 µg/l, respectively, in the controls. White cell count was 24% higher in the workers than in the controls which is presumably due to the higher number of smokers in this group. There was no statistically significant difference in any of the immunological parameters assayed between the groups. A small but significant positive correlation was found between blood mercury levels and elevated PHA-induced IL-2 and IFN-gamma production ($r = 0.4$ and 0.28, respectively).

Conclusions: Our results provide no indication that occupational mercury exposure adversely affects the human immune system or produces reduced immunocompetence.

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EFFECTS OF INORGANIC AND ORGANIC ARSENIC COMPOUNDS ON INTESTINAL ABSORPTIVE FUNCTIONS

G. Hunder, P.T. Nguyen, G. Strugala*

The toxicity of arsenic differs between organic and inorganic compounds and depends on its state of valence. Although the gastrointestinal tract is a target organ in arsenic toxicity and a major site of its absorption, little is known about the effects of different arsenic species on intestinal absorptive functions. In the present experiments, we investigated in vitro the effects of arsenic trioxide (As_2O_3), arsenic pentoxide (As_2O_5) and the organic oxophenylarsine (PhAsO) on intestinal absorption of water, glucose and electrolytes in isolated luminally perfused jejunal segments of rats according to the method of Fisher and Parsons.

As_2O_3 (2.5; 25; 250 µmol/l perfusion fluid), As_2O_5 (25; 250; 2500 µmol/l) and PhAsO (2.5; 10; 25 µmol/l) decreased in a dose dependent manner intestinal water transfer to 25% of the corresponding controls. The intestinal transfer of glucose was decreased and reached only 10% of the control values, at the highest dose level. Serosal to mucosal concentration ratio of glucose (S/M) decreased to 1 whereas in controls a concentration ratio of about 4 was determined. Depending on the transmural waterflux, the transfer of Na^+, K^+ and Ca^{++} was decreased, but the S/M concentration ratio of these electrolytes was not changed.

The most toxic compound tested was PhAsO. Its inhibitory effect was about 10 times higher than that of As_2O_3, which in turn was 10 times more potent than As_2O_5.

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EFFICACY OF DITHIOL COMPOUNDS GIVEN i.p. OR ORALLY IN ACUTE ARSENIC POISONING.

H. Kreppel, B. Schäfer, F.X. Reichl and L. Szinicz

The efficacy of DL-2,3-dimercaptopropanol (BAL), DL-2,3-dimercaptopropanesulfonate (DMPS), and meso-2,3-dimercaptosuccinic acid (DMSA) in reducing lethality and arsenic content of organs was investigated in mice. Additionally, the efficacy of BAL dissolved in peanut oil and in saline was compared. NMRI mice were injected with 85 µmol/kg arsenic trioxide (containing 76-As, organ study) or 130 µmol/kg (survival study). 30 min later the animals were given i.p. or orally equimolar doses (0.7 mmol/kg) of the dithiols or saline (controls). 0.5 h, 2 h, 4 h, 6 h, and 8 h, respectively, after the arsenic injection the 76-As content of several organs (heart, lung, liver, spleen, kidneys, small intestine, large intestine, brain, testes, blood, skin, and muscle) was quantified using a gamma counter. In the 130 µmol/kg group lethality was recorded for 30 days. DMPS, both i.p. and orally, was the most effective dithiol in reducing the arsenic content in tissues, followed by DMSA and BAL/saline; BAL/oil was less effective in reducing the arsenic content in organs. Remarkably, DMPS reduced even faster (within 2 h) than the other drugs the arsenic content of organs. The arsenic content of the brain was significantly higher in both, BAL/oil and BAL/saline treated animals, than in controls and the other experimental groups. In reducing the lethal effects of arsenic, DMPS and DMSA, given i.p., were superior to BAL. Given orally, however, BAL/saline was markedly superior to BAL/oil and the other antidotes, tested. The vehicle (of BAL) as well as the route of administration of the antidotes seem to be important for its antidotal efficacy in the organism.

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INFLUENCE OF NICKEL ON THE INTRACELLULAR FREE CALCIUM IN STIMULATED HEART VENTRICULAR CELLS

A. Stampfl

$NiCl_2$ is known to diminish the force of contraction of a papillary muscle in a concentration of 2 mmol/l (Kaufmann u. Fleckenstein, Pflüg. Arch 282, 290-297, 1965). We investigated the influence of lower concentrations (300 µmol/l and 150 µmol/l) in heart ventricular cells of adult guinea-pigs. We measured the Ca^{++} transients after stimulation using the fluorescence dye Fura 2 under control and experimental conditions in the same cell. The fura loaded cells were placed in a temperature controlled (35 ° C; 0.5 ml)) chamber on a Zeiss IM 35 inverted fluorescence microscope. The chamber was perfused with O_2 saturated modified Krebs Henseleit Buffer (1.2 mM Ca^{++}; 1 ml/min) with or without $NiCl_2$. The fluorescence of one cell was selected with an adjustable rectangular diaphragm. We used an Amko Delta-Scan dual-wavelength fluorimeter for excitation and data acquisition. The measurement of the fluorescence was triggered by the stimulation pulse. The ratio of 340/380 nm excitation represents the free intracellular Ca^{++}. After addition of 300 µmol/l Ni^{++} at 0.5 Hz stimulation frequency, we found a decrease of the ratio by 50% during 82 s. With a stimulation frequency of 0.2 Hz decreased the ratio by 43% during 104 s. After adding 150 µM $NiCl_2$ at 0.5 Hz stimulation frequency we observed a decrease of 20 % during 65 s. Both concentrations of Ni^{++} had no influence on the fluorescence at 360 nm excitation. This is the isobestic point of Fura, where the fluorescence is independent of the Ca^{++} concentration and depends only from the quench.

The results suggest that the effect of Ni^{++} is action dependent and therefore possibly an intracellular effect is involved.

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INFLUENCE OF CIRCADIAN RHYTHM, FEEDING RHYTHM AND FAT CHARGE ON LIPID PEROXIDATION UNDER PEROXISOME PROLIFERATION BY NAFENOPIN W.W.Huber, B.Kraupp-Grasl, C.Gschwentner

Oxidative stress, possibly caused by enhanced peroxisomal ß-oxidative fatty acid degradation, has been discussed as the carcinogenic mechanism of peroxisome proliferators. However, in a recent long term experiment with nafenopin (NAF) there was no association between tumor rate and indicators of oxidative stress (e.g. thiobarbituric acid reactive substances = TBARS). The influences of daytime and fatty acid supply on TBARS formation under NAF were investigated. Male wistar rats received NAF in the diet (100 mg/d/kg b.w.) for 10 days. There were three feeding protocols: 1) ad libitum, 2) food 5 hours per day, 3) as (2) but a single dose of corn oil (i.g, 20ml per kg b.w.) instead of food on the last day. The following parameters were determined at 5-7 time points during 24 hours: a) TBARS, b) peroxisomal ß-oxidation, c) carnitin-acetyl-transferase, d) rel. liver wt., e) serum and liver triacylglycerol. NAF enhanced all indicators of peroxisome proliferation (b-d), reduced triacylglycerol in serum and liver but did not induce any increase in TBARS. Major variations with regard to time point were only found after corn oil treatment. Corn oil alone led to a strong increase in hepatic triacylglycerol associated with a slight increase in TBARS. Pretreatment with NAF markedly reduced the corn oil induced increase in triacylglycerol and prevented any significant increase in TBARS. Thus, any synergistic effect of peroxisome proliferator treatment and a high fat diet on carcinogenesis seems unlikely.

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APPLICATION OF A NEW ESR-METHOD TO DETECT CHANGES OF GSH TISSUE LEVELS AS A CONSEQUENCE OF TEMPORARY ORGAN ISCHEMIA H. Nohl

Generation of highly reactive oxygen species was repeatedly suggested to cause organ-injury following reperfusion of ischemic organs. However, methodological problems to detect these short lived compounds do not allow a clear recognition of their existence and pathogenetic significance. Glutathione (GSH) is considered a sensitive indicator of imbalanced oxidant generation in the tissue. Thus, the continous monitoring of GSH-levels was taken as a reliable approach to estimate the existence of an "oxidative stress" following reperfusion of the ischemic heart. Methods frequently used to determine tissue levels of GSH require homogenation of the respective organs. We applied instead a sensitive ESR-method recently developed (1). The essential of this method is a GSH-induced disruption of a biradical to a stable mono-radical. Both types of radicals exhibit different ESR-signals allowing qualitative and quantitative estimation of GSH. In the present paper we tested the validity of this method to detect alterations of heart GSH-levels. Interestingly we observed the appearence of the paramagnetic split product in the perfusate following heart-perfusion with the GSH-trapping biradical. This was due to differences in the partition coefficient favouring accumulation of the biradical preferentially in the extravascular system and redistribution of the monoradical into the vascular system. Monitoring of the paramagnetic reaction product in the effluent perfusate elicited significantly lower and also faster decreasing GSH-levels in rat hearts following ischemia as compared to the controls.

(1) V.V. Khramtsov et al., Anal. Biochem. 182, 58-63, (1989).

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CARDIOTOXIC EFFECTS OF NITROFURANTOIN AND t-BUTYL-HYDROPEROXIDE IN ISOLATED RAT HEARTS
Bettina Biel, H. Brasch and M. Younes

Redox cycling compounds which produce free oxygen radicals were used to study the importance of oxidative stress for the development of myocardial damage.
Isolated rat hearts were perfused in a nonrecirculating manner (Langendorff technique) with Krebs-Henseleit solution (4 ml/min; 37° C; 2.5 mmol/l $CaCl_2$) and were stimulated at a rate of 3.33 Hz. In 7 control hearts the force of contraction (43.5 ± 1.6 mN) and the efflux rates of the enzymes CPK and LDH (140 ± 40 and 56 ± 5 mU/g*min, respectively) and of the reduced form of glutathione (GSH; 0.6 ± 0.1 nM/g*min) varied little during a 75 min observation period. Oxidized glutathione (GSSG) was not detected in the efflux from control hearts.
Nitrofurantoin (0.25 and 0.5 mmol/l) had a negative inotropic effect. Perfusion with 0.5 mmol/l for 75 min decreased the force of contraction from 47 ± 2 to 4 ± 1 mN. Simultaneously, the efflux of GSH rose from 0.6 ± 0.2 to 5.3 ± 0.4 nM/g*min and GSSG (1.9 ± 0.6 nM/g*min) appeared in the effluent. The release of CPK and LDH increased to maximum values of 730 ± 221 and 611 ± 199 mU/g*min, respectively. At the end of the experiments the tissue concentration of glutathione was smaller (5.5 ± 0.63 nmol/mg protein) than in the control hearts (9.98 ± 0.78 nmol/mg protein) while the concentration of malondialdehyde was significantly increased (16.60 ± 1.46 versus 9.00 ± 0.75 nmol/mg protein). Similar results were obtained with t-butylhydroperoxide (0.25 mM).
Addition of either catalase (100 mU/ml) or catechin (0.5 mmol/l) to the perfusion fluid did not attenuate the negative inotropic effect of nitrofurantoin or the associated increase of enzyme efflux but it prevented the appearance of GSSG in the effluent.
These results show that redox cycling compounds can produce oxidative stress in isolated rat hearts. But apparently oxygen radicals are not the main cause for their cardiotoxic effects.

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THE VITAMIN K CYCLE AS A CELLULAR ANTIOXIDANT

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The cellular vitamin K cycle comprises 1) the reduction to the hydroquinone, 2) the oxidation to the epoxide, 3) the reduction to the quinone. In the hepatocyte the epoxidation is linked to the gamma-carboxylation of glutamic acid residues in clotting factor proteins. Also for some non-hepatic tissues vitamin K dependent proteins are known, such as bone Gla protein and matrix Gla protein excreted by osteoblasts. Functions of the vitamin K cycle in other tissues so far are unknown. We investigated whether the vitamin K cycle may serve as a radical scavenger in biosystems. Micro-somal peroxidation stimulated by either Fe^{2+}/ascorbate, NADPH/ATP/Fe^{2+}, or NADPH/CCl_4 was strongly suppressed by an operating vitamin K cycle; for instance, 4.5 μM vitamin K with 1 mM DTT as dithiol d onor for the microsomal vitamin K reductase suppressed the NADPH/CCl_4 induced lipid peroxidation almost completely. The vitamin K effect could be inhibited by warfarin. Evidence for an in-vivo antioxidant function was obtained by observing warfarin to potentiate the paracetamol hepato-toxicity in rats; paracetamol, 200 mg/kg, given to methylcholanthrene-induced rats was lethal within 20 hr when co-administered with warfarin (0.2 mg/kg).

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INHIBITION OF NO_2 INDUCED DNA SINGLE STRAND BREAKS IN V 79 CELLS BY ANTIOXIDATIVE VITAMINS

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Humans are exposed to NO_2 by inhalation e.g. of tobacco smoke or by endogenous formation of this compound as a metabolite of NO, which itself is either generated as a physiologically essential substance or as a metabolite of N-nitroso compounds. NO_2 is able to damage DNA, proteins and lipids and was active as a tumor promoter. Previously we could show that NO_2 induces DNA single strand breaks (SSBs) in cultivated chinese hamster lung (V 79) cells. Antioxidative vitamins are supposed to protect cells against NO_2 induced damages.

Therefore V 79 cells were incubated with micromolar concentrations of various tocopherols, retinol, ß-carotene or ascorbic acid for 30 minutes. Then cells were treated for 10 minutes with 200 ppm NO_2. The rate of SSBs was measured by the alkaline elution assay, the amount of DNA by a fluorimetric assay.

The results show, that τ-tocopherol inhibits the rate of NO_2 induced SSB to 40%, ß-carotene and ascorbic acid to 25%. None of these vitamins had any effects on DNA or the viability of cells. When the experimental conditions were slightly changed, retinol was also able to inhibit the NO_2 induced SSBs to 35%. However, in high concentrations, retinol itself induced SSBs and reduced the cell viability.

In conclusion τ-tocopherol and less ß-carotene and ascorbic acid may have the potential to play an important role in protecting cells against NO_2 induced DNA-damage. However, retinol can pass as a protective substance only with reservation, since the toxic and protective effects of this vitamin are strictly concentration dependent.

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RADICAL-SCAVENGING PROPERTIES OF FLAVONOIDS IN HUMAN GRANULOCYTES.

C.-P. Siegers, A. Röbke, B. Steffen, Z. Guo and R. Pentz

Flavonoids are potent radical-scavengers against activated oxygen species, as evidenced by their ability to depress lipid peroxidation in a model of glutathione-depletion-induced spontaneous lipid peroxidation in rat liver homogenates (Planta medica 43, 240-244, 1981). In a model of phorbol-myristate-acetate-stimulated (PMA) superoxide anion generation (O^-_2) the influence of free and glycosidic-bound flavonoids was investigated; O^-_2 was measured indirectly by the oxidation of cytochrome C. PMA alone evoked a 10-fold increase in O^-_2-formation in granulocytes (basal value: 1-3 nmol/10^6 cells); the solvent for all tested compounds, propandiol decreased the PMA-stimulated O^-_2-generation by 20%. (+)-Catechin showed a concentration-dependent inhibition of the O^-_2-formation with an IC_{50}-value of 11 µmol/l. Quercetin was also very active with an IC_{50}-value of 26 µmol/l. The glycosidic forms of quercetin, quercitrin and rutin, were less active as evidenced by higher IC_{50}-values of 179 and 66 µmol/l, respectively. The known degradation products of quercetin, occurring after oral application of quercetin or rutin in the systemic circulation, 3,4-dihydroxyphenylacetic acid and 4-methylcatechol, inhibited the O^-_2-formation with IC_{50}-values of 95 and 125 µmol/l, respectively. Naringenin lacking a 3,4-dihydroxy-grouping was ineffective in this respect indicating that this molecular structure seems to be an essential prerequisite for the scavenging properties of flavonoids against oxygen free radicals.

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ANTIOXIDANTS INCREASE LACTATE AND PYRUVATE PRODUCTION AND THE FORMATION OF CARBON DIOXIDE FROM GLUCOSE AND GLYCEROL IN HEPATOCYTES.

A.Bredy, C.Guthardt, and R.Kahl

The influence of two antioxidants, the hydroxyl radical scavenger dimethyl sulfoxide (DMSO) and the iron chelator o-phenanthroline (OP), on glycolysis in freshly isolated rat hepatocytes was examined. Both antioxidants increased the production of lactate + pyruvate in the presence of 5.5 mM glucose in the medium by about 40% within a 30 min incubation period (DMSO: 142 ± 14 %; OP 138 ± 7 % of control). The formation of carbon dioxide from glucose labeled at C1 was also stimulated by both agents (DMSO: 122 ± 7 % ; OP: 138 ± 9 % of contro). Only part of this increase was due to protection of glycolysis (carbon dioxide formation from glucose labelled at C6: DMSO: 117 ± 6 %; OP: 118 ± 3 % of control). When a tracer dose of radioactively labelled glycerol was administered to the hepatocytes instead of glucose, a much higher increase of carbon dioxide formation by the antioxidants was observed than that detected with glucose as the substrate (DMSO: 195 ± 40 %; OP: 527 ± 57 % of control). These results suggest that the glycolytic pathway as well as the hexose monophosphate pathway in freshly prepared hepatocytes are moderately suppressed by oxidative stress and that the inhibition can be relieved by antioxidants. In addition, the basal rate of other reactions of carbohydrate and/or fat metabolism may also be reduced by reactive oxygen.

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DETERMINATION OF 8-HYDROXYDEOXYGUANOSINE IN DNA OF HEPATOCYTES AFTER TREATMENT OF RATS WITH 2-NITROPROPANE, PHORONE OR N-NITROSOMORPHOLINE.

M.Dahlhaus, R.Kramer, J.LaSoe

Oxidative damage to DNA is believed to be a major contributor to aging and other associated degenerative processes. Also the genetoxicity of several mutagens and carcinogens is considered to be caused by the effects of reactive oxygene species (ROS) on the genome. The determination of the oxidised nucleoside 8-hydroxydeoxyguanosine (8-OH-dG), one of the ≈ 20 known oxidative DNA damage products, is generally used to record the attack of e.g. hydroxy radicals on cellular DNA.

The aim of this study was to determine the formation rate of 8-OH-dG after administration of various compounds, for which generation of ROS during their metabolism can be assumed. The test compounds were administered by i.p. injection to male F 344-rats. After 6 hours rats were killed, livers exised and immediately homogenised. DNA was isolated and hydrolysed by nuclease P1 and alkaline phosphatase. Deoxynucleosides were separated by HPLC and detected using UV- and electrochemical detection systems. The ratio of 8-OH-dG/dG indicates the extend of DNA damage.

The results show, that administration of 2-nitropropane (2-NP), a widespread industrial solvent and hepatocarcinogen, led to a 5-6 -fold increase in the amount of 8-OH-dG. Phorone, which is able to deplete glutathione, was slightly effective. The combined administration of Phorone and 2-NP led to no measurable increase of 8-OH-dG compared with 2-NP treatment alone. Rats treated with the liver carcinogen N-nitrosomorpholine (NMOR) in various doses showed no significant increase of 8-OH-dG formation above control values.

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THE DETECTION OF PERFERRYL-HEME COMPOUNDS BY CHEMILUMINESCENCE K. Stolze

In a previous study we have postulated the transient existence of a perferryl species during the formation of MetHb from hydroxylamine derivatives and butylated hydroxyanisole (BHA). The strong oxidizing property of this compound was proven by the formation of a phenoxyl type radical from BHA in the presence of MetHb and H_2O_2 (1). More detailed studies of this compound revealed transient light emission during MetHb generation from hydroxylamine and, to a lesser extent, from BHA. Chemiluminescence was also observed when other ferriheme compounds were mixed with H_2O_2. Simultaneously paramagnetic species were generated suggesting the following set of reactions:

1) $Met(HX\text{-}Fe^{III}) + H_2O_2 \longrightarrow Met(^{\cdot}X\text{-}Fe^{IV}\text{-}OH) + H_2O$

2) $Met(^{\cdot}X\text{-}Fe^{IV}\text{-}OH) + H \longrightarrow Met(HX\text{-}Fe^{IV}{=}O) + H^{+} + light$

The identity of the light emitting species is unclear. The absence of oxygen decreases light intensity and affects its generation and decay kinetics. Analysis of the light spectra using interference and cut off filters does not exclude singlet oxygen as one main light source but other types of chemiluminescence generation cannot be excluded. The main subject of our investigation is to clarify whether the observed chemiluminescence is characteristic for ferryl compounds so that it can be used for their detection.

(1) K. Stolze and H. Nohl, Methemoglobin Formation from Butylated Hydroxyanisole and Oxyhemoglobin. Comparison with Butylated Hydroxytoluene and p-Hydroxyanisole. Free Radical Res. Comms. (1991), in press

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HEMOGLOBIN BINDING OF AROMATIC AMINES: MOLECULAR DOSIMETRY AND QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP OF THE N-OXIDATION, G. Sabbioni

We have established the hemoglobin binding index (HBI) [(mmol compound / mol Hb) / (mmol compound / kg body weight)] of several aromatic amines in female Wistar rats.: aromatic amine (HBI): 4-fluoro- (33), 4-iodo- (296), 4-bromo- (341), 4-trifluoromethyl- (148), 4-ethyl- (5.8), 4-methylmercapto- (2.5), 3-chloro- (18), 3-ethyl- (12.7), 2-chloro- (0.5), 2-ethyl- (5.1), 2,4-dichloro- (2.3), 2,6-dichloroaniline (not detected.), 2,4-difluoro- (32), 3-chloro-4-fluoro- (31), 2,5-dimethyl- (7.3), 2,6-dimethyl- (1.1), 3,4-dimethyl- (0.7), 3,5-dimethyl- (14) and 2,4,6-trimethylaniline (0.1).
For our studies of quantitative structure activity relationships we included HBI's determined by other researchers in the same rat strain. The logarithm of hemoglobin binding (log HBI) was plotted against the following parameters: the sum of the Hammett constants ($\Sigma\sigma = \sigma_p + \sigma_m$), pKa, log P (octanol/water), the half wave oxidation potential ($E_{1/2}$) and the electronic descriptors of the amines and their corresponding nitrenium ions obtained by semiempirical calculations (MNDO, AM1 and PM3), such as atomic charge densities, energies of the HOMO and LUMO and their coefficients, the bond order of C-N, the dipole moments and the 'reaction enthalpy' [MNDOHF, AM1HF or PM3HF = Hf(nitrenium) - Hf(amine)]. The amines were classified in three groups: Group 1, all para substituted amines: log HBI correlates with $\Sigma\sigma$, AM1HF, $E_{1/2}$, pKa and logP with r=0.84, 0.71, 0.73, -0.69 and 0.60 , respectively.
Group 2, all amines with halogens: log HBI correlates with pKa, $\Sigma\sigma$, MNDOHF, $E_{1/2}$ and logP with r=0.81, -0.80, -0.55, -0.46, and -0.20, respectively.
Group 3, all amines with alkyl groups: log HBI correlates with with $E_{1/2}$, PM3HF, $\Sigma\sigma$, pKa and logP with r=0.92, 0.89, 0.76, 0.24, and 0.12, respectively.

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EFFICACY OF TWO NEW OXIMES IN SOMAN POISONED GUINEA PIGS

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The oxime HI 6 (1-[[[4-(aminocarbonyl)-pyridino]-methoxy]-methyl]-2-(hydroxyimino)methyl]pyridinium dichloride) proved to be quite effective in the therapy of soman (SO) poisoning but is less effective in tabun poisoning. Recently, HLö 7 (1-[[[4-aminocarbonyl)pyridinio]methoxy] methyl]-2,4-bis-[hydroxyimino)methyl]-pyridinium diiodide) was shown to reactivate acetylcholinesterase (AChE) inhibited by SO and tabun. We now investigated the efficacy of HLö 7 dimethane sulfonate and HI 6 on cardiorespiratory pattern in guinea-pigs poisoned by 10 x LD50 SO.
Female Pirbright-white guinea-pigs were anesthetized with urethane and the a. carotis, v. jugularis and trachea were cannulated. After base line measurements SO (160 ug/kg = 10xLD50) or saline (both i.v.) were injected. 2 min later saline, atropine (AT, 10 mg/kg), HI 6 (30 umol/kg) + AT or HLö 7 (30 umol/kg) + AT (all i.v.) were administered. Circulatory (heart rate and mean arterial pressure) and respiratory data (respiratory rate and minute volume) were recorded for 60 min or until death. Blood was taken before drug administration and at the end of the experiment for measurement of AChE activity.
SO administration resulted in a rapid respiratory arrest followed by circulatory failure (mean survival time 7.8 min). In the SO + AT group a significant but transient improvement of circulation was observed. Due to respiratory arrest the circulation failed after a mean of 9.5 min. HI 6 + AT improved circulation almost completely but respiration only to about 40% of base line and the animals died after a mean of 31.4 min. The therapy with HLö 7 + AT restored circulation completely, too, but improved respiration only partially and the survival time increased to 52.8 min.
AChE was totally inhibited by SO and was not reactivated by HI 6 or HLö 7.
The results of this study indicate that the therapy of superlethal SO poisoning with AT alone is insufficient. The combination of HI 6 or HLö 7 with AT may significantly improve respiration. These data suggest that the oxime HLö 7 is at least as efficient as HI 6 in the therapy of SO intoxication.

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LIQUID/SOLID/LIQUID-EXTRACTIONS WITH DIFFERENT SORBENTS: A COMPARATIVE STUDY

W. Dünges, H. Jolaei-Moghadam and G. Özgül

In chromatographic or gentoxicological investigations of drugs or toxicants in biological or environmental fluids liquid/solid/liquid-extraction is the standard sample preparation procedure.

For hplc analysis of drinking water the first step is sorption of the analytes on surface modified silica (particle size 40 µm, "reversed phase material", rpm), then: treatment with a solvent, followed by concentration of the desorbate to 1 ml, up to 1/4 of which is analyzed by hplc. In 2 l samples (bed volume: 2 ml) pesticides were thus quantitated down to 20 ppt-concentrations, G. Werner, DVGW-Schriftenreihe Wasser, 65 67-87 (1988).

For assaying mutagenicity, drinking water is passed through columns with a macroporous polystyrene resin (particle size 700 µm, e.g. XAD 2). After desorption with an organic solvent and further concentration, aliquots are investigated with Ames' technique, see e.g. S. Onodera, J. Chromatogr. 557 413-427 (1991).

For gc analysis 20 to 100 ml water samples are passed through a column with 0,2 ml sorbent, desorption with ether, concentration to 5 µl. With rpm as sorbent we have recovered pesticides (40 ppt-range) in high yields, W. Dünges, H. Muno and F. Unckell, DVGW-Schriftenreihe Wasser, 108 187-208 (1990).

Using our micro method we have examined the extraction efficiency of rpm and of resins with comparable particle size. The recovery of a test mixture consisting of chemically different compounds (ppb-range) was not satisfactory for all compounds in many of the investigated systems.

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SURAMIN - A CHROMATIN-INTERACTIVE POLYANION

A. Ignatius, K. Tempel, and M. Hund

Although the polyanion suramin (SA) has recently entered clinical trials as a novel antitumor compound, the postulated mechanism(s) of action are still controversial (La Rocca, R.V., et al.: J. Steroid Biochem. Mol. Biol. 37: 893, 1990).
Therefore, potentially nucleotoxic effects of SA were studied in primary cultures of rat and chick embryo cells by using some short-term tests. At therapeutically relevant serum concentrations the cells were exposed to SA for 30-60 min. The principal results may be summarized as follows:
At concentrations of > 20-40 µg/ml, SA inhibited scheduled and unscheduled (UDS) DNA synthesis, enzymatic DNA breakdown (DNase I), and a DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase. An increase in activity was observed in poly(ADP-ribose)polymerase. Within a lower concentration range (< 20 µg/ml), SA slightly stimulated UDS. At concentrations of > 40-60 µg/ml, SA enhanced nucleoid sedimentation and diminished the viscosity of alkaline cell lysates.
The present results suggest that SA is able to interfere either directly (because of its polyanionic nature) or indirectly (because of chromatin condensation) with enzyme systems critical to cellular proliferation.

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DNA MODIFICATIONS INDUCED BY PHOTOSENSITIZATION

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Specific repair endonucleases were used to quantify various types of DNA modification induced under cell-free conditions by photosensitization (acridine orange, proflavin and hematoporphyrin in the presence of visible light, acetone in the presence of UV^{330}). The DNA damage profiles thus obtained were compared with those generated by direct excitation of DNA, chemically generated singlet oxygen and hydroxyl radicals.

While the DNA damage profile induced by hydroxyl radicals is dominated by single strand breaks and sites of base loss (sensitive to exonuclease III and endonuclease IV), the damage profiles produced by both singlet oxygen and by the photosensitizers in the presence of visible light consist predominantly of base modifications sensitive to FPG protein (formamidopyrimidine-DNA glycosylase), a repair endonuclease which so far has been demonstrated to recognize 8-hydroxyguanine and formamidopyrimidines. Excitation of acetone by UV^{330} generates pyrimidine dimers (sensitive to UV-endonuclease from M. luteus) and base modifications sensitive to FPG protein in a ratio of 2:1.

A modified alkaline elution assay was used to quantify endonuclease-sensitive DNA modifications in L1210 (mouse leukemia) cells treated with acridine orange plus visible light. High levels of FPG-sensitive modifications were detected. We conclude that for this dye the DNA damaging mechanism in the cells is largely the same as that found under cell-free conditions.

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DIFFERENT BINDING OF METHYL BROMIDE TO BLOOD MACROMOLECULES DUE TO CONJUGATOR STATUS

A. Müller, U. Jorritsma, B. Gansewendt

In human erythrocytes, an enzymatic polymorphism has been described for the conjugation of methyl bromide to glutathione. 70 % of the human population are conjugators, 30 % lack this enzyme activity (non-conjugators). Preliminary experiments showed marked differences between conjugators and non-conjugators in the distribution of ^{14}C-methyl bromide in blood compartments . Following the same dose of methyl bromide, the non-conjugators showed significantly higher levels of radioactivity in lymphocytes. After incubation of whole blood samples of conjugators and non-conjugators with the same dose of methyl bromide, higher SCE rates could be observed in the group of the non-conjugators.
The genotoxic potential of methyl bromide has been attributed to the direct alkylation of DNA. The results of these preliminary experiments suggest that differences in DNA-alkylation due to this polymorphism are responsible for the observed disparity in genotoxic effects.
In the study presented here, whole human blood samples (9ml) of conjugators and non-conjugators were incubated in gas-tight headspace-vials (22 ml) with different doses of ^{14}C-methyl bromide at 37° C under constant rolling. After 6 h, plasma was separated and lymphocytes were isolated by density centrifugation. DNA was isolated by phenol extraction and human serum albumin was separated by affinity chromatography.
Striking differences were determined concerning the covalently bound radioactivity in lymphocytes; the group of non-conjugators showed clearly higher values. Accordingly, higher levels of alkylation were observed in serum albumin of non-conjugators. After hydrolysis and HPLC-separation, S-^{14}C-methyl-cysteine was determined in this protein. These differences in alkylation of target macromolecules caused by the described enzyme polymorphism influence the genotoxic risk of methyl bromide. The results presented here demonstrate a coincidence concerning alkylation levels of lymphocyte-DNA and serum albumin. The measurement of methylated serum albumin appears to be a suitable parameter for biomonitoring of workers exposed to methyl bromide.

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BAY-REGION DIOL EPOXIDE-DNA ADDUCTS OF DIBENZ[a,h]-ANTHRACENE AFTER METABOLIC ACTIVATION IN VITRO

J. Fuchs and J. Mlcoch

The absolute stereochemistry of various bay-region diol epoxide-DNA adducts of dibenz[a,h]anthracene (DBA) was assigned. DNA adducts were obtained after metabolic activation with liver microsomes from Aroclor 1254 induced male Sprague-Dawley rats in vitro and were separated by reverse phase HPLC chromatography. The absolute configurations of the adducts were determined by coelution with adducts from the reaction of the (+)- and (-)-3,4-dihydroxy-dihydro-DBA after activation and also from the reaction of the anti- and syn-3,4-dihydroxy-1,2-epoxy-tetrahydro-DBA with DNA or with individual deoxyribonucleotides. The main bay-region DNA adduct of DBA was formed via (-)-3R,4R-dihydroxy-3,4-dihydro-DBA and was identified as an anti-3S,4R-dihydroxy-1R,2S-epoxy-1,2,3,4-tetrahydro-DBA derived deoxyguanosine adduct. One minor deoxyguanosine adduct originated from the anti-1,2-epoxide of (+)-3S,4S-dihydroxy-3,4-dihydro-DBA. Syn-1,2-epoxides of the two enantiomeric dihydrodiols of DBA formed four different deoxyguanosine adducts to a lesser extent. The absolute stereochemistry for six of probably eight different deoxyadenosine adducts could be assigned.
Using ^{14}C-labeled DBA the amount of bay-region anti-dihydroxy-epoxy-tetrahydro-DBA adducts of deoxyguanosine was calculated to be 18% of the total binding, syn-diol epoxide-deoxyguanosine adducts of DBA represented a fraction of 6%. Bay-region diol epoxide-deoxyadenosine adducts were responsible for 4% of adduct associated radioactivity. Bay-region diol epoxide-DNA adducts represented only 25% of the total DNA adducts. The major proportion of adduct related radioactivity eluted at more polar conditions.

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IN VITRO STUDIES OF THE GENOTOXIC EFFECT OF TOLUENE DIISOCYANATE (TDI)

B. Marczynski, A. B. Czuppon, E. Lammich, W. Marek, and X. Baur,

Toluene diisocyanate (TDI) used especially for the production of polyurethanes is known to induce chromosome aberrations, base-pair substitution and frameshift mutation after metabolic activation. Inhalation of TDI vapours is associated with immediate type hypersensitivity, direct toxic effects and allergic alveolitis. The effect of TDI immediately upon genomic DNA has not yet been investigated. Following treatment of human blood by TDI, the isolated DNA was analysed by anion-exchange chromatography (FPLC), and DNA from white blood cells (WBC) was analysed by alkaline and neutral filter elution and pulsed-field gel electrophoresis (PFGE).
The results show that TDI induces single and double strand breaks in DNA of WBC in vitro. The elution rates calculated after alkaline filter elution were significantly increased after TDI treatment. TDI induced DNA fragments of around 200 kbp. Denaturation and renaturation of TDI treated DNA indicated that DNA could be cross-linked by TDI. Purified DNA treated with TDI in buffer alone showed none of the double strand breaks as assessed by FPLC. These findings suggest that DNA damages could be induced by TDI after biotransformation of the latter. Further studies are needed to correlate these results with the phenomenon of "apoptosis" which is considered to be due to the activation of endogenous endonucleases in cells such as lymphocytes in response to a variety of adverse conditions.

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MUTAGENICITY OF AROMATIC AMINE-DNA-ADDUCTS

R.S. Kerdar, D. Wild

The discovery that certain carcinogenic heterocyclic aromatic amines occur in fried fish, fried beef and beef extract has attracted renewed attention to aromatic amines. Although their genotoxic effects have been studied in various ways, so far they cannot be connected with each other or with the chemical structure of the amine. One main question concerns the mutagenicity of adducts: is it the mere presence of an adduct which determines the mutagenic outcome or does the specific chemical structure of the amine bound to DNA play a role? We aimed to study the two consecutive effects, DNA-adducts and mutations in *Salmonella typhimurium* and to relate these to chemical structure. For this purpose, the ultimate reactive species of several aromatic amines (arylnitrenium ions) were generated by the arylazide photolysis technique, DNA-adducts were assayed by the ^{32}P-postlabelling technique, mutations were assayed by the Salmonella reversion test.

The nitrenium ions derived from the heterocyclic cooked food mutagens MeIQ and IQ produced the highest levels of DNA-adducts; the levels decreased in the sequence 2-aminofluorene, NI, MeIQx, 1-aminopyrene, PhIP, 4-aminobiphenyl (see Wild and Dirr, Mutagenesis 4, 446, 1989 for full names and chemical structures). The nitrenium ions of MeIQ and IQ were also the most mutagenic, those of PhIP and 4-aminobiphenyl the least mutagenic. These and other data suggest that the DNA-adducts derived from these amines are equally mutagenic. However, the adducts derived from 2-aminofluorene appear to be relatively weakly mutagenic, those derived from 1-aminopyrene highly mutagenic. Thus the yield of mutations does not only depend on the frequency of adducts but also on their aromatic ring structure.

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THE POSSIBLE ROLE OF α,β-UNSATURATED CARBONYL COMPOUNDS IN CARCINOGENESIS: GENOTOXIC ACTIVITIES, DNA-ADDUCT FORMATION, AND DEVELOPMENT OF SENSITIVE DETECTION METHODS FOR DNA-ADDUCTS.

S. Scheckenbach, C. Deininger, C. Hoffman and E. Eder

α,β-Unsaturated carbonyl compounds are chemically reactive, industrially important and environmentally ubiquitous compounds. They are natural products which are formed endogenously, e.g. during lipid peroxidation, and are considered to play an important role in mutagenesis and carcinogenesis. We established structure-mutagenicity relationships and investigated their genotoxic effects in the SOS-chromotest as well as their strand-breaking abilities using the alkaline elution technique. The underlying mechanism for genotoxic effects is most probably their formation of DNA-adducts. We identified two types of regioisomers of 1,N^2-cyclic deoxyguanosine adducts, 7,8-cyclic adducts, 7-linear adducts and 1,N^2,7,8-biscyclic adducts as well as 1,N^2-cyclic, 7-linear bisadducts. Although endogenously formed acrolein congeners are a constant source of DNA-damage, no clear data are available for the extent of this damage in humans. For a more precise assessment of the DNA-damage sensitive detection methods for such DNA-adducts are necessary in order to evaluate the role of these compounds in human carcinogenicity. Optimized hydrolyzation methods of the modified DNA and of the enrichment of DNA-adducts led to an improvement in the detection. In particular, separation of modified nucleosides by Sephadex LH-20 chromatography prior to HPLC-analysis resulted in a clear increase in detectability. Nevertheless the detection sensitivity is not sufficient for an in vivo analysis of adducts. Therefore, we are at present developing a highly sensitive ^{32}P-postlabelling technique using HPLC-separation for adduct enrichment.

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INFLUENCE OF ETHANOL ON ORGANSPECIFIC GENOTOXIC EFFECTS OF N-NITROSONORNICOTINE IN MICE.

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The influence of ethanol on genotoxic effects of the tobacco specific nitrosamine N-nitrosonornicotine (NNN) was investigated in animal mediated DNA repair tests with *E. coli* K-12 strains differing in repair capacity (uvrB/recA vs. uvr+/rec+). Mixtures of the strains were given iv. into mice. 2 hrs later, induction of DNA damage was measured by determination of the strain survival rates. NNN (120-24 mg/kg i.v.) caused dose related effects in all organs (liver≫lungs>kidneys>blood>spleen). When ethanol (8-2 g/kg) was given orally 24 hrs before the NNN (60 mg/kg), a substantial increase of genotoxicity was found (20-40% at the highest dose) in all organs. A similar effect was observed with acetone (3,6 g/kg; given 15 hrs before NNN). On the contrary, ethanol administration 1 hr before NNN treatment resulted in a reduction of DNA damage. Biochemical mechanisms responsible for the dual effects of ethanol (modulation of P450IIE1) are discussed.

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TIME DEPENDENT INCREASING MUTAGENICITY OF CHLOROPRENE

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Chloroprene (2-chloro-1,3-butadiene) is used as an intermediate for the production of chemical resistant plastics (e. g. Neoprene™) and as an ingredient of glues. Due to inconsistent data the substance has not yet been classified by the IARC (International Agency for Research on Cancer) in regard to its carcinogenic potential. The close structural relationship to vinyl chloride and 1,3-butadiene, which both are proven carcinogens in animal experiments and the inconsistant data prompted us to reinvestigate the chemical in the Ames- and the SCE- Test (Sister Chromatid Exchange).

Pure chloroprene is unstable. The substance therefore was purchased as a 50% solution in xylene and freshly distilled prior to each experiment. Due to the volatile character of chloroprene the Ames Test with *S. typhimurium* TA 100 was carried out as a preincubation test in gas tight screw cap vials with or without addition of S9 Mix or glutathione. Cytotoxicity of chloroprene in TA 100 was tested in parallel experiments with one single dilution of an aliquot of the stock culture.

Under the conditions discribed above freshly prepared chloroprene was only slightly mutagenic. Addition of S9 Mix had no effect. However, a mutagenic effect occured linearly with increasing age of the distilled substance. The revertants per µmol induced by aged chloroprene were the same whether the substance was incubated gasthight or not. The mutagenicity of "aged" chloroprene was doubled with the addition of S9. Glutathione in physiological concentrations (with and without S9 Mix) reduced the mutagenicity and the cytotoxicity independent of the age of the substance. The increase of mutagenicity with aging of the distillate was far slower when the substance was kept under nitrogen or argon.

Suprisingly the distillate could be splitted in fractions of different activity without change of the boiling point. The last fraction was by far the most active in regard to the time dependent increase of mutagenicity. To check if these findings correlate to the occurence of an additonal substance in the distillate, the fractions were analysed by gas chromatography. It revealed three peaks in the aged last fraction of the distillate, which were not to be seen when the same sample was analysed immediately after distillation. These peaks were hardly visible in aliquots of these samples kept under argon.

SCE determination according to Perry & Wolf was performed in isolated human lymphocytes. Following 24 hours of cultivation, chloroprene was added and incubated for 48 hours. A dose dependent enhancement of the rate of SCE was found.

Our experiments show that chloroprene induces sister chromatid exchange in isolated human lymphocytes. Mutagenicity in the Ames-Test is low with freshly distilled chloroprene and not inducable with S9 but increases with aging of the instable chemical. Thus mutagenicity of chlorprene is due to a succsessive product rather than to the substance itself.

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GENOTOXIC AND CELL-TRANSFORMING PROPERTIES OF 5-AZACYTIDINE IN THE SYRIAN HAMSTER EMBRYO CELL SYSTEM

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5-azacytidine (5-AC) is an effective antineoplastic compound which has been used in the treatment of leukemia. It induces tumors in several organs of rats and mice. The mechanisms of these effects are still poorly understood although it is known that 5-AC can interact with DNA synthesis and DNA methylation. Furthermore the known data on its clastogenic and/or gene mutation inducing potential are still controversial. Therefore we have investigated which kind of genotoxic effects are caused by 5-AC in Syrian hamster embryo (SHE) fibroblasts. It is possible to assay three biological endpoints (micronucleus formation; unscheduled DNA synthesis = UDS; cell transformation) under identical conditions of metabolism and dose at target in this cell system. 5-AC induces morphological transformation of SHE cells (0.5 to 2 µM), but no DNA excision repair (UDS). Therefore, 5-AC seems to be devoid of direct DNA damaging potential. Furthermore, our studies revealed that 5-AC is a potent inducer of micronuclei in the SHE system at low concentrations (µM). This indicates that 5-AC is able to cause changes at the chromosomal level. The possible relationship of these findings to chromosomal damage and/or aneuploidy is currently under investigation.

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INTERACTION-STUDY ON METABOLISM AND MUTAGENICITY OF METRONIDAZOLE AND ETHANOL

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The 5-nitroimidazole derivate metronidazole is widely used in the treatment of trichomonas - and anaerobic bacterial infections and parasitic infestations.Although therapeutic benefits are beyond question metronidazole has been shown to be mutagenic in bacterial assays and can cause cancer in experimental animals. In mice metronidazole potentiates the teratogenicity of alcohol. In this study investigations on the interaction of metronidazole and ethanol were performed in order to detect an increase of mutagenicity in dependence on the different pretreatment. One group of NMRI-mice was pretreated orally with metronidazole in three different concentrations (100, 400, 1600 mg/kg b.w.) for four days, another group additionally received water or 10 % ethanol (w./v.), the control groups received water or 10 % ethanol, respectively. The effect of pretreatment was tested on induction of microsomal enzymes (Schulte-Hermann and Parzefall: Chem.-Biol. Interactions 31, 297, 1980) and on mutagenicity in the Salmonella/mammalian microsome assay (Ames-test) by a slightly modified protocol (Prival and Mitchell: Mut. Res. 97: 103, 1982).Liver-S9 (separated liver homogenate containing microsomal enzymes) of the differenty pretreated animals was used as metabolizing system and was tested on enzyme activity. The data demonstrate that the oxidative metabolism of metronidazole can be induced by pretreatment with metronidazole and more distinctly with metronidazole in combination with ethanol. In the Ames-test metronidazole demonstrated a strong mutagenic activity with TA 98 and TA 100 strain of Salmonella. However compared to control mouse-liver S9 the metabolic activation mediated by pretreated mouse-liver S9 caused neither enhancement nor reduction of the response. These results postulate that alcohol-consumption during metronidazole-therapy does not increase the known mutagenic effect of metronidazole.

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H-RAS GENE MUTATIONS IN RAT LIVER TUMORS PRODUCED BY CARCINOGENIC AROMATIC AMINES

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2-Acetylaminofluorene (AAF) is a complete liver carcinogen in rats. 2-Acetylaminophenanthrene (AAP) and trans-4-acetylaminostilbene (AAS) initiate liver cells but do not produce liver tumors in adult Wistar rats. In an initiation-promotion experiment with newborn Wistar rats and phenobarbital (500 ppm in the drinking water) as a promoter, it has now been confirmed that all three chemicals initiate the generation of liver tumors. Tumor incidence after 18 and 24 months in males and females, respectively: 18 mo.: AAF (1.5 mmol/kg) 0/3, 1/3; AAP (1,5 mmol/kg) 2/4, 1/4; AAS (0.15 mmol/kg) 4/5, 4/6. 24 mo.: AAF 3/6, 7/7; AAP 5/5, 6/6; AAS 3/7, 6/6.

The H-ras gene was analyzed in 32 tumors. The base pairs 1091-1403 including codon 12 and 1438-1611 including codon 61 were sequenced. In addition all exons and most of the introns were sequenced in 12 of the tumors. This provides the first comprehensive information about the H-ras gene in Wistar rats, which differs somewhat from that e.g. in Fischer rats. Point mutations could not be found in any one of the tumors. In accordance with most but not all other published results, point mutations of the H-ras gene, therefore, seem not to play a role in the formation of rat liver tumors by initiating aromatic amines.

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DIFFERENCES IN GAP- (GTPASE ACTIVATING PROTEIN) ACTIVITY BETWEEN LIVER TUMORS AND NORMAL LIVER TISSUE IN MICE. O.Müller, and M.Schwarz

The oncogenes H-*ras*, K-*ras* and N-*ras* encode for small membrane bound proteins of molecular mass 21 kD that bind guanine nucleotides. The cellular p21 proteins ($p21_c$) are believed to be active as signal transducers in their GTP-complexed form. GTP-complexed $p21_c$ is converted into its inactive GDP-complexed form by means of an intrinsic GTPase activity. This intrinsic GTPase activity of $p21_c$ is strongly increased by the two cytosolic proteins p120-GAP and NF1-GAP. The GAP-mediated stimulation of $p21_c$ GTPase activity was measured in cytosols obtained from carcinogen-induced liver tumors and normal liver tissues of mice of two strains, namely C3H/He and C57BL/6J. Liver cytosolic extracts mediated an increase in the GTPase activity of wild-type $p21_c$. There were great differences between tumor and normal tissues in the maximal velocity V_{max} and in the apparent Michaelis constant K_M of the $p21_c$ GTPase reaction. Both V_{max} and apparent K_M were decreased in the liver tumors. Cytosolic extracts isolated from liver tumors that harbored point mutations in codon 61 of the c-Ha-*ras* gene did not differ in their activity from extracts obtained from non-mutated liver tumors. Since both GAP proteins are important cellular regulators of the *ras* signalling pathway and probably also effectors of *ras* p21 the observed differences in GAP activity may be of relevance for the tumorigenic process in mouse liver.

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ANALYSIS OF MUTATIONS IN THE P53 AND THE C-HA-*RAS* GENES IN RODENT AND HUMAN LIVER TUMORS S.Kress, J. König, R. Bauer-Hofmann and Y. Zhu-yuan

Alterations of the p53 and the c-Ha-*ras* genes by point mutation are frequently observed in human cancers. While the p53 gene is regarded to function as tumor suppressor gene and is inactivated by point mutations, *ras*-protooncogenes become activated and assume oncogenic potential by the introduction of point mutations.
We comparatively analyzed the mutation frequencies in both the p53 and the c-Ha-*ras* gene in a total of 93 carcinogen-induced liver tumors of three different mouse strains, in 19 cell lines derived from some of these experimental tumors and in 8 human hepatocellular carcinomas (HCC) stemming from patients of the eastern part of China. The DNA sequences of interest within the two genes were amplified *via* PCR. The mutation analyses were carried out by single-strand conformation polymorphism (SSCP) analysis, direct sequencing and allele-specific oligonucleotide hybridization of the amplified DNA fragments.
While 2 of the 8 HCC cases and 3 of the 19 murine hepatoma cell lines showed p53 mutations, none of the 93 primary mouse liver tumors was mutated in this gene. On the contrary, none of the human tumors and none of the murine cell lines analyzed was mutated in the c-Ha-*ras* gene while activating mutations at codon 61 of this protooncogene were frequent in liver tumors of some strains of mice but absent in others. Those strains of mice which showed a high frequency of c-Ha-*ras* mutations are also characterized by a high background incidence of spontaneous liver tumors and a high sensitivity towards liver carcinogens and tumor promoters. Our results now suggest that different molecular genetic mechanisms may be operative in the development of liver tumors in these particular strains of mice and in humans. This may impede or even render impossible the extrapolation of liver carcinogenicity data from mouse to man and contradicts the use of the highly sensitive mouse strains for liver carcinogenicity assays.

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ANTIOXIDANTS INHIBIT THE ENHANCEMENT OF MALIGNANT CELL TRANSFORMATION INDUCED BY 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD)

D.Wölfle, E.Becker, C.Schmutte and H.Marquardt

The mechanisms of the tumor promoting activity of TCDD were studied using as in vitro-model the enhancement of malignant transformation of C3H/M2 mouse fibroblasts. TCDD, at the very low concentration of 1.5 uM, was found to enhance the cell transformation induced by N-methyl-N'-nitro-N-nitrosoguanidine and 3-methylcholanthrene as effectively as the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). This effect was strongly inhibited by mannitol, a scavenger of hydroxyl radicals, or antioxidants, i.e., ascorbic acid and tocopherol. Thus, oxygen radicals appear to play an important role in tumor promoter-enhanced malignant transformation.
In addition, the role of protein kinase C (PKC) activation was studied. A PKC inhibitor, i.e., 1-(isoquinolinesulfonyl)-2-methylpiperazine (H-7), markedly reduced malignant transformation enhanced by TPA but not that enhanced by TCDD. Thus, the results suggest that TPA and TCDD enhancement of cell transformation is dependent on a common mechanism, possibly induced by oxygen radicals, and, in addition, on a further mechanism that may involve signalling pathways and is agent-specific.

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DOWN-REGULATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR BY THE HEPATOCARCINOGEN 2-ACETYLAMINOFLUORENE IN MALE WISTAR RATS IN VIVO AND IN VITRO

W. Muster and P. Cikryt

The epidermal growth factor (EGF) receptor is a transmembrane glycoprotein with intrinsic tyrosine kinase activity. The EGF receptor is involved in the control of cellular proliferation both in normal and in neoplastic cells. Activation of the cell surface EGF receptor is initiated by the binding of EGF or the transforming growth factor α (TGFα) which is functionally equivalent to EGF to the extracellular domain. One of the initial cellular events triggered by receptor activation is the autophosphorylation of the cytosolic domain of the EGF receptor. However, the sequence of EGF receptor-stimulated signal transduction from the plasma membrane to the nucleus is still not known in detail. The concentration of the EGF receptor seems to be of critical importance in the regulation of cellular proliferation. It has been shown that the EGF receptor concentration is modulated by a number of diverse chemical tumor promotors such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, phenobarbital, 12-O-tetradecanoylphorbol-13-acetate and ethinyl estradiol.

In our study we have addressed the question of whether the EGF receptor is involved in the mechanism of tumor promotion by the complete hepatocarcinogen 2-acetylaminofluorene (AAF). We have shown that the EGF receptor was down-regulated as early as 2 days after treatment of male Wistar rats with 0.02 % AAF in the diet. The EGF receptor concentration did not recover during a 3 weeks treatment period. Using cultured rat hepatocytes, we have extended our studies of the underlying mechanism of EGF receptor down-regulation by AAF. The protein kinase C (PKC) seems not to be involved because the PKC inhibitor staurosporin did not prevent the EGF receptor down-regulation. On the other hand, it was demonstrated in competitive binding experiments that AAF did not interact directly with the EGF receptor. The influence of AAF treatment on EGF and TGFα production in cultured rat hepatocytes will be presented and a possible mechanism of cellular EGF receptor down-regulation by AAF will be discussed.

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ISOLATION,BIOCHEMICAL CHARACTERISATION AND LONG-TERM CULTURE OF OVAL CELLS FROM CARCINOGEN-FED RATS. P. Steinberg,R. Pack,R. Heck and F. Oesch

Oval cells(OC) are liver epithelial cells that proliferate during hepatocarcinogenesis and chemically-induced severe liver injury.It is likely that they derive from cholangioles or cells located near these structures in the portal spaces of the rat liver.The fate of OC regarding the histogenesis of hepatocellular carcinomas is a matter of considerable controversy.In the present study we isolated,biochemically characterised and established long-term cultures of OC from rats fed a choline-deficient/DL-ethionine-supplemented diet(CDE) for 6,14 or 22 weeks.OC isolation was performed as follows:the liver first underwent a two-step collagenase perfusion;then the tissue was minced and incubated with collagenase,pronase E and DNase I.A single-step discontinuous Nycodenz gradient was used to obtain a nonparenchymal cell fraction and OC were purified from this fraction by centrifugal elutriation.The freshly isolated OC (30-70 million/rat liver,viability and purity ⩾90%) had a diamter of 12-15 µm,were γ-glutamyltranspeptidase(GGT)-positive,cytokeratin (CK) 7, 8,18 and 19-positive,peroxidase-negative and expressed lactate dehydrogenase (LDH) isoenzymes 1-5; interestingly,60 % OC also expressed albumin, a marker for liver parenchymal cells.A low glucose-6-phosphatase(G6PASE) and relatively high GGT and alkaline phosphatase(AP) activities(when compared to parenchymal cells) were detected in OC.Primary OC cultures were established by allowing frshly isolated OC to attach to uncoated Petri dishes;OC lines were obtained by cloning after selective removal of contaminating fibroblasts with EDTA.OC cultures contained small and large epithelial-like cells replicating to form uniform monolayers with a cobblestone appearance.Mononucleated giant cells were also present in the different cell lines,being more numerous in cultures of OC from rats fed CDE for 22 weeks.After 22 passages OC expressed LDH isoenzymes 2-5,CK 8 and CK 18, while only 15 % expressed CK 7 and CK 19 was undetectable;GGT and AP activities were strongly reduced,that of G6PASE remained unchanged.All OC lines at passage 22 showed anchorage-dependent growth.In conclusion,isolated OC express both bile duct epithelial as well as parenchymal cell-specific markers. The OC lines will now be used in transformation studies to determine if oval cells can give rise not only to cholangiocellular but also to hepatocellular carcinomas in the rat liver.

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EFFECTS OF MAGNETIC FIELDS ON RAT MAMMARY TUMOR DEVELOPMENT INDUCED BY 7,12-DIMETHYLBENZ(A)ANTHRACENE (DMBA)

M. Mevissen, U. Wahnschaffe, and S. Buntenkötter

A series of epidemiological studies indicated associations between exposure to magnetic fields (MF) produced by alternating current (AC) power transmission lines and a variety of cancers, including breast cancer. In order to test the possibility that MF acts as a cancer promoter, 4 separate experiments have been conducted in rats in which the effects of chronic exposure to MF on the development of mammary tumors induced by DMBA were determined. Female rats were exposed in magnetic coils for 91 days to either AC(50 Hz)-MF (with magnetic flux densities of 30 mT or 1 µT) or to direct current(DC)-MF (15 mT). DMBA (5 mg) was administered per os at the onset of MF-exposure. The administration of DMBA was repeated thrice at intervals of 1 week. Per experiment, 18-36 animals were exposed in 6 magnetic coils. The same number of rats was used as control. These control animals were placed in dummy coils and treated with DMBA but were not exposed to MF. The experiment with AC-MF at 30 mT was repeated once. At the end of the exposure or sham-exposure period, tumor number and sizes were determined at necropsy. In one experiment (AC-MF at 1 µT), blood was collected for melatonin determination. Results were as follows: In sham-exposed animals, the tumor incidence varied between 50 and 78% in the 4 experiments. The number of mammary tumors per animal (mean ± S.D.) was 2.2 ± 2.0, 1.6 ± 0.73, 1.7 ± 2.5 and 2.1 ± 1.2 in the 4 experiments, respectively. In none of the experiments, MF significantly altered tumor incidence or number of tumors per animal when statistical calculations were done with non-parametric two-sided tests. Although there was a tendency to increased tumor incidence and number of tumors per animal in the experiment with AC-MF at 30 mT, this tendency could not be substantiated when the experiment was repeated. In conclusion, these experiments do not indicate that MF acts as a promoter of breast cancer.

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HISTOCHEMICAL PHENOTYPING OF DIETHYLNITROSAMINE-INDUCED LIVER LESIONS OF MALE C3H/He, B6C3F1 AND C57BL/6J MICE

A. Buchmann

Male C3H/He, B6C3F1 and C57BL/6J mice were given a single i.p. injection of diethylnitrosamine (20µg/g body wt.) on day 15 after birth and animals were killed between 17-29 (C3H/He and B6C3F1) and 29-46 weeks (C57BL/6J) after treatment. Carcinogen-induced liver lesions were identified by a deficiency in the marker enzyme glucose-6-phosphatase and the enzymatic phenotypes of these lesions were studied by enzyme- and immunohistochemical methods using serial liver sections stained for seven additional histochemical markers. In all three mouse strains, liver lesions were characterized by an increased basophilia and a decreased expression of UDP-glucuronosyl-transferase, microsomal epoxide hydrolase and NADPH-cytochrome P450 reductase, while the cytochrome P450 isoenzymes 1A2, 2C6 and 2E1 were virtually unexpressed. Quantitative analyses revealed that throughout all periods of investigation on average more than 70% of the glucose-6-phosphatase-deficient lesions occupying up to 99% of the total volumetric fraction expressed concomitant alterations in at least one of these additional marker stainings. Upon determination of the phenotypic complexity levels, between 70-90% of lesions were found to contain alterations in at least 6 of the markers analysed, while alterations in less than 3 markers were comparatively infrequent. On the background of previous observations in the rat liver system, the relative homogeneity of enzyme phenotypes and the apparent lack of time-dependent changes in enzyme expression suggest that the majority of lesions of all three mouse strains possess an increased neoplastic character already from the very early beginning of the carcinogenic process in liver.

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INITIATION AND PROMOTION OF ENZYME-ALTERED LIVER FOCI IN RATS BY DIFFERENT TYPES OF MUTAGENIC METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS

G. Werle-Schneider[1,2] and H.R. Glatt[1];

Different metabolites of polycyclic aromatic hydrocarbons such as dihydrodiol epoxides, phenol dihydrodiol epoxides, benzylic esters and quinones have been demonstrated to be mutagenic in cell culture. In order to estimate the relative importance of different classes of metabolites *in vivo* some proximate or ultimate mutagenic representatives such as 6-hydroxymethylbenzo[*a*]pyrene (6-hydroxymethyl-BP), BP-1,6-quinone, *trans*-7,8-dihydroxy-7,8-dihydro-BP (BP-7,8-diol) and 1-hydroxy-BP-7,8-diol as well as pyrene-1,6-quinone and 1-hydroxymethylpyrene (HMP) were examined for their initiating and promoting activities in rat liver and compared with their purely aromatic congeners benzo[*a*]pyrene (BP) and pyrene. The initiating potency was tested by a single i.p. injection of the compound into rats within one day after birth, followed by continuous exposure to the promotor phenobarbital beginning at weaning. The promoting activity was analysed by continuous treatment of the rats with BP-1,6-quinone and HMP subsequent to a single dose of diethylnitrosamine. As parameters of hepatocarcinogenic response, number and volume fraction of adenosine triphosphatase deficient foci were determined.

Three compounds markedly enhanced number and volume fraction of enzyme-altered foci, the activity following the order BP ≥ BP-7,8-diol > HMP. Animals treated with 6-hydroxymethyl-BP at the same dose died within four weeks. All other compounds led to statistically significant, but very small, increases in the number of enzyme-altered foci. HMP additionally possessed promoting activity in the liver of female but not of male rats.

The observation that BP-7,8-diol did not exceed the activity of BP suggests that other activation pathways are of importance. These other activation pathways do not appear to involve any of the other examined metabolites, since none showed sufficient carcinogenic activity. Nevertheless, some benzylic alcohols proved to be bio-logically active: HMP exhibited both initiating and promoting activity and 6-hydroxymethyl-BP was highly toxic. The sex-dependence of the promoting activity of HMP can be explained by the different expression of sulfotransferase activity in liver between adult female and male rats.

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SELECTION OF APPROPRIATE CONDITIONS FOR A MOUSE LIVER FOCI BIOASSAY Barbara Ostermeier, E. Deml, Doris Oesterle

The "Rat Liver Foci Bioassay" (RLFB) has been developed as an in vivo short term test system to detect carcinogens. For risk assessment in long term carcinogenicity studies two rodent spezies are used. We investigated with diethylnitrosamine (DEN) as initiating agent whether the experimental conditions described in the RLFB according to Oesterle and Deml (J Cancer Res Clin Oncol 105: 141-147, 1983) would be suited for a Mouse Liver Foci Bioassay. 21-31 days old male and female mice of the strains C3H and the hybrids between C3H (males) and C57 (females), the B6C3F1 (inbreeding, Neuherberg) were used. Male C3H mice are described to be especially sensitive towards chemical carcinogens. C57 mice contribute vitality and longevity. The B6C3F1 hybrids are commonly used in long term studies. The following results were obtained:
Male mice were more sensitive to DEN than female mice. B6C3F1 mice were more sensitive than C3H mice. The appropriate histochemical marker for preneoplastic foci is the loss of glucose-6-phosphatase. In contrast to rats, loss of adenosine-5'-triphosphatase and emergence of gamma-glutamyltranspeptidase are not suited. Applying a standard scheme with 1x10 or 1x30 mg DEN/kg body weight for initiation and 10 mg of the PCB-mixture Clophen A50 /kg body weight twice a week for 11 consecutive weeks for promotion, the yield in foci was lower than 1 per cm^2 liver section after 12 weeks. Single enzyme-deficient cells were observed which might be interpreted as initiated cells. The prolongation of the promoting period up to 23 weeks resulted in a dose- and time-dependent enhancement of foci incidence up to 16 per cm^2.
In conclusion: For the use as a short term test, the promoting period should be shortened by using a more efficient promoter. Under the experimental conditions given, male mice were more sensitive than females and less sensitive than rats.

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TISSUE-SPECIFIC REGULATION OF G-PROTEIN α-SUBUNITS BY THYROID STATUS. M. Michel-Reher and G. Gross[1]

Thyroid status is known to affect the responsiveness of various receptors acting through G-proteins. Therefore, we have compared the effects of hyperthyroidism (T_3) and hypothyroidism (PTU) on the expression of G-protein α-subunits in four rat tissues, heart, vas deferens, liver and cerebral cortex. T_3 was induced by treatment with triiodothyronin (0.5 mg/kg/d for 7 days) and PTU was induced by treatment with 6-propyl-2-thiouracil (0.05% in the drinking water for 6 weeks). G-Protein α-subunits were quantified by pertussis toxin (PTX)-catalyzed ADP-ribosylation and by Western blotting using the specific antisera AS/7 (recognizing the α-subunits of G_{i1} and G_{i2}) and GC/2 (recognizing the α-subunit of G_o). The amount of PTX substrates in cardiac membranes was largest in PTU (200±8 fmol/mg protein, n =9) and smallest in T_3 (102±4 fmol/mg protein, n=8, $p<0.001$ in one-way analysis of variance); similar data were obtained in vas deferens (112±7 vs. 90±4 fmol/mg protein, $p<0.05$). On the other hand, PTX-substrates did not differ among groups in liver (≈63 fmol/mg protein) or cerebral cortex (≈430 fmol/mg protein). This tissue difference could be caused by the expression of different G-proteins among tissues. However, immunodetectable α-subunits of both G_i and G_o were elevated in cardiac membranes from PTU compared to T_3 rats whereas both remained unchanged in cerebral cortical membranes. We conclude that tissue-specific factors regulate the expression of G-protein α-subunits by thyroid hormone.

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ISOPRENALINE INCREASES TRANSCRIPTION OF mRNA ENCODING THE INHIBITORY G-PROTEIN ALPHA-SUBUNIT IN THE HEART
F.U. Müller, W. Schmitz

In failing human hearts due to idiopathic dilative cardiomyopathy the inhibitory G-protein alpha-subunit (Giα-2) and its mRNA are increased while the stimulating G-protein alpha-subunit (Gsα) and its mRNA remain unchanged (Feldman et al., J Mol Cell Cardiol 21, 359 - 365, 1989). Similar alterations in G-protein expression have been found in hearts of rats treated with isoprenaline (ISO) for 4 days (Eschenhagen et al., Naunyn Schmiedeberg's Arch Pharmacol 343, 609 - 615, 1991). In order to study whether these changes are due to enhanced transcription or to increased stability of the mRNA, run-on transcription assays were performed in ventricular nuclei of rats treated with a 4-day infusion of NaCl 0.9% (CTR) or ISO (2.4 mg/kg·d). Ventricular nuclei were isolated and run-on transcriptions were performed as described earlier (Müller et al., Naunyn Schmiedeberg's Arch Pharmacol 344 (Suppl), R 57, 1991). Compared to CTR ISO increased incorporation of 32P-UTP into specific transcripts encoding Giα-2 by 40% (ISO: 38±4 ppm, CTR: 27±2 ppm, n=7, SEM, $p<0.05$) while transcriptional activity of Gsα remained unchanged (ISO: 34±3 ppm, CTR: 33±2 ppm, n=7). No change in total 32P-UTP incorporation into nascent RNA was observed.
In conclusion, increase of Giα-2 expression after ß-adrenergic stimulation in rat hearts is at least partly due to increased transcriptional activity of Giα-2. The unchanged transcriptional activity of Gsα supports previous results of unchanged Gsα mRNA levels under the same experimental conditions. Further investigations using run-on transcription assays should allow further insights in the regulation of G-protein expression and the possible role of cyclic AMP in this regulation. (Supported by the DFG.)

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Goα-mRNA EXPRESSION PATTERN IN HUMAN AND RAT HEART. EVIDENCE FOR ß-ADRENOCEPTOR-MEDIATED UPREGULATION IN RAT HEART
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Although the exact role of the pertussis toxin sensitive G-protein Go is still not defined there is evidence that it may mediate inhibition of neuronal Ca-channels through muscarinic receptors, stimulation of neuronal and cardiac atrial K-channels and stimulation of phospholipase C. Two α-subunits of Go (Goα1 and Goα2) with a MW of 40 and 39 kDa, respectively, have been described recently to be encoded by at least three alternatively spliced mRNAs (Hsu et al J Biol Chem 265:18576, 1990). Conflicting data exist concerning the expression of Goα in the heart. We used a single stranded 32P-labeled 550 bp cRNA derived from the 3' noncoding region of the rat Goα1 cDNA as a hybridization probe for northern blot analysis of total RNA which was extracted from human or rat hearts. Overall expression levels of Goα-mRNA were low in the heart as compared to rat brain. In atria from nonfailing and failing human hearts the Goα-probe detected at least two Goα-mRNAs at about 4.6 and 3.4 kb. In contrast, Goα-mRNA was not detectable in human ventricles. In hearts from 2-month old rats two predominant Goα-mRNA bands at about 4.3 and 3.4 kb and two faint bands at about 2.4 and 1.6 kb were detected. Expression levels of all four mRNA subtypes were increased by infusion of isoprenaline (2.4 mg/kg·d) by maximally 75% (n=6, $p<0.05$) after 8 days and remained elevated over a 4-week infusion period. The time course of Goα-mRNA upregulation resembled the time course of ß-adrenoceptor mediated upregulation of Giα2- and Giα3-mRNA levels in rat heart. - We conclude that (1) in the adult human heart expression of Goα1 is probably restricted to the atria, (2) there might be more than three Goα-splice variants in rat heart and (3) regulation of the molecular expression of Goα1 might participate in adaptation to chronic ß-adrenergic stimulation in vivo. (Supported by the DFG)

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INHIBITION OF Ca^{2+} CHANNELS IN BOVINE ADRENAL MEDULLA CELLS BY α_2-ADRENERGIC AND OPIATE AGONISTS.

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Catecholamine (CAT) release from bovine adrenal medulla, which largely depends on $[Ca^{2+}]_i$, has been observed to be voltage-dependend and dihydropyridine-sensitive. The physiologically evoked rise in $[Ca^{2+}]_i$ is initially localized exclusively to the entire subplasmalemmal area. Thus, Ca^{2+} influx via voltage-dependent Ca^{2+} channels is thought to be involved in CAT secretion. Since α_2-adrenoceptor agonists inhibit secretion of CATs and decrease Ca^{2+} uptake in bovine chromaffin cells, we studied α-adrenergic effects on Ca^{2+} channels in this cell type, using the patch-clamp technique. Whole-cell Ba^{2+} currents were recorded in a Na^+-free solution. Adrenaline (AD) (10μM) in the presence of propranolol (1μM) reduced the Ca^{2+} channel current in chromaffin cells by 35.3±8.2% (SEM; n=8). Yohimbine (100μM) suppressed this inhibitory effect, whereas prazosin (100μM) was inactive. Clonidine (10μM) mimicked the effect of AD. The opioid D-Ala^2-D-Leu^5-enkephalin (DADLE) (5μM) caused an inhibition of the Ca^{2+} channel current by 23.7±9.7% (SEM; n=9), which was reduced by the receptor antagonist, naloxone (100μM). The effects of AD and DADLE progressively declined during infusion of GDPβS into the cell and were abolished by treatment of cells with pertussis toxin (PT). These data point to an inhibition of Ca^{2+} channels via receptors coupled to PT-sensitive G-proteins.

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ALTERATIONS IN THE EXPRESSION OF GTP-BINDING PROTEINS IN 3T3-L1 CELLS AFTER DIFFERENTIATION TO THE INSULIN-SENSITIVE PHENOTYPE

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Murine 3T3-L1 fibroblasts differentiate to an insulin-sensitive, adipocyte-like phenotype after growth to confluence and treatment with dexamethasone, isobutylmethylxanthine and insulin. In these cells, glucose transport activity is insensitive to insulin before differentiation, and is increased 8-10 fold by the hormone after differentiation. Furthermore, the response of adenylate cyclase to catecholamines is markedly increased. In addition, an increase in heterotrimeric GTP-binding proteins has been observed after differentiation. In the present study we have further studied the expression of GTP-binding proteins in 3T3-L1 cells in order to find specific alterations of their overall expression or of their subcellular localization correlated with the metabolic changes occurring during differentiation. Differentiated and undifferentiated 3T3-L1 cells were homogenized, and membrane fractions (plasma membranes and low-density microsomes) were isolated by differential centrifugation. Subunits of heterotrimeric GTP-binding proteins were detected by immunoblotting with peptide-derived antisera (obtained from Dr. K. Spicher, Institut für Pharmakologie der FU, Berlin), Ha-*ras* with polyclonal antiserum. After differentiation, the abundance of the α_i-subunit, of the α_o-subunit, of the β-subunit of heterotrimeric G-proteins, and of Ha-*ras* as normalized per protein was reduced by approximately 50%. The abundance of a 47 kDa α_s-subunit was reduced by approximately 20%, whereas that of a 43 kDa α_s-subunit was increased about 3-fold after differentiation. When normalized per cell, the abundance of α_i, α_o, α_s (47 kDa), β, and Ha-*ras* was moderately increased, whereas the abundance of the 43 kDa α_s-subunit as normalized per cell was markedly increased, since the total membrane protein per cell increased significantly during differentiation. Cholera toxin-catalyzed ADP-ribosylation of both the 47 and 43 kDa α_s-subunit was significantly increased after differentiation. The abundance of all of the GTP-binding proteins in the low-density microsomes was reduced rather than increased by differentiation. In summary, the data show a moderate increase of the G-protein subunits α_i, α_o, α_s (47 kDa), and β as normalized per cell, and a large increase of α_s (43 kDa). The specific increase in the abundance of the 43 kDa α_s-subunit might be responsible for the previously described increase in adenylate cyclase responsiveness to catecholamines after differentiation of 3T3-L1 cells.

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ASSIGNMENT OF G_o-TYPE G PROTEINS CONTAINING DIFFERENT β SUBUNITS TO TRANSMEMBRANE RECEPTORS.

C. Kleuss*, H. Scherübl, B. Wittig*, and G. Schultz

G proteins constitute a family of heterotrimeric signal transducers attached to the cellular membrane. Suppression of a single G_o-protein α subunit blocks the agonist-induced inhibition of voltage-sensitive Ca^{++} channels (Kleuss et al. Nature (1991) 353, 43-48). We tried to inhibit the expression of G-protein β-subunits by intranuclear injection of antisense oligonucleotides into rat pituitary GH_3 cells. Injection of an oligonucleotide targeting the mRNAs of all 3 known β subunits reduced the modulation of Ca^{++} channels induced by somatostatin or carbachol. This effect was observed 18h after oligonucleotide injection and lasted one further day. An oligonucleotide targeting the mRNA of the β_3 subtype selectively blocked the carbachol-induced inhibition of Ca^{++} currents. The inhibitory effect of somatostatin on Ca^{++} currents was impaired by injection of a β_1-type antisense oligonucleotide. An oligonucleotide targeting the mRNA of the β_2-type G-protein subunit was ineffective. Our previous and present results indicate that in GH_3 cells a G_o protein containing α_{o1}- and β_3-subtype subunits couples the muscarinic receptor to the voltage-sensitive Ca^{++} channel; a G_o protein containing α_{o2} and β_1 subunits couples the somatostatin receptor to the same effector.

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REVERSE INTRINSIC ACTIVITY OF ANTAGONISTS ON G PROTEIN-COUPLED RECEPTORS.

W. Schütz

The concept that an antagonist may have biological effects opposite to those of an agonist because of its ability to stabilize an antipodal conformation of the receptor ("reverse agonism") is well known for ethyl-β-carboline-3-carboxylates on the $GABA_A$/benzodiazepine receptor. At G protein-coupled receptors, biological effects observed with an antagonist are usually interpreted as the result of its ability to block receptor activation produced by an endogenous agonist. However, considerable evidence has been accumulated that antagonists not only bind to the receptor, but also induce a conformational change which favors uncoupling of the receptor from its G protein. Findings obtained by us and others which are in support of this concept are the following: *(i)* agonist and antagonist binding to muscarinic cholinergic, D_2-dopamine, μ-opioid, and A_1-adenosine receptors is reciprocally modulated by guanine nucleotides; *(ii)* the guanine nucleotide-dependent increase in antagonist receptor binding is lost upon purification and reestablished following reconstitution of the purified receptor with G protein; *(iii)* in contrast to agonist-bound receptors, which accelerate nucleotide exchange and thus GTPase activity on the G protein molecule, antagonist-bound δ-opioid receptors inhibit GTPase activity of their associated G protein; *(iv)* in functional reconstitution systems, the basal rate of association of [^{35}S]GTPγS to G protein as well as GTPase activity is increased even in the presence of the unliganded receptor and, for the β_2-adrenergic and A_1-adenosine receptor, this increase is shown to be abolished by antagonists. Hence, the existence of antagonists with reverse intrinsic activity seems a more general feature of G protein-coupled receptors and requires a re-evaluation of biological effects produced by competitive antagonists.

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G Protein Regulation in Thyroid Cells

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The molecular mechanisms underlying the development of thyroid autonomous adenomas are still poorly understood, although abnormalities in G protein function have been implicated. Stimulation of growth by serum factors involves G_i-controled pathways in many cells. We have therefore used thyroid tissues as a model system to investigate the regulation of $G_{i\alpha}$-expression: (i) membranes from autonomous adenomas contain two pertussis toxin-substrates, which can be identifed as $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$ using subtype-specific antibodies; $G_{i\alpha\text{-}1}$ is absent or barely detectable in membranes derived from the corresponding extranodular tissue, whilst levels of $G_{s\alpha}$ are comparable. (ii) The increased amounts of $G_{i\alpha\text{-}1}$ in autonomous adenomas are of functional relevance since basal adenylyl cyclase activities are significantly reduced when compared with the control membranes from the paired, undiseased tissue (10.5 ± 1.65 vs 20.28 ± 1.68 pmol cAMP.min^{-1}.mg^{-1} protein; n=11). (iii) Thyroid membranes from normothyreotic patients contain both $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$. This suggests that hyperthyroidism produced by the autonomus adenoma is associated with a loss of $G_{i\alpha\text{-}1}$ in the remainder of the gland. In primary cell culture, normal human thyroid cells do not express $G_{i\alpha\text{-}1}$ unless TSH (thyroid stimulating hormone) is added to the culture medium. In thyroid cells derived from autonomous adenomas, $G_{i\alpha\text{-}1}$ levels are not regulated by TSH. Likewise, in a rat thyroid epithelial cell line (FRTL-5), TSH induces the expression of $G_{i\alpha\text{-}2}$, suggesting that species differences do exist. We conclude that TSH regulates the activity in G_i-linked pathways in the thyroid gland. This control may modify the activity of other mitogenic stimuli. In autonomous adenoma $G_{i\alpha\text{-}1}$ escapes TSH control and is apparently expressed constitutively. This constitutive expression of $G_{i\alpha\text{-}1}$ may be causally related to autonomous growth of thyroid cells.

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FUNCTIONAL EXPRESSION OF THE HUMAN $5HT_{1A}$-RECEPTOR IN E.COLI

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The $5HT_{1A}$-receptor has been suggested to couple to both G_i and G_s since both $5HT_{1A}$-receptor-mediated activation and inhibition of adenylyl cyclase have been obesrved in the same tissue. We have investigated the functional coupling of the human $5HT_{1A}$-receptor using an *in vitro* reconstitution system based on the expression of recombinant receptor and G protein α-subunit in *E.coli*. The gene coding for the $5HT_{1A}$-receptor was ligated into a plasmid that allowed for the accumulation of active receptor in the inner bacterial membrane upon induction. Binding of the agonist radioligand [^{3}H]-OHDPAT [8-hydroxy-(2-N-dipropylamine)-tetralin] to intact bacteria was saturable ($K_D \sim 8$ nM, $B_{max} \sim 120$ sites/cell) and showed the appropriate pharmacological profile. Incubation of *E.coli* membranes with purified bovine brain $G_{o,i}$ reconstituted guanine nucleotide-sensitive high-affinity binding of [^{3}H]-OHDPAT to the receptor ($K_D \sim 0.7$ nM). Titration of the receptor with increasing amounts of rG_α-subunits (G protein α-subunits expressed in and purified from *E.coli*) in the presence of purified bovine brain βγ-subunit showed the following rank order of potency in reconstituting high-affinity agonist binding: $rG_{i\alpha\text{-}3} > rG_{i\alpha\text{-}2} > rG_{i\alpha\text{-}1} > rG_{o\alpha}$; neither $rG_{s\alpha\text{-}s}$ nor $rG_{s\alpha\text{-}L}$ were capable of interacting with the receptor. This results show that the $5HT_{1A}$-receptor can discriminate among the various subtypes of $G_{i\alpha}$ and $G_{o\alpha}$. In addition, based on the inability of the receptor to couple to $rG_{s\alpha\text{-}s}$ and $rG_{s\alpha\text{-}L}$, we postulate the existence of an additional molecular species of serotonin receptor, which has a pharmacological profile similar to the $5HT_{1A}$-receptor but is capable of stimulating adenylyl cyclase via G_s.

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DIRECT ACTIVATION OF PERTUSSIS TOXIN-SENSITIVE G-PROTEINS BY BASIC HISTAMINERGIC AGONISTS AND ANTAGONISTS

R. Seifert

Amphiphilic basic substances such as mastoparan, substance P and compound 48/80 directly activate regulatory heterotrimeric guanine nucleotide-binding proteins (G-proteins), presumably by mimicking the structure of the third cytoplasmic domain of agonist-occupied receptors (Higashijima et al. (1990) J Biol Chem **265**:14176-14186). We studied the effects of basic histaminergic agonists and antagonists on GTPase activity of α-subunits of G-proteins in membranes of dibutyryl cAMP-differentiated HL-60 leukemic cells. Basic substances activated GTP hydrolysis in the order of effectiveness 2-[2-(3-chlorophenyl)-4-imidazolyl]ethanamine (chlorophenylhistamine) (H_1-agonist) > 2-[2-(3-fluorophenyl)-4-imidazolyl]-ethanamine (fluorophenylhistamine) (H_1-agonist) = diphenhydramine (H_1-antagonist) = chlorpheniramine (H_1-antagonist) = arpromidine (H_2-agonist and H_1-antagonist) = impromidine (H_2-agonist and H_3-antagonist) > dimaprit (H_2-agonist) > histamine. The EC_{50} values for GTPase activation ranged from 0.28-0.55 mM, and maximum effects were reached at 1-3 mM. In membranes from pertussis toxin-treated cells, the stimulatory effect of the chemotactic peptide, *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (fMet-Leu-Phe), was greatly reduced, and basic substances inhibited GTP hydrolysis. Clemastine (H_1-antagonist) and famotidine (H_2-antagonist) did not reduce GTPase stimulations caused by histamine and H_1- and H_2-agonists. Basic substances enhanced the effect of fMet-Leu-Phe at a maximally effective concentration on GTP hydrolysis. *N*-Ethylmaleimide diminished GTPase activations by basic substances and by fMet-Leu-Phe but enhanced the relative extent of synergistic interaction between chlorophenylhistamine and the chemotactic peptide. Our data suggest that basic histaminergic agonists and antagonists directly activate pertussis toxin-sensitive G-proteins, i.e. through a mechanism different from that of fMet-Leu-Phe.

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G-PROTEINS INVOLVED IN THE RECEPTOR-MEDIATED INHIBITION OF INSULIN-SECRETION

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Inhibitory modulators of insulin secretion include adrenaline, somatostatin and galanin, which exert their effects *via* cell surface receptors coupled to pertussis toxin-sensitive G-proteins. In order to identify the G-proteins involved in the inhibitory control of insulin secretion, we analyzed them in membranes from SV40-transformed pancreatic B-cells from hamster (HIT-T15); membranes from a rat insulinoma cell line (RINm5F) were used for comparison. The proteins of the two cell lines, which were ^{32}P-ADP-ribosylated by pertussis toxin, differed considerably in their electrophoretic mobility. Immunoblotting of membranes with antibodies against pertussis toxin-sensitive G-proteins showed that both cell lines possess the two forms of G_o (G_{o1} and G_{o2}) and G_{i2}. G_{i1} was detected in RINm5F cells but not in HIT-T15 cells. G_{i3} appeared to be present in HIT-T15 but not in RINm5F cells. In membranes of HIT-T15 cells, adrenaline and clonidine stimulated a pertussis toxin-sensitive high-affinity GTPase. The hormone also stimulated incorporation of the photoreactive GTP analogue, [α-^{32}P]GTP azidoanilide, into proteins comigrating with the immunologically identified α-subunits of G_{o1}, G_{o2} and G_{i2}. Similar effects on photolabeling of proteins were observed with somatostatins 14 and 28 and with galanin. The data indicate that inhibitory modulators of insulin secretion act *via* multiple G-proteins which belong to the G_i and G_o families. The assignment of these G-proteins to the known transduction pathways i.e. inhibition of adenylyl cyclase and Ca^{2+} channels and stimulation of K^+ channels remains to be clarified.

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SK&F 96365 blocks agonist-induced inward currents through nonselective cation channels in HL-60 cells

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Biophysical characteristics and Pharmacological modulation of nonselective cation (NSC) channels in neutrophil-like cells are poorly understood. We studied NSC channels under whole-cell voltage-clamp conditions in HL-60 leukemic cells. In these cells no voltage dependent inward currents were observed. When HL-60 cells were differentiated to the granulocyte type with 200 µM dibutyryl cyclic AMP (for 48 h), ATP (30 µM) and *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (fMLP, 100 nM) induced inward currents at negative potentials in a reversible manner. The effects of both agonists were not additive. In undifferentiated cells ATP induced only about half the current observed in differentiated cells. NSC currents were blocked by compound SK&F 96365 (1-{ß-[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl}-1H-imidazole hydrochloride) in a dose-dependent manner, with an IC_{50} of about 3 µM. Pretreating the cells with pertussis toxin produced a complete inhibition of fMLP-induced currents and about 80% inhibition of ATP-induced currents. Infusion of GDPßS (guanosine 5'-O-[2-thio]diphosphate, 500 µM) *via* the patch pipette abolished the agonist-induced currents. By varying the extracellular ionic conditions, we found Na^+ to be the main charge carrier of fMLP-induced inward current. Ca^{2+} to a small extent also participated in the agonist-induced inward current. We conclude that activation of membrane purinergic receptors and fMLP receptors induces currents through nonselective cation channels *via* guanine nucleotide-binding protein (G-protein)-dependent mechanisms.

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CROSS-TALK BETWEEN CHEMOTACTIC RECEPTORS SHARING THE SAME G PROTEIN POOL IN MEMBRANES OF HL 60 CELLS

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Differentiated human leukemia (HL 60) cells contain high numbers of receptors for the chemotactic factors, fMet-Leu-Phe (fMLP) and complement factor 5 a (C5a), both coupled to pertussis toxin-sensitive guanine nucleotide-binding regulatory proteins (G protein). Agonist activation of either receptor stimulated binding of the GTP analog, guanosine 5'-[γ-thio]triphosphate (GTP[S]), to membrane G proteins by a similar maximal extent. In the presence of both agonists, no additive stimulation was observed. Guanine nucleotides inhibited the high affinity binding of the radiolabelled agonists [^{3}H]fMLP and [^{125}I]C5a in a similar manner with the potency order GTP[S] > guanosine 5'-[β,γ-imino]triphosphate (GppNHp) >> GDP. The possible interaction of the two receptors was studied by measuring agonist binding to one receptor in the presence of the other receptor agonist. fMLP and C5a had no effects on [^{125}I]C5a and [^{3}H]fMLP receptor binding, respectively, when studied in the absence of guanine nucleotides. However, in the presence of 10 nM GTP[S], fMLP reduced the binding of [^{125}I]C5a in a concentration-dependent manner (by maximally about 30 %). The same extent of reduction was observed when the binding of [^{3}H]fMLP was studied under similar conditions at increasing concentrations of C5a. The potency of GTP[S] and GppNHp to inhibit [^{125}I]C5a binding was increased about 3-fold by fMLP (10 µM), while the potency of GDP was not changed. Similar data were obtained when the influence of C5a (10nM) was studied on binding of [^{3}H]fMLP in the presence of increasing concentrations of guanine nucleotides. On the other hand, the potency of Na^+ ions, which also inhibit agonist receptor binding and receptor-G protein coupling in HL 60 membranes, probably by an action on the receptor moieties, was not enhanced by addition of another receptor agonist. The data suggest that fMLP and C5a receptors share the same G protein pool in membranes of HL 60 cells. Activation of these G proteins by one of the two receptors in the presence of metabolically stable GTP analogs apparently decreases the availability of G proteins required for the high affinity agonist binding state of the other receptor.

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ROLE OF GDP IN RECEPTOR-STIMULATED BINDING OF GUANOSINE 5'-[γ-THIO]TRIPHOSHATE IN MEMBRANES OF HL 60 CELLS

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Membranes of myeloid differentiated human leukemia (HL 60) cells contain a high number of receptors for the chemotactic peptide, fMet-Leu-Phe (fMet, *N*-formylmethionine), which stimulate phospholipase C via a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein(s) (G protein). Stimulation of the receptors leads to an increase in binding of the radiolabelled GTP analog, guanosine 5'-[γ-thio]triphosphate ([^{35}S]GTP[S]), to membrane G proteins, however only in the presence of exogenously added GDP when membranes were incubated at 30°C. In contrast, at 0°C fMet-Leu-Phe stimulated the binding of [^{35}S]GTP[S] to G proteins without addition of GDP. Furthermore, exogenous GDP only decreased receptor-stimulated binding of [^{35}S]GTP[S] at an incubation temperature of 0°C. When HL 60 membranes were preincubated at 30°C in the presence of Mg^{2+} followed by binding of [^{35}S]GTP[S] at 0°C, control binding was significantly increased, while the extent of agonist-stimulated binding of [^{35}S]GTP[S] was reduced. The effect of preincubation at 30°C was mimicked by incubation at 0°C with alkaline phosphatase, an enzyme capable of degrading GDP. Therefore, the release of [^{3}H]GDP from prelabelled HL 60 membranes was studied under similar conditions as used for binding of GTP[S]. At 30°C, [^{3}H]GDP was rapidly released, with only 50 % of total [^{3}H]GDP remaining bound to the membranes after 10 min of incubation. In contrast, at 0°C the binding of [^{3}H]GDP was very stable, with about 80 % still being bound after 60 min of incubation. Addition of fMet-Leu-Phe did not significantly alter the observed basal release of [^{3}H]GDP under the conditions studied. Furthermore, the degradation of [^{3}H]GDP in prelabelled membranes was studied. About 50 % of the bound [^{3}H]GDP was degraded to [^{3}H]GMP when membranes were incubated for 60 min at 30°C in the presence of Mg^{2+}, while it was stable at 0°C. A similar extent of GDP degradation was obtained by addition of alkaline phosphatase to the 0°C incubation. The data suggest that in native membranes of HL 60 cells the G proteins are initially in a GDP-liganded form and that GDP is rapidly released and degraded when the membranes are incubated at or near a physiological temperature. GTP[S] will rapidly bind to these unliganded G proteins, a reaction not further facilitated by agonist-activated receptors. In order to keep the target G proteins under control of formyl peptide receptors, GDP has to be added. In contrast, at low temperature GDP remains apparently bound to the respective G proteins, which state allows the receptor action.

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SELECTIVE PHOSPHORYLATION OF G PROTEIN ß, SUBUNITS IN MAMMALIAN MEMBRANES

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It is generally believed that G protein activation involves a GDP/GTP exchange at G protein α-subunits in the presence of βγ-dimers. Recently Wieland et al. (Eur J Biochem **196**:707, 1991) reported evidence for an additional mechanism of information transfer by intermediately thiophosphorylated transducin ß-subunits. The present report evaluates whether this reaction is common among three distinct ß-subunits ($ß_1$-$ß_3$) present in mammalian membranes.

HL-60 cell and porcine brain membranes were incubated with [γ-^{32}P]-GTP in the presence of $MgCl_2$. Reaction was terminated by adding Laemmli's buffer. Samples were subjected to SDS-PAGE with and without 6 M urea. Resolved proteins were transferred to nitrocellulose followed by identification of ß-subunits using subtype-specific peptide antibodies from rabbits against three mammalian ß-subunits ($ß_1$-$ß_3$). Subsequently, immunoblots were exposed to X-ray films in order to visualize phosphorylated protein bands.

Both membranes tested contained at least two immunoreactive bands at 35 and 36 kD which were sensitive to $ß_2$- and $ß_1$- specific peptide antibodies. Additionally, in porcine brain a third band was identified at 32-33 kD using a peptide antibody specific for the $ß_3$-subunit. Protein-incorporated radioactivity comigrated only with the 35/36 kD immunoreactive bands. Using SDS-PAGE in the presence of 6 M urea, ß-subunits shifted to a molecular weight range of 40-43 kD. Furthermore, resolution of the 35/36 kD bands ($ß_2$ and $ß_1$) were achieved, whereas a third band immunoreactive to $ß_3$ comigrated with $ß_2$. Radioactivity comigrated only with a $ß_1$-immunoreactive doublet whereas neither $ß_2$- nor $ß_3$-subunits were radioactively labelled.

From the results obtained it is concluded that only the $ß_1$-subunit of mammalian G proteins undergoes transient GTP-dependent phosphorylation, whereas $ß_2$- and $ß_3$-subunits are not phosphorylated at least under the experimental conditions chosen.

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$G_s\alpha$ IN NEUROBLASTOMA x GLIOMA HYBRID CELLS (NG108-15) UPON OPIOID EXPOSURE

H. Ammer and R. Schulz

The literature provides data suggesting a selective interaction of anti-G protein antibodies with the function of specific G protein subunits in situ. On the basis of these studies we report investigations on the function of $G_s\alpha$ in particulate membrane fractions of NG108-15 cells, using a site specific G protein antibody as well as purified G_s. The amount of cAMP produced served as a functional parameter for adenylate cyclase activity which is regulated by both inhibitory and excitatory receptors via G proteins. The antibody employed was raised against a decapeptide resembling the extreme C-terminus of $G_s\alpha$, a region suggested to be involved in receptor coupling. For in situ application, the antibody was affinity-purified and its specificity was verified by Western blot analysis, using recombinant G proteins expressed in E. coli. Preincubation of NG membranes with anti-α_s antibodies inhibited dose-dependently the PGE_1-stimulated production of cAMP but failed to affect opioid action on adenylate cyclase activity. Preimmune IgG did not show this effect. However, concomitant exposure of the antibody together with its cognate peptide as well as heat inactivation of the antibody failed to restore PGE_1-action, although binding ex situ to isolated α_s-subunits was completely eliminated. We, thus, turned to study $G_s\alpha$ function in membrane preparations exposed to low pH. This material fails to respond to PGE_1 but substitution with G_s dose-dependently reconstitutes PGE_1- as well as forskolin-mediated stimulation of adenylate cyclase activity. Pretreatment of G_s with the anti-α_s antibody prevented functional reconstitution as indicated by failure of PGE_1 to stimulate cAMP production. Controls with free peptide as well as heat inactivated antibodies eliminated this effect. Moreover, opioids proved fully active in both low pH pretreated and reconstituted membranes.

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PURIFICATION OF A G-PROTEIN INHIBITOR FROM BOVINE BRAIN

M.J. Lohse

Crude cytosolic preparations from bovine brain containing ß-adrenoceptor kinase (ßARK) are capable of inhibiting $ß_2$-adrenoceptor (ßAR) function. This inhibitory effect can in part be attributed to ßARK-mediated ßAR-phosphorylation followed by binding of the protein ß-arrestin. When such crude cytosolic fractions are further purified on anion exchange columns, the inhibitory effects of ßARK-mediated phosphoryl–ation are lost. This was thought to be due to separation of the kinase and ß-arrestin on these columns. Therefore, it was attempted to restore the inhibitory effect by combining the fractions containing the kinase with other fractions from the same column. Fractions eluting at ≈250 mM NaCl from a DEAE-Sephacel (Pharmacia) column were found to inhibit the GTPase activity of G_s stimulated by phosphorylated purified ßARs. These fractions were further purified by several chromatographic procedures. The final product was a protein of an apparent molecular weight of 33,000 as determined by gel electrophoresis. This protein turned out to inhibit the GTPase of G_s even in the absence of receptors. Therefore, this protein was termed G-protein inhibitor protein (GIP). Half-maximal inhibition of the GTPase of G_s occurred at a stoichiometry of GIP:G_s of about 1:1, suggesting that inhibition is effected by formation of a complex between the two proteins. To determine the sequence of this protein, 3 µg of the purified protein were digested with CNBr, and the fragments were purified by reverse phase chromatography. Gas-phase sequencing of two fragments allowed the determination of sequences containing 9 and 7 amino acids, respectively. These sequences are identical to two stretches contained in the retinal protein phosducin. This suggests that GIP is identical to or a close homologue of phosducin.

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PHOSDUCIN - A REGULATOR OF G-PROTEIN MEDIATED SIGNAL TRANSDUCTION

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Phosducin has been known as a retinal phosphoprotein which binds to the βγ–subunits of the retinal G-protein G_t. We show here that phosducin can act as a regulator of different G-protein activities.

The cDNA of phosducin was isolated both from bovine retina and from bovine brain via amplification using the polymerase chain reaction (PCR). Both products were sequenced and found to be identical. The PCR-product from bovine brain was then cloned into an inducible bacterial expression vector. After induction of transformed *E. coli* and preparation of a cytosolic extract, a protein with an apparent molecular weight of about 33,000 D was identified in Coomassie stained sodium-dodecyl-sulfate polyacrylamide gels. This protein represented a significant fraction of the cytosolic proteins of the induced bacteria, and was not present in control bacteria. It was identified as phosducin by means of specific anti-peptide antibodies. After purification of the expressed product to 95 % homogeneity, the effects of this phosducin on various purified G-proteins were determined by measuring their GTPase and GTPγS-binding activities. Phosducin inhibited the GTPase activity of G_s, G_o and G_i holoproteins. The GTPase activities of isolated $G\alpha_o$- and $G\alpha_s$-subunits were not affected, and the inhibitory activity of phosducin depended on the addition of isolated βγ-subunits.

Purified phosducin was an excellent substrate for cAMP-dependent protein kinase (PKA). This phosphorylation abolished the inhibitory effect of phosducin on G-protein function. Since PKA is activated via G_s and inhibited via G_i, phosducin may act as a negative feedback regulator for G_i-mediated signals but as a positive feedback regulator for G_s-mediated signals. Thus, phosducin seems to play an important role in G-protein mediated signal transduction.

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THROMBIN STIMULATES TYROSINE PHOSPHORYLATION IN BC_3H1 CELLS

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Thrombin is a potent mitogen for different cell types, including BC_3H1 cells. In this study, the effect of thrombin on tyrosine phosphorylation was examined using anti-phosphotyrosine antibodies. Thrombin was found to induce tyrosine phosphorylation of 65/70- and 110/120-kDa proteins in BC_3H1 cells. The effect of thrombin was concentration-dependent, being half-maximal and maximal at concentrations of 0.01 and 0.5 U/ml, respectively. The thrombin-induced increase in phosphorylation was rapid (≤10 s) and transient with a peak response after about 90 s. Preincubation of cells with pertussis toxin (PT) had no effect on thrombin-induced tyrosine phosphorylation. Epidermal growth factor (EGF) stimulated tyrosine phosphorylation of proteins of identical molecular masses to those phosphorylated in response to thrombin. Additionally, EGF induced tyrosine phosphorylation of 170- and 55/60-kDa proteins. The phorbol ester, 12-myristate 13-acetate (PMA), also stimulated tyrosine phosphorylation of proteins identical to those phosphorylated by thrombin, suggesting that activation of protein kinase C (PKC) is sufficient to induce tyrosine phosphorylation. However, the PKC inhibitor, staurosporine, and down-regulation of PKC activity by prolonged exposure to phorbol ester completely inhibited tyrosine phosphorylation induced by PMA but not by thrombin. In BC_3H1 cells permeabilized by streptolysin-O, the non-hydrolysable guanine nucleotide GTPγS stimulated tyrosine phosphorylation of proteins of identical molecular masses to those phosphorylated after exposure of cells to thrombin. These results suggest that thrombin stimulates tyrosin phosphorylation via a PT-insensitive G-protein in a manner not necessarily dependent on PKC.

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IMMUNOCHEMICAL CHARACTERIZATION OF PHOSPHOLIPASE C ISOZYMES OF HUMAN GRANULOCYTES

M. Camps, A. Carozzi*, P. Schnabel, P.J. Parker*, and P. Gierschik

We have previously reported that human HL-60 granulocytes contain a soluble phospholipase C (PLC), which is markedly stimulated by purified G-protein $\beta\gamma$-subunits. G-protein $\beta\gamma$-subunits also stimulated soluble PLC from human peripheral neutrophils, as well as membrane-bound, detergent-solubilized PLC from HL-60 cells. Partial purification of soluble HL-60 PLC revealed the existence of at least two distinct PLC isozymes in granulocytes, designated PLC-I and PLC-II. Only one of these forms (PLC-II) was sensitive to stimulation by $\beta\gamma$-subunits. To characterize the PLC isozymes present in granulocytes and to identify the $\beta\gamma$-sensitive form of the enzyme, we have performed an immunochemical analysis of granulocyte PLCs using antisera raised against synthetic peptides corresponding to sequences predicted by various PLC cDNAs. Here, we report that antibodies reactive against the PLC-β_1 isozyme, which is known to be stimulated by members of the α_q subfamily of G-protein α-subunits in other cells, fail to detect immunoreactive proteins in HL-60 cells or human neutrophils. In contrast, a serum raised against a PLC-β_2 peptide was strongly and specifically reactive with a $\approx$ 140 kDa and a $\approx$ 100 kDa polypeptide present in both membraneous and soluble fractions of HL-60 cells and peripheral granulocytes and designated β_2' and β_2'', respectively. Since the PLC-β_2 cDNA encodes a 1181 amino acid residue protein, it is likely that β_2' corresponds to the native PLC-β_2 polypeptide and β_2'' represents a proteolytic fragment. Interestingly, β_2' is much more abundant in membranes than in cytosol, whereas β_2'' is the predominant soluble form of β_2, suggesting that proteolysis of PLC-β_2 may alter the subcellular distribution of PLC-β_2. Most importantly, however, both β_2' and β_2'' are constituents of the partially purified, $\beta\gamma$-sensitive PLC-II preparation, but absent from the $\beta\gamma$-resistant PLC-I preparation. Taken together, our results show that the PLC-β_2 is the predominant PLC-β isozyme in granulocytes and that this protein is a likely candidate for the PLC isozyme regulated by G-protein $\beta\gamma$-dimers in this system.

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MASS MEASUREMENTS OF INOSITOL 1,4,5-TRISPHOSPHATE AND INOSITOL 1,3,4,5-TETRAKISPHOSPHATE IN A NEURONAL CELL LINE. DESENSITIZATION OF THE INOSITOLPHOSPHATE RESPONSE TO BRADYKININ.

G. Reiser

The second messenger inositol 1,4,5-trisphosphate (Ins-P_3), which releases Ca^{2+} from internal stores can be inactivated by dephosphorylation to Ins 1,4-P_2, or, alternatively, be phosphorylated to Ins 1,3,4,5-P_4. Ins-P_4 has been suggested also to be involved in Ca^{2+} metabolism, yet by a still unknown mechanism.

We have found high-affinity receptors for Ins-P_4 in porcine cerebellar membranes [Donié, F. & Reiser, G. (1989) FEBS Lett. 254, 155-158]. The receptor which we have isolated and purified from brain [Donié, F. & Reiser, Biochem. J. 1991, 275, 453] displays a high selectivity for Ins 1,3,4,5-P_4. Binding protein preparations from pig cerebellum (bovine liver) were used to quantify cellular contents of Ins-P_4 (Ins-P_3) in tissue samples.

In a neuronal cell line (108CC15, NG108-15) the levels of $InsP_3$ and $InsP_4$ rise transiently after stimulation with bradykinin (EC_{50} approx. 150 nM). Maximal $InsP_3$ level of 354 pmol/mg protein (15-fold basal level) is obtained at 10 - 15 s after addition of bradykinin, the $InsP_4$ level rises maximally to 78 pmol/mg protein (14-fold basal level) at 20 - 30 s. The bradykinin-dependent rise of the inositolphosphate levels is diminished with reduced extracellular Ca^{2+} concentration. However, depletion of internal Ca^{2+} stores by treatment with ionophores does not affect the bradykinin-induced rise in $InsP_3$ and $InsP_4$ levels. Pretreatment of the cells with La^{3+} or Ni^{2+} to inhibit Ca^{2+} fluxes prevented the rise of $InsP_3$ levels induced by bradykinin. Homologous desensitization to bradykinin occurs in the signal transduction pathway already at the production of inositolphosphates, since after a 2 min stimulation with bradykinin the rise in cellular masses of $InsP_3$ and $InsP_4$, inducible by a following second bradykinin stimulus, is substantially reduced.

Thus, we have obtained some evidence for the mode of activation of phospholipase C by bradykinin in neural cell lines which might be a useful system to study the physiological role of $InsP_4$.

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SEQUESTRATION PERMITS REACTIVATION OF DESENSITIZED β_2-ADRENOCEPTORS

S. Pippig

Agonist-stimulation of β_2-adrenoceptors (β_2AR) causes disappearance of the receptors from the cell surface. This process is called receptor sequestration.

Sequestered β_2ARs remain accessible for hydrophobic ligands, but are inaccessible for hydophilic ligands. Upon fractionation of plasma membranes on sucrose gradients, sequestered receptors are found in a "light vesicle fraction".

Sequestration was originally proposed to be a mechanism of receptor desensitization. However, after treatment of cells with Concanavalin A (Con A), which prevents β_2AR sequestration, agonist induced desensitization is little affected. Therefore the hypothesis was investigated that receptor sequestration may be important in receptor reactivation after stimulation and desensitization of β_2AR.

β_2AR desensitization has been shown to be triggered by receptor phosphorylation, and reactivation of the receptors after removal of agonist is accompanied by receptor dephosphorylation. When cells were pretreated with Con A and then stimulated with the β_2AR agonist Isoproterenol (Iso), β_2AR remained phosphorylated even after removal of the agonist.

Receptor function was measured under the same conditions by determining β_2AR-stimulated adenylyl cyclase activity in cell membranes. Receptor function decreased by 50 to 60 % after a 10 min desensitization with Iso. After removal of the agonist, receptor function of control cells returned to control levels over a period of 60 min. In contrast, in Con A-pretreated cells receptors remained desensitized. These data indicate that sequestration of desensitized receptors permits their dephosphorylation and that this is a necessary step in their reactivation.

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DO NEUTROPHILS HAVE FUNCTIONAL α_2-ADRENOCEPTORS?

I.F. Musgrave

Neutrophils possess ß$_2$-adrenoceptors which play a role in the inhibition of *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (FMLP)-stimulated superoxide anion ($O_2^{\cdot-}$) production (Seifert & Schultz, Rev Physiol Biochem Pharmacol **117**: 159 (1991)). Human neutrophils have been reported to possess α_2-adrenoceptors (Panosian & Marinetti, Biochem Pharmacol **32**: 2234 (1983)). However, the functional role of these receptors is unknown. We therefore investigated the effect of α_2-adrenoceptor agonists on FMLP-induced $O_2^{\cdot-}$ production. 5-Allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepin-dihydrochloride (BHT 920) and 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304), structurally unrelated non-catecholamine full α_2-adrenoceptor agonists, by themselves had no effect on $O_2^{\cdot-}$ production. Surprisingly, neither BHT 920 nor UK 14304 had any effect on FMLP-induced $O_2^{\cdot-}$ production at concentrations up to 10 μM, despite evidence for tonic elevation of cyclic 3',5' adenosine cyclic monophosphate (cyclic AMP) by endogenous adenosine. The ß-adrenoceptor agonist isoprenaline significantly reduced FMLP-induced $O_2^{\cdot-}$ production. However, this effect of isoprenaline was not affected by either BHT 920 or UK 14304 at 10 μM. The apparent discrepancy between our functional studies and the binding studies of Panosian & Marinetti (1983) may be due to the presence of platelets in their neutrophil membrane preparations. We therefore examined the binding of the selective α_2-adrenoceptor antagonist [^{3}H]-(2-[2,3-dihydro-2 methoxyl-1,4-benzodioxin-2-yl]-4,5-dihydro-1H-imidazole hydrochloride) ([^{3}H]-RX821002) to membranes of HL-60 myeloid cells, an accepted model for neutrophils. Membranes from undifferentiated, dibutyryl cyclic AMP- or dimethyl sulfoxide-differentiated HL-60 cells showed no saturable high affinity binding of [^{3}H]-RX821002, whereas such binding was clearly seen in membranes from human erythroleukemia (HEL) cells which are known to possess α_2-adrenoceptors.

Taken together the above results suggest that the lack of functional response to α_2-adrenoceptor agonists in human neutrophils is due to the absence of α_2-adrenoceptors in these cells.

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A MEMBRANEOUS COFACTOR PRESENT IN VARIOUS CELL TYPES ENHANCES SUPEROXIDE PRODUCTION IN A CELL-FREE SYSTEM FROM DIFFERENTIATED HL-60 CELLS

I. Schwaner

The superoxide-producing NADPH-oxidase system in phagocytes consists of different cytosolic components and one membraneous component, the cytochrome b_{558} (Seifert, R. & Schultz, G. (1991) Rev. Physiol. Biochem. Pharmacol. 117:1). We studied the effects of various types of membranes from myelocytic cells on the superoxide production in a cell-free system from dimethyl sulfoxide-differentiated HL-60 cells. Membranes from the human erythroleukemia cell line HEL enhanced arachidonic acid-induced superoxide production in a concentration-dependent manner by up to about 80 %. Intact HEL cells, cell-free systems derived from these cells and HEL membranes plus cytosol from differentiated HL-60 cells did not produce superoxide. Cytosol from HEL cells did not potentiate superoxide formation in the cell-free system from differentiated HL-60 cells. The inhibitor of protein isoprenylation lovastatin substantially reduced the effect of HEL membranes. Neither pertussis toxin- nor cholera toxin-treatments nor heating of the HEL membranes affected the enhancing effect. The enhancing effect was independent from the presence or absence of the stable guanine nucleotide GTPγS. Membranes from the human myelocytic cell lines K-562 and U-937 and from human thrombocytes and erythrocytes (the latter being devoid of cytochrome b_{558}) enhanced superoxide production as well. The effect of membranes was not mimicked by various phospholipids and diacylglycerols. In conclusion, we have found a membraneous cofactor of NADPH-oxidase activation which (1) is heat-stable, (2) is present in various myeloid cell types, (3) is not a guanine nucleotide, (4) is not a GTP-binding protein, (5) is not cytochrome b_{558} and (6) is not a defined lipid.

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ISOLATION AND EXPRESSION OF A HEAT-STABLE ENTEROTOXIN RECEPTOR cDNA FROM A HUMAN COLONIC CELL LINE.

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Heat-stable enterotoxins (STs) are small peptides secreted from enterotoxigenic strains of E. coli, Y. enterocolitia and V. cholerae. These peptides induce acute diarrhea by binding to and activation of a membrane form of guanylate cyclase. Recently one form of this type of guanylate cyclase has been cloned from rat tissue. (Schulz S et al.(1990) Cell 63:941). We now have isolated another form of ST-sensitive guanylate cyclase from a human colonic cell line (T84).
Methods: A bacteriophage lambda UNI-ZAP-XR library was screened with a catalytic domain probe from rat GC-B. A full-length clone was obtained by the 5`-rapid amplification of cDNA (RACE) protocol. A full-lenght cDNA was ligated to the expression vector pSVL and COS-7 cells were transiently transfected using DEAE-dextran. Accumulation of cyclic GMP was measured by stimulating the monolayers with different concentrations of STa in culture medium containing IBMX. Cyclic GMP was measured by RIA.
Results: Sequence analysis revealed a protein with a predicted molecular weight of 120 858, an extracellular ligand binding region, a single transmembrane domain and a cytoplasmatic catalytic domain. Sequence comparison between ST-sensitive guanylate cyclase from rat tissue (GC-C) and human source (STaR) indicated 81 % sequence identity for the whole molecule, but only 71 % identity for the extracellular ligand-binding domain. Expression of STaR in COS-7 cells and stimulation of the transfected cells with STa showed a dose-depenent accumulation of cGMP with a half-maximal response of 15-20 nM. Compared to the published results for rat GC-C, the human StaR appears to be about 20-times more sensitive to STa.
The sequence diversity of the extracellular ligand binding domain may account for this increased sensitivity of the human receptor.

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ACTIVATION OF ADENYLYL CYCLASE BY PITUITARY ADENYLATE CYCYLASE ACTIVATING POLYPEPTIDE (PACAP) IN MEMBRANES OF PORCINE HEARTS

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PACAP is a novel hypothalamic peptide present in two forms with 38 and 27 amino acid residues which shows 68% homology with vasoactive intestinal peptide (VIP). VIP-receptor mediated activation of cardiac adenylyl cyclase (AC) has already been shown in membrane preparations of a number of species except guinea pig+rabbit (Chatelain et al.,1980). We used highly purified sarcolemmal membranes of porcine hearts to measure AC activation by PACAP-38, PACAP-27, VIP, and l-isoprenaline. AC activating potency of both of the PACAP's was equipotent and was found to surpass that of l-isoprenaline 2-3 times. VIP was slightly but not significantly less active than PACAP. At pmolar concentrations PACAP activated AC via a high affinity class of receptors (Kact≈10 pM). At higher concentrations PACAP seems to occupy VIP receptors since no additive effects has been observed. Guanylyl imidodiphosphate, a nonhydrolyzable GTP derivative, potentiated the PACAP-induced AC activation indicating that Gs protein(s) are coupled to these peptide receptors. In order to search for a physiological function of the PACAP/VIP receptors in cardiac sarcolemma,cultured neonatal rat cardiomyocytes were exposed to PACAP. The peptide was found to increase the beating rate to about 30 beats/min at maximum. The dose-response curve was biphasic in shape and resembles in this respect to the PACAP-induced activation of AC in porcine sarcolemma.
The findings suggest that PACAP's may have specific function(s) in regulation of cardiac activity via distinct VIP and PACAP receptors.

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DETECTION OF HORMONAL ACTIVATION OF PROTEIN KINASE A IN BOVINE CORONARY ARTERY IS DEPENDENT ON THE PH-BUFFER USED FOR HOMOGENIZATION. R. Fermum and K.-U. Möritz

A major problem encountered with studies on the hormonal regulation of cyclic-AMP-dependent protein kinase in tissues is the preservation of the activation state of the enzyme after homogenization.
After preincubation of isolated bovine coronary arteries with either isoproterenol (ISO) or L-thyroxine (T), the activity of protein kinase A (PK-A) of the 30000 x g supernatant fraction was investigated. When homogenization was performed at physiological ionic strength of 0.16 M in a buffer solution containing 20 mM phosphate, no significant increases in kinase activity ratios were observed in coronary arteries treated with ISO or with T. These results agree with those of SILVER et al. (Am. J. Physiol. **242**, H177 **1982**) and VEGESNA and DIAMOND (Can. J. Physiol. Pharmacol. **62**, 1116 **1984**) for ISO. In contrast, homogenates prepared in buffer solutions containing MOPS or imidazole showed that stimulation with ISO or T was associated with a marked increase in activity ratio. Isozymic forms of PK-A in the supernatant were idendified by DEAE cellulose chromatography. ISO activated predominantly the subtype II isoenzyme. This effect was also only detectable when MOPS was substituted for phosphate in the homogenization buffer. The results suggest that MOPS has a preserving effect on the activation state of PK-A after hormonal stimulation in situ.

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NITRIC OXIDE SYNTHASE-CATALYZED FORMATION OF HYDROGEN PEROXIDE B. Heinzel

An FAD- and FMN-mediated electron-transfer from NADPH to molecular oxygen is apparently involved in the brain NO synthase-catalyzed conversion of L-arginine into NO and L-citrulline [Mayer et al., FEBS Lett. 288, 187-191, 1991]. Here we show that this oxygen reduction results in the formation of hydrogen peroxide at low concentrations of L-arginine or tetrahydrobiopterin (H_4biopterin) due to an uncoupling of oxygen reduction. This generation of hydrogen peroxide was strictly dependent on the presence of Ca^{2+}/calmodulin and was inhibited by L-arginine and H_4biopterin with half-maximal effects observed at 11 μM and 0.3 μM, respectively. The L-arginine analogues N^{ω}-nitro-L-arginine, its methylester and N^{ω}-monomethyl-L-arginine (L-NMMA) inhibited the synthesis of L-citrulline from 0.1 mM L-arginine with half-maximal effects at 0.74 μM, 2.8 μM and 15 μM, respectively. The N^{ω}-nitro-derivatives also blocked the formation of hydrogen peroxide, whereas L-NMMA did not affect this reaction. These results indicate that Ca^{2+}-dependent activation of brain NO synthase may lead to the formation of hydrogen peroxide and/or reactive oxygen species instead of NO at pathophysiologically low levels of intracellular L-arginine or H_4biopterin.

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CEREBELLAR NO SYNTHASE IS A Ca^{2+}/CALMODULIN-DEPENDENT CYTOCHROME C REDUCTASE B. Mayer

Nitric oxide (NO) is formed from L-arginine by differently regulated NO synthase isoenzymes with NADPH, tetrahydrobiopterin and molecular oxygen as essential cofactors. Previously, we have shown that a soluble NO synthase purified from porcine cerebellum catalyzes an FAD- and FMN-mediated electron-transfer from NADPH to molecular oxygen in a Ca^{2+}/calmodulin-dependent fashion [Mayer et al., FEBS Lett. 288, 187-191, 1991]. Similarily, the NADPH-dependent enzymatic reduction of cytochromes is mediated by FAD- and FMN-containing reductases, and Bredt et al. [Nature 351, 714-718, 1991] reported on considerable sequence homologies between rat brain NO synthase and cytochrome P_{450} reductase. We investigated whether brain NO synthase may be a soluble isoform of cytochrome c (P_{450}) reductase. It was found that the purified enzyme catalyzed a reduction of cytochrome c which was to 90 % dependent on the presence of Ca^{2+}/calmodulin (K_m = 34.1 μM cytochrome c; v_{max} = 10.2 μmol x mg^{-1} x min^{-1}). Thus, cytochrome c reduction proceeded about 10-fold faster than the formation of L-citrulline. Precipitation of NO synthase by cytochrome c agarose and lack of inhibition by superoxide dismutase indicate a direct interaction of these proteins. Our results suggest that brain NO synthase is a Ca^{2+}/calmodulin-dependent cytochrome c reductase which may participate in intracellular electron-transfer processes.

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STUDIES ON THE SUBSTRATE SPECIFICITY OF NITRIC OXIDE SYNTHASE *M. Hecker, D.T. Walsh, A. Zembowicz, and J.R. Vane*

Nitric oxide (NO) is a potent vasodilator and antithrombotic agent, and plays an important role in the cytotoxicity of activated macrophages and as a neurotransmitter. Its formation from L-arginine (L-Arg) is catalyzed by a NADPH-dependent dioxygenase, referred to as NO synthase (NOS), which can exist in at least two distinct forms, a constitutive Ca^{2+}/calmodulin-dependent NOS (NOS_c) and a Ca^{2+}-independent inducible NOS (NOS_i). An important way of elucidating the chemical mechanism of the biosynthesis of NO is to characterise the substrate specificity of these isoforms of NOS. We have, therefore, investigated the substrate specificity of NOS preparations from cultured endothelial cells (EC) and lipopolysaccharide-activated J774.2 monocyte/macrophages by monitoring the NO-mediated increase in intracellular cyclic GMP in LLC-PK_1 pig kidney epithelial cells. NOS_c activity in EC was largely membrane-bound, whereas NOS_i activity in J774.2 cells was equally distributed among cytosol and membrane(s). The cytosolic NOS_c in EC and the membrane-bound NOS_i in J774.2 cells were strictly Ca^{2+}-dependent, whereas the membrane-bound NOS_c in EC and the cytosolic NOS_i in J774.2 cells were not. Interestingly, the capacity of these isoforms of NOS to produce NO was very similar, suggesting that the biosynthesis of NO by EC, in contrast to the continuous production of NO by cytokine-activated macrophages, is tightly controlled by intracellular signals. L-Homoarginine and L-Arg-containing small peptides, such as L-Ala-L-Arg, L-Arg-L-Arg, L-Arg-L-Phe or L-Arg-L-Arg-L-Arg, could replace L-Arg as a substrate for the NOS_c in EC and the Ca^{2+}-independent NOS_i in J774.2 cells. In contrast, the Ca^{2+}-dependent NOS_i accepted only L-Arg, N^G-hydroxy-L-arginine and L-Arg-L-Arg as substrates. Thus, the active site of NOS seems to be flexible enough to accomodate molecules different from L-Arg in size, charge and hydrophobicity. These findings also suggest that the availability of L-Arg-containing small peptides may represent an important regulatory factor for the biosynthesis of NO, e.g. by EC. We conclude that, irrespective of their intracellular localisation, at least three isoforms of NOS exist, which can be differentiated by their substrate specificity and Ca^{2+}-dependency (*Supported by a grant from Glaxo Group Research Ltd.*).

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Immunohistochemical detection of NO synthase type I suggests co-localization with NADPH-diaphorase but not soluble guanylyl cyclase, and para-neuronal functions for nitrinergic signal transduction

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Nitrinergic signaltransduction is based on two protein families. Nitrogen oxides (NO) synthases (NOS), which catalyze the conversion of L-arginine to NO and citrulline but may also have other oxido-reductase functions. NO activates the soluble isoforms of guanylyl cyclase (GC-S), which catalyze the conversion of GTP to the second messenger molecule cyclic GMP. Polyclonal antiserum (6761-8) to rat cerebellum type I NOS (160 kDa-dimer) and monoclonal antibody to rat lung GC-S (B_4) were generated in order to localize both signal transduction proteins in various rat tissues. NOS was also visualized by its NADPH-diaphorase (NADPH-d) properties, that is NADPH-dependent nitroblue tetrazolium formazan formation.Throughout the rat, NOS and NADPH-d were co-localized. Staining was found both in neuronal cells, *i.e.* in the cerebellar cortex (granular cells), hippocampus (CA, dentate gyrus), pituitary, peripheral neurons and ganglia (duodenum), but also in non-neuronal cells, *i.e.* in pancreas (islets of Langerhans), kidney (macula densa), and epithelial cells (lung, uterus, stomach). Western blot analysis revealed that the NOS present in epithelial cells and pancreatic B-cells, may differ in molecular mass (180 and 150 kDa) from the rat brain NOS-I. These type I-related NOS may have important para-neuronal functions such as mediating the renal tubulo-glomerular feedback, bronchodilatation, and peptide-hormone (*e.g.* insulin) secretion. In the brain, NOS and GC-S were not co-localized in the same cells but were found in adjacent cell layers. In the brain, NO is, therefore, likely to function as a inter-cellular messenger or neurotransmitter generated in specialized neurons (granular cells) regulating GC-S activity in specific target cells (Purkinje and CA pyramidal cells). Since we and others recently succeeded to purify a human NOS from cerebellum, there is evidence that nitrinergic signal transduction does also occur in man.

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PARTICULATE AND SOLUBLE ENDOTHELIAL NITRIC OXIDE SYNTHASES HAVE SIMILAR PROTEIN STRUCTURES WHICH DIFFER FROM BRAIN NITRIC OXIDE SYNTHASE

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Constitutive, calcium/calmodulin-regulated nitric oxide synthases (NOS) have been identified in brain and endothelial cells. Clearly both of these enzymes are different from the calcium-independent, induced isoform of NOS found in macrophages. On the other hand, it has been inferred from the immunohistochemical staining of endothelial cells by a polyclonal antibody against brain NOS that brain and endothelial NOS are identical (Snyder and Bredt, Trends Pharmacol. Sci. 12: 125-127, 1991). In our experiments, we found brain NOS (isoform I) exclusively in the cytosolic fraction, whereas about 95% of the total endothelial NOS activity (isoform III) was particulate and only about 5% was soluble. We purified soluble NOS from rat brain and particulate NOS from bovine aortic endothelial cells using affinity chromatography on 2'5' ADP Sepharose followed by Superose 6 gel permeation chromatography. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, brain NOS migrated as a single band corresponding to a M_r of 155 kDa, whereas purified, particulate NOS from native and cultured endothelial cells had a denatured molecular mass of 135 kDa. Brain NOS had chromatographic properties different from the two endothelial enzymes: Brain NOS was eluted from DEAE anion exchange columns with 0.1M KCl, whereas elution of both the soluble and the particulate endothelial NOS required 0.2M KCl. On Superose 6 gel permeation chromatography, brain NOS eluted according to its native molecular weight of approximately 300 kDa, whereas both endothelial enzymes eluted anomalously near the total included volume. In Western blot analyses, polyclonal antibodies generated against purified, denatured brain NOS did not cross-react with endothelial NOS. Conversely, monoclonal antibodies generated against purified, denatured, particulate endothelial NOS recognized both the particulate and the soluble endothelial NOS as single bands corresponding to a molecular weight of 135 kDa, but did not cross-react with brain NOS. Immunohistochemical experiments showed that our antibodies against particulate, endothelial NOS, but not our antibodies against brain NOS, stained endothelial cells. These data indicate that soluble and particulate endothelial NOS may represent post-translational modifications of the same protein, whereas brain NOS is a structurally different protein.

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TETRAHYDROBIOPTERIN REGULATES THE FORMATION OF NITRIC OXIDE IN CULTURED ENDOTHELIAL CELLS.

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In mammalian tissues, there are at least two isoforms of NO generating enzymes which catalyze the conversion of L-arginine into L-citrulline and NO: a constitutively expressed, Ca^{2+}/calmodulin regulated enzyme present in the brain and in endothelial cells, and a Ca^{2+}-independent, cytokine-inducible enzyme found in activated macrophages. While the latter has been found to be regulated by tetrahydrobiopterin (H_4biopterin), a possible cofactor role of this pteridine for the Ca^{2+}/calmodulin dependent NO synthase is still discussed.

In the present study we demonstrate that inhibition of H_4biopterin biosynthesis in intact endothelial cells reduces their ability to produce NO. Under control conditions, endothelial cells responded to addition of bradykinin or the Ca-ionophore A 23187 with an enhanced formation of NO as detected spectrophotometrically by the conversion of oxyhemoglobin to methemoglobin or by an increase in intracellular cGMP levels. Preincubation of endothelial cells with 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, reduced in same concentration range intracellular H_4biopterin levels and the formation of NO. Addition of sepiapterin, a pteridine which leads to the biosynthesis of H_4biopterin via a salvage pathway, elevated intracellular H_4biopterin levels and restored the ability of endothelial cells to produce NO.

These data indicate that the biosynthesis of EDRF depends on the presence of intracellular H_4biopterin, which, in addition to Ca^{2+}, may represent a further (patho)physiological regulator of endothelial NO biosynthesis.

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NITRIC OXIDE STORES IN VASCULAR TISSUE

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The identity of endothelium-derived relaxing factor, the nitric oxide (NO)-containing vasodilator released from endothelial cells, is still obscure. Though NO is generated by endothelial NO synthase intracellularly, a paracrine action of free NO is questionable, because of its lability. Instead, NO could be stabilized by formation of a nitrosyl-complex with ferrous iron bound to thiolate groups. We studied the biological activity, stability, and interaction of dinitrosyl-iron(II)-di-L-cysteine (DNIC) with vascular tissue. DNIC potently activated purified soluble guanylyl cyclase (GC) and relaxed noradrenaline-precontracted segments of endothelium-denuded rabbit femoral artery (EC_{50}'s 10 nM). DNIC (1 μM) lost its biological activity and decomposed in Tyrode's solution at 37°C with a half life of 30 s, as measured by UV and electron spin resonance (ESR) spectroscopy. Pre-incubation (5 min; 37°C) of endothelium-denuded rabbit aortic (RA) segments with DNIC resulted in a rapid formation of a stable (> 2 h) ESR-detectable dinitrosyl-iron(II) complex with aortic protein thiol groups. A labile vasodilating and GC activating factor was released from pre-incubated RA segments for up to 2 h of perfusion. N-acetyl-L-cysteine enhanced the release of the GC activating factor and enabled the detection of paramagnetic DNIC in the superfusate. We conclude that NO produced by, or acting on vascular cells can be stabilized and stored as a dinitrosyl-iron(II) complex with protein thiols, and can be released from cells in the form of a low molecular weight dinitrosyl-iron(II)-dithiolate by intra- and extracellular thiols.

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THE ROLE OF NITRIC OXIDE (NO) FOR THE POTENTIATION OF THE ANTIPLATELET EFFECTS OF NITRATES BY CULTURED ENDOTHELIAL CELLS

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The antiaggregatory effect of organic nitrates such as glyceryl trinitrate (GTN) has been shown to be markedly potentiated by cultured vascular smooth muscle cells and plasma via the formation of NO.The aim of the present study was to investigate whether endothelial cells (EC) also express this biotransformation pathway and to elucidate its role for the antiaggregatory action of nitrates in vitro.

RESULTS: The formation of NO from superfused aortic EC cultured on microcarrier beads was measured continuously by means of difference-spectrophotometry. Infusion of GTN over the cells (1-300 μM, 1 min) resulted in the immediate and concentration-dependent generation of NO. Preincubation of platelet-rich plasma (PRP) with GTN (0.1-200 μM) for 5 min resulted in a concentration-dependent inhibition of ADP-induced aggregation.In the presence of EC in suspension ($2.8x10^5$ cells/ml) the concentration response curve for GTN was shifted to the left. This potentiation was completely reversed by oxyhaemoglobin (10 μM) indicating that the effect was mediated via NO. Similar results were obtained with isosorbide dinitrate and isosorbide 5-mononitrate. The potentiation of the antiaggregatory effect of GTN by EC was even more pronouced in washed platelets.

CONCLUSION: These data demonstrate that EC metabolize organic nitrates to NO. This NO generation is responsible for the potentiation of the inhibitory effects of nitrates on platelet aggregation in vitro. Our results suggest that the biotransformation of organic nitrates in EC is likely to play an important role for the modulation of blood cell function in vivo.

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EFFECTS OF ILOPROST AND SIN-1 ON cAMP AND cGMP LEVELS IN WASHED HUMAN PLATELETS

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The guanylate cyclase activator NO and the adenylate cyclase activator PGI_2 show a synergistic inhibitory effect on platelet aggregation in vitro and in vivo by elevating platelet cGMP or cAMP. The dose- and time-dependent alterations in cGMP and cAMP levels after coincubation with the NO-donor SIN-1 and the PGI_2-analog iloprost were studied, in order to investigate possible interactions of the second messengers. cAMP levels increased with iloprost 3-30 ng/ml for 1-30 min from 22.7±5.6 to 250±52 pmol/10^8 platelets; cGMP levels were not altered. Incubation with SIN-1 3-30 µg/ml for 1 min showed a dose dependent rise of cGMP levels from 1.6±0.3 to 6.2±1.4 pmol/10^8 platelets, 10 min incubation with SIN-1 30 µg/ml increased cGMP levels from 2.0±0.3 to 13.2±1.4, and 30 min from 1.9±0.3 to 15.4±2.4 pmol/10^8 platelets. cGMP levels were not significantly altered by coincubation of SIN-1 with iloprost and vice versa, cAMP levels were unaltered by coincubation with SIN-1. Thus we conclude that the synergistic inhibition of platelet aggregation is not due to synergistic alterations in cAMP or cGMP levels.

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MOLSIDOMINE, THE NOVEL SYDNONIMINE CAS 936 AND THEIR METABOLITES INHIBIT INTRACELLULAR FREE CALCIUM IN STIMULATED HUMAN PLATELETS

I. Baumann and J. Baumann

Recent studies have indicated that molsidomine (MOL), SIN-1, SIN-1A and the novel analogues CAS 936, C87 3754 and C87 3786 may alter cell activity independent of providing NO (BOHN et al.: J. Cardiovasc. Pharmacol. 18, 522, 1991). Since cytosolic free Ca^{++} may be considered as a common denominator of cellular activity this study investigates the effects of these compounds on this parameter.
Washed human platelets were incubated with the agents for 2 min in Tyrode-HEPES buffer (pH 7.4; $[Ca^{++}]_e$: 2 mM, 37°C) and then stimulated with calcimycin (3 µM), thrombin (0.5 IU/ml) or arachidonic acid (AA; 10 µM). Changes in cytosolic Ca^{++} were monitored fluorimetrically using INDO-1AM (1.5 µM). In comparison to solvent-treated controls (inhibition: 0 %), the percent inhibition of the $[Ca^{++}]_i$-signal subsequent to the addition of the test drugs was as follows:

	concentration [µM]	calcimycin inhibition [%]	thrombin	AA
MOL	100	100	100	100
MOL	10	56 ± 4	64 ± 4	57 ± 3
CAS 936	100	100	100	100
CAS 936	10	73 ± 2	79 ± 2	79 ± 2
SIN-1	100	13 ± 5	58 ± 2	88 ± 4
C87 3754	100	39 ± 6	54 ± 5	75 ± 5
SIN-1A	100	34 ± 5	55 ± 1	59 ± 4
C87 3786	100	7 ± 2	41 ± 4	73 ± 5

x ± SEM (n=4) or respective stimulus controls
The data suggest an inhibition of stimulated cytosolic Ca^{++} by both NO-donators and (inactive) prodrugs.

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ISCHEMIA AND REPERFUSION-INDUCED EARLY ARRHYTHMIAS IN VIVO: BENEFICIAL EFFECTS OF THE ACTIVE METABOLITES OF MOLSIDOMINE (SIN 1;SIN 1A)

T.F.Krzemiński

The sudden restoration of coronary blood flow can lead to reperfusion - induced ventricular fibrillation and sudden death in man. The effects of two molsidomine active metabolites (SIN 1;SIN 1A) against ischemia- and reperfusion-induced arrhythmias and creatine kinase (CPK) release were investigated using anaesthetized rats (60mg/kg pentobarbiton,i.p.) with transient (7min) coronary artery occlusion (improved method according to Seley et al.,Angiology,1960;11;398-407).Moreover,the hemodynamic parameters (heart rate(HR), systolic and diastolic blood pressures(BPs,BPd), tension-time index (TTI) were measured. The test drugs or vehicle (controls) were administered (i.v.) 10min prior to occlusion (bolus and continuous infusion).
Reperfusion-induced ventricular fibrillations (VF) were observed in 100% of control animals (n=16), whereas such episodes occured in 54 and 53% of the animals treated with 6µg/ml/kg+3.6µg/ml/kg/h (n=16) or 20µg/ml/kg+12µg/ml/kg/h (n=16) SIN 1. This effect of SIN 1A (n=16) in the same doses was less pronounced.
The mean duration of reperfusion-induced VF and ventricular tachycardias (VT) were not changed by both drugs, if occured.
The mortality index was reduced from 43% in control to 31 and 18% by SIN 1, while SIN 1A decreased this ratio to 12 and 25% respectively.
SIN 1 was more effective in dose dependently reducing of the CPK release caused by reperfusion. The mean CPK value in the plasma of the higher dose SIN 1 treated group (7.7±1.6 U/g Prot. n=10) after reperfusion was not different from the value of the sham operated group (8.1±1.2 U/g Prot., n=8).
These evidences may enlarge present clinical indications for long-treatment with molsidomine (as the NO-releasing drug) in preventing severe rhythm disturbances caused by sudden coronary artery reperfusion.

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PHARMACOLOGICAL PROPERTIES OF SPM 3672, A NITROVASODILATOR DIRECTLY LIBERATING NITRIC OXIDE.

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The activity of organic nitrates is blunted by the development of tolerance during long-term therapy. On a molecular basis nitrate tolerance is thought to be associated with reduced intracellular concentrations of substances containing SH-groups such as cysteine. Therefore, several new compounds were developed (Noack et al.: 2nd Int. Meeting "Biology of nitric oxide", London Oct 1, 1991) combining both, a nitrate- and a thiol-group. In the case of SPM 3672 (N-[3-Nitratopivaloyl]-1-cysteineethylester) the intramolecular thiol moiety derives from the covalently bound cysteine molecule. Isolated guanylate cyclase (GC), partially purified from the cytosolic fraction of human platelets by DEAE-sepharose-chromatography is half-maximally stimulated by 1,54±0,16 nM of SPM 3672, measured by radioimmunassay for cyclic GMP. A nearly 10-fold potentiated stimulation of GC was observed with glycerol trinitrate (GTN), but only in the presence of 5 mM cysteine. The vasorelaxing activity of SPM 3672 was examined in isolated rat aorta and porcine right coronary artery rings precontracted with norepinephrine (1µM) or. $PGF_{2\alpha}$ (50µM) resp.. Presence of intact endothelium was checked by the addition of acetylcholine (10µM) or substance P (3nM) resp.. Dose-response-curves for SPM 3672 were performed via cumulative drug application (10nM-0,1mM). In order to evaluate a development of tolerance a second dose-response-curve was added, or the vessels were preincubated with GTN (30 min, 0,1mM). Halfmaximally vasodilating concentrations (IC_{50}) of SPM 3672 slightly increased following repeated drug application in rat aorta (IC_{50}: 1,21±0,09 and 2,02±0,37 µM resp., n=5).In porcine coronary artery SPM 3672 exhibited an IC_{50}-value of 1,05±0,18 µM (n=8). Preincubation of the artery rings with GTN, performed to elicit nitrate tolerance, significantly potentiated the vasorelaxing activity of SPM 3672 (IC_{50}: 0,51±0,09 µM, n=8). By contrast, repeated exposure of these vessels with GTN resulted in a pronounced and significant reduction of its vasodilating activity [IC_{50}(GTN): 0,40±0,07 resp. 6,97±0,56 µM, n=5]. These results demonstrate that SPM 3672 is a powerful, approximatly GTN-equieffective vasodilator, activating isolated human GC without the requirement of additional thiols. Furthermore, the activity of this compound is not diminished by tolerance and is not only fully retained but even slightly enhanced in porcine coronary arteries tolerant to GTN. Thus, in future SPM 3672 could represent a therapeutical alternative in long-term nitrate treatment.
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SEPIAPTERIN INCREASES TETRAHYDROBIOPTERIN LEVELS AND INFLUENCES BLOOD PRESSURE IN SPONTANEOUSLY HYPERTENSIVE RATS.

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Among several abnormalities observed, an impairment of endothelium dependent relaxation was shown in aortae from SHR (Experientia 45,705-708,1989). Furthermore, tetrahydrobiopterin synthesis is lower in SHR than in normotensive Wistar Kyoto rats (WKY). It has been shown recently, that nitric oxide synthase requires tetrahydrobiopterin for full activity (FEBS letters 277,215-219,1990) and that intracellular levels of tetrahydrobiopterin regulate the amount of nitric oxide (NO) formed from L-arginine (J.Exp.Med.172: 1599-1607). Thus, decreased tetrahydrobiopterin might lead to decreased NO synthesis and hence contribute to hypertension in SHR. We treated SHR (n=6) with sepiapterin (10mg/kg) which is converted to tetrahydrobiopterin by a salvage pathway. Controls received the same dose of pterin-6-carboxylic acid (n=6), which is not metabolized to tetrahydrobiopterin, or vehicle (n=5).
Blood pressure of SHR treated with sepiapterin was lower than that of SHR treated with pterin-6-carboxylic acid or vehicle. This effect lasted for two days. While NO synthase activities in various brain areas and organs remained unchanged by the treatment, sepiapterin but not pterin-6-carboxylic acid increased the tetrahydrobiopterin levels. Significant increases were seen in the brainstem, cerebellum, liver and serum. These results demonstrate that the increase in tetrahydrobiopterin levels by sepiapterin leads to a transient delay in the development of hypertension in SHR. Further experiments will show whether this effect is due to peripheral or central mechanisms.

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EDRF INFLUENCES PERIPHERAL SYMPATHETIC ACTIVITY IN MYOCARDIUM Klaus Addicks, & Wilhelm Bloch

There is discussion about the influence of regulational factors of the vascular wall such as EDRF/NO on the vascular reaction induced by sympathetic innervation.
We studied isolated perfused rat-hearts at a constant coronary flow of 9ml/min. and added NO(10^{-8}M) to analyse the effects of EDRF/NO on the intrcardiac sympathetic nerve fibres. Coronary and left ventrikular pressure were monitored. The intraxonal catecholamines were visualised by fluorescence microscopy and measured.

	Time	NO/EDRF, 10^{-8} M % of control
Area of intraaxonal catecholamines	3 min.	126%
	20 min.	125%
coronary pressure	3 min.	69%
	20 min	66%

The NO-antagonist L-nitro-arginin causes a decreased fluorescence of intracardiac nerve fibres and a rise in coronary perfusion pressure. These results demonstrate a strong inffluence of EDRF on peripheral sympathetic activity. The observed vascular resistence is consequence of the direct effects of EDRF on the walls of coronary vessels and the observed modulation of sympathetic activity.

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STABLE EXPRESSION OF cGMP-DEPENDENT PROTEIN KINASE IN CHO CELLS SUPPRESSES CALCIUM TRANSIENTS

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The cDNA of the Iα cGMP kinase (cGK) was stably transfected into CHO cells which lack endogenous cGK. After amplification of the integrated gene the concentration of cGK in these cells was 3 μM. The recombinant cGK showed cGMP activation and binding characteristics identical with the enzyme purified from bovine lung. To study the effect of the cGK on the cytosolic calcium concentration ($[Ca^{2+}]_i$), calcium transients in CHO cells were investigated. Thrombin at a concentration of 30 nM stimulated $[Ca^{2+}]_i$ in CHO wild type cells (CHO- WT) from 127 nM to 492 nM and in CHO cells expressing cGK (CHO-cGK) from 120 nM to 412 nM. Preincubation with 1 mM 8-Br-cGMP almost completely reduced thrombin induced calcium transients in the CHO-cGK cells, but not in CHO-WT cells. In contrast to 8-Br-cGMP, 1 mM 8-Br-cAMP did not affect the calcium transients in both cell clones.
These results suggest that the activation of the cGK indeed lowers cytosolic calcium concentration. The mechanism of this effect remains to be resolved. However, the target protein(s) being phosphorylated by cGMP kinase are obviously present also in CHO cells which do not endogenously express cGK.

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CHARACTERIZATION OF AN ENDOGENOUS NITRIC OXIDE (NO)-STIMULATED ADP-RIBOSYLATION S. Dimmeler and B. Brüne

An endogenous ADP-ribosyltransferase is present in different tissues including platelet cytosol. Agents known to release NO are able to activate the ADP-ribosylation of a 39 kD-protein in a cGMP independent way. Recently we demonstrated that NO, derived from it's physiological source L-arginine by the enzymatic activity of the soluble NO-synthase, stimulates the ADP-ribosylation in rat cerebellum as well as in DMSO-differentiated HL-60 cells. Using platelet cytosol we observed a dramatically increased ADP-ribosylation by addition of the cofactors (1 mM NADPH, 250 μM $CaCl_2$, 10 μM tetrahydrobiopterin, 10 μg/ml calmodulin and 3 μM L-arginine) known to activate the cytosolic NO-synthase. Unfortunatelly, the increased incorporation of ADP-ribose from NAD was not inhibited by known NO-synthase inhibitors N-nitro-L-arginine or N-methyl-L-arginine. We now studied the effect of each cofactor alone and detected an increase of ^{32}P-ADP-ribose incorporation in the presence of NAPDH whereas all other cofactors were ineffective. The stimulatory effect was concentration dependent reaching a maxium with 1 mM NADPH. NADPH not only enhanced the basal acitivity. It increased also the NO-stimulated ADP-ribosylation synergistically. Since the stimulatory action was only achieved in the presence of another reduced cofactor like DTT, we suppose that the ADP-ribosylation may be linked to the cellular redox status. NADPH may attribute to the physiological significance of this reaction by shifting the dose-response curve of nitric oxide to the left.

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EFFECTS OF THE ADENOSINE RECEPTOR AGONISTS NECA AND CCPA ON LYSOSOMAL ENZYME RELEASE AND ADHESION TO ENDOTHELIAL CELLS OF HUMAN NEUTROPHILS.

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Human neutrophils (PMN) play an important role in atherosclerosis due to their adhesion to the blood vessel wall and the release of enzymes and reactive oxygen species. We investigated the influence of the A_2-receptor agonist NECA [5' N-ethyl-carboxamido-adenosine] and the A_1-receptor agonist CCPA [2-chloro-N^6-cyclopentyl-adenosine] on adhesion to cultured porcine aortic endothelial cells (PAE) and ß-glucuronidase release of PMN stimulated by phorbol 12-myristate 13-acetate (PMA) or n-formyl-methionine-leucine-phenylalanine (FMLP). NECA (10^{-7} to 10^{-5} mol/l) reduced the adhesion of PMA (10 ug/l) stimulated PMN to a minimum of 69 % and inhibited the enzyme release of FMLP (30 nmol/l) stimulated PMN to 75 % of control. CCPA (10^{-7} to 10^{-5} mol/l) enhanced the adhesion to a maximum of 132 % and slightly inhibited the enzyme release of PMN to 90 % of control. Additionally, NECA increased the cyclic AMP formation of isobutylmethylxanthine (0.25 mmol/l) treated PMN to a maximum of 411 % and CCPA to 145 % of control. These findings suggest that A_2-receptors mediate inhibitory effects on enzyme release and adhesion of stimulated human PMN. These effects are partly explained by the activation of adenylate cyclase.

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PURIFICATION OF A HIGH AFFINITY ADENOSINE BINDING SITE FROM BOVINE BRAIN

A. Lorenzen and U. Schwabe

It has been shown previously that bovine brain membranes do not only contain adenosine receptors, but also two other proteins of unknown function with different affinities for 5'-N-ethylcarboxamido[^{3}H]adenosine([^{3}H]NECA). A high affinity NECA binding protein can be distinguished from A_2 adenosine receptors and the low affinity adenotin-like NECA binding protein by its characteristic pharmacological profile.

Bovine brain membranes were solubilized with the zwitterionic detergent CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulonate), and the solubilized extract was subjected to ion exchange chromatography on a QAE sepharose column. [^{3}H]NECA binding sites were absorbed completely to the gel, and the high affinity NECA binding sites were selectively recovered almost completely by elution with a KCl gradient (20-300 mM). Solubilization and ion exchange chromatography purified high affinity NECA binding sites approximately 70-80fold compared to membranes. These fractions were adsorbed to a NECA affinity matrix produced by coupling adenosine 5'-carboxylic acid to an amino terminal agarose. High affinity NECA binding sites were eluted with 10 mM adenosine (purification factor of NECA agarose: 200). Subsequent polyacrylamide gel electrophoresis revealed the enrichment of a polypeptide with an apparent molecular weight of 53 KDa.

In summary our results show a 16,000fold purification of a 53 KDa high affinity NECA binding protein by ion exchange and affinity chromatography.

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COMPARISON OF THE EFFECTS OF ADENOSINE AGONISTS ON CORONARY AND AORTIC ENDOTHELIAL CELLS

J. O. Schiele

There is increasing evidence that endothelium from different vessels exhibits different functional properties. In cultivated endothelial cells adenosine has been shown to increase cAMP levels via adenosine A_2 receptors. Conflicting results have been reported on the extent of adenylyl cyclase stimulation by adenosine in endothelium from different tissues and species. Therefore, we compared the effect of several adenosine receptor agonists on cAMP levels in both macro- and microvascular endothelial cells of the same species.

Macrovascular endothelial cells were cultivated from guinea pig aorta. Coronary endothelial cells were prepared from guinea pig heart and were mainly of microvascular origin. Cells were subcultivated on microcarrier beads, and stimulated in the presence or absence of the phosphodiesterase inhibitor 4[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724, 500 μM). cAMP content was determined by radioimmunoassay.

In coronary endothelium, 5'-N-ethylcarboxamidoadenosine (NECA), R-N^6-phenyl-isopropyl-adenosine (R-PIA) and the new A_2 receptor selective agonist 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamido adenosine (CGS 21,680) stimulated cAMP formation 8-, 3-, and 2 fold, respectively. In the presence of Ro 20-1724 basal levels were unchanged, NECA-, R-PIA- and CGS 21,680 stimulated cAMP levels were 37-, 21-, and 16 fold. Forskolin and isoproterenol increased cAMP levels in presence of Ro 20-1724 40- and 5 fold. The time course showed an initial steep increase of the cAMP level, reaching a plateau after 10 to 15 min. The NECA-induced cAMP increase was competitively blocked by the adenosine receptor antagonist xanthine amine congener (XAC).

In aortic endothelium, NECA and forskolin increased cAMP level in presence of Ro 20-1724 more than 200 fold. CGS 21,680, R-PIA and isoproterenol increased cAMP levels in the presence of the phosphodiesterase inhibitor 52-, 87- and 5 fold. In both endothelial cell preparations the observed rank order of potency was typical for an adenosine A_2 receptor: NECA > CGS 21,680 > R-PIA.

In aortic and coronary guinea pig endothelium NECA is the most efficacious adenosine analogue. CGS 21,680 and R-PIA are partial agonists for the adenosine A_2 receptor. The large differences in the maximal response to stimulation with adenosine agonists in coronary versus aortic endothelium may either be due to different activities of adenylyl cyclase or phosphodiesterase or to different adenosine receptor densities.

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CHARACTERIZATION OF A NOVEL ADENOSINE-BINDING PROTEIN FROM THE CYTOSOLIC FRACTION OF HUMAN PLATELETS

M. Schreiner, T. Fein

Previous studies have shown that human platelets contain two different ([^{3}H]NECA) binding proteins. An adenotin-like binding site has been seperated from A_2 adenosine receptors and partially purified by affinity chromatography. In this study we describe an additional NECA-binding protein obtained from the cytosolic fraction of human platelets, which can be distinguished from the membrane-bound adenotin-like NECA-binding protein by several criteria. Gel filtration of the cytosolic fractions of human platelets yielded a single peak in which two distinct NECA-binding sites were observed. The first part of the peak exhibited adenotin-like binding properties with adequate affinity for NECA and 2-chloroadenosine whereas in the second part of the peak [^{3}H]NECA binding was displaced by NECA but not substantially by 2-chloroadenosine used for the definition of nonspecific binding. A separation of these two NECA-binding proteins was obtained by ion exchange chromatography on DEAE-Sepharose CL 6B. A first NECA-binding component was not retarded by the column, whereas a second binding component was eluted by a NaCl-gradient from 200-400 mM. The second binding peak showed the typical binding characteristics of the adenotin-like binding site with the order of potency 5'-N-methylcarboxamidoadenosine (MECA)>NECA>2-chloroadenosine>adenosine. In contrast, the first peak had the potency order NECA>MECA>adenosine>2-chloroadenosine. In addition, 5-50fold differences in the binding affinities for several adenosine analogues were observed. Our results show that human platelets contain an additional cytosolic NECA-binding protein which can be distinguished from A_2 adenosine receptors and adenotin-like binding protein present in platelet membranes.

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CHARACTERIZATION OF MEMBRANE-BOUND HIGH AFFINITY NECA-BINDING SITES FROM BOVINE CEREBRAL CORTEX

M. Nitsch-Kirsch

In addition to adenosine receptors, bovine cortical membranes contain low affinity adenotin-like NECA (5'-N-ethylcarboxamidoadenosine) binding sites and high affinty NECA binding sites of unknown function. For detection of these high affinity binding sites in membranes, it was necessary to remove metabolites with high affinity, e.g. adenosine by addition of adenosine deaminase and inosine by addition of nucleoside phosphorylase. The pore-forming peptide antibiotic alamethicin further increased binding of [^{3}H]NECA to this site by more than 100%. In contrast to A_2 adenosine receptors and the low affinity NECA binding protein, this site was sensitive against treatment with the sulfhydryl alkylating agent N-ethylmaleimide. This site was characterized by competition experiments which demonstrated a pharmacological profile distinct from the A_1 and A_2 adenosine receptors and the adenotin-like NECA binding protein. This site did not bind adenosine receptor antagonists, but had a relatively high affinity for adenine nucleotides and the inosine derivative NBTI (S-4-nitrobenzyl-6-thioinosine). Saturation with [^{3}H]NECA revealed a K_D value of 53.7 nM and a binding capacity of 796 fmol/mg protein. These pharmacological data show that the previously described solubilized high affinity NECA binding site and the site characterized in membranes are identical.

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DECREASED AFFINITY OF CENTRAL A_1 ADENOSINE RECEPTORS IN SPONTANEOUSLY HYPERTENSIVE RATS

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Defective adenosine mechanisms have been proposed to be involved in the pathophysiology of arterial hypertension. Thus, it has been observed that adenosine has a lower activity to depress stimulation-induced noradrenaline release and vasoconstriction in arteries of spontaneously hypertensive rats (SHR). In the present study we have used radioligand binding for a direct characterization of A_1 adenosine receptors in brain membranes of Wistar Kyoto rats (WKY) and SHR rats.

Saturation experiments with [^{3}H]-2-chloro-N^6-cyclopentyladenosine ([^{3}H]-CCPA) demonstrated a higher affinity in 5 weeks old SHR in the "prehypertensive" state, whereas 48 weeks old SHR showed a lower affinity in comparison with age-related WKY rats. No gross changes in receptor density were seen in both groups of animals. In the presence of GTP, the low-affinity binding site shows a lower affinity in 5 weeks old SHR and no difference in 48 weeks old SHR in comparison with controls. This result appears to be of functional importance since the effects of adenosine are mediated by the low affinity state of the receptor.

Saturation studies with the antagonist radioligand [^{3}H]-1,3-dipropyl-cyclopentylxanthine ([^{3}H]-DPCPX) showed no significant difference both in affinity and number of receptors in 5 weeks old rats, but a marked reduction in affinity and B_{max} values was found in 48 weeks old SHR in comparison with age-matched WKY rats. Competition experiments for [^{3}H]-DPCPX binding to A_1 adenosine receptors by R-N^6-phenylisopropyladenosine (R-PIA) showed a significant decrease of both high and low affinity binding sites in 48 weeks old SHR rats. This difference was mitigated by the addition of GTP. B_{max} values for both groups were not different.

In conclusion, our results show a decreased affinity of central A_1 adenosine receptors in SHR in comparison with WKY rats, which is already detectable in the "prehypertensive" phase. These changes might be due to an alteration of receptor structure or to a deficient G protein-receptor coupling.

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COUPLING OF PERTUSSIS TOXIN SENSITIVE G PROTEINS TO A1 AND NON-A1 ADENOSINE RECEPTORS

Angela Ameri

In a previous study it was demonstrated that the adenosine agonists R-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxamidoadensosine (NECA) inhibit CA1 neurones in rat hippocampal slices by binding to A1 receptors and excite these neurones by acting on adenosine receptors other than the A1 receptor (Ameri and Jurna, 1991, Brain Res., 546, 69-78). The aim of the present study was to investigate the involvement of GTP-binding proteins (G proteins) in the transmembranal signal transduction activated by PIA and NECA. For this purpose, pertussis toxin (PTX) was injected intracerebroventricularly. For control experiments, two groups of rats were injected either with the vehicle or with heat-inactivated PTX. Intracellular recording were made from CA1 neurones of rat hippocampal slices 4-10 days after the pretreatment. The inhibition (i.e. hyperpolarization and decrease in neuronal input resistance) produced by PIA (0.5 μmol/l) and NECA (1 μmol/l) in unpretreated neurones and neurones pretreated with the vehicle or inactivated toxin was significantly reduced in PTX-pretreated neurones. Also when applied in higher concentration (1-2.5 μmol/l), PIA failed to increase the hyperpolarization and the decrease in membrane resistance. An excitatory effect of PIA (i.e. depolarization and increase in neuronal input resistance) was observed in about 50% of the unpretreated neurones when PIA was applied in a high concentration (2.5 μmol/l) or it was produced by PIA at lower concentrations in all neurones when the A1 receptor was blocked by DPCPX. After pretreatment with PTX, the excitatory effect of PIA was absent.

It is concluded that PTX-sensitive G proteins are involved in the inhibitory action of PIA and NECA mediated by A1 receptors and in the excitatory action mediated by non-A1 receptors.

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THE TRIAZOLOPYRIMIDINE TRAPIDIL ONLY MODERATELY INTERACTS WITH THE A_1-ADENOSINE RECEPTOR OF BRAIN AND HEART

E.-G. Krause, H. Tenor

Trapidil (5-methyl-7-dimethylamino-s-triazolo[1,5α]pyrimidine; TRAP) is a nonspecific inhibitor of PDE isoenzymes (Tenor et al.: Biomed.Biochim.A. 46:749,1989) that is clinically applied, especially in Japan, in early stages of ischemia-related heart failure due to its positive inotropic and cardioprotective effects. As it was suggested from hemodynamic studies, that TRAP did not affect the regulatory function of adenosine, the interaction of TRAP with the A_1 adenosine binding sites was studied in membrane preparations from rat brain and bovine heart muscle tissue using [^{3}H]-PIA and [^{3}H]-DPCPX as radioligands. In a competing assay the K_i value for TRAP of brain membrane was calculated at 517 $\pm$ 25 μM which reflects a 32-fold less affinity as it was additionally measured for theophylline (K_i 15,9 $\pm$ 5 μM). The K_i value of unlabelled PIA was found at 0.89 $\pm$ 0.1 μM, almost idendical with its K_d value. In cardiac membrane fraction the affinity of the A_1 adenosine receptor (B_{max}: 14.5 fmol/mg protein; K_d for DPCPX: 0.11 $\pm$ 0.02 nM) for TRAP was found in the mM range (K_i: 1.4 $\pm$ 0.2mM) which seems to be without pharmacological importance. In comparison, theophylline interferes with a respective 160 times higher affinity (K_i: 8.7 $\pm$ 1.9 μM) with that binding site. As any suppression of the beneficial effects of endogenous adenosine at the level of A_1 receptors, as it was recently demonstrated for some specific PDE inhibitors, may be deleterious during ischaemia the lack of such side effect of TRAP may explain, at least partly, its efficacy in the treatment of heart failure.

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EFFECTS OF AN A2-ADENOSINE RECEPTOR AGONIST ON CONTRACTILE RESPONSE AND cAMP CONTENT IN VENTRICULAR CARDIOMYOCYTES FROM GUINEA PIGS

B. Stein, A. Drögemüller, C. Seeland

There are conflicting reports concerning the existence of cAMP-increasing A2-adenosine receptors on cardiomyocytes. Therefore, we investigated the effects of the selective A2-adenosine receptor agonist 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamid-adenosine (CGS 21680C) alone and in the presence of isoprenaline (ISO) on contractile response (extent of cell shortening in % of cell length) and cAMP content (pmol/mg protein) in isolated ventricular cardiomyocytes from guinea pigs. Cardiomyocytes were electrically stimulated at 1 Hz.

CGS 21680C alone (10 nmol/l - 10 μmol/l) did not change contractility, but concentration-dependently increased cAMP content from 2.77 ± 0.14 to 4.43 ± 0.39 at the highest concentration investigated (10 μmol/l, n=10-12, $p<0.05$). ISO (10 nmol/l) increased contractility from 1.81 ± 0.07 to 3.45 ± 0.12 and cAMP content from 2.37 ± 0.15 to 3.47 ± 0.13 (n=11-14, $p<0.05$). Additionally applied CGS 21680C concentration-dependently attenuated contractility from 3.45 ± 0.12 to 2.09 ± 0.09 at 10 μmol/l (n=11-15, $p<0.05$). However, CGS 21680C in the presence of ISO increased cAMP content from 3.43 ± 0.24 to 4.58 ± 0.31 (n=12-15, $p<0.05$). The A1-adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.3 μmol/l) antagonized the contractility-decreasing effects of CGS 21680C and led to a further increase of the CGS 21680C-stimulated cAMP content.

We conclude that A2-adenosine receptors exist on ventricular cardiomyocytes which increase cAMP content without affecting contractility. (Supported by the DFG.)

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INTRALUMINAL PGE_2 CONTRACTS EPITHELIUM-DENUDED GUINEA PIG TRACHEA

Duft S.[1], Wilkens J.H.[2], Becker A.[1], Machmüller C.,[1] Ilg, S.[1]

Airway epithelium derived PGE_2 has been proposed to act as airway smooth muscle relaxing factor (for review see Morrison K. J. et al., Am. J. Physiol. 258, L258 - L262, 1990). However, constricting effects of PGE_2 have also been observed (Gardiner P. J. and Collier H. O. J. Prostaglandins, 819 - 841, 1980). In all of these experiments ring or strip bioassays have been used.

In order to study the role of PGE_2 more closely related to the in vivo situation, we established a novel sensitive in vitro system that allows perfusion of intact, epithelium denuded guinea pig trachea (GPT) tubes. GPT responses were assessed as diameter changes by computerized video-microscopy (resolution 15 µm). Response to added PGE_2 was expressed as percentage of the maximal change in diameter induced by methacholine (100 µM) in the same preparation.

Unexpectedly, PGE_2 dose-dependently (0.1 nM - 1 µM) **contracted** tracheal tubes (maximal contraction 59 ± 7 %; n=6). Pretreatment of the GPT with the cyclooxygenase (CO) inhibitor diclofenac (10µM) did not affect maximal contraction (65 ± 14 %, n=4).

In contrast, in epithelium denuded tracheal ring preparations under basal tone or precontracted with methacholine (1 µM), PGE_2 caused a dose dependent **relaxation** (-37 ± 6 %, n=6 and -59 ± 8 %, n=5). However, after pretreatment with diclofenac (10µM) PGE2 **contracted** tracheal rings. A bell-shaped dose response curve was observed with its maximal contraction at 10 nM PGE_2 (46 ± 9 %, n=9).

The effect of PGE_2 depends critically on the experimental approach. Relaxation to PGE_2 in ring preparations appears to be CO dependent, whereas the response of GPT tubes to exogenous PGE_2 is not affected by CO inhibition. We conclude that endogenously released prostanoids influence the effect of exogenous PGE_2 .

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CALCIUM-DEPENDENT REGULATION OF PHOSPHOLIPASE A_2 IN THE HUMAN MONOCYTIC TUMOR CELL LINE THP-1.

W.Rehfeldt and K.Resch

Phospholipase A_2(PLA_2), which selectively hydrolizes the sn-2 fatty acyl ester bond of membrane phospholipids, plays an important role in generating arachidonic acid, the precursor for the biological active eicosanoids. Compared to the intensively characterized secretory and extracellular forms of the enzyme, little is known about the role of the new class of intracellular high molecular weight phospholipases A_2($cPLA_2$).

We have purified a PLA_2 from the cytosol of the human monocytic tumor cell line THP-1 to homogeneity, which exhibits a molecular mass of 100 kDa.

The enzyme shows a striking preference for polyunsaturated fatty acids in the sn-2 position of phospholipids. It is activated at physiological calcium concentrations with an EC_{50} of approximately 400 nM Ca^{2+}.

Reconstruction experiments with partially purified PLA_2 and 100.000* g (microsomal) fractions, led to a Ca^{2+}-dependent association of the enzyme with the particulate fraction at Ca^{2+}-concentrations of 300 - 500 nM.

To investigate the influence of intracellular calcium-levels on PLA_2 activity, the enzyme activity was measured in Thapsigargin treated cells (10^{-6}M). Thapsigargin is a non 12-O-tetradecanoylphorbol-13-acetate (TPA)- type tumor promoter, which raises intracellular calcium by specific inhibition of microsomal Ca^{2+}-ATPases.

Short term incubation of THP-1 cells (0-5h) induced a significant increase in PLA_2 activity after 10 minutes, being maximally enhanced after 1 hour.

The enzyme activity was determined in vitro in the presence of mM concentrations of calcium. The activation was thus due to a calcium- dependent modification of the enzyme in vivo.

These data illustrate the diverse role of calcium on the regulation of the activity of this intracellular $cPLA_2$. On the one hand mobilization of intracellular calcium activates $cPLA_2$ directly and induces translocation of the enzyme to the membrane, which represents its natural substrate. One the other hand, PLA_2 activity may be regulated by Ca^{2+}-dependent modification perhaps due to Ca^{2+}/CaM- dependent kinases.

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PROSTANOID SYNTHESIS OF HUMAN MONOCYTES: EFFECT OF ACETYL SALICYLIC ACID

J.Fauler

Monocytes are involved in the inflammatory response. We found that monocytes isolated from blood of normal volunteers synthesized thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2) in response to fMLP and A23187. However, it is not clear from this and previous investigations whether monocytes are able to synthesize TXA_2 themselves or whether TXA_2 originates from contaminating platelets. We, therefore, investigated this issue by using acetylsalicylic acid (ASS), an irreversible inhibitor of cyclooxygenase. Monocytes and platelet rich plasma (PRP) were prepared at 0, 6, 12, and 24 hours after treatment of healthy volunteers with 500mg ASS. TXA_2 and PGE_2 synthesis was assessed by radioimmuno assay. The identity of TXB_2 was unequivocally demonstrated by gas chromatography mass spectrometry. Values in the table were obtained from at least 6 independent experiments and expressed as ng TXB_2 / 10^6 monocytes.

Time after treatment (hrs)	0	6	12	24
Stimulus				
None	57	68	58	57
A23187 (5µM)	132	112	112	112
fMLP (1µM)	124	122	116	128

The amounts of PGE_2 were under all conditions less than 5% of the amounts of TXB_2. Synthesis of TXA_2 (Tab.) and PGE_2 by monocytes in the absence and in the presence of A23187 or fMLP was unchanged during the entire observation period ($p>0.05$). In contrast TXA_2 synthesis by PRP stimulated by A23187 was suppressed by more than 95% ($p<0.05$) throughout the 24 hour interval compared to the synthesis before ASS. The present study, therefore, conclusively shows that TXA_2 is the main prostanoid metabolite of monocytes.

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AVAROL RESTORES THE ALTERED PROSTAGLANDIN AND LEUKOTRIENE METABOLISM IN MONOCYTES INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS.

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Infection of monocytes with human immunodeficiency virus (HIV-1) [strain Ada-M] caused increased levels of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in vitro. These two products result from the activities of the two enzymes 5-lipoxygenase and cyclooxygenase. Addition of Avarol [Müller et al., Cancer Res. 45: 4822, (1985)], an HIV inhibitor, strongly reduced the levels of LTB_4 and PGE_2 via inhibition of both lipoxygenase and cyclooxygenase in monocytes. The 50% inhibition concentrations (IC_{50}) for the enzymes were determined to be 1.97 µM (lipoxygenase) and 2.26 µM (cyclooxygenase). A 50% reduction of the extent of LTB_4 and PGE_2- production in HIV-infected monocytes was measured at a concentration of 0.9 µM Avarol, a dose which caused an 80% anti-HIV effect in vitro [50% inhibition of virus release from infected cells: 0.3 µM]. These data show that Avarol inhibits the enzymes lipoxygenase and cyclooxygenase and suggest that, in general, inhibitors of these enzymes are promising anti-HIV compounds.
Moreover, we present evidence that Avarol displays also an antiinflammatory effect via inhibition of lipoxygenase and cyclooxygenase and hence should be considered as a promising agent for treatment of inflammatory skin diseases, e.g. psoriasis vulgaris.

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REGULATION OF PROSTACYCLIN-DEPENDENT SIGNAL TRANSDUCTION IN ENDOTHELIAL CELLS H. Schröder, C. Machunsky, K. Schrör

Whereas nitric oxide is known to produce an autocrine stimulation of endothelial cGMP, conflicting results have been published on whether or not prostacyclin (PGI_2) has a similar autocrine function in endothelial cells. Using porcine aortic endothelial cells (PAEC), the present study investigates if the release of PGI_2 may cause elevations of cAMP in the endothelial generator cells. In PAEC at passage 1, the 37-fold PGI_2 stimulation by 3 µM calcimycin was not accompanied by an increase in intracellular cAMP. Correspondingly, the PGI_2 mimetic iloprost (10 pM-10 µM) did not change cAMP levels in these cells. Under the same conditions, forskolin (1-100 µM) and prostaglandin (PG) E_2 (0.1-10 µM) produced concentration-dependent increases in cAMP with a 9-fold and 8-fold stimulation at 100 µM forskolin and 10 µM PGE_2, respectively. In PAEC at passage 6, basal PGI_2 formation was reduced by 80% as compared to control cells at passage 1. Under these conditions, iloprost (0.1-10 µM) increased cAMP with a 6-fold elevation at 10 µM. cAMP stimulation by iloprost (10 µM) was further enhanced up to 11-fold over basal, when PAEC (passage 6) were cultured for 48 h in the presence of indomethacin (10 µM) or diclofenac (10 µM) prior to the experiment. These results demonstrate that in endothelial cells, basal PGI_2 formation may cause a complete homologous desensitization of adenylate cyclase, presumably via down-regulation or uncoupling of PGI_2 receptors.

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CYCLOOXYGENASE-RELATED AND NON-CYCLOOXYGENASE-RELATED EFFECTS OF IBUPROFEN ENANTIOMERS ON HUMAN PLATELET AND POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS M. Villanueva, M. Palmér

This study investigates the effect of racemic ibuprofen (IBU ±) and its S-(+) (IBU +) and R-(-) (IBU -) enantiomers on human platelet and polymorphonuclear leukocyte (PMN) functions in vitro. IBU ± and IBU + inhibited collagen (2.5 µg/ml)-induced aggregation (AGG) and TXB_2 generation of platelet rich plasma (PRP) and washed platelets (WP), while IBU - was up to 100 times less active:

	PRP (IC_{50}, µM)		WP (IC_{50}, µM)	
	AGG	TXB_2	AGG	TXB_2
IBU+/-	134 ± 63	50 ± 27	1.31 ± 0.33	0.27 ± 0.03
IBU +	87.5 ± 44	26 ± 15	0.57 ± 0.44	0.16 ± 0.02
IBU -	1260 ± 670*	380 ± 60*	33.0 ± 6.80*	15.6 ± 1.20*

(n = 3-6), * $p < 0.01$ (IBU - vs. IBU ±)

In contrast, no significant differences between the isomers were found with respect to inhibition of FMLP-induced O_2^- generation, β-glucuronidase release and A-23187-induced LTB_4 formation in human PMN (n = 4-9):

	O_2^- (IC_{50}, mM)	β-GLUC (IC_{50}, mM)	LTB_4 (IC_{50}, mM)
IBU+/-	0.60 ± 0.13	0.44 ± 0.08	0.26 ± 0.10
IBU +	0.50 ± 0.11	0.53 ± 0.12	0.14 ± 0.07
IBU -	0.43 ± 0.08	0.58 ± 0.09	0.22 ± 0.02

The data suggest non-stereoselective actions of IBU on PMN function in vitro, eventually related to its chellating properties. These effects required higher concentrations than inhibition of platelet function which is clearly due to inhibition of cyclooxygenase. These data suggest antiinflammatory actions of IBU which are independent of inhibitory cyclooxygenase product formation.

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INHIBITION OF STIMULUS INDUCED ACTIVATION OF HUMAN POLYMORPHONUCLEAR NEUTROPHILS (PMN) BY SC 41930, A LTB_4 RECEPTOR ANTAGONIST R. Heckenberger

SC 41930 - 7-[3-(4-Acetyl-3-methoxy-2-propenylphenoxy)propoxy]-3,4-dihydro-8-propyl-2H-1-benzopyran-2-carboxylic acid - is a new, orally active LTB_4 receptor antagonist which selectively inhibits LTB_4 induced PMN chemotaxis and granular enzyme release (Djuric et al., J Med Chem 32: 1145, 1989). This study investigates the effects of SC 41930 on FMLP (30 nM) or Calcimycin (10 µM) induced activation of human PMN.
SC 41930 concentration dependently inhibited O_2^- generation (IC_{50} = 5.9 ± 1.5 µM), β-glucuronidase release (IC_{50} = 7.6 ± 1.0 µM) and LTB_4 release (IC_{50} = 3.8 ± 0.5 µM). Preincubation of PMN with SC 41930 (10 µM) shifted the FMLP dose response curves to the right. The increase of cytosolic Ca^{2+}-level in PMN following stimulation was also concentration dependently inhibited by SC 41930 (IC_{50} ≈ 10 µM). In additional experiments SC 41930 (100 µM) inhibited 5-lipoxygenase product formation from linoleic acid (100 µM) by 53% but not at lower concentrations. In contrast nordihydroguaretic acid inhibited the enzyme at concentrations of 30 µM by 35%.

These data demonstrate an inhibition of receptor - and nonreceptor mediated PMN activation by SC 41930. Some additional inhibition of the 5-lipoxygenase enzyme at higher concentrations may contribute to this effect.

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ORAL CICAPROST PREVENTS IMPAIRMENT OF ENDOTHELIUM-DEPENDENT RELAXATION IN THE CORONARY CIRCULATION OF HYPERCHOLESTEROLEMIC RABBITS I. Woditsch, H. Strobach

Cholesterol enriched diet (1%, 3 months, CHOL) resulted in a pronounced reduction of endothelium-dependent relaxation in Langendorff-perfused (22 ml/min) rabbit hearts (NZW) as compared to hearts of a control group (CON): Infusions (3 min) of bradykinin (BK), substance P and carbamoylcholine at equieffective concentrations into the coronary inflow tract resulted in a significantly reduced decrease of coronary perfusion pressure (CPP). Total release of endothelium-derived NO (oxyhemoglobin-technique) and prostacyclin (6-oxo-$PGF_{1\alpha}$, RIA) into the coronary effluent induced by the vasodilators was enhanced in hypercholesterolemia. Oral cicaprost given together with cholesterol diet (CHOL+CICA) (5 µg/kgxd, withdrawn 72 h before exp.) largely prevented this impairment. This is examplified with BK (0.05 µmol/l):

group	Δ CPP [mmHg]	Δ NO [nmol]	Δ 6-oxo-$PGF1_{\alpha}$ [nmol]
CON	5.4 ± 0.6	1.5 ± 0.2	0.09 ± 0.02
CICA	4.4 ± 0.5	1.6 ± 0.3	0.21 ± 0.04*
CHOL	1.1 ± 0.5*	2.3 ± 0.3*	0.24 ± 0.04*
CHOL+CICA	3.8 ± 1.0+	1.5 ± 0.3+	0.17 ± 0.03

The data are mean ± SEM of 9 experiments in each group. *)p<0.05 vs. CON, +)p<0.05 vs. CHOL

Basal mediator release and basal levels of CPP were not significantly different in all groups. The data demonstrate an impairment of endothelium-dependent relaxation in the coronary microcirculation of hypercholesterolemic rabbits which is not accompanied by diminished mediator release and which can largely be reversed by an oral prostacyclin mimetic.

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ORAL CICAPROST PREVENTS FUNCTIONAL PLATELET DESENSITIZATION IN CHOLESTEROL-FED RABBITS Th. Hohlfeld, A. Weber, M. Braun

PGI_2 mimetics may reduce the pro-atherogenic effects of platelets. However, platelets are known to become less sensitive to PGI_2 in hypercholesterolemia. This study investigates the effect of long-term oral treatment with the PGI_2 mimetic cicaprost (CICA) on platelet function in experimental hypercholesterolemia.

Male New Zealand White rabbits (1.8-2 kg) were fed a cholesterol-enriched diet (1% for 3 months, CHOL), resulting in severe aortic plaque formation. CICA treatment (5 µg/kg x d, orally, during CHOL feeding) was withdrawn 3 days before measurements in order to exclude direct CICA effects on platelet function. CICA did not change serum levels of cholesterol. Platelet reactivity, measured by ex vivo collagen (0.6 µg/ml)-induced aggregation (CIA), was markedly increased by CHOL feeding in comparison to a control group fed standard diet (CON). Platelet sensitivity to PGI_2, determined by the IC_{50} of iloprost for inhibition of ADP-induced platelet ATP secretion, increased 3-fold in CHOL rabbits. CICA completely prevented this hypercholesterolemia-related desensitization of platelets.

Group	n	CIA [mm/min]	IC_{50} iloprost [nM]
CON	9	33 ± 12	14.6 ± 2.4
CON-CICA	9	31 ± 13	21.9 ± 4.1
CHOL	8	161 ± 8*	41.6 ± 8.4*
CHOL-CICA	9	59 ± 16+	9.5 ± 3.0+

* p<0.05 vs CON; + p<0.05 vs CHOL

Binding studies with rabbit platelet membranes using ^{3}H-iloprost as ligand showed a markedly reduced affinity (4-fold increase of K_d) and an increase (2-fold) of binding sites in CHOL fed rabbits, which was not reversed by CICA treatment. The data confirm the desensitization of platelets to PGI_2 in hypercholesterolemia and demonstrate an improvement towards normal by oral CICA. This action may involve a mechanism independent of a regulation of specific binding sites.

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INHIBITORY EFFECTS OF THE NEW PGD_2 ANALOG ZK 118.182 IN PLATELETS AND NEUTROPHILS

H.Darius

PGD_2 is a labile arachidonic acid product involved in mast cell activation, bronchial smooth muscle tone and the regulation of sleepwake rhythm. The chemical instability and rapid metabolism in vivo hampered studies on the physiologic role and possible therapeutic properties of PGD_2. The pharmacologic properties of ZK 118.182 [(5Z,13E)-(9R,11R,15F)-9-Chloro-15-cyclohexyl-15-hydroxy-16,17,18-19,20-pentanor-3-oxa-5,13-prostadienoic acid], a chemically stable PGD_2-analog were studied in human platelets (PL) and neutrophils (PMN). ADP-induced PL aggregation was dose dependently inhibited by ZK 118.182, PGD_2 and iloprost exerting IC_{50}-values of 14.3±1.9, 65.4±8.5 and 3.3±1.4 nmol/l (n=6-9). Collagen-induced aggregation and ATP release from PL were inhibited by ZK 118.182 with IC_{50} values of 42.2±12.0 and 21.9±6.4 nmol/l. Platelet cAMP levels increased dose dependently from 9.7±6.5 to 227±84 pmol/10^8 pl. following incubation with ZK 118.182 3 µmol/l. Isolated human PMNs were stimulated by FMLP (30 nmol/l) or PAF (3 µmol/l). Preincubation with 2.8 µmol/l ZK 118.182 inhibited oxygen radical release by 80±25 % when FMLP and by 58±17 % when PAF were used for stimulation. PGD_2 and PGE_2 were significantly less potent. ß-Glucuronidase release was inhibited dose-dependently, with ZK 118.182 being more potent than PGD_2 and PGE_2. ZK 118.182 is a chemically stable PGD_2-analog with potent PL and PMN inhibitory effects. The inhibitory effects are mediated via intracellular cAMP elevation.

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EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON PLATELET PROSTACYCLIN RECEPTOR DESENSITIZATION

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Long term exposure of platelet (PL) prostacyclin receptors to the agonist results in a dose and time dependent down regulation of the membrane receptors. The exact mechanism of this desensitization process is unknown. The influence of diminished cAMP degradation by phosphodiesterase (PDE) inhibition on the prostacyclin analog induced receptor desensitization was studied in human PL. Incubation of PL with the prostacyclin analog iloprost (ILO; 100 nmol/l) for 1 min to 12 hrs at 21° C resulted in a decrease in ^{3}H-ILO binding by 50 % of the respective vehicle treated control value 270±60 fmol/10^9 pl. Binding decreased dose-dependently from 361±17 to 352±29, 220±15, and 181±19 fmol/10^9 pl. following incubation with ILO 1,10, and 100 nmol/l for 4 hrs. When PL were coincubated with ILO 1 nmol/l and the PDE-inhibitor piroximone 150-500 µmol/l ^{3}H-ILO binding was reduced to 269±33 fmol/10^9 pl. This augmentation of PL desensitization by the PDE inhibitor was not clearly dose-dependent in the concentration range studied. Platelet cAMP levels increased dose-dependently following incubation with iloprost 0.1-3 nmol/l from 24±5 to 649±93 pmol/10^9 pl. Coincubation with piroximone (150-500 µmol/l) for 4 hrs at RT did not significantly alter ILO induced increase in cAMP levels. Desensitization of PL ^{3}H-ILO binding is not altered by simultaneous inhibition of cAMP degradation by phosphodiesterase inhibition.

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ACE-INHIBITORS STIMULATE THE ENDOTHELIAL PROSTACYCLIN FORMATION – INFLUENCE OF A THRESHOLD CONCENTRATION OF BRADYKININ.

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Inhibitors of angiotensin converting enzyme (ACE-I) and bradykinin (Bk) are known to induce the formation of vasorelaxant prostacyclin (PGI_2) in various vascular preparations. Whether the effect of ACE-I is due to the diminished degradation of endogenous Bk or a direct effect of the ACE-I on the vessel wall is undecided.
We investigated the role of prostacyclin formation induced by the ACE-inhibitors captopril and fosinoprilate in ring preparations of rabbit aorta.
Rings were incubated for 15 min. with ACE-I (10 nM – 100 µM), exogenous bradykinin (100 nM or 0,1 nM), or with ACE-I (100 µM) and Bk (0,1nM). Prostacyclin formation was determined by radioimmunoassay (RiA) as its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$.

Captopril and fosinoprilate increased the formation of prostacyclin dose-dependently in the range from 10 nM – 1 µM. Higher concentrations of the ACE-I had no further stimulating effect.
In the presence of a threshold concentration of Bk (0,1 nM) the formation of prostacyclin by ACE-I (1 µM) was significantly increased versus basal by captopril and by fosinoprilate.

	[% of basal release]	
treatment	captopril	fosinoprilate
ACE-I (10 nM)	389,4±98,0	187,8±47,1
ACE-I (1 µM)	694,9±122,3	547,9±97,7
ACE-I (100 µM)	245,0±80,0	149,4±21,0
Bk (100 nM)	613,8±121,0	305,1±42,6
ACE-I (1µM) + Bk (0,1nM)	1284,0±285,1	379,0±118,7

These results indicate that the stimulation of prostacyclin formation in the vessel wall by ACE-I may be at least partly due to the diminished degradation of Bk. Captopril had a stronger stimulating effect on prostacyclin formation in rabbit aorta than fosinoprilate. This may be due to a direct stimulation possibly related to the SH-group of captopril.

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COMPARISON OF INHIBITIONS OF LYSO-PAF-TRANSACETYLASE AND 5-,12-,15-LIPOXYGENASES BY 2,6-DI-TERT BUTYLPHENOL DERIVATES OF NAPHTHOQUINONES AND RELATED COMPOUNDS

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Since we have found dual inhibitors of 5-lipoxygenase [EC 1.13.11.34; LOX] and acetyl CoA: lyso-PAF (platelet-activating factor) acetyltransferase [EC 2.3.1.67; Lyso-PAF-TA] within bicatecholic structures (v. Bruchhausen et al., Arch. Pharmacol. 342, R6, 1990) and highly hydroxylated compounds (Querner et al., Arch. Pharmacol. 343, R82, 1991) we extended the comparison to naphthoquinones with or without substituents of 2,6-di-tert butylphenol. Oxydized products of ^{14}C labelled arachidonic acid, or PAF (platelet-activating factor) from ^{3}H labelled acetyl CoA, respectively were separated after selective extraction by suitable thin layer chromatography and counting.

Most of the 16 substances used inhibited 5-LOX, fewer 12-LOX and only that which had an aliphatic residue at the 4th position of the 2,6-di-tert butylphenol inhibited the 15-LOX preparation. An inhibitory potency against Lyso-PAF-TA especially had two naphthoquinones with an 2- or 3-positioned hydroxyl group, irrespectively to the presence of a tert butylphenol residue (TBP-R). Probucol with two TBP-R also had no such inhibitory action. The 2 compounds mentioned possessed a 5-LOX inhibitory potency, i.e. were dual inhibitors. These compounds were: 1.) 2-(3,5-di-tert butyl-4 hydroxyphenyl)-3-hydroxy-1,4-naphthoquinone, 2.) 2-hydroxy-1,4-naphthoquinone. They both did not inhibit purified prostaglandin synthetase (Kalkbrenner, Med. Thesis, Berlin, 1989). It remains to be seen whether these compounds also act on the cellular level.

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CICLOSPORIN (CS) SUPPRESSES THE THROMBOXANE B_2 (TXB_2) PRODUCTION OF HUMAN GLOMERULAR MESANGIAL CELLS (HMC)+

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In vivo data suggest that CS therapy leads to an increased renal TXB_2 production and a fall of renal blood flow and glomerular filtration rate in >50% of the treated patients.
In the present study with cultured HMC starting from a comparable basal secretion (TXB_2: 1.66 ± 0.027 ng/mg protein vs PGE_2: 2.5 ± 0.536 ng/mg protein) interleukin 1-β (IL-1-β) dose dependently enhanced the TXB_2 release up to 3-fold, while PGE_2 was raised up to 16-fold. CS at doses from 0.01 to 1 ng/ml dose dependently reduced basal and IL-1-β induced TXB_2 secretion by >92%, but PGE_2 release was only diminished by 40%. A significant inhibition of the IL-1-β induced release of PGE_2 by CS (1 ng/ml) was observed after a lag period of 12 hrs (up to 48 hrs), while TXB_2 secretion was significantly reduced already after 6 hrs. In parallel, determining the enzymatic activity in particular fractions of HMC CS at these low concentrations caused a reduction of the IL-1-β induced cyclo-oxygenase/thromboxane synthase activity (IL-1-β + CS: 2.51 vs. IL-1-β: 8.27 ng TXB_2/mg prot./30 min). However, Western blots using specific cyclooxygenase (COX) antibodies revealed that neither basal nor IL-1-β induced COX protein expression of HMC was affected by CS.
These results with cultured HMC determined at very low CS concentrations are in contrast with in vivo findings during longterm CS therapy. Our in vitro data suggest that CS might have specific effects on the cytochrome P450 III enzyme, thromboxane synthase at concentrations far below the immunosuppressive level.

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5-LIPOXYGENASE METABOLITES IN SELENIUM DEFICIENT LEUCOCYTES

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Selenium dependent glutathione peroxidases are known to reduce various types of peroxides and to play an important role in the cellular defense system against oxidative stress. The role of the two known selenium containing enzymes, glutathione-peroxidase (GPx) and phospholipid-hydroperoxide-glutathione-peroxidase (PH-GPx) in the regulation of the 5-lipoxygenase-activity (5-LOX) in leucocytes was examined.
Rat basophilic leucemia cells (RBL-1) were cultured for 5 days in a selenium deficient medium, or in control medium supplemented with Na_2SeO_3.
In this *in vitro* system the activity of 5-LOX was enhanced 6-fold in selenium deficient cells. This observation was confirmed by results obtained from *ex vivo* experiments with polymorphnuclear gronulocytes from selenium deficient rats. In both cases HPLC analysis of 5-LOX products showed a product shift in favour of increased hydroperoxy-eicosatetraenoic acid (HPETE) formation in selenium deficient cells.
Since the activation of 5-LOX occurs via translocation to the membrane, we propose that its regulation by the peroxide tone is controlled by the membrane-associated selenoenzyme PH-GPx.

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IDENTIFICATION OF A PUTATIVE PEPTIDOLEUKOTRIENE RECEPTOR BY AN ANTI-IDIOTYPIC ANTIBODY STRATEGY. J.A. van Hilten, and *J. Mollenhauer

Peptidoleukotrienes (LTC4, LTD4 and LTE4) are involved in induction of biological activity that is believed to be mediated via putative LT receptors. Therefore the characterization of LT receptors in peritonal macrophages using anti-idiotypic antibodies (AIA) raised against an anti-LT monoclonal antibody (LTmAb) was tried. AIA recognition of proteins expressing the LTmAb epitopes could include a putative LT-receptor. (1), AIA activity against the functional LTmAb epitope in two immunized rabbit sera was demonstrated by (i) high titers (1:4000) against $F(ab')_2$ of the LTmAb in an ELISA and (ii) by inhibition of ^{3}H-LTE4 binding to LTmAb by 1:300 antisera dilutions. (2), we found binding of AIA on macrophages by immunofluorescence and (3), a three-fold increase in AIA-reactivity against membrane proteins as compared with the original homogenate in an ELISA with native proteins. In confirmation with the ELISA, the strongest signals were also found with denatured and blotted membrane proteins: protein conformations of a Mr of 236, 200, 118, 100, 75, 25 and 18 KDa were detected. SH-reduced proteins gave signals at 25 and 18 KDa. More specifically, overlay assays with ^{3}H-LTD4, ^{3}H-LTE4, ^{3}H-PGE2 and ^{3}H-TXB2 favor protein conformations of the putative LTD4/E4-receptor of 236 and 75 KDa. These data suggest the presence of a oligomeric protein composed of 25 and 18 KDa subunits with intramolecular disulphide bridges all bearing the functional LTmAb, located almost exclusively in plasma membranes of murine peritoneal macrophages.

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INHIBITION OF LEUKOTRIENE (LT) RELEASE FROM FMLP-STIMULATED RAT NEUTROPHILS AND OF HUMAN PLATELET AGGREGATION BY PROSTAGLANDIN (PG) E_2 AND 3-MORPHOLINOSYDNONIMINE (SIN-1) D. Pallapies, K.-U. Jirmann, A. Dembińska-Kieć, and B.A. Peskar

Nitric oxide (NO) has been shown to interact synergistically with PGE_1 and PGI_2 with regard to inhibition of platelet activation (Radomski et al., Br. J. Pharmacol. 92, 639-646, 1987; Sinzinger et al., Lancet i, 627-628, 1990). However, in human and animal tissues much less PGE_1 than PGE_2 is formed under normal conditions. In addition, PGE_2 concentrations in inflammatory exudates have been demonstrated to be higher than those of PGI_2. We have now examined the effects of PGE_2 and the NO donor SIN-1 on LT release from rat peritoneal neutrophils (RPN) stimulated by FMLP and on aggregation of human platelets induced by thrombin. RPN elicited by i.p. injection of glycogen were incubated in Tyrode solution ($1x10^7$ cells/ml) at 37°C for 20 min in the absence or presence of SIN-1 (30 - 300 µM), PGE_2 (1 - 100 nM) and FMLP (100 nM). Aggregation of 1.0 ml washed human platelet suspensions (2.5×10^8 cells/ml) induced by thrombin (20 - 30 mU/ml) was measured for 4 min. SIN-1 and/or PGE_2 were added 90 s or 60 s before thrombin, respectively. It was found that basal release of LTB_4 (0.48 ± 0.09 ng/10^7 cells) and cysteinyl-LT (< 54 pg/10^7 cells) from RPN was significantly stimulated by FMLP to 12.1 ± 4.7 ng/10^7 cells ($p < 0.001$) and 424 ± 129 pg/10^7 cells ($p < 0.01$), respectively. Both PGE_2 and SIN-1 inhibited release of LTB_4 and cysteinyl-LT in a concentration-dependent manner. The combination of both compounds resulted in an effect not greater than expected by summation. Platelet aggregation was inhibited by SIN-1 (0.5 or 1 µM), while PGE_2 (0.3 - 5.0 µM) had only marginal effects. Nevertheless, PGE_2 potentiated the inhibition of platelet aggregation by SIN-1 concentration-dependently. We conclude that PGE_2 and NO, probably by increasing cyclic AMP and cyclic GMP levels, respectively, may exert differential regulatory effects on cellular functions of RPN and platelets.

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ISCHEMIA AND REPERFUSION-INDUCED EARLY ARRHYTHMIAS IN VIVO: CARDIOPROTECTIVE EFFECTS OF DEFIBROTIDE

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The ability of defibrotide to increase the generation of prostacyclin (PGI_2) may play important role for the prevention and treatment of ischemia- and reperfusion-induced arrhythmias.
The effects of defibrotide and PGI_2 in different doses against ischemia- and reperfusion-induced arrhythmias and creatine kinase (CPK) release were compared using anaesthetized rats (thiopentone i.p.) with transient (7min) coronary artery occlusion (improved method according to Seley et al., Angiology, 1960;11; 398-407). The test drugs or vehicles were administered (i.v.) 10min prior to occlusion (bolus and continuous infusion). Reperfusion-induced ventricular fibrillations (VF) were observed in 71% in control group (n=28), whereas such episodes occured in 40, 33 and 43% of the animals treated with 0.96mg/ml/kg+0.96mg/ml/kg/h (n=10), 32mg/ml/kg+32mg/ml/kg/h (n=15) and 96mg/ml/kg+96mg/ml/kg/h (n=16) respectively. PGI_2 given in two doses: 100ng/ml/kg/h (n=16) and 100ng/kg/min (n=16) reduced the incidence of VF to 43% in higher dose only. The mean duration of VF and ventricular tachycardias (VT) were not effected by defibrotide, if occured. In contrary, PGI_2 dose dependently prolonged VF duration, whereas exerted no influence on VT occurrence and duration. Mortality index (MI) was not significantly reduced by defibrotide in all doses The lower dose of PGI_2 reduced MI to 0%, while the higher dose increased to 20% compared to control (10%). Defibrotide in the highest dose decreased the CPK release similary to PGI_2 in higher dose. PGI_2 in lower dose significantly reduced CPK release (8.6 ± 0.7U/g Prot., n=16) compared to control (13.6 ± 1.3U/g Prot., n=28).
The appearance of the rhythm disturbances depends on the amount of PGI_2 released, so the proper defibrotide dosage may have a crucial importance for new clinical indications.
In conclusion, the cardioprotective effects of defibrotide (e.g.VF prevention) might extend the potential therapeutic implications of this substance.

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POLYAMINE MODULATORS OF MITOCHONDRIAL CALCIUM TRANSPORT

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The polyamine spermine has a dual effect on the mitochondrial Ca^{2+} uptake, an inhibition of the initial velocity of mitochondrial Ca^{2+} uptake followed by a subsequent increase of mitochondrial Ca^{2+} accumulation. This dual effect could be observed also with other, chemically different polyamine cations such as aminoglycosides, polylysine, and protamine.
The IC_{50} values of the various polyamines differed by more than 10^3, ruthenium red being the most potent inhibitor of uptake velocity with an IC_{50} of 0.19 µM, while the efficacy of Ca^{2+} uptake inhibition differed by a factor of less than three. Generally, a high potency of a compound was accompanied by a high efficacy. Cyclic polyamines were completely ineffective. Particularly effective stimulators of mitochondrial Ca^{2+} accumulation were the aminoglycosides tobramycin and gentamycin as well as spermine. The EC_{50} values for stimulation of mitochondrial Ca^{2+} accumulation also differed by more than 10^2 between the various agents. However, potency and efficacy were not in parallel, an increase in potency over gentamycin (EC_{50} 28 µM) was accompanied by a marked loss of efficacy. Ruthenium red did not stimulate mitochondrial Ca^{2+} accumulation.
Thus the present results allow to define a group of polyamine modulators of mitochondrial Ca^{2+} transport including ruthenium red and spermine which are usually regarded as functional antagonists. Structurally, not only charge but also the spatial distribution of the amino groups along a flexible backbone seem to be of importance.

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CHEMOSENSITIZER PROPERTIES OF CYCLOSPORIN A IN MULTIDRUG RESISTANT FRIEND LEUKEMIA CELLS.

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Cyclosporin A (CsA), used clinically as an immonosuppressant drug, has been reported to restore sensitivity to certain anticancer drugs by blockade of p-glycoprotein (pgp) 170-mediated extrusion of cytostatics in vitro and is a current candidate for clinical trial (Nooter, K. et al., Int J Cancer 45, 263-268, 1990). However, pharmacodynamic properties of CsA as a chemosensitizer require further definition.
We report on in vitro chemosensitizing effects of CsA including two possible solvent systems for CsA, i.e. cremophor EL (CREL) or Tween 80/Ethanol (TW/E) as compared to the experimental chemosensitizer R,S-verapamil (VER). Murine leukemia cells strain F4-6RADR expressing pgp170, were tested in a daunorubicin (DNR) accumulation assay with a silicone filtration method. Cells were preincubated with drugs for 10 min, followed by 60 min incubation with radiolabelled DNR (1 μmol/l), and centrifuged into trichloroacetic acid. CREL (up to 10 ml/l) increased DNR content in F4-6RADR as well as in sensitive F4-6P. Therefore, TW/E (maximum concentration 0.3/1.0 ml/l) was used as the solvent system for CsA studies. CsA (0.01-10 μmol/l) elevated total DNR content in resistant F4-6RADR with first effects observed at 0.3 μmol/l CsA. In contrast to VER, the potency of CsA in reversing resistance-associated deficits for DNR in F4-6RADR could not be defined unequivocally: At 10 μmol/l CsA, elevating effects on DNR content in F4-6RADR were also found in TW/E solvent controls (pmol/mg cell protein, n=4, SEM, F4-6RADR controls: 178±11; CsA: 958±125; TW/E alone 709±90). Furthermore, unlike VER, CsA elevated DNR content in sensitive cells also (pmol/mg cell protein, F4-6P controls: 704±67; CsA: 959±114). Indicative of functional synergism in pgp170 inhibition, the concentration-dependent response to CsA was increased in the presence of 1 μmol/l VER in a more than additive way. In proliferation assays CsA, but also TW/E or CREL increased the sensitivity of F4-6RADR cells to cytostatic effects of DNR.
In conclusion, chemosensitizing effects of CsA were superimposed in part by effects of the solvent system required to test CsA in the experimental setup. Interaction of VER with CsA is compatible with different mechanisms of action on pgp170.

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MOLECULAR MODE OF ACTION OF CYTOTOXIC THIOETHER PHOSPHOLIPIDS.

D.B.J. Herrmann, and H.G. Opitz

Thioether phospholipids (TLPs) such as Ilmofosine (1-hexadecylthio-2-methoxymethyl-1,3-propanediol-phosphochline, BM 41.440) are synthetic analogues of the cell membrane component lysophosphatidylcholine. TLPs represent a new family of anticancer compounds with an unique pharmacological profile. The purpose of this study was to determine the correlation between the antineoplastic activity of Ilmofosine and related substances and their specific effect on cell membrane phospholipid metabolism in sensitive and resistant MethA fibrosarcoma cells in vitro. TLPs exerted a concentration- and time-dependent inhibition of sensitive but not of resistant MethA sarcoma growth. Accordingly, a specific inhibition and stimulation of phosphatidylcholine (PC) and triacylglycerol (TG) synthesis, respectively, was observed in sensitive cells. These biochemical changes preceeded cellular destruction for 6 h, indicating that they are not trivial consequences of tumor cell death. All other lipid fractions investigated were not influenced by TLPs. Determination of lysophosphatidylcholine acyltransferase (ACTF) activities and affinities to TLPs in cell-free systems of TLP-sensitive and -resistant MethA cells revealed differences in inhibitor constants (sensitive cells: K_i = 423 μM; resistant cells: K_i = 13 μM). These data suggest that susceptibility of tumor cells to TLP-induced cytolysis is correlated to a selective inhibition of fatty acid incorporation into the PC fraction of cellular membranes. Differences in affinities of TLPs to ACTF can explain these results on a subcellular level.

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INTESTINAL ABSORPTION OF ß-LACTAM ANTIBIOTICS AND OLIGOPEPTIDES: III. DIFFERENCES IN THE UPTAKE OF ZWITTERIONIC α-AMINO-CEPHALOSPORINS OF THE CEPHALEXIN-TYPE AND DIANIONIC CEPHALOSPORINS OF THE CEFIXIME TYPE

W. Kramer, U. Gutjahr, F. Girbig

The uptake of two types of orally active ß-lactam antibiotics of different chemical structure - the zwitterionic α-amino-ß-lactam antibiotic cephalexin and the dianionic α-carboxymethoxyimino-ß-lactam antibiotic cefixime - was investigated using brush border membrane vesicles from rabbit small intestine. The uptake of both classes of orally active ß-lactam antibiotics was stimulated by an inwardly directed H^+ gradient; the stimulating effect of such a pH-gradient on the uptake of cefixime was much greater than on the uptake of α-amino-ß-lactam-antibiotics. In contrast, the modification of histidine residues by diethylpyrocarbonate greatly inhibited the uptake of α-amino-ß-lactam antibiotics, whereas the uptake of cefixime was only slightly inhibited. The uptake of cephalexin was inhibited by cefixime and dipeptides and vice versa the uptake of cefixime was inhibited by cephalexin and dipeptides. The uptake of α-amino-ß-lactam antibiotics was markedly impaired upon heat pretreatment of brush border membrane vesicles whereas the uptake of cefixime remained unchanged. Organic anions like palmitate or the organic transport inhibitor DIDS clearly inhibited the uptake of cefixime and had no or only a slight inhibitory effect on the uptake of cephalexin; bile acids, folate or dicarboxylic acids did not inhibit the uptake of both, cephalexin and cefixime. Competition photoaffinity labeling experiments with [^{3}H]benzylpenicillin as photoaffinity probe demonstrated a direct interaction of cefixime with the peptide transporter of M_r 127 000.
These results suggest that dianionic cephalosporins like cefixime bind to the intestinal peptide transport system responsible for the uptake of orally active α-amino-ß-lactam antibiotics and oligopeptides. Their transport across the intestinal brush border membrane, however, seems to occur by an additional transport system for organic anions. It may be speculated that this transport system physiologically transports fatty acids.

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TWO DIFFERENT mRNA'S ARE CODING FOR THE HEPATOCELLULAR TRANSPORT PROTEINS FOR TAUROCHOLATE AND BUMETANIDE

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The transport proteins for hepatocellular uptake of taurocholate and bumetanide were expressed in Xenopus laevis oocytes by injection of rat liver poly A^+-RNA. A twofold induction of the (^{3}H)-taurocholate uptake (in comparison to non-injected oocytes) was accompanied by an only 20% increase in the uptake of (^{3}H)-bumetanide. Oocyte uptake of both compounds was inhibited by the non-labeled substrates. Furthermore uptake could be reduced to the control levels by addition of competitive inhibitors of the transport systems. Size fractionation of the poly A^+-RNA by sucrose gradient centrifugation yielded two different mRNA-fractions which either accounted for taurocholate transport (fraction 21, 400-800% increase) with only a slight increase in bumetanide uptake, or for bumetanide transport (fraction 18, 160-200% increase) with concomitant small increase in taurocholate uptake. Both mRNA-fractions are clearly different in their size. The mRNA-fraction which contains the sequence information for the taurocholate transporter has a size of 0.4 to 1.8 kb, whereas the second mRNA-fraction (bumetanide transporter) has a size of 0.6 to 2.5 kb. Uptake of cholate was induced by both mRNA-fractions with an almost 2.5 fold higher expression by the bumetanide fraction. In conclusion, the bumetanide transporter mRNA has a size of more than 1.8 kb and is clearly different from the mRNA for the taurocholate transport protein. This transporter accepts cholate even better than the taurocholate transporter. Both mRNA-fractions were used for the construction of cDNA-libraries. Expression tests of pools of the cDNA-clones are under progress in order to isolate the cDNA-clones which are coding for the different transport proteins.

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TISSUE-SELECTIVE ACTION OF PRAVASTATIN DUE TO HEPATOCELLULAR UPTAKE VIA A SODIUM-INDEPENDENT BILE ACID TRANSPORTER.

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Pravastatin (PRA), a hydrophilic metabolite from P. citrinium, is a specific inhibitor of the microsomal enzyme 3-hydroxy-3-methyl-glutaryl-coenzym A reductase, which has a key function in the biosynthesis of cholesterol. PRA inhibits sterol synthesis selectively in liver and in intestine, the major sites of cholesterogenesis, but not in non-hepatic cells. This appears to be due to the high enrichment in the liver and the excretion via the bile, whereas there is no penetration into cells of extrahepatic tissues. The molecular mechanisms of this predominant hepatocellular uptake of PRA remain unclear. The aim of the present study was therefore, to characterize the uptake mechanisms of PRA into isolated rat liver cells.

We investigated the effect of PRA on the hepatocellular uptake of substrates of endogenous transport systems in the basolateral membrane of liver cells.

PRA competitively inhibits the sodium-independent hepatocellular uptake of cholate, taurocholate and ouabain, whereas the total uptake of cholate is non-competitively blocked. The sodium-dependent taurocholate transport is, however, only effected in millimolar concentrations. PRA has no affinity to other transport systems in liver cells such as those for long-chain fatty acids, amino acids, bilirubin and bivalent organic cations.

Therefore, we conclude that PRA is a foreign substrate of a sodium-independent transport system for bile acids. The tissue selectivity of PRA is due to the uptake via a transport system which predominantly exists in liver cells.

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SENSITIVITY OF SIALIDASE AND HUMAN SIALYLTRANSFERASE TOWARDS DIFFERENT CALCIUM ANTAGONISTS

G. Werner and W. Gielen

Removal of external cellular sialic acid modulates the action of calcium channel ligands through an inhibitory effect on transmembrane calcium influx and/or by a decrease of external calcium availability. A functional role of sialic acid in the regulatory mechanism of calcium channels may additionally be discussed, (G. Werner et al., Biochem. Pharmacol.,in press).Thus, it would be interesting to know whether the enzymes of the sialic acid metabolism, the hydrolyzing sialidase (EC 3.2.1.18), prepared from Clostridium perfringens and the transferring sialyltransferase (EC 2.4.99.1/4/6), isolated by affinity chromatography (CDP-hexanolamine coupled to a Sepharose 4B matrix) from human serum, are influenced by different calcium antagonists. Tissue sialic acid release was measured according to Warren (J. Biol.Chem. 234, 1971-1975, 1959), transfer of ^{14}C-N-acetyl-neuraminic acid was performed on asialofetuin. The incubation of the enzyme solutions with nisoldipine, gallopamil, diltiazem and fendiline (10^{-5}-10^{-7} mol/l; 2-60 min) resulted in an enzyme activity of 90,0-106,7% (sialidase) and 97,5-106,2% (sialyltransferase) compared to control. From these experiments it is concluded that the sialic acid metabolism is not affected by calcium antagonists, and that after sialidase treatment the regeneration of the surface sialic acid can be achieved even in the presence of calcium antagonists.

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A METHOD TO DETERMINE LOCAL ANAESTHETIC EFFICACY IN ISOLATED FROG NERVE

B. Bräunig

An in vitro method is described to assay local anaesthetic acting drugs in isolated sciatic nerves of Rana esculenta. Drug actions were quantified by measuring amplitude and conduction velocity of electrically induced summation potentials. Experiments were performed in a humidified chamber which was constructed and accomodates several nerves of maximal lengths (95mm) under optimal physiological conditions. With the use of nerves of maximal lengths, it was possible to perform measurements at an electrode distance of 15mm, which is optimal for recording of summation potentials. Precision of measurements in the threshold range was improved by electronical averaging before recording the potentials.
Dose response curves were obtained for procaine and lidocaine with cumulative technique, using amplitude and conduction velocity of A_α-fibers as parameters. Determination of ED_{50} revealed that procaine has a 6.6-fold higher efficacy on the amplitude and a 6.5-fold on the conduction velocity than lidocaine.
The comparison of the conduction of excitation of all types of A-fibers shows that the A_δ-fibers are blocked earlier and with smaller concentrations of the local anaesthetic drug.

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COMPARTMENTALIZATION OF THE INTESTINAL METABOLISM AND METABOLITE TRANSPORT OF p-NITROPHENOL IN THE GUINEA PIG IN VIVO

E. Fischer* and F. Lauterbach

Two different compartments were found in the isolated mucosa of the guinea pig jejunum in vitro for the metabolism of drugs and for the transport of their conjugates (Lauterbach et. al. (1989), Progr. in Pharmacol. and Clin. Pharmacol. 7/2: 231). The present experiments were designed to study the intestinal metabolism of p-nitrophenol (PNP) and the transport of p-nitrophenyl sulphate (PNP-S) and p-nitrophenyl glucuronide (PNP-G) in the jejunum of the guinea pig in vivo and to compare these results with data obtained in the isolated mucosa.
The isolated jejunal loop was recirculated or single pass perfused with isotonic medium containing 0 mM, 1 mM or 10 mM sulphate; p-nitrophenol was used in a concentration of 20 µM, 100 µM and 500 µM.
PNP was metabolized in the jejunum and the metabolites were rapidly transported back on the luminal side. A steady state in the luminal concentration of conjugates at single pass perfusion was observed already in 5 or 10 minutes. Luminal appearance of PNP-S tended to saturability, e.g. 5 times higher PNP concentration (500 µM) produced only about 50 % increase in the luminal PNP-S compared to the rate (1050 nmole in 45 min) measured at 100 µM PNP concentration. The absence of sulphate in the incubation medium decreased the luminal appearance of PNP-S and stimulated that of PNP-G. These effects were more pronounced at the single pass perfusion of PNP. However, higher sulfate concentration (10 mM) did not change significantly the luminal appearance of PNP-S.
Similary to the results of the in vitro experiments PNP underwent preferentially sulphoconjugation and the metabolites were released mainly on the luminal side in vivo as well. About 50 - 60 % of the perfused amount of PNP could be detected in 90 min as sulphoconjugate and only 10 % or less was present as glucuronide on the luminal side when PNP was recirculated in the jejunum.
The data obtained in vivo thus emphazise the importance of luminal sulphate for the conjugation reaction observed previously in vitro and show the importance of the protective excretory function of the intestinal tract.

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EFFECT OF CICLETANINE, A PROSTACYCLIN SYNTHESIS STIMULATING DRUG, ON KIDNEY FUNCTION.

J. Greven, A. Rahn, and E. Brändle

The furopyridine derivative cicletanine [1,3-dihydro-3-(4-chlorophenyl)-7-hydroxy-6-methyl-furo(3,4-c)pyridine] is a new antihypertensive drug which acts directly on vascular smooth muscle by increasing prostacyclin sythesis. At doses higher than those having an antihypertensive action cicletanine exerts natriuretic effects. The aim of the present study was to characterize the renal effects of cicletanine by means of clearance and micropuncture techniques in anaesthetized rats. The drug was applied intravenously at doses of 15, 30 and 60 mg/kg b.w. Urine flow and renal NaCl excretion increased dose-dependently. Renal potassium-excretion was also enhanced but showed no dose-dependency. At the maximum effective dose cicletanine decreased fractional tubular Na-reabsorption by 5 %. By micropuncture of proximal and distal tubules an action of cicletanine in the distal tubule could be established. Thus, cicletanine shares the tubular site of action with that of thiazide diuretics. However, in contrast to thiazides, cicletanine did not decrease renal plasma flow and GFR and, again in contrast to thiazides, dose-dependently diminished arterial blood pressure in these short-termed experiments. It is concluded that cicletanine, independently from its vasodilating properties, may increase renal NaCl excretion by inhibiting NaCl reabsorption of the distal tubular epithelium.

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PHARMACOLOGICAL CHARACTERIZATION OF HISTAMINE RECEPTORS IN THE URETER OF THE DOG

R. Dodel, D. Hafner

Histamine receptors were characterized pharmacologically in the isolated ureter of the dog. Histamine and the H_1-receptor agonist 2-(2-pyridyl)-ethylamine produced a concentration dependent contraction of isolated strips of the ureter (EC_{50} for histamine $5.0 \times 10^{-5} \pm 1.0 \times 10^{-5}$ mol/l). This effect was competitively antagonized by the H_1-receptor antagonist dimethindene (pA_2: 8.30). The H_2-antagonist cimetidine in a concentration of 10^{-5} mol/l was without action on the concentration response curve for histamine. However, a relaxant effect of the H_2-receptor agonist impromidine could be shown after precontraction with various spasmodic agents ($PGF_{2\alpha}$, serotonin, carbachol). Contractile force of the ureter was increased in the order: $PGF_{2\alpha}$ < potassium < carbachol < serotonin < histamine.
It is concluded that there are two types of histamine receptors in the canine ureter: (1) H_1-receptors, mediating contraction and (2) H_2-receptors, mediating relaxation in the precontracted preparation.

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PHARMACODYNAMIC INTERACTION BETWEEN LOOP AND THIAZIDE DIURETICS WITH THE ACE-INHIBITOR RAMIPRIL.

M. Hropot, T. Fabian, R.H.A. Becker and W. Fabian*

The combination of ACE-inhibitors with diuretics is well established in the treatment of hypertension and cardiac insufficiency and has an additive effect on the blood pressure lowering potency of both classes of drugs. Aim of this study was to evaluate the influence of the ACE-inhibitor ramipril (RA) on diuresis and saluresis induced by furosemide (FU), piretanide (PI), and hydrochlorothiazide (HCT) in dogs. For this purpose experiments were performed in male Beagle dogs with body weights of 17 to 20 kg. The dogs were treated with RA, 10 mg/kg p.o., simultaneously either with FU, 5 mg/kg p.o., PI, 1.56 mg/kg p.o., or HCT, 12 mg/kg p.o. The salidiuretic effects were measured hourly for up to 6 hours and in a collection period of 7 to 24 hours after diuretic treatment. Blood samples were taken hourly up to 6 hours and at 24 hours after the administration of diuretics. Urine electrolytes, plasma aldosterone (PA) and plasma renin activity (PRA) were measured. Oral administration of RA caused mild diuretic and natriuretic effects with no change in potassium excretion. Simultaneous oral administration of PI and RA produced an additive natriuresis and chloruresis with little change in urine and potassium excretion. PRA significantly increased after RA and PI. Simultaneous oral administration of FU and RA caused also an additive and a prolonged natriuresis and chloruresis. The potassium excretion was not changed. RA exerted the same favourable effects on HCT-induced natriuresis, thus Na^+/K^+ ratio being increased. PA was markedly decreased and PRA increased by RA in combinations with both loop diuretics.
In conclusion, the ACE inhibitor RA favourably influences the saluretic profile of diuretics by increasing Na^+/K^+ ratio and reducing serum PA. The rebound effects following furosemide are attenuated.

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DOES DEOXYCORTICOSTERONE ACETATE (DOCA) AFFECT RENAL DOPAMINE EXCRETION DURING ACUTE SALT LOADING?

B. Mühlbauer, S. Lang, H. Osswald

In a recent study [Mühlbauer et al., Winter Meeting DGPT, Hannover 1991] we demonstrated that renal dopamine (DA) was not increased after expansion of the extracellular volume (1% NaCl as drinking water plus DOCA administration 1 mg/kg, for five days). Since it has been suggested earlier [Häberle et al. In: The Juxtaglom. App., Elsevier, pp 177-188, 1988] that an inhibitory principle in the tubular fluid in salt loaded rats, presumably DA, was depressed by DOCA treatment, we wanted to examine whether DOCA treatment can inhibit renal DA excretion in a model of acute salt loading in conscious Sprague-Dawley rats.
Group I (CON) kept on normal diet (Altromin C 1324) served as controls. Group II rats (HS8) were salt loaded with 8% NaCl containing rat chow (Altromin C 1051) 24 h before the experiment. Group III (HS8+DOCA) was treated as group II plus 1 mg/kg body weight DOCA s.c. Thereafter, rats were housed in metabolic cages for 24 hours. Urine was collected and concentrations of sodium, creatinine (Crea), and DA were measured by photometry and by HPLC with ECD, respectively. The following results were obtained (means ± SD):

	DA [nmol/kg/d]	Na [mmol/kg/d]	Crea [mol/kg/d]	n
I (CON)	55.5 ± 12.2	7.2 ± 3.0	269 ± 36	6
II (HS)	68.5 ± 9.5 (n.s.)	15.8 ± 4.0	251 ± 12	6
III (HS+DOCA)	67.5 ± 4.9 (n.s.)	17.9 ± 3.4	256 ± 11	6

The data show (1) that DA excretion in 24 h urine remains unchanged in spite of a 2.5-fold increase in sodium excretion and (2) that DOCA administration did not affect DA excretion. Our experiment does not support the concept that DA is a dominant natriuretic factor in the renal response to acute salt loading.

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CLONING AND FUNCTIONAL EXPRESSION OF THREE CALCIUM CHANNEL β SUBUNITS FROM HEART, AORTA AND BRAIN

R.Hullin, M.Freichel, D.Singer-Lahat*), N.Dascal*) M.Biel, V.Flockerzi

The L-type calcium channel is the major pathway for voltage-gated calcium entry in heart and most kinds of smooth muscle. In these tissues calcium currents are differentially modulated by phosphorylation and G-proteins. However, the responsible sites of the channel for this modulation are unknown and might reside in one of the four subunits α1, α2, β or γ, which form the oligomeric channel complex. cDNAs of these subunits have been cloned from skeletal muscle. So far only α1 and α2 have been identified in cardiac and smooth muscle but not β and γ. As very similar α1 and α2 exist in both tissues, β and γ may be responsible for tissue specific channel function and modulation. We now have isolated cDNAs from three novel and distinct β subunits (CaB2a, CaB2b, CaB3) which are expressed in different abundance in heart, smooth muscle and brain. Their deduced amino acid sequence is homologous to the β subunit originally cloned from skeletal muscle (CaB1). CaB2a and CaB2b are splicing products of a common primary transcript (CaB2). A partial DNA sequence complementary to a third variant of the CaB2 gene, subtype CaB2c, has also been cloned from rabbit brain. Coexpression of CaB2a, CaB2b and CaB3 with α1 heart enhances not only the expression in the oocyte of the channel directed by the α1 alone, but also effects its macroscopic characteristics such as drug sensitivity and kinetics. These results together with the known α1 heterogeneity, suggest that different types of calcium currents and their distinct hormonal regulation may depend on channel subunit composition.

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CELL SURFACE LOCALIZATION OF THE ATP-DEPENDENT CALCIUM STORE IN AN HUMAN ISLET TUMOR CELL LINE (HIT).

K. Lange and u. Brandt.

A hydrodynamic shearing technique was used for the isolation of a cell surface-derived vesicle fraction containing microvilli and other cell surface protrusions. This surface-derived vesicle fraction was shown to contain the major portion of the total cellular ATP-dependent Ca++ storing activity of HIT cells. The properties of this Ca++ store are identical with those described for the microsomal Ca++ stores of various other cell types. Moreover, it was shown that omission of the shearing procedure prior to the homogenization step resulted in the recovery of this surface derived vesicle fraction in the microsomal fraction. Electron microscopic evidence is presented for the involvement of microvilli in the action PI response agonists and other effectors of the intracellular Ca++ store in HIT cells.

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IMMUNOREACTIVITY OF Ca^{2+} CHANNEL ANTIBODIES AGAINST MEMBRANES AND CELLS FROM SKELETAL MUSCLE AND HEART

H. Haase, W. Nastainczyk*, T. Schneider*, and R. Vetter

Monoclonal antibodies directed against the 165 kDa α_1-subunit (mAb 8B7) and the 55 kDa ß-subunit (mAb 7C3) of the skeletal muscle Ca^{2+} channel were produced by hybridoma technique. The antibodies immunoprecipitated 1,4-dihydropyridine (DHP) sensitive Ca^{2+} channels from rabbit skeletal muscle that had been prelabeled with (+)-[methyl-^{3}H]isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate {(+)-[^{3}H]PN200-110} and solubilized with digitonin. Immunoprecipitation was half maximal at 10 nM and 3 nM for mAb 8B7 and mAb 7C3, respectively. The mAb 8B7 and mAb 7C3 were able to immunoprecipitate 52% ± 5% and 93% ± 3% of the DHP-labeled Ca^{2+} channels (mean ± SEM, n=3), respectively. Both mAb 8B7 and 7C3 immunoprecipitated phosphorylated Ca^{2+} channels from skeletal muscle membranes in a dose-dependent manner.

By contrast, the (+)-[^{3}H]PN200-110 labeled receptor from solubilized cardiac sarcolemmal membranes was only poorly precipitated by mAb 8B7. It was not precipitated by mAb 7C3.

However, incubation of cultured spontaneously beating rat heart myocytes with mAb 8B7 caused a dose-dependent positive chronotropic effect. This positive chronotropy was 74% of that obtained with 10 μM (-)isoprenaline and was not reversed by the ß-adrenergic and α-adrenergic agonists (-)-propranolol (1μM) and phentolamin (1μM), respectively. Under the same conditions, the antibody mAb 7C3 did not influence the basal beating rate amounting to 150-180 min^{-1}.

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MITOCHONDRIAL Ca^{2+} ANTAGONIST RECEPTORS IN HUMAN LEUCOCYTES

G.Zernig, W.Sperl**, A.Mayr** and M.Widschwendter***

The demonstration of beneficial effects of Ca^{2+} antagonists in recent large controlled clinical trials of myocardial infarction has again strengthened the interest in the molecular mechanisms of the long-observed anti-ischemic effects of certain members of this drug class (for a recent review see Zernig, 1991, Trends Pharmacol Sci 12(12),439).

One of the putative target systems for the anti-ischemic effect might be the mitochondrial Ca^{2+}-antagonist-receptor which is associated with an inner mitochondrial membrane anion channel (Zernig et al., 1990, Mol Pharmacol 38,362). This receptor has been partially purified (Zernig and Reider, 1991, Mol Pharmacol, in press). A major experimental advance for the complete elucidation of its proposed (patho)physiological role would be its characterization in easily accessible tissues from patients suffering from ischemia.

Saturable binding of the dihydropyridine (DHP) Ca^{2+} antagonist (±)-[^{3}H]nitrendipine could indeed be demonstrated in human leucocytes. Granulocytes and lymphocytes/monocytes were obtained from adult volunteers according to Boyum, 1968, Scand J Clin Lab Invest 21(S97),77. Binding was stimulated by $CdCl_2$ (1-100 mM) or NaCl (up to 500 mM tested). To exclude any contribution by L-type Ca^{2+} channels, the cells were preheated at 56°C for 15 min. Saturation experiments were performed in presence of 0.1 mM $CdCl_2$ to prevent any possible (±)-[^{3}H]nitrendipine interaction with the DHP binding domain of the nucleoside transporter of contaminating erythrocytes (Striessnig et al., 1985, Eur J Pharmacol 150,67). In presence of 500 mM NaCl at 37°C, (±)-[^{3}H]nitrendipine saturably bound to the lymphocyte-monocyte preparation with a K_D of 588 ± 138 nM (mean ± SEM, n=3) and a B_{max} of 44.7 ± 3.7 pmol/mg protein (determined acccording to Bradford, 1976, Anal Biochem 72,248), corresponding to 1.55 ± 0.12 pmol/10^6 cells. Granulocytes displayed a K_D of 710 ± 244 nM and a B_{max} of 58.8 ± 15.2 pmol/mg protein (n=4), corresponding to 1.95 ± 0.60 pmol/10^6 cells. Experiments are under way to assess the potential usefulness of these easily obtainable human preparations for the elucidation of ischemia-related cellular events.

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VASCULAR ACTIVITY AND INOTROPIC ACTION OF SOME DIHYDROPYRIDINE-TYPE CALCIUM CHANNEL BLOCKERS SUBSTITUTED IN POSITION 2

J. Günther and U. Fricke

The vascular selectivity of the 1,4 Dihydropyridine (DHP)-type calcium channel blockers is well established. Both, high vascular activity and minimal cardiodepressive effects, are highly influenced by the substitution of the DHP-nucleus in position 2 and 3. While alteration in the ester side chain increases potency, substitution in position 2 is said to decrease the activity of the DHPs (Bossert et al.,Med.Res.Rev,Vol 9, 1989). In order to study the latter aspect further, we investigated the action of (±)3-ethyl-5-methyl-1,4-dihydro-2-ethyl-6-methyl-4-(3-nitrophenyl)-3,5-pyridindicarboxylat (Bay F 1779), its 2-trifuormethyl-derivative (Bay K 9593), a 2-(2'-hydroxyethyl)-derivative (Bay R 8885) and a 2-(N'-phthalimidomethyl)-derivative (Bay I 1990) on porcine ventricular trabeculae as well as on coronary and basilary arteries. Nitrendipine (NTD) was used as reference. Isolated trabecular muscles (resting tone (RT) = 0,5 g; Krebs-Henseleit-solution, pH 7,4; 30°C) were stimulated at 1 Hz, isolated right coronary (RT= 2,0 g) and basilary arteries (RT= 0,7 g) bathed in Krebs-Henseleit-solution (pH 7,4; 37°C) were precontracted with 60 mmol/l KCl. The calcium antagonists were added cumulatively. Half maximum negative inotropic effects and vasorelaxation were obtained at the following drug-concentrations (nmol/l; n=5-9).

Drugs	Trabeculae	A. coranaria	A. basilaris
NTD	93,2 ± 13,6	28,2 ± 2,3	41,6 ± 5,6
BAY F 1779	67,6 ± 3,8	131 ± 15	103 ± 15
BAY K 9593	95,7 ± 6,3	330 ± 49	97,4 ± 7,5
BAY R 8885	45,2 ± 4,7	209 ± 35	25,7 ± 1,8
BAY I 1990	129,0 ± 16,1	2190 ± 320	80,7 ± 8,1

The data show that, increasing the side chain in position 2 markedly reduces vasodilator effects in coronary arteries compared to nitrendipine. However in basilary arteries bulky side chains rather increase vascular activity.

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DECREASE IN FREE INTRACELLULAR CALCIUM CONCENTRATION IN CARDIAC MYOCYTES AFTER AN INCREASE IN EXTRACELLULAR MAGNESIUM CONCENTRATION

W. Vierling

Intracellular calcium concentration was measured in isolated myocytes of guinea-pig hearts by means of the calcium-sensitive fluorescent dye fura II.

There was no detectable $[Mg^{2+}]_o$-dependent decrease of the calcium transient elicited by stimulation at 0.2 Hz in the presence of 1.2 or 2 mmol/l $[Ca^{2+}]_o$. This is in contrast to the findings that $[Mg^{2+}]_o$ diminishes the force of contraction in isolated papillary muscles and decreases the calcium inward current in isolated myocytes (Dichtl, Vierling, Eur. J. Pharmacol., 1991, in press). In the presence of isoprenaline (10 nmol/l), there was only a Mg^{2+}-dependent shortening but also no decrease in the calcium transient. However, if additionally ryanodine was present to deplete intracellular calcium stores, a significant decrease in the calcium signal was found after increasing $[Mg^{2+}]_o$ from 1.2 to 9.6 mmol/l.

If the $[Ca^{2+}]_i$ was increased during rest by elevating $[K^+]_o$, there was only a slight reduction in $[Ca^{2+}]_i$ at 60 mmol/l K^+ (by about 15%), but a strong decrease in the presence of 30 mmol/l K^+ (by about 65%).

The results show that, in spite of the negative inotropic effect of magnesium in multicellular tissue, it is difficult to demonstrate a magnesium-induced decrease in the calcium transient after stimulation in isolated ventricular myocytes. However, under certain conditions, there is evidence for a Mg^{2+}-dependent decrease of $[Ca^{2+}]_i$ in the myocyte.

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RADIOLABELED CALMODULIN LIGANDS: THEIR LOW AFFINITY AND HIGH LIPOPHILICITY MAY LEAD TO ARTEFACTS IN BINDING STUDIES

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When we had developed a new radioligand (fig. 1) for calmodulin to study the interaction of a new series of calmodulin antagonists, using a calmodulin-agarose complex for establishing the affinities, some peculiar results were obtained. The unexpected results could readily be explained by a high binding of the labelled compound and the investigated ligands to the walls of the test tubes used. Such results were not only found with our new radioligand ([^{3}H]-VUF 4576) but also when [^{3}H]-chlorpromazine was applied.

In literature comparable findings have been published. For explaining such results it has been suggested to assume the influence of positive co-operativity or irreversible binding. We propose that not only in our study but also in some published investigations binding of radioligand and/or the cold compounds under investigation may have had a strong influence. We suggest to be very careful in interpreting displacement data obtained with ligands which combines a rather low affinity and a high degree of lipophilicity. This care should be taken not only for binding to calmodulin but for any other system.

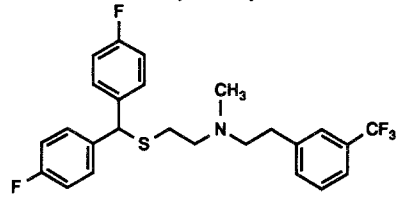

Fig. 1 The structure of VUF 4576; in the related radioligand, the tritium isotope is located in the methyl moiety connected to the nitrogen.

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DIAZOXIDE-SENSITIVITY OF THE ATP-DEPENDENT K^+ CHANNEL IN PANCREATIC B-CELLS

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The potency of diazoxide to activate ATP-sensitive potassium channels (K-ATP channels) in isolated membrane patches of insulin-secreting cells depends on the presence of MgATP. MgATP might act by serving as substrate for kinases phosphorylating the channel proteins. In the present study we used the inside-out configuration of the patch-clamp technique to study the control of the diazoxide-sensitivity of the pancreatic B-cell. Diazoxide (10-400 µmol/l) failed to significantly activate K-ATP channels in the absence of any nucleotides. In the presence of 30 or 100 µmol/l MgATP, channel activity was reduced to 40 or 8 % of controls and was restored to 80 or 48 %, respectively, by addition of maximally effective concentrations of diazoxide (EC_{50} 90-100 µmol/l). When the bath solution was supplemented with MgADP (1mmol/l), the channel activating potency of diazoxide (300 µmol/l) was even stronger than in the presence of MgATP. Moreover, in conjunction with MgADP diazoxide (50-300 µmol/l) strongly antagonized the channel-inhibitory effect of tolbutamide. It is concluded that protein phosphorylation is not the sole mechanism by which cytosolic nucleotides control the diazoxide-sensitivity of the K-ATP channel in B-cells.

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MAGNESIUM ATP ENHANCES PINACIDIL - INDUCED DISPLACEMENT OF GLIBENCLAMIDE FROM THE SULPHONYLUREA RECEPTOR IN A PANCREATIC B-CELL LINE AND CEREBRAL CORTEX

M. Schwanstecher, S. Behrends, Ch. Brandt and U. Schaupp

The effects of blockers and openers of K^+ channels on binding of [^{3}H]-glibenclamide to microsomes obtained from a pancreatic B-cell line (HIT-T15) or rat cerebral cortex were examined. The blockers quinine, chlorpromazine and thiopentone and the openers cromakalim and minoxidil sulphate did not significantly interact with the sulphonylurea receptor of HIT-cells both at phosphorylating (presence of MgATP) and dephosphorylating (absence of MgATP) conditions. In the absence of MgATP pinacidil insignificantly displaced [^{3}H]-glibenclamide binding to microsomes from HIT-cells. This displacement of [^{3}H]-glibenclamide binding was strongly enhanced by MgATP and was due to a decrease in the number of high affinity binding sites for glibenclamide. MgATP enhanced pinacidil-induced inhibition of [^{3}H]-glibenclamide binding to microsomes from rat cerebral cortex. The effect of MgATP on pinacidil-induced inhibition of [^{3}H]-glibenclamide binding was maintained after solubilization of the membranes from HIT-cells or rat cerebral cortex.

It is concluded that the sulphonylurea receptor is regulated not only by sulphonylureas but also by the K^+ channel openers diazoxide and pinacidil and by protein phosphorylation. The binding sites for sulphonylureas and these K^+ channel openers are not identical, but appear to be located at a single protein or at tightly associated proteins.

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EFFECTS OF GLIBENCLAMIDE, CHARYBDOTOXIN, $[ATP]_i$ AND $[Ca^{2+}]_i$ ON CROMAKALIM-INDUCED MEMBRANE CURRENTS IN VASCULAR SMOOTH MUSCLE CELLS

D. Pfründer and I. Anghelescu

The target channels for the hyperpolarizing action of K^+ channel openers such as cromakalim have not been finally established in vascular smooth muscle. Using the whole-cell clamp technique, we recorded steady-state membrane currents at a holding potential of +20 mV for 20-40 min in enzymatically dispersed cells of guinea pig portal vein and rabbit aorta. The drugs under investigation were added to the extracellular medium, while intracellular variations of ATP and/or Ca^{2+} content were done by respective modifications of the pipette solution, which replaces the cytosol after rupture of the membrane patch. - Cromakalim ($3x10^{-6}$ or 10^{-5} mol/l) induced an increase of the membrane current, which was antagonized by 10^{-5} mol/l glibenclamide, a known antagonist to the vasorelaxation by cromakalim, and was reversible upon washout of the drug. Only in few cells, glibenclamide had an effect of its own on the membrane current at +20 mV, while all cells responded to cromakalim. The augmentation of the steady-state current by cromakalim was observed regardless of whether ATP (5 mmol/l) was present in the pipette solution or not, suggesting that the presence of ATP-sensitive K^+ channels is not a necessary prerequisite for the action of K^+ channel openers in vascular smooth muscle cells. - Cromakalim-induced membrane currents were independent of the intracellular level of Ca^{2+} ($2x10^{-8}$ or $2x10^{-6}$ mol/l). Prior application of charybdotoxin (10^{-7} mol/l), an assumed blocker of large-conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}), however, reduced the increase of the steady-state current evoked by cromakalim. The significance of these data remains to be established.

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EVIDENCE THAT NORADRENALINE-INDUCED ^{86}Rb EFFLUX FROM RABBIT AORTA DOES NOT OCCUR VIA BK_{Ca} CHANNELS

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In vascular smooth muscle, noradrenaline and other agonists increase the efflux of ^{86}Rb, a marker of K^+ ions. Pharmacological regimens aiming at a reduction of the intracellular Ca^{2+} level interfere with this agonist-induced augmentation of ^{86}Rb efflux, and make it apparently suitable to study mechanisms involving intracellular Ca^{2+} modulation. Regarding the abundance of large-conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) found in vascular smooth muscle by the patch clamp technique, we took so far for granted that agonist-induced ^{86}Rb efflux be mediated by this channel type. However, in patch clamp studies on isolated vascular smooth muscle cells, we found that tedisamil (10^{-6} - 10^{-4} mol/l) reduced the open-probability of BK_{Ca} channnels, while it had no effect on noradrenaline-induced ^{86}Rb efflux. In addition, noradrenaline-stimulated ^{86}Rb efflux was neither inhibited by charybdotoxin (10^{-7} mol/l), which is assumed to be a selective inhibitor of BK_{Ca} channels, nor by tetraethylammonium (20 mmol/l), which also inhibits BK_{Ca} channels in isolated membrane patches. On the other hand, noradrenaline-induced ^{86}Rb efflux was partially inhibited by the K^+ channel blockers Ba^{2+} (5 mmol/l), 4-aminopyridine (5 mmol/l) and quinidine (10^{-4} mol/l). The most potent inhibitors, however, were organic nitrates and atrial natriuretic factor (which raise intracellular cGMP), 8-bromo-cGMP, and trifluoperazine. In rabbit aorta made nominally Ca^{2+}-free, noradrenaline-induced ^{86}Rb efflux was suppressed, but the validity of this data is obscured by the fact that the basal ^{86}Rb efflux was substantially raised under these conditions. - Our findings suggest that augmentation of ^{86}Rb efflux by noradrenaline is probably not the result of an activation of BK_{Ca} channels, but its true nature remains to be established.

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CHARACTERIZATION OF A SPECIFIC BINDING SITE FOR K^+ CHANNEL OPENERS IN RAT AORTA

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The K^+ channel openers (KCOs) form a heterogeneous group of compounds which relax smooth muscle by opening K^+ channels in the plasmalemma. At present it is not known whether these drugs elicit their effects by binding to the same target (presumably the K^+ channel) and whether they bind to the same site of this target. To approach these questions we have developed a binding assay for KCOs in a vascular smooth muscle preparation.

In intact rat aortic strips, the novel tritiated KCO, ^{3}H-P1075 (^{3}H-N-cyano-N'-(1,1-dimethylpropyl)-N''-3pyridylguanidine), a potent pinacidil analogue shows saturable specific binding of high affinity (K_D = 6± 1 nM, B_{max} = 21 ± 3 fmol/mg tissue wet weight). Specific binding of ^{3}H-P1075 is stereospecifically inhibited by representatives from all major families of K^+ channel openers with a rank order of potencies that correlates well with the potencies of eliciting $^{86}Rb^+$ efflux from rat aorta. Glibenclamide, an inhibitor of the KCOs, also inhibits ^{3}H- P1075 binding and increases the rate of dissociation of ^{3}H-P1075 from the tissue in a concentration-dependent manner, indicating a negative allosteric coupling between the binding sites for glibenclamide and ^{3}H-P1075. In addition, lowering temperature (from 37°C to 2°C) and decreasing intracellular ATP levels by metabolic poisoning, attenuates specific ^{3}H-P1075 binding by reducing B_{max}.

The data demonstrate the existence of a specific and functionally relevant binding site for the KCO ^{3}H-P1075 in rat isolated aorta.

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EFFECTS OF THE POTASSIUM CHANNEL OPENERS HOE 234 AND LEMAKALIM IN RAT AORTA

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In rat aorta with intact endothelium HOE 234 ((3S,4R)-3-Hydroxy - 2,2 - dimethyl - 4 - (2 - oxo - 1 - pyrrolidinyl) - 6 - phenylsulfonylchroman hemihydrate) and Lemakalim exert dose dependent relaxations of KCl (20 mM) induced contractions with IC_{50}-values of $1.5 \cdot 10^{-10}$ M and $8.8 \cdot 10^{-9}$ M, respectively. Bradykinin at 10^{-7} M and HOE 234 at 10^{-11} M only marginally reduced KCl induced contractions by 9 % and 15 % (n=4) when tested in separate experiments. However the combined addition of Bradykinin (10^{-7} M) and HOE 234 (10^{-11} M) inhibited contractions by 48 % (n=4). Bradykinin at 10^{-6} M and HOE 234 at $5 \cdot 10^{-11}$ inhibited KCl induced contractions by 25 % (n=7) and 41 % (n=8) when tested in separate experiments. In this case the combined addition produced a 67 % (n=8) reduction. The antispasmogenic effects of combined HOE 234 and Bradykinin were sensitive to blockade of ATP dependent potassium channels by glibenclamide (10^{-5} M, 96 % and 90 % reductions of antispasmogenic effect, n=4, respectively), inhibition of NO-synthetase by nitroarginine (10^{-4} M, 71 % and 94 % reduction, n=4, respectively) and to inhibition of Bradykinin B_2-receptor by HOE 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin, 10^{-7} M, 83 % and 61 % reduction, n=4). Lemakalim at 10^{-9} M, $3 \cdot 10^{-9}$ M and 10^{-8} M inhibited the antispasmogenic effects of 20 mM KCl by 20 % (n=4), 40 % (n=4) and 66 % (n=6). At these concentrations Bradykinin (10^{-6} M) failed to augment the relaxation induced by lemakalim. Nevertheless the 43 % (n=12) relaxation induced by the combined addition of lemakalim (10^{-8} M) and Bradykinin (10^{-6} M) was almost completely abolished by either glibenclamide (10^{-5} M, n=6), or nitroarginine (10^{-4} M, n=5) or HOE 140 (10^{-7} M, n=6). These results show that at low concentrations potassium channel openers may interfere with Bradykinin induced NO formation in endothelial cells. Glibenclamide sensitive potassium channels are most likely to mediate these effects.

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STRYCHNINE ACTIVATES POTASSIUM CHANNELS IN VASCULAR SMOOTH MUSCLE

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Besides its well-known blockade of glycine receptors, strychnine appears to interact with potassium channels of vascular smooth muscle cells (VSMC) as suggested by the following findings. Measurements of the membrane potential with intracellular glass microelectrodes revealed a hyperpolarization of VSMC within 3-6 min when strips of rabbit main pulmonary artery were exposed to strychnine. The hyperpolarization was concentration-dependent over the range of 10^{-4} to 10^{-3} mol/l strychnine. It was accompanied by a decrease in space constant λ indicating a strychnine-induced decrease in membrane resistance and, therefore, suggesting an opening of potassium channels by strychnine. The hyperpolarization was antagonized by tetraethylammonium (TEA), but not by glibenclamide. The decrease in space constant λ produced by strychnine was antagonized by TEA in a competitive manner. Strychnine inhibited the contraction in response to noradrenaline both in the rabbit main pulmonary artery and the rabbit aorta. The inhibition occurred in the same range of strychnine concentrations that hyperpolarized the membrane of VSMC of rabbit main pulmonary artery. There was both a rightward shift by strychnine of the noradrenaline concentration-response curve as well as a depression of its maximum. In VSMC of rabbit main pulmonary artery incubated in the presence of strychnine, noradrenaline-induced contractions appeared only when the agonist had depolarized the membrane from the hyperpolarized state to a value which was some mV more positive than the resting membrane potential. Quaternary strychnine was inactive, suggesting an effect of strychnine on a component of the potassium channel located near the inner side of the surface membrane.

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PATHOPHYSIOLOGICAL ELECTRICAL AND MOTOR REACTIONS OF ISOLATED URINARY BLADDER PREPARATIONS (GUINEA PIG) ON CYPERMETHRIN

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The evaluation of functional disturbances of the urogenital tract on toxicants is an important task for the medical and eco-toxicology. Normal functions depend on electrical and motor activities of this tract (Proc. Int. Un. Physiol. Sci. 16, 117, 1986 & 17, 529, 1989). Cypermethrin (a pyrethroid) influences these activities of vesical preparations (guinea pig). This toxicant (10-100 µM) had a negative chrono- and inotropic effect on the spontaneous fast (1-5/min) and slow (0.1-0.5/min) contractions (isotonically recording) of detrusor and trigonum preparations: Contraction amplitudes and frequency were decreased (to 50% of the initial values; n=8, $p < 0.002$). The contractions of detrusor after electrical neural stimulation (100 Hz, 0.3 ms, 3 s) were also diminished (to 70%; n=4, $p < 0.01$). At normal conditions detrusor myocytes generate in vitro electrical (intracellular recording) spikes (S: 40%), bursts (B: 22%) and burst-plateaus (BP: 38%) (n=112), which can be transformed by changes of ionic concentrations. Cypermethrin (1-50 µM) (n=8) transformed S in B and/or BP similar to effects after increase of $[K^+]$ or decrease of $[Ca^{++}]$; it induced in cells with initial BP-activity a strong decrease of BP-amplitudes and -duration (more than twice of the initial value) without visible changes of the membrane potential. All effects were concentration-dependent and only partially reversible (after washing up to 1 h). It is suggested that cypermethrin could also induce pathophysiological motor reactions of the urinary tract (the motility of pyeloureter was also inhibited after cypermethrin) in vivo and that the action mechanism may be based on disturbances of K^+- and/or Ca^{++}-channels of the detrusor myocytes. Investigations with Patch Clamp could clear finally the membrane action mechanisms.

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REVERSE USE-DEPENDENCE OF d-SOTALOL IS CONSISTENT WITH SLOW ON- AND OFF-KINETICS OF A PERIODICAL INTERACTION WITH POTASSIUM CHANNELS.

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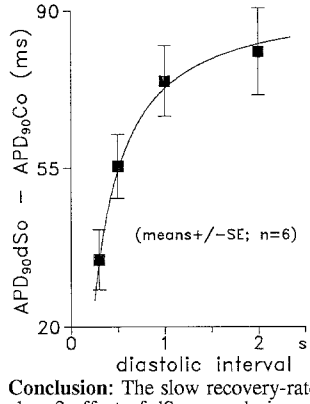
The frequency-dependence of the class-3-action (APD_{90}-prolongation) of d-sotalol (dSo) was examined on electrically stimulated guinea pig papillary muscles. According to a reverse use-dependence (block of closed K^+-channels) dSo (50µM) prolongs APD_{90} at low frequencies (0.5Hz) from 222±10 ms to 293±12 ms (means±SE; n=6; $p < 0.05$) whereas the effect is reduced at high stimulation rates (3Hz) with 159±8 ms vs 189±9 ms. The use-dependent APD_{90}-prolongation at 0.5 Hz disappears with slow recovery-rates of 0.026±0.001 and 0.021±0.004 AP^{-1} upon switching the stimulation frequency from 0.5Hz to 2Hz or to 3Hz, respectively. Although the relationship between APD-prolongation and K^+-channel blockade induced by dSo is complex, the use-dependent steady-state prolongation of APD (APD_{90}dSo -APD_{90}Co) is consistent with an algorithm for periodical ligand binding (J. Weirich and H. Antoni, J. Cardiovasc Pharmacol 15, 1990) after modifications: instead of equilibrium blockade, br_∞ and bd_∞, a minimal and maximal APD-prolongation and a t_{on}-interval equal to the diastolic interval is used. According to this periodical interaction of dSo with K^+-channels the on- and off-kinetics are in the order of several seconds (τ_{on}=17s and τ_{off}=27s). These kinetics result in an effect-frequency-relation with saturation characteristics at low frequencies (continuous curve) and recovery-rates (0.029 AP^{-1} and 0.019 AP^{-1} at 2Hz and 3Hz) in agreement with the experimental values.



Conclusion: The slow recovery-rates might be important for preserving the class-3-effect of dSo even during runs of up to 10 closely coupled extrasystoles, thus preventing them from degeneration into ventricular fibrillation.

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CARDIAC CALCIUM CHANNEL REGULATION BY ACETYLCHOLINE DEPENDS ON PROTEIN PHOSPHATASE ACTIVITY

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The increase in cardiac contractile force and in L-type calcium channel current observed after ß-adrenergic stimulation is partially antagonized by cholinergic agonists. Calcium channel activity, and hence channel phosphorylation, could be physiologically regulated not only by the cAMP-dependent phosphorylation rate but also by modulation of protein phosphatase activity. Thus, the adrenergic/cholinergic interaction was assessed in the absence and presence of three different phosphatase inhibitors.

Using the whole-cell patch clamp technique in isolated guinea pig ventricular myocytes, calcium currents were elicited every 10sec (300ms-pulses from -40mV to +20mV). When the effect of isoprenaline (10nM) on the current was established (a two- to about fivefold increase), acetylcholine (10/uM) rapidly and reversibly reduced this effect by 64±5% (n=9). This inhibition was abolished (2.4±5%, n=4) when cells were treated with 3mM external NaF, which non-selectively inhibits phosphatases. Internal dialysis with okadaic acid (an inhibitor of type 2A and 1 phosphatases) led to a concentration-dependent inhibition of the acetylcholine response (3/uM: 45±7%, n=5; 9/uM: 15±3%,n=4). Cell dialysis with the specific type 1-phosphatase inhibitor protein I_2 (from rabbit skeletal muscle, 1000U/ml, n=4) suppressed (7.6 ±8.2%) the acetylcholine effect.

These results suggest that the activity of cellular protein phosphatases - possibly a type 1 phosphatase - is involved in the cholinergic regulation of the cardiac calcium channel.

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EVIDENCE FOR PHOSPHATASE ACTIVATION BY M-CHOLINOCEPTOR AGONISTS AND ADENOSINE RECEPTOR AGONISTS IN THE HEART J. Neumann, G. Kaspareit

In various cardiac preparations M-cholinoceptor (M) agonists and adenosine receptor (Ad) agonists reduce isoprenaline (Iso)-stimulated phosphorylation of regulatory proteins independently of changes in cAMP content. However, type 1 phosphatase activity in the heart is decreased by Iso and increased by M-agonists and A-agonists. If this phosphatase activation is of functional importance the effects of M-agonists and Ad-agonists in the heart should be modified by addition of phosphatase activity inhibitors. NaF inhibits type 1 and 2A, 2B and 2C phosphatase activity whereas okadaic acid (OA) inhibits type 1 and 2A phosphatase activity only. We tested the effects of carbachol (C) and the Ad-agonist (-)-N^6-phenyl-isopropyladenosine (PIA) on force of contraction (FC) in guinea-pig papillary muscles in the presence of the phosphatase activity inhibitors NaF (3 mM) or OA (10 μM). The IC50 of C or PIA for the negative inotropic effect (NIE) in the presence of Iso was 1.4 μM and 1.2 μM, respectively. In the additional presence of NaF the IC50 was > 1000 μM for C and > 100 μM for PIA. The NIE of C (1 μM, 2 min) in the presence of Iso was reduced from 46 % to 22 % by 10 μM OA. At 3 mM NaF and at 1 μM OA inhibited guinea-pig phosphorylase phosphatase activity which comprises type 1 and 2A phosphatase activity by 43 % and completely, respectively. It is concluded that non-specific (NaF) and specific (OA) phosphatase activity inhibitors can diminish the effects of a M-agonist and an Ad-agonist on FC at concentrations where they inhibit cardiac phosphatase activity. This supports the hypothesis that activation of type 1 and conceivably 2A phosphatase are involved in the cardiac effects of M- and Ad-agonists. (Supported by the DFG.)

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MAPPING OF VENTRICULAR β-ADRENOCEPTOR SUBTYPES IN THE SEVERELY FAILING HUMAN HEART.

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A decrease in cardiac β-adrenoceptors (AR) is a general feature of chronic heart failure; it is, however, not known, whether β-AR are uniformely decreased in the failing ventricles or whether regional variations exist. To answer this question we assessed β-AR number [by (-)[^{125}I]-iodocyanopindolol (ICYP) binding] and the relative amount of β_1- and β_2-AR [by analyzing competition curves of the β_2-AR antagonist ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) with ICYP binding] in ventricular segments (obtained by cutting transversal rings of 2 cm from the midventricular region) of explanted hearts from 2 patients with end-stage dilated cardiomyopathy (DCM) and 1 patient with end-stage ischemic cardiomyopathy (ICM). In all three hearts ventricular β-AR number was very low (15-25 and 8-13 fmol ICYP bound/mg protein in DCM, 10-20 fmol/mg protein in ICM) and evenly distributed over the whole ventricular regions. Similarly, β_1- and β_2-AR were uniformely distributed: In DCM the β_1:β_2-AR ratio was 65-70: 35-30%, in ICM it was 75-80:25-20%. These results demonstrate a) that in failing human ventricles β-AR are homogeneously down-regulated and b) that in end-stage DCM the loss in β-AR is mainly due to a loss in β_1-AR, while in end-stage ICM the reduction in β-AR is due to a concomitant decrease in β_1- and β_2-AR.

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IBOPAMINE MEDIATES POSITIVE INOTROPIC EFFECTS VIA ß$_1$-AND ß$_2$-ADRENOCEPTORS IN THE HUMAN MYOCARDIUM

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Inotropic and vasodilatory actions of dopamine derivates may improve cardiovascular function in congestive heart failure patients. We studied the effects of epinine (EPI 1-1000 μmol/l), the biologically active metabolite of ibopamine on isometric force of contraction (FOC) in electrically driven (1 Hz, 37°C) papillary muscle strips (PAP) from nonfailing human myocardium (NF, brain death, n=3), moderately failing (NYHA II-III, n=8) and terminally failing myocardium (NYHA IV, n=7) as well as right auricular trabeculae (AUT, ACVB, n=9). The inotropic effects (IE in mN) after prestimulation with forskolin (FOR, 0.3 μmol/l), milrinone (MIL, 100 μmol/l), ICI 118.551 (ICI, 50 nmol/l), CGP 207.12A (CGP, 300 nmol/l) or ICI+CGP and of isoprenaline (ISO, 0.01-10 μmol/l) were also studied. In PAP and AUT (IE: +6.7±0.2 mN) EPI concentration dependently increased FOC (PAP: IE: NF: +6.9±0.5 mN, NYHA II-III: +3.9±0.2 mN, NYHA IV: +2.8±0.2 mN). The potency of EPI was higher after FOR (NYHA IV: +3.9±0.3 mN; EC_{50} 9.32 μmol/l) or MIL (NYHA IV: +3.7±0.3 mN, EC_{50} 12.23 μmol/l) compared to EPI alone (EC_{50} 37.7 μmol/l). In contrast ICI (AUT: EC_{50} 11.4 μmol/l), CGP (EC_{50} 16.3 μmol/l) and ICI+CGP (EC_{50} 49.6 μmol/l) shifted the concentration response curve of EPI (EC_{50} 5.7 μmol/l) to the right. In radioligand binding experiments (^{125}I-CYP) EPI has higher affinity to ß$_2$-than to ß$_1$-adrenoceptors.

(1) EPI has similar efficacy but lower potency than ISO to increase FOC, (2) the positive IE of EPI decreases parallel to the degree of heart failure, (3) EPI increases FOC via activation of ß$_1$-and ß$_2$-AR.

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DESENSITIZATION OF CARDIAC ADENYLATE CYCLASE IN SPONTANEOUSLY HYPERTENSIVE RATS

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In order to study whether alterations of the inhibitory guanine-nucleotide binding protein α-subunits (Giα) contribute to alterations of adenylate cyclase (AC) regulation in hypertensive cardiomyopathy of spontaneously hypertensive rats (SHR) without heart failure, we measured pertussis toxin substrates (PTS). The amount of Giα was quantified by competition of rat myocardial membrane extracts to DS 4 antiserum binding to the ^{125}I-radiolabeled C-terminus of retinal transducin α (^{125}I-KENLKDCGLF).The DS4 antiserum was raised against the same decapeptide (KENLKDCGLF). Cardiac ß-adrenoceptors (ß-AR), m-cholinoceptors (m-Ch) as well as isoprenaline (ISO)-, guanine-nucleotide (Gpp(NH)p)- and forskolin (FORS)-stimulated AC activity and the inotropic responses (IR) to ISO- and carbachol were studied in SHR and age-matched Wistar-Kyoto-rats (control).

In SHR, there was a 40 % increase of pertussis toxin substrates. The radioimmunological quantification of Giα revealed a 41 % increase in membrane extracts of SHR. In addition, ß-AR were reduced by 36 % and m-Ch were diminished by 14.4 % in SHR compared to WK. Basal AC, ISO-, Gpp(NH)p- and FORS-stimulated AC activities were also reduced. However, in the presence of 5 mmol/l $MnCl_2$ no differences in adenylate cyclase activities between SHR and controls were detected under either condition. In isolated, electrically driven papillary muscle strips, the IR to ISO was reduced while the indirect negative inotropic effect of carbachol was unchanged.

The present study shows that the amount of Giα-proteins and not only pertussis toxin substrates are increased in membranes of hypertrophic hearts from SHR. Increased Giα-expression and reduced ß-adrenoceptor number might have functional relevance in the regulation of AC activity and force of contraction in SHR, whereas the activity of the catalyst appears to be unchanged. The data of this study raise the possibility that an increase of Giα expression might play a pathophysiological role not only in heart failure, but also in hypertrophic cardiomyopathy.

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DESENSITIZATION OF ß$_1$-ADRENOCEPTORS OF RAT CARDIOMYOCYTES BY PLASMA OF PATIENTS WITH SEPTIC SHOCK

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In cardiogenic shock endogenous and exogenous catecholamines are known to desensitize cardiac ß-adrenoceptor (ß-AR)-adenylyl cyclase (AC) stimulation. In hyperdynamic septic shock catecholamines are often needed to maintain arterial blood pressure. The aim of the present study was to estimate the desensitization of cardiac ß-AR in patients with hyperdynamic septic shock treated with noradrenaline (NA) or dopamine (DA). As an in-vitro model the desensitization of rat cardiomyocyte ß-AR by treatment with plasma of patients with hyperdynamic septic shock was studied.

Exposure of rat cardiomyocytes for 48 h to plasma of critically ill patients without shock treated with DA in a dosage causing renal vasodilatation (200 ug/min, n=5) did not significantly alter ß-AR number and isoproterenol-stimulated AC-activity (decrease by 13 and 2 %, resp.). A pronounced decrease of ß-AR (by 30 %) and of AC-activity (by 32 %) was found following incubation of the cells with plasma of patients with cardiogenic shock treated with adrenaline (mean 20 ug/min, n=6). Exposure to plasma of patients with septic shock treated with NA (mean 13 ug/min, n=6) even decreased ß-AR and AC-activity by 40 and 50 %, resp. In contrast, incubation of the cells with plasma of septic shock patients treated with DA (mean 780 ug/min, n=6) did not significantly alter ß-AR number and AC-activity (decrease by 2 and 19 %, resp.).

Conclusion: Exposure of rat cardiomyocytes to plasma of septic shock patients treated with NA, but not to plasma of septic shock patients treated with DA, desensitized ß-AR-stimulated AC-activity in rat cardiomyocytes. The data suggest that the ß-AR desensitization by exogenous NA is one of the reasons for the early cardiac tolerance to NA in patients with hyperdynamic septic shock.

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CARDIAC β-ADRENOCEPTORS IN ATENOLOL-TREATED HYPERTHYROID RATS. G Amos, L Brown, C Marchant, C Sernia.

In hyperthyroidism, cardiovascular changes possibly related to β-adrenoceptor up-regulation are prominent. We have determined whether administration of the selective β_1-adrenoceptor antagonist, atenolol, modifies these cardiovascular changes. Male, 10 week old Wistar rats were given either saline (S rats) or triiodothyronine (T_3; 1 mg/kg/day sc) for 7 days (T7 rats) or T_3 for 14 days (T14 rats) together with atenolol (100mg/day) for the last 7 days (A rats). In A rats, heart rate was decreased (S, 251±5; T7, 355±15; T14, 506±8; A, 447±20 beats/min), but not plasma T_3 (S, 1.0±0.1; T7, 19.3±0.4; T14, 29.7±3.8; A, 38.0±4.5 ng/ml), right atrial (RA) weight (S, 68±2; T7, 83±3; T14, 148±5; A, 139±8 mg), left ventricular (LV) weight (S, 778±36; T7, 917±14; T14, 1104±22; A, 1024±36 mg), systolic blood pressure (S, 122±6; T7, 135±9; T14, 176±7; A, 182±11 mmHg), basal rate of contraction of isolated RA (S, 239±3; T7, 310±6; T14, 310±4; A, 304±9 beats/min) or basal force of contraction of isolated LV papillary muscles. Maximal chronotropic responses to noradrenaline were reduced (S, 196±7; T7, 168±11; T14, 158±11; A, 133±7 beats/min). Positive inotropic responses to noradrenaline were decreased (S, 3.1±0.5; T7, 2.2±0.5; T14, 1.0±0.3 mN); LV papillary muscles from A rats showed toxicity at low noradrenaline concentrations. The increase in LV β_1-adrenoceptor density measured by ^{125}I-cyanopindolol binding (S, 30.0±10.3; T7, 60.8±10.8; T14, 285±23.2 fmol/mg protein) was not altered by atenolol treatment. Thus, most T_3-induced cardiovascular changes, especially hypertrophy and hypertension, occur independently of β-adrenoceptor changes; only tachycardia is partly due to β-adrenoceptor activation. In isolated tissues, atenolol treatment does not reverse the decreased responses of hyperthyroid tissues nor the marked increase in ventricular β_1-adrenoceptor density.

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Differential influence of phosphodiesterase III and IV inhibition on the positive inotropic response to isoprenaline in rat and guinea-pig left atria.

D. Wilhelm, M. Ebbert, and A. Leidig

R 80122 ((E)-N-cyclohexyl-N-methyl-2-[[[phenyl (1,2,3,5-tetrahydro-2 oxoimidazo [2,1-b]-quinazolin-7-yl) methylene] aminoloxy] acetamide) is a cardiotonic compound which preferentially inhibits phosphodiesterase (PDE) III. In the present study, the PDE IV inhibitor rolipram was used to examine whether inhibition of PDE IV modifies the mechanical effects of PDE III inhibition by R 80122. The experiments were performed in guinea-pig and rat left atria to study the species difference in the distribution of PDE isoenzymes. In isolated guinea-pig left atria, pretreatment with R 80122 (0.1 - 1 µmol/l) dose-depently shifted the concentration-response curve of isoprenaline (IPN) to the left. R 80122 (0.1, 0.3, 1 µmol/l) increased force of contraction by 7 ± 5%, 26 ± 7%, and 43 ± 5%, respectively ($\bar{x}$ ± SEM, n = 6). Rolipram (1 µmol/l) had a small positive inotropic effect (10 ± 2%) and did not influence the concentration-response curve of IPN. The combination of R 80122 (0.3 or 1 µmol/l) and rolipram (1 µmol/l) shifted the concentration-response curve further to the left than R 80122 alone.

In isolated rat left atria, pretreatment with R 80122 (1 µmol/l) did not influence the concentration-response curve of IPN. R 80122 (1 µmol/l) did not significantly increase contractile force (6 ± 3%). Rolipram (1 µmol/l) augmented force of contraction by 80 ± 8% and depressed the maximal response to IPN. The combination of R 80122 and rolipram (both 1 µmol/l) did not further enhance contractile force (94 ± 11%) and abolished the positive inotropic response to IPN.

The results are consistent with a role of both PDE III and IV in rat and guinea-pig atria. With respect to contractile force the differential response to rolipram and R 80122 suggests a dominant role of PDE IV in rat left atria and of PDE III in guinea-pig left atria.

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ROLIPRAM DIFFERENTIALLY AFFECTS THE CARDIOTONIC PROFILES OF THE PHOSPHODIESTERASE (PDE) III-INHIBITORS R 80122, ADIBENDAN, MILRINONE, AND ENOXIMONE IN AN ACUTE HEART FAILURE MODEL. J. Schneider and E. Beck

We have recently reported that the higly selective phosphodiesterase III-inhibitor R 80122 ((E)-N-cyclohexyl-N-methyl-2-[[[phenyl (1,2,3,5-tetrahydro-2 oxoimidazo [2,1-b]-quinazolin-7-yl) methylene] amino] oxy] acetamide) induces marked cardiotonic effects. The present study was performed to evaluate the contribution of PDE IV-inhibition by rolipram additional to PDE III-inhibition by R 80122. The selective PDE III-inhibitors adibendan, milrinone, and enoximone served as reference compounds.

The model used was the guinea-pig heart-lung preparation with hexobarbital-induced acute heart failure. Cumulative addition of the PDE III-inhibitors dose-dependently improved cardiac function in the following order of cardiotonic potency: R 80122 > adibendan > milrinone > enoximone.

Pretreatment with the PDE IV-inhibitor rolipram (10^{-4} M) neither had any effects on functional parameters nor prevented the loss of cardiac function after induction of heart failure.

After pretreatment with rolipram (10^{-4} M) the effects of enoximone on LVP, $+LVdP/dt_{max}$, $-LVdP/dt_{max}$, heart rate (HR), stroke volume (SV) and cardiac output (CO) were hardly influenced. The same pretreatment enhanced the positive chronotropic actions of milrinone and caused positive chronotropic effects of adibendan and consequently potentiated the increases of the contractile parameters elicited by both drugs. For R 80122 the same picture was obtained, although the distance between chronotropic and inotropic actions was more pronounced and no increase in SV and CO was observed.

In conclusion, the effects of PDE IV-inhibition additional to PDE III-inhibition seem to be primarily mediated by interactions with HR. The increase in HR elicited by R 80122, in the presence of rolipram only, was not able to further enhance the marked increases in SV and CO induced by this drug.

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SELECTIVITY OF DIFFERENT PHOSPHODIESTERASE INHIBITING AGENTS FOR PHOSPHODIESTERASE III AND IV FROM PORCINE VENTRICULAR HEART TISSUE
H. Wenzlaff, H. Scholz, W. Zimmermann

Recent studies reported a positive inotropic effect of phosphodiesterase (PDE) IV inhibitors after pretreatment with the PDE III inhibitor milrinone. These results suggest a synergism of PDE III and IV inhibition. Therefore, we tested whether the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX), the selective PDE III inhibitors milrinone (MIL), saterinone (SAT) and UD-CG 212 Cl ((2-(4-hydroxyphenyl)-5-(5-methyl-3-oxo-4,5-dihydro-2H-6-pyridazinyl)-benzamidazole HCl); UD-CG) and the selective PDE IV inhibitor rolipram (ROL) equally inhibited PDE III and IV activity. In homogenates from porcine ventricular heart tissue three different PDE activities could be separated using a DEAE sepharose chromatography. According to Reeves et al. (Biochem. J. 241, 535-541, 1987) the isozymes could be identified as PDE II, III and IV. A calmodulin-activated PDE (PDE I) was not found.

IC_{50} values for the PDE inhibitors were as follows:

IC_{50} values (μmol/l) and selectivity

Agent	II	III	IV	III/IV
IBMX	27.1	4.30	9.59	0.49
MIL	293	1.58	6.79	0.31
SAT	394	0.06	1.00	0.06
UD-CG	300	0.16	22.2	0.01
ROL	>1000	616	3.20	193

n=4 each

It is concluded that among the so-called selective PDE III inhibitors there are differences in PDE IV inhibiton, with UD-CG 212 Cl being the most selective inhibitor of PDE III. (Supported by the DFG.)

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HEMODYNAMIC EFFECTS OF THE PHOSPHODIESTERASE INHIBITOR PIROXIMONE IN MAN
M. Buerke, T. Cyrus, R. Erbel

Phosphodiesterase (PDE) inhibitors are new drugs for the therapy of congestive heart disease. PDE inhibitors increase contractile force by inhibition of cAMP degradation and elevation of Ca^{2+} in cardiac muscle cells. We measured the hemodynamic effects of Piroximone bolus administration by a Swan-Ganz catheter in patients with very severe congestive heart disease (NYHA III-IV). Piroximone (0.25, 0.5, 1, 2 mg/kgbw) was tested vs placebo. Piroximone bolus injection resulted in dose related increase in cardic index (CI), decrease in pulmonary capillary wedge pressure (PCWP), mean pulmonary arterial pressure (MPAP) and right atrial pressure (RAP). Heart rate (HR) and mean arterial blood pressure (MABP) were not significantly altered.

	before bolus	0.5	2 mg/kgKG	PIR
CI	1.8±0.1	2.4±0.2*	3.2±0.2*	[l/m^2]
PCWP	23±1.5	14±10	9±2.2*	[mmHg]
MPAP	38±4	32±5*	23±2*	[mmHg]
RAP	13±3	8±3*	9±5*	[mmHg]
HR	100±10	96±5	130±15	[b/min]
MABP	101±4	86±5	75±6	[mmHg]

*p<0.05

Piroximone exerted potent inotropic effects, which resulted in an elevation of CI and a decrease of PCWP, MPAP and RAP. In patients with congestive heart disease the administration of Piroximone might be an effective way to treat the failing heart.

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THE EUDISMIC RATIO OF THE STEREOSELECTIVE DRUG ASOCAINOL DEPENDS ON THE EXPERIMENTAL CONDITIONS: EVIDENCE AGAINST THE GUARDED RECEPTOR HYPOTHESIS
J. Gödicke, A. Mescheder, and F. Steinke

The dependence of antiarrhythmic drug potency upon the experimental conditions is explained in terms of two models: the modulated receptor hypothesis envisages a binding site on Na^+ channels the affinity of which varies, e.g. among channel states. The guarded receptor hypothesis assumes the binding site to have a constant affinity, but the access of drug and its removal from a critical compartment vary according to factors determining drug diffusion, protonation and its mobility in the electric field.

Since enantiomers are identical with regard to physicochemical properties, the eudismic ratio (ER) should remain unaffected by conditions which change absolute potency, if the guarded receptor hypothesis is valid. We investigated (+)-asocainol and its less active enantiomer in various models of Na^+ channel function in guinea pig myocardium. Stimulus frequency and diastolic potential were varied in order to change absolute potencies by up to one decade. The ER obtained measuring rectangular pulse threshold in atria increased from 2.5 to 3.1 when raising $(K^+)_o$. Similarly, for the use-dependent depression of $\dot{V}_{max}$ in papillary muscles, ER increased from 0.9 to 2.0. Use-dependent inhibition of whole-cell Na^+ current in isolated myocytes displayed ERs which ranged from 3.4 to 9.1, depending on pulse frequency and holding potential.

In all assays, there was an increase of ER with elevation of absolute potency. These findings are consistent with the modulated receptor hypothesis, but they contradict the guarded receptor hypothesis.

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MOLECULAR ELECTROPHYSIOLOGY OF VERATRIDINE ACTION AT THE CARDIAC SODIUM CHANNEL

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Veratridine (pK_a=9.6) modification of Na current was examined in single dissociated ventricular myocytes from late-fetal rats using the whole-cell configuration of the patch clamp technique. Pulses to -30 mV for 50 ms were applied every 2 or 5 s from a holding potential of -100 mV (20 °C; $[Na]_o$=70 mM), and amplitude, I_{tail}, and time constant, τ_{tail}, of the post-repolarization inward tail current induced by the alkaloid were measured. Increasing the pH of a 30 μM veratridine superfusate from 7.3 to 8.3 increased I_{tail} by a factor of 2.5±0.5 (mean±SEM; n=3). Intracellular (pipette) application of 100 μM veratridine at pH 7.3 or 8.3 produced small I_{tail}s suggesting transmembrane loss of alkaloid. If this was compensated for by simultaneous extracellular application of 100 μM veratridine at a pH identical to intracellular pH, I_{tail} (measured relative to the maximum amplitude obtainable in the same cell) at pH_i=7.3 did not significantly differ from that at pH_i=8.3 (84±4 % vs. 70±6 %; n=3 each). Results from 6 control cells and 5 cells subjected to extra- and/or intracellularly increased viscosity by addition of 0.5 or 1 *m* sucrose showed that increasing intracellular viscosity 1.6- and 2.5fold increased τ_{tail} 1.5- and 2.3fold, respectively, while a selective 2.5fold increase of extracellular viscosity did not significantly affect τ_{tail}.

We conclude on the basis of the extracellular and intracellular pH and viscositiy effects that extracellularly applied veratridine diffuses in its free base form through the sarcolemma and activates the Na channel at an intracellular site in its protonated form. Chemical complementarity of veratridine to its binding site suggests the intracellular presence of a negative charge as an essential component of the veratridine recognition site at the Na channel macromolecule. (Supported by D.F.G., Sandoz-Stiftung, and F.C.I.)

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THE DIFFERENT INFLUENCE OF PROPAFENONE, DIPRAFENONE, HYDROXYFENONE AND FLECAINIDE ON CARDIAC ELECTRICAL ACTIVITY.

U.Stark, B.Vogt, P.Philipp, K.Stoschitzky and W.Lindner

The aim of the present study was to evaluate whether antiarrhythmic agents belonging to the same class, according to the classification of Vaughan Williams, similarily affect cardiac electrical activity. The influence of propafenone (P), diprafenone (D), hydroxyfenone (H) and flecainide (F) on cardiac conduction and refractoriness of isolated Langendorff perfused guinea pig hearts were studied, using an ECG recording technique of high resolution. Effective refractory periods (ERP_s) and rate dependent refractory periods (ERP_c) of the AV-nodal, His-bundle conduction and of the atrial and ventricular myocardium were evaluated by programmed stimulation. Equimolar concentrations (3μM) of either drug induced a similar reduction of the spontaneous sinus rate. P, D and H similarily prolonged the AV-nodal conduction interval, whereas F did not alter this parameter. D and P induced a prolongation of the His-bundle and intraventricular conduction interval to the same degree. H was significantly higher effective, whereas F had the weakest effects on these parameters. On the AV-nodal refractoriness, all four compounds showed a similar rate dependency of their effects ($AV\text{-}ERP_c > AV\text{-}ERP_s$). D, H and P exerted a comparable and strong rate dependent prolongation of the atrial myocardial refractoriness, whereas $A\text{-}ERP_s$ was as prolonged as $A\text{-}ERP_c$ by F (+60±7% and +62±7%, respectively, both n=6, $p<0.01$). D, H and P induced a marked prolongation of $V\text{-}ERP_c$ and this was significantly higher than $V\text{-}ERP_s$. The strongest effect on $V\text{-}ERP_c$ was exerted by P (+117±9%; n=8, $p<0.01$). F had the weakest effect on the ventricular myocardial refractoriness and also showed the weakest rate dependency on this parameter, compared to D, H and P ($V\text{-}ERP_c$: +46±9% and $V\text{-}ERP_s$: +28±6%, both n=6, $p<0.01$). On the His-bundle refractoriness a rate dependent effect was only present under the influence of H and P.
The present results show, that D, H, P and F although they belong to the same class of antiarrhythmic agents differently influence cardiac electrical activity. H and P show a strong rate dependency of their effects on all parts of the conduction system and the myocardium. D lacks this effect on the His-bundle. F showed a strong rate dependency on the AV-node, but this effect was not as expressed on the atrial and ventricular myocardium.

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THE INFLUENCE OF PROPAFENONE, LIDOCAINE AND (+)-SOTALOL ON CARDIAC STIMULATION THRESHOLDS

H.Markert, P.Lang, G.Fachbach, H.A.Tritthart

Excitability must be rendered abnormal for premature ventricular contractions or more complex ventricular rhythms. We studied whether lidocaine, propafenone or (+)-sotalol alter the pattern of stimulation thresholds in a fashion which might produce the conditions for the appearance of reentrant and ectopic arrhythmias. Experiments were performed on isolated Langendorff perfused guinea-pig hearts. At fourty points, located on the epicardiac surface, eight in the horizontal direction, in angles of 45 degrees around the heart, beginning at the intraventricular septum and fife in the vertical direction in regular distances from the basis to the apex, stimulation threshold was determined on untreated hearts at a stimulation rate of 300 beats/min. During control conditions the values of stimulation threshold around the heart were between 0.8±0.1mV and 1.6±0.2mV (n=18; $\bar{X}$±SEM). There was no significant difference from one point to the contiguous point. The influence of 100μM lidocaine induced a homogeneous and highly significant elevation of the stimulation threshold on most points of measurements. Values ranged between 1.6±0.2mV and 2.5±0.1mV (n=5). In 2.5% of all points there was a significantly different stimulation threshold between two contiguous points. (+)-sotalol at a concentration of 100μM induced only slight changes of the stimulation threshold and in only 10% it was significantly elevated against control values (values between 0.9±0.1mV and 1.7±0.2mV; n=5). As lidocaine also (+)-sotalol induced at only 2.5% a significantly different stimulation threshold between neighboured points. Propafenone (3μM) however induced at 70% of the points of measurements a significant change of the stimulation threshold, (values ranged between 1.1±0.1mV and 2.3±0.1mV; n=10) and at 12.5% there was a significantly different stimulation threshold between contiguous points indicating an inhomogenity of the cardiac excitability. In summary (+)-sotalol had only minor effects, propafenone was stronger acting and lidocaine more significantly influenced cardiac excitability threshold. Lidocaine and (+)-sotalol maintained a rather homogeneous distribution of the stimulation threshold whereas propafenone induced a slight dispersion of the excitability. The inhomogenity of changes of excitability in the epicardiac surface is likely important for the likelyhood of proarrhythmogenic effects of class Ic antiarrhythmic compounds.

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EFFECT OF GE 68 (1-[3-(2-PHENYLETHYL)-2-BENZOFURYL]-2-(PROPYLAMINO)-ETHANOL) ON THE SODIUM CURRENT OF GUINEA-PIG VENTRICULAR MYOCYTES

R. Lemmens-Gruber and C. Studenik

The effect of the propafenone derivative GE 68 on macroscopic and single-channel sodium current (I_{Na}) of guinea-pig ventricular myocytes was studied with the patch-clamp technique using the cell-attached mode. GE 68 (1 and 10 µmol/l) added to the bathing solution caused a reduction of the open state probability by increasing the number of sweeps without acivity. This increase in the number of records without sodium channel openings was twofold at a drug concentration of 1 µmol/l (n=5) and sixfold at 10 µmol/l GE 68 (n=2), while the single-channel conductance remained unchanged.
At a stimulation rate of 1, 2 and 3 Hz peak I_{Na} and time integral of I_{Na} were reduced in a frequency-dependent way (1 µmol/l: n=3; 10 µmol/l: n=2). The mean current per record was decreased by GE 68 to the same extent at all measured holding potentials at a stimulation rate of 2 Hz (1 µmol/l: E_h=-120 mV, 25.0 ± 9.35%, n=5; E_h=-100 mV, 20.0 ± 5.31%, n=7; E_h=-90 mV, 25.11 ± 10.3%, n=9; E_h=-80 mV, 25.75 ± 6.14%, n=4; 10 µmol/l: E_h=-120 mV, 66.0 ± 8.17%, n=3; E_h=-100 mV, 67.8 ± 6.55%, n=6; E_h=-90 mV, 69.0 ± 8.26%, n=4; E_h=-80 mV, 72.0 ± 6.36%, n=3).
The shape of the steady-state current-voltage relationship was not significantly changed by GE 68 (1 µmol/l: n=3, 10 µmol/l: n=1).
After a period of 15 minutes washout the effects of GE 68 on the I_{Na} were completely reversible (n=2).
In conclusion GE 68 decreases I_{Na} by increasing sweeps without activity while the amplitude of the unitary current was not changed.

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ELECTROPHYSIOLOGICAL EFFECTS OF GE 68 (1-[3-(2-PHENYLETHYL)-2-BENZOFURYL]-2-(PROPYLAMINO)-ETHANOL) ON VARIOUS GUINEA-PIG HEART MUSCLE PREPARATIONS

H. Marei

The effects of the propafenone derivative GE 68 on the action potential of isolated papillary muscles and spontaneously beating sinoatrial nodes have been studied using the intracellular microelectrode technique. In papillary muscles continuously driven at a rate of 1 Hz, GE 68 (10 µmol/l, n=5) decreased $\dot{V}_{max}$ until a new steady state was reached within 120 min. However, when stimulation of the preparation was interrupted at the time of the admission of the drug, and resumed after a period of 120 min, the $\dot{V}_{max}$ of the first action potential showed no significant changes as compared to the control values; then it decreased until a new steady state was reached after 7 ± 1.1 action potentials. The time constant of recovery of $\dot{V}_{max}$ from the action of GE 68 (10 µmol/l, n=8) during a pause in stimulation was calculated to be 4.1±1.1s. GE 68 (1 µmol/l, n=5) did not cause significant changes of the sinoatrial node action potential parameters. At a concentration of 3 µmol/l, GE 68 (n=6) only produced a significant ($P<0.05$) decrease in spontaneous rate. 10 µmol/l GE 68 (n=5), however, produced a significant decrease in rate of spontaneous activity, $\dot{V}_{max}$, slope of slow diastolic depolarization and maximum diastolic potential, increased action potential duration ($P<0.001$), decreased amplitude ($P<0.01$), and abolished spontaneous sinoatrial node activity after a period of 65 min. The data obtained show that GE 68 caused a use-dependent block of the sodium channels and a negative chronotropic effect on the sinoatrial node.

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PROPRANOLOL REDUCES THE PROARRHYTHMIC ACTIVITY OF FLECAINIDE S.Dhein, R.Gerwin and A.Müller

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The aim of this study was to find out whether the high proarrhyhtmic risk of flecainide can be reduced by additive treatment. For this purpose, in isolated perfused rabbit hearts (prepared according to the Langendorff-technique, perfused at constant pressure of 70 cm H_2O with Tyrode solution equilibrated with 95% CO_2 and 5% O_2, paced at the right atrium with 3 Hz) a computer assisted 256 channel epicardial potential mapping (4kHz/channel, 1mm interelectrode distance, 0.04 mV resolution) was performed in order to analyse geometry and timecourse of the epicardial activation and repolarization process. Clinically relevant concentrations of flecainide (0.1, 0.5, 1.5 µmol/l) were perfused alone or in the presence of 0.01 µmol/l propranolol. The activation process was analysed by determination of the local activation times (dU/dt_{min}), giving the activation sequence. The repolarization timepoints were defined as dU/dt_{max} during the T-wave of the potentials. From these data the local potential duration (ARI) was calculated and the dispersion of ARI (DISP) was determined as the standard deviation of ARI. We found that the activation process was significantly disturbed and deteriorated by flecainide in a concentration dependent manner, an effect which was accompanied by a parallel increase in DISP, finally resulting in arrhythmia. In the presence of 10 nmol/l propranolol the deteriorating influence of flecainide on the activation patterns was attenuated and no increase in DISP could be observed anymore. No sustained arrhythmias were registered. Propranolol (0.002, 0.01, 0.1 µmol/l) alone led to a stabilization of the activation patterns and to a reduction of ARI-dispersion.
From these data it is concluded (a) that flecainide may lead to arrhythmia by increasing ARI-dispersion and (b) that this arrhythmogenic effect can be antagonized by propranolol.
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POTASSIUM CHANNEL OPENERS ABOLISH EARLY AFTERDEPOLARIZATIONS AND EXTRASYSTOLES INDUCED BY SOTALOL OR DISOPYRAMIDE IN PURKINJE FIBRES OF GUINEA-PIG HEARTS

S. F. Brosch and P. Heistracher

The effect of nicorandil on sotalol-induced and the action of pinacidil and lemakalim on disopyramide-induced early afterdepolarizations and extrasystoles were investigated in spontaneously beating Purkinje fibres by means of the intracellular microelectrode technique.
The potassium channel opener nicorandil (50 µmol/l; 100 µmol/l; 500 µmol/l) suppressed early afterdepolarizations and extrasystoles, induced by sotalol hydrochloride (80 or 160 µmol/l), at a potassium concentration of the bathing solution of 2,7 mmol/l, in 4 out of 6, in 6 out of 6, and in 3 out of 3 experiments, respectively. At a potassium concentration of the bathing solution of 1,35 mmol/l, disopyramide-induced (10, 20 or 30 µmol/l) early afterdepolarizations and extrasystoles were abolished by pinacidil at a concentration of 3 µmol/l in 4 out of 8, at 30 µmol/l in 6 out of 6 and at 100 µmol/l in 6 out of 6 experiments, by lemakalim at a concentration of 3 µmol/l in 2 out of 5, at 10 µmol/l in 5 out of 5 and at 30 µmol/l in 5 out of 5 experiments, respectively. On readmission of sotalol- or disopyramide-containing, potassium channel openers-free bathing solution, early extrasystoles and afterdepolarizations reappeared.
In conclusion, the three potassium channel openers mentioned above concentration-dependently decrease or abolish early afterdepolarizations and extrasystoles and thereby exert antiarrhythmic effects.

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CHARACTERIZATION OF TRANSIENT OUTWARD CURRENTS MODIFIED BY α_1-ADRENOCEPTOR STIMULATION IN RAT CARDIAC MYOCYTES. G. Hauser, E. Wettwer and U. Ravens

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Alpha$_1$-adrenoceptor stimulation by phenylephrine reduces transient outward currents (I_{to}) in rat heart myocytes (Wang et al. 1991, J Pharmacol Exp Ther, **259**). In order to characterize the membrane currents influenced by α_1-stimulation, we have studied the effects of phenylephrine under conditions which modify I_{to}. Single-electrode voltage clamp studies (300 ms long clamp steps from holding potentials V_h of -80 mV or -40 mV to various potentials) were performed at room temperature (20 - 22°C). Beta-adrenoceptors were blocked by propranolol (1 µM), Ca^{2+}-currents were blocked with Cd^{2+} (0.1 mM). Phenylephrine (PE, 30 µM) decreased both the peak (I_p) and the late (I_l) component of I_{to}. These effects depended on V_h: in a clamp step to +60 mV, PE reduced I_p from 4.8 ± 0.5 nA to 4.4 ± 0.5 nA (V_h -80 mV) and from 3.4 ± 0.7 nA to 2.6 ± 0.5 nA (V_h -40 mV; means ± SEM, n>5). I_{to} was reduced by substitution of extracellular Na^+ with N-methyl-D-glucamine, by tetrodotoxin (10 µM, V_h -40 mV) and by 4-aminopyridine (3 mM, V_h -80 mV). Phenylephrine still decreased I_l in the presence of tetrodotoxin but not in the presence of 4-aminopyridine. When the inactivation of I_{to} was accelerated by tedisamil (10 µM), PE reversibly reduced I_l. It is concluded that the PE-induced reduction of I_{to} is potential dependent and that I_{to} may be composed of different current components.

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MODULATION OF IONIC CURRENTS AND CONTRACTILITY BY NEUROPEPTIDE Y IN RAT VENTRICULAR MYOCYTES

T. Weis, B.J. McDermott*, B.C. Millar*, and H.M. Piper

Neuropeptide Y (NPY) has been shown to inhibit contractile response of isoprenaline-stimulated rat ventricular myocytes. In order to clarify the underlying mechanism the action of NPY on cell contraction and ionic currents in voltage clamped cells (whole cell configuration) was measured. NPY (10^{-7} M) increased the fast component of transient outward current (i_{to}) to 165 % ± 9 (n=5) and slow inward Ca-current to 151 % ± 8 (n=5) of control. Negative inotropic effects of NPY in the presence of isoprenaline could be abolished by the selective i_{to} blocker 4-aminopyridine (4-AP, 0.5 · 10^{-3} M completely inhibiting i_{to}) or by pretreatment of the cells with pertussis toxin. In 4-AP pretreated cells NPY caused an increase in contractile response which was abolished by the Ca-antagonist verapamil (10^{-8} M) but not by pertussis toxin.
In conclusion, negative and positive contractile responses of NPY may be explained by G-protein-mediated activation of i_{to} and activation of i_{si}, respectively.

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R 59494 STEREOSPECIFICALLY DEPRESSED VERATRIDINE-INDUCED $^{45}Ca^{2+}$ UPTAKE IN LEFT ATRIA OF THE RAT

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Activation of Na^+ channels by veratridine was shown to result in an increased Na^+ and Ca^{2+} uptake in heart tissue. The Na^+ Ca^{2+} overload inhibitor R 59494 (N-methyl-N-[1-(4-phenoxybutyl)-3-pyrrolidinyl]-2-benzothiazolamine) was reported to potently inhibit veratridine-induced $^{45}Ca^{2+}$ uptake. Since R 59494 contains an asymmetric carbon atom, the effect of the (-)-enantiomer (R 83572) and the (+)-enantiomer (R 83573) of R 59494 on $^{45}Ca^{2+}$ uptake elicited by veratridine (10^{-4} M) in electrically driven (1 Hz) left isolated rat atria was investigated.
Tetrodotoxin (10^{-7}-10^{-6} M) but not nifedipine (10^{-7}-10^{-6} M), amiloride (6 x 10^{-3} M) and phentolamine (10^{-5} M) significantly reduced veratridine-induced $^{45}Ca^{2+}$ uptake. R 59494 (10^{-9}-10^{-5} M) and R 83572 (10^{-9}-10^{-5} M) potently inhibited veratridine-induced $^{45}Ca^{2+}$ uptake, whereas R 83573 (10^{-9}-10^{-5} M) was only poorly effective. However, R 83573 (10^{-8}-10^{-5} M) was more potent than R 83572 (10^{-7}- 10^{-5} M) in depressing the K^+-induced $^{45}Ca^{2+}$ uptake in isolated rat aorta, indicating that the Ca antagonistic properties of the compounds are not related to their inhibitory effect on veratridine-induced $^{45}Ca^{2+}$ uptake.
In summary, R 59494 and R 83572 but not R 83573 were found to be potent inhibitors of veratridine-induced $^{45}Ca^{2+}$ uptake in isolated left atria of the rat. This protective effect of R 59494 and R 83572 is not related to their Ca^{2+} antagonistic properties. However, the veratridine-induced $^{45}Ca^{2+}$ uptake was also antagonized by the Na^+ channel blocker tetrodotoxin. Hence, the effect of R 59494 on veratridine-induced $^{45}Ca^{2+}$ uptake seems to be due to a stereospecific inhibition of veratridine-activated Na^+ channels, thereby depressing both veratridine-induced Na^+ and Ca^{2+} load.

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Na^+-Ca^{2+} EXCHANGE IN NORMOXIC AND ISCHAEMIC RAT LANGENDORFF HEARTS

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In normoxic hearts, reducing extracellular $[Na^+]$ inhibits and can also reverse Na^+-Ca^{2+} exchange, thus resulting in a rise in intracellular (i) $[Ca^{2+}]$. Similarly, the increased $[Na^+]_i$ found after ischaemia may contribute to the rise in total $[Ca^{2+}]_i$ which is usually observed after ischaemia in the heart.
In order to explore this possibility we determined the intracellular Na^+, K^+ and Ca^{2+} concentrations in rat Langendorff heart preparations by means of atomic absorbance spectrophotometry. The tissue samples were taken from preparations which were subjected to normoxic conditions, 30 min of ischaemia or ischaemia (30 min) plus reperfusion (30 min). In addition, we monitored the left ventricular pressure (LVP) in the rat Langendorff heart in response to decreasing Na^+ concentrations. The first Na^+ reduction was performed under normoxic conditions and in a second set of experiments repeated after 30 min of reperfusion following 30 min of ischaemia.
Under normoxic conditions the intracellular ion contents amounted to $Na^+ = 12.4 \pm 0.4$, $K^+ = 99.0 \pm 3.1$ and $Ca^{2+} = 0.64 \pm 0.02$ mmol/kg cell (mean ± s.e.m., n=7). $[Na^+]_i$ increased twofold after ischaemia as compared to the normoxic situation, an effect which was even more pronounced (4 fold increase) after ischaemia plus reperfusion. The opposite effects were observed for $[K^+]_i$, with a 25 % decrease after ischaemia and a 40 % decrease after ischaemia plus reperfusion. $[Ca^{2+}]_i$ was increased only after ischaemia plus reperfusion (6 fold) as compared with the normoxic controls.
In the normoxic heart decreasing of $[Na^+]$ in the perfusion fluid from 125 mM to 25 mM increased LVP from 50.0 ± 3.5 mm Hg to 154.4 ± 6.4 mm Hg (n=8; $p<0.05$). After ischaemia and reperfusion, the increase in LVP by decreasing of $[Na^+]$ was virtually abolished (n=6).
In conclusion, in the rat Langendorff heart the ischaemia-induced rise in $[Na^+]_i$ precedes the rise in $[Ca^{2+}]_i$. Decreasing $[Na^+]$ after ischaemia did no longer increase LVP. These results suggest that the Na^+-Ca^{2+} exchange is already inhibited or even reversed after ischaemia and during reperfusion; this constellation cannot be substantially changed by reducing $[Na^+]$ during reperfusion.

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CHANGES IN INOTROPIC RESPONSE OF HEARTS OF SHR TO CA^{2+}-INFLUX AND CA^{2+}-SENSITISERS.

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In connection with a programme on the effects of inotropic drugs on hypertrophied hearts of hypertensive rats, we studied the effects of elevated extracellular Ca^{++}-concentrations ($[Ca^{2+}]_o$), the calcium-entry promoter BayK 8644 and the Ca^{2+} sensitiser AR-L115BS on isolated hearts and papillary muscles of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto control rats (WKY). The hearts were isolated, perfused according to Langendorff with a Tyrode solution of 37°C and paced at a frequency of 5 Hz. The force of contration was measured by means of an intraventricular balloon and expressed as left ventricular pressure (LVP). Preparations of papillary muscle were isometrically mounted in organ baths and paced with 1 Hz. Results are expressed as % increase of basal values. The papillary muscles of SHR showed an increased responsiveness to elevated $[Ca^{2+}]_o$ compared to those of WKY (97.7 ± 9.5% and 62.3 ± 8.2%, respectively), and this effect was repeated with BayK 8644 (94.4 ± 9.7% and 59.6 ± 7.3%, respectively). Also in isolated hearts the contractile response to BayK 8644 was stronger in SHR than in WKY (93.1 ± 9.6% and 57.9 ± 6.8%, respectively), but a further elevation of the LVP to $[Ca^{2+}]_o$ was prevented by a sharp decrease in coronary flow in the SHR-hearts.
In contrast, the contractile reponse of SHR hearts to the Ca^{2+}-sensitiser AR-L115BS was decreased compared to that of WKY hearts (10.9 ± 2.3% and 35.4 ± 1.2%, respectively).

These results indicate that there is a discrepancy in the contractile reponse of SHR hearts to Ca^{2+} influx and the Ca^{2+} mediated intracellular signal transduction.

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ANALYSIS OF THE POSITIVE INOTROPIC EFFECT OF MONENSIN IN GUINEA-PIG HEART MUSCLE PREPARATIONS

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In guinea-pig heart muscle preparations, the Na^+ ionophore monensin (10 µmol/l) exerted a pronounced but transient increase in force of contraction (F_c). The action potential (AP) was shortened in the ventricle, but remained virtually unchanged in the atrium. In the presence of $BaCl_2$ (0.4 mmol/l), the addition of monensin caused a significant hyperpolarization of the membrane both in atrial and ventricular preparations. $^{86}Rb^+$ efflux was enhanced both in resting and in beating (1 Hz) atrial preparations. To further clarify the mechanisms which underly the positive inotropic effect of monensin, Ca^{2+} current (I_{Ca}) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using the fluorescent dye fura-2 were measured. In single atrial and ventricular myocytes, monensin did not affect I_{Ca} but evoked a transient increase in $[Ca^{2+}]_i$. The effects of monensin on $[Ca^{2+}]_i$ were eliminated in Ca^{2+}-free or Na^+-free solutions, whereas TTX (30 µmol/l) or D600 (10 µmol/l) did not change the response to monensin. It is concluded that a monensin-induced increase in the intracellular Na^+ concentration ($[Na^+]_i$) causes a rise in $[Ca^{2+}]_i$, presumably via activation of the Na^+/Ca^{2+} exchange mechanism; thereby, F_c is increased. The changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ by monensin may have additional effects on Na^+/K^+ pump, K^+ conductance and AP configuration thus counteracting the positive inotropic effect and resulting in the transient changes in F_c.

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FOSINOPRILATE AUGMENTS THE SLOW INWARD CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES

H.F. Räcke and P. Lemke

Various ACE-inhibitors cause antiarrhythmic (Dietz et al.1990, J.mol.cell.Cardiol,22(Suppl.III):PF 53) and positive inotropic (del Monte et al., 1991, Eur. Heart J. 12 (suppl.):1498) effects. To explore the electrical properties of these compounds, the new ACE-inhibitor Fosinoprilate (FAT) was studied under voltage clamp conditions using whole cell patch clamp (PC) in guinea pig ventricular myocytes. FAT prolonged the action potential duration (APD_{50} and APD_{90}) to a considerable extent and often exerted irregular low voltage oscillations and slow responses confirming the data of Koppers and Hauswirth (this meeting) in rabbit Purkinje fibres. PC-experiments in the plateau range showed that FAT increased the slow inward current (i_{si}) from a control value of 0.94 ±0.1 nA to 1.2 ±0.05 nA (0.1µM), to 1.32±0.2 nA (0.3µM),to 1.57±0.2 nA (1µM) and to 1.72±0.1 nA (3 µM; n=5). Although the voltage of the maximal inward currents at 3.33±2.35 mV and the location of the steady state activation (d_∞) were unchanged (V_h = -17,5±1.4 mV), steady state inactivation (f_∞) was shifted up to 15 mV (3µM). V_h of f_∞ was displaced from a control of -23.8±0.9 mV to -16.2±1.1 mV (0.1µM), to -13.1±2.1 mV (0.3µM), to -10.5±1.2 mV (1µM) and to -8.6±1.4 mV (3µM; n=5). The steepnesses of d_∞ and f_∞ showed constant k - values of 4.6±0.6 mV and 3.0±0.5 mV, respectively. Computer reconstructions using the OXSOFT-HEART model with the present experimental data injected show, that the prolonged APD as well as the slow responses and the oscillations at the plateau are well explained by our findings.

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CAPTOPRIL, FOSINOPRIL AND FOSINOPRILATE PROLONG THE ACTION POTENTIAL DURATION IN RABBIT PURKINJE FIBRES

D. Koppers and O. Hauswirth

ACE-inhibitors exert antiarrhythmic (Dietz et al., 1990, J.mol.cell.Cardiol.22,(Suppl.III):PF 53) and positive inotropic (del Monte et al.1991, Eur.Heart J. 12 (Suppl.):1498) effects. Mechanisms underlying these actions have been studied using the double microelectrode voltage clamp technique(VC).Captopril (CAP), Fosinopril (FOS) and Fosinoprilate (FAT) prolonged APD_{50} and APD_{90} without significant changes of the RP (resting potential). CAP (1µM) extended the APD_{50} from 326±34 ms to 436±50 ms (n=3).FOS(1µM) lengthened the APD_{50} from 513±41 ms to 633±41 ms and the APD_{90} from 666±110 ms to 993±155 ms (n=3).In two experiments, low voltage oscillations occurred. The RP nonsignificantly increased from -81.0±4.6 mV to -86±2.8 mV. FOS (5µM) lengthened the APD_{50} to 685±49 ms whereas FOS again shortened the APD_{50} to 563±32 ms (10µM) and to 50 ms (30µM), respectively.With 50 µM and 100 µM, the impulse turned into a short spike resembling a nervous action potential. FAT prolonged the APD_{50} from 685±49 ms to 700 ms (0.1µM; n=1) and to 940 ms (1µM; n=1). Up to 3µM, CAP, FOS and FAT leave V_{max} practically unchanged (n=3), whereas CAP and FOS beyond 5µM both cause tonic block. Phasic block was absent. VC-experiments with the purpose to study i_K are being done. VC-studies in ventricular myocytes (Räcke and Lemke, this meeting) revealed that Fosinoprilate augments the slow Calcium inward current (i_{si}). Computer simulations using the OXSOFT HEART model show that these findings are sufficient to explain the markedly prolonged plateau not only in guinea pig myocytes, but also in rabbit Purkinje fibres.

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THE EFFECT OF RYANODINE ON INTRACELLULAR Ca^{2+}-TRANSIENTS IN GUINEA-PIG VENTRICULAR MYOCYTES

GX. Wang, R. Schmied & M. Korth

Ryanodine has been shown to strongly reduce Ca^{2+}-transients (Ca^{2+}T) in voltage-clamped (holding potential -40 mv) guinea-pig ventricular myocytes (Barcenas-Ruiz & Wier, Circ Res 61:148,1987;Beuckelmann & Wier, J Physiol 405:233,1988). Since ryanodine interferes specifically with the Ca^{2+} release channel of the sarcoplasmic reticulum (SR), it was concluded that Ca^{2+}T and hence contraction strongly depend on SR Ca^{2+}-release.

In the present study, the effect of ryanodine on Ca^{2+}T elicited in polarized guinea-pig ventricular cells loaded with the fluorescent Ca^{2+}-sensitive dye Fura-2/AM (2-3 µM) was investigated. At a stimulation rate of 0.5 Hz, 1µM ryanodine prolonged the time to peak Ca^{2+}T by 432±162% (n=7), produced a small decrease of the Ca^{2+}T amplitude (by 18±11%,n=7) and increased diastolic Ca^{2+} from 115±48 to212±61 nM (n= 7). Ryanodine concentrations ranging from 0.1 to 10µM, produced nearly identical steady-state effects on Ca^{2+}T. Ca^{2+}T augmented by 10nM isoprenaline, 1µM forskolin or 100µM IBMX exhibited an early and a late component, the amplitude of the latter being about 80% of the former. Ryanodine abolished the early component without affecting the amplitude of the late peak. In addition, after a rapid (within 100ms) decline of the Ca^{2+}T to 30% of its maximum, a slowly decreasing Ca^{2+}T which lasted for about 3 s could be detected. Caffeine (10 mM), applied after the Ca^{2+}T had declined to the steady-state level, did not induce Ca^{2+}-release from the SR. Ca^{2+}T elicited in the absence of ryanodine after a rest period of 15 min, showed the same prolongation of time to peak Ca^{2+}T as those with ryanodine under continuous stimulation.

It is concluded, that 1) ryanodine up to 10µM depletes SR Ca^{2+} stores probably by locking Ca^{2+}-release channels in an open state and 2) the amount of Ca^{2+} entering the cell during an action potential is sufficient to maintain a high Ca^{2+}T. From the difference between the present results and those obtained in depolarized cells, it can be speculated that Na-Ca exchange plays an important role in maintaining Ca^{2+}T in the presence of ryanodine.

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INFLUENCE OF POTASSIUM ON THE INTERACTION OF BRETYLIUM WITH GUINEA PIG MYOCARDIAL Na^+-K^+-ATPASE

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Bretylium, a class III antiarrhythmic agent which also possesses positive inotropic properties, is a potent inhibitor of myocardial Na^+-K^+-ATPase activity. In this study, we investigated the influence of varying the incubation K^+ concentration on its inhibition of this enzyme system in guinea pig heart preparations. The hearts were homogenized and centrifuged at 14000 g. The microsomal Na^+-K^+-ATPase fraction was separated on TSK Toyopearl HW-55F gel column and dialyzed using 100 mM imidazole buffer. The actions of bretylium on Na^+-K^+-ATPase were studied in mediums containing 2.5, 5.0 and 10.0 mM K^+, respectively. At these concentrations, bretylium inhibited the enzyme activity in a concentration-dependent fashion, with IC_{50} values of 0.92 ± 0.03, 1.4 ± 0.2 and 2.2 ± 0.4 mM (mean ± s.d.; n = 8) respectively. Thus, reducing the K^+ concentration from 5.0 to 2.5 mM enhanced the inhibitory action of bretylium, showing a right-to-left shift in inhibitory effective concentration ranges. This effect was particularly significant at low concentrations. Increasing the K^+ concentration to 10 mM on the other hand reduced its inhibitory potency. Accordingly, the inhibition of myocardial Na^+-K^+-ATPase activity by bretylium depends on [K^+]. These interactions may be pertinent to the mechanism(s) responsible for some of its cardiac actions, such as positive inotropism, inducing or enhancing cardiac arrhythmias.

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AGGREGATES OF CARDIAC MYOCYTES AS A SUBSTRATE FOR THE IN VITRO ASSESSMENT OF CARDIAC GLYCOSIDE POTENCY

H.D. Lehmann, J. Hupe and D. Seemann

The new method was developed in order to replace the method of Knaffl-Lenz which requires guinea pigs for the assessment of the pharmacological potency of plant cardiac glycosides according to DAB9. In this method hearts of 11-day-old chicken embryos are used. Single heart cells are dissociated using trypsin and cultured for 4 days in non adherent microbiological plastic dishes. During this time-period the single cells form small aggregates (diameter 100 µm) which spontaneously contract like beating hearts. In Tyrode solution containing pure or mixed glycosides from plants the aggregates cease to contract after a certain time (5-60 min) depending on the glycoside concentration. While high concentrations of glycosides provoke a permanent arrest (e.g. 10^{-5} - 10^{-6} M Cymarin), lower concentrations cause only a temporary arrest (e.g. $5 \cdot 10^{-7}$ M Cymarin). Therefore, arrest for a 10 s period was defined as the criterion to assess potency. The concentration-response relationship can be described by a hyperbolic function which was transformed to a straight line in a double reciprocal manner (x-axis: 1/log concentration, y-axis: 1/time). The transformation allows the calculation of EC_{15min} values: the glycoside concentration which causes an arrest after 15-min exposure.

Glycoside	Cymarin	Oleandrin	Convallatoxin	Proscillaridin
EC_{15min} [M]	$4.4 \cdot 10^{-7}$	$6.5 \cdot 10^{-7}$	$3.1 \cdot 10^{-7}$	$1.5 \cdot 10^{-7}$
$\bar{x}$ ± SD (N=6)	$\pm 1.1 \cdot 10^{-7}$	$\pm 2.7 \cdot 10^{-7}$	$\pm 1.1 \cdot 10^{-7}$	$\pm 4.3 \cdot 10^{-9}$

For the assessment of cardiac glycoside potency in plant preparations every glycoside preparation of plant origin has to be tested in comparison to a corresponding pure glycoside. The ratio of both EC_{15min} values represents the potency of the plant preparation. The values calculated display the good reproducibility and establish a basis for the replacement of the in vivo Knaffl-Lenz method by an in vitro method.

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TOXIC CONCENTRATIONS OF DIGOXIN RELEASE ADENINE NUCLEOTIDE CATABOLITES FROM GUINEA-PIG HEARTS.

W. Bernauer

In isolated perfused guinea-pig hearts digoxin concentrations of 0.1, 0.3 and 1.0 µmol|l provoked the appearance of tachyarrhythmias, with ventricular fibrillation occurring in 28.6, 92.0, and 100 %, respectively, of the experiments. Simultaneously, a considerable release of adenine nucleotide catabolites (ANCs) into the perfusion fluid was observed. Whereas normal control hearts released 581 ± 32 nmol|g dry weight during 30 min, a value of 4024 ± 209 was obtained, for instance, with 1.0 µmol|l digoxin ($P<0.001$). The metabolite pattern was: Adenosine 2.6 %, inosine 89.9 %, hypoxanthine and xanthine 6.9 %, and uric acid 0.6 %. Especially, the appearance of fibrillation was associated with the release of large amounts of ANCs. When ventricular fibrillation was elicited electrically in hearts without digoxin, also a high release of ANCs was observed. Increasing the potassium concentration in the perfusion fluid from 2.7 to 8.1 mmol|l almost completely prevented arrhythmias and ANC-release in experiments with 0.3 µmol|l digoxin. A similar antiarrhythmic effect was obtained with 60 µmol|l lidocaine. The ANC-release, however, was only partially antagonized (2334 ± 289 nmol|g dry weight; vs 3215 ± 231 in experiments without lidocaine; $P<0.05$). Surprisingly, digoxin-intoxicated hearts which fibrillated in spite of the application of lidocaine (10, or 30 µmol|l), released significantly greater amounts of ANCs than they did without the antiarrhythmic.- (Supported by the Deutsche Forschungsgemeinschaft).

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EFFECTS OF ADENOSINE ON CONDUCTION AND REFRACTORINESS OF ISOLATED GUINEA PIG HEARTS

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For the antiarrhythmic action of adenosine on supraventricular tachyarrhythmias a negative chronotropic and dromotropic effect seems to be responsible.

To gain further information about the antiarrhythmic activity of adenosine on supraventricular arrhythmias, the effects on heart rate, conduction and refractoriness, as well as the time course of onset of AV-nodal conduction slowing and of sinus rate reduction were evaluated in isolated guinea pig hearts perfused by the method of Langendorff.

At a concentration of 3 and 10 µM adenosine sinus rate was reduced and AV-nodal conduction significantly prolonged. Up to 10 µM intraventricular and His-bundle conduction were not altered, whereas QT duration was slightly but insignificantly shortened. The onset of the maximal action of adenosine on the sinus node and AV-nodal conduction occurred after 111 ± 35 and 636 ± 109 (mean ± SEM) beats, respectively.

During programmed stimulation, adenosine led to a prolongation of the effective refractory period of the AV-nodal conduction which was not rate dependent. In the atrial myocardium, however, adenosine caused a rate dependent prolongation of the effective refractory period.

These observations explain the efficacy of adenosine against supraventricular tachyarrhythmias proposed the AV-node forms a part of a reentrant circuit or the reentrant circuit is located in the atrium.

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CARDIAC ISCHEMIA AND REPERFUSION: EFFECT OF VERAPAMIL AND LIDOCAINE

E. Scheufler and A. Mozes

The effect of the Ca antagonist verapamil on an ischemic parameter (onset of the ischemic contracture) and on a parameter of reperfusion damage (incidence of postischemic fibrillations) was compared to the respective effects of the sodium channel inhibitor lidocaine. The antiischemic and antifibrillatory properties of the drugs were determined in isolated guinea pig Langendorff heart preparations perfused with constant flow. Left ventricular pressure (LVP) was assessed by an intraventricular balloon. Drugs were given 45 min before ischemia. Ischemia was elicited by reducing the perfusion flow to 0.1 ml/min for 60 min. During ischemia the onset of the ischemic contracture was defined as the time, at which the diastolic pressure was raised by 2 mmm Hg. After 60 min of reperfusion the incidence of irreversible fibrillations was monitored.

Verapamil reduced the LVP between 0.01 and 0.3 µmol/l in normoxic hearts. At 0.3 µmol/l only 9 ± 2 % of the initial contractility was maintained. The onset of the ischemic contracture was delayed from 28 ± 5 min to 42 ± 5 min at the same concentration (0.3 µmol/l), whereas the the incidence of fibrillations during reperfusion was only reduced to 42 % from 83 % in nontreated controls.

Lidocaine did not influence the normoxic LVP up to 3 µmol/l. At 30 µmol/l the LVP was only reduced to 58 ± 5 % of intial levels (untreated controls: 83 ± 7 %). The onset of the ischemic contracture was not altered up to 30 µmol/l. However in marked contrast to verapamil treatment, the incidence of fibrillations during reperfusion was already dose-dependently reduced between 3 and 30 µmol/l. At 30 µmol/l fibrillations were entirely abolished.

In conclusion: interference with Ca channels by verapamil leads to marked negative inotropic effects and only to a minor reduction of the incidence of fibrillations at a concentration which nearly abolishes contractility. Interference with the fast sodium channel by lidocaine has only minor cardiodepressant effects at concentrations which reduce or abolish postischemic fibrillations. Therefore, interference with sodium channels instead of calcium channels seems to be by far the more promissing principle in ameliorating the damage caused by ischemia and in particular reperfusion.

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INFLUENCE OF DILTIAZEM ON HYPOPERFUSION-INDUCED LOCALIZED ISCHEMIAS AND INCREASES IN TISSUE NADH-FLUORESCENCE IN THE RAT HEART F. Vetterlein, M. Prange, N. Tran, and G. Schmidt

The incomplete occlusion of a coronary artery induces anoxia in part of the affected myocardium while other parts remain completely oxygenated. The question studied was whether such inhomogeneities may be reduced by pharmacological means. In anaesthetized, thoracotomized rats the left coronary artery was cannulated and connected with a flowprobe-containing autoperfusion system. Reduction in coronary perfusion pressure to 50 mmHg led to a decrease in coronary blood flow from 3.4 to 1.9 ml/min. Capillary blood flow was studied in this state by the method of timed plasma labeling, rapid freezing of a myocardial biopsy and histological study of capillary labeling. Tissue anoxia could be detected from increases in cellular NADH-fluorescence. During restricted flow localized areas of ischemia and of increased tissue NADH fluorescence were found. In these samples anoxic cells were observed to occur prior to the development of ischemia. When diltiazem was infused during the phase of occlusion, left coronary flow increased to 2.8 ml/min and the localized areas of ischemia as well as those of raised NADH-fluorescence nearly disappeared (17.8% and 0.4% of the tissue was ischemic in exps. without and with diltiazem treatment, respectively; the corresponding data for cellular anoxia were 57.9% and 9.0%). The observations point to the ability of diltiazem to ameliorate tissue supply even when autoregulatory adaption processes have become exhausted.

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THE ANTI-ISCHEMIC EFFECT OF NIFEDIPINE IN THREE DIFFERENT TYPES OF ISCHEMIA IN THE RAT WORKING HEART PREPARATION.

A.J. Pijl

Cardiac ischemia can be induced by different methods in animal models and in isolated organs. Accordingly, three different procedures were followed to find the most sensitive model for the analysis of the anti-ischemic activity of calcium antagonists. The experiments were performed in the isolated working heart preparation of the rat, paced at a frequency of 5 Hz and perfused with tyrode solution at 37°C.

Global ischemia was achieved by closing off the supply of the perfusion medium and surrounding the heart with tyrode solution of 37°C gassed with N_2; low flow ischemia was achieved by reducing the cardiac afterload from 51.5 mmHg to 11.0 mmHg; ligation of the left descending coronary artery (L.A.D.) was applied in order to achieve regional ischemia.

Nifedipine was applied in a concentration (EC_{50}) known to reduce contractile force by half of its basal value.

The following five parameters were determined after 15 minutes of nifedipine pretreatment and at the end of the experiment, respectively; LVP (Left Ventricular Pressure, dP/dt, AO (Aortic Output), CF (Coronary Flow) and CO (Cardiac Output). From the data obtained the percentages of recovery were calculated. All values in the table are given in % of the initial value. The values marked with an asterix are significantly higher than the control data.

	LVP	dp/dt	AO	CF	CO
global ischemia					
control	5.0 ± 0.8	8.4 ± 0.5	4.2 ± 1.9	7.2 ± 3.3	5.9 ± 2.9
nifedipine	9.5 ± 2.5	16.9 ± 3.4*	8.4 ± 2.0	25.1 ± 6.7*	15.8 ± 3.9
LAD ligature					
control	6.4 ± 0.6	7.8 ± 1.1	7.1 ± 1.0	19.3 ± 6.9	12.6 ± 3.8
nifedipine	9.9 ± 0.6*	11.3 ± 1.1*	6.2 ± 0.8	10.4 ± 2.3	8.1 ± 1.2
low flow ischemia					
control	6.0 ± 0.8	8.3 ± 1.0	7.9 ± 3.6	10.4 ± 2.8	8.5 ± 3.1
nifedipine	34.7 ± 7.3*	32.2 ± 8.3*	27.7 ± 7.5*	49.8 ± 16.3*	36.0 ± 9.8*

Nifedipine caused a significant improvement in the functional recovery of most of the parameters studied. This improvement, however, was much more pronounced in the model of low flow ischemia, which is obviously more sensitive to the anti-ischemic activity of calcium antagonists than the other experimental procedures studied.

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BENEFICIAL EFFECT OF AMRINONE ON THE SIZE OF AN ACUTE REGIONAL ISCHEMIA IN ISOLATED RABBIT HEARTS.

A.F.E. Rump, R. Rösen, B. Sigmund, J. Fuchs, W. Klaus.

The application of positive inotropic drugs in the initial phase of acute myocardial infarction is problematic since the concomitant increase of myocardial oxygen demand might induce additional ischemic damage. However, inotropic drugs differ in their effects on coronary vessels and thus on the O_2-supply of the myocardium. Therefore, we have studied the effects of amrinone in comparison to ouabain and isoprenaline in non arrhythmogenic concentrations in isolated perfused rabbit hearts after coronary ligation using NADH-fluorescence photography for quantitation of the ischemic area and intensity. All substances increased contractility. Coronary flow was decreased by ouabain and increased by isoprenaline and particularly by amrinone. Neither ouabain nor isoprenaline did change the intensity or the distribution pattern of NADH-fluorescence, whereas the size of the ischemic zone was significantly reduced by amrinone, an effect probably related to the marked vasodilation induced by this substance.

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CARDIOPROTECTIVE EFFECTS OF PROSTAGLANDIN- AND ß-CASOMORPHIN-RELATED SUBSTANCES

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A cytoprotective property of some prostanoids (PGI_2, PGE_1) and related compounds has been described in the stomach, gut, kidney, liver and heart subjected to damaging injuries. The efficacy of these substances is characterized by little specific action, but an improvement of morphological integrity and function of the injured organs. For further elucidation of this problem we investigated the influence of some prostanoids and peptides on the damaged myocardium under in vivo- and in vitro-conditions.
Ischemia, loading by aortic stenosis and pacing with high frequency, anaphylaxia and cardiotoxic agents (CCl_4, PAF) induced a reduction of the cardiac function and a marked increase of the myocardial biosynthesis of 6-oxo-PGF_{1alpha} and TXB_2. Application of iloprost or PGE1 diminished the cardiac injury and the enhanced PG-formation. In a similar manner the inhibitory effect of indomethacin on the PG-biosynthesis is paralleled by a reduction of several damages of the heart. Comparable cardioprotective effects in the hypoxic and intoxicated myocardium could be observed with some peptides related to ß-casomorphin.
The mechanism of the cardioprotective effects is not fully understood. An improved membrane stabilisation should be regarded as a main cause including a reduced PG-formation by a diminished degradation of phospholipids and alterations of the cellular embrane with changes of ion fluxes and receptor susceptibility.

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ISCHEMIA- AND REPERFUSION-INDUCED CARDIAC INJURY: EFFECTS OF TWO FLAVONOID CONTAINING PLANT EXTRACTS POSSESSING RADICAL SCAVENGING PROPERTIES

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The effects of standardized extracts of Ginkgo biloba (EGb 761) and Hawthorn (Crataegutt forte®) against ischemia- and reperfusion-induced arrhythmias and creatine kinase (CPK) release were compared using anaesthetized rats with transient (7 min.) coronary artery occlusion (method according to Seley et.al. Angiology; 1960; 11; 398-407). The test drugs or vehicle (controls) were administered (i.v.) 5 min. prior to occlusion.
Reperfusion-induced ventricular fibrillations (VFib) were observed in 88 % of control animals (n=25), whereas such episodes occured in less than 20 % of the animals treated with 0,5 (n=16) or 5 mg/kg (n=17) crataegutt extract. The mean duration of reperfusion-induced arrhythmias (VFib + tachycardias) were also significantly reduced in the crataegutt treated groups. This extract did not influence reperfusion-induced CPK release.
Effects of EGb 761 were quantitatively and qualitatively different from those of the Hawthorn extract. Significant effects of EGb 761 on VFib were observable only after higher doses (3-5 mg/kg) and the durations of arrhythmias were not effected by this extract. It was, however, very effective in dose dependently reducing the CPK release caused by reperfusion. The mean CPK value in the plasma of the 5 mg/kg EGb 761 treated group (5,7 ± 0,40 U/g Prot. n=18) after reperfusion was not different from the value of the sham operated group. This value for the control group with ischemia and reperfusion was 13,1 ± ,1,15 U/g Prot. (n=24).
Such observations clearly indicate that the beneficial effects of the two extracts studied (and for that matter of plant extracts in general) do not solely depend on their flavone contents or on their in vitro potencies to scavenge free radicals. The observed effects of Hawthorn extract against reperfusion-induced arrhythmias is in agreement with the known antiarrhythmic activities of the extracts of Crataegus oxyacanthae and flavones, whereas the extract of Ginkgo biloba seems to possess additional constituents having cytoprotective activities.

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HIGH-DENSITY LIPOPROTEIN PROTECTS SOLUBLE GUANYLYL CYCLASE AGAINST DESENSITIZATION BY MODIFIED LOW-DENSITY LIPOPROTEIN AND LYSO-LECITHIN.

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We previously demonstrated that a reduced responsiveness of soluble smooth muscle guanylyl cyclase may contribute to the impaired relaxant response of atherosclerotic blood vessels to vasodilators (Schmidt, K. et al., Biochem. Biophys. Res. Commun., 172, 614-619, 1990).
The present study shows that the inhibitory effect of low-density lipoprotein (LDL) modified either by copper-induced oxidation (LDLox) or by treatment with phospholipase A_2 (PLA_2 - LDL) on sodium nitroprusside stimulated soluble guanylyl cyclase can be antagonized by co-incubation of the enzyme with high-density lipoprotein (HDL). At a concentration of 200 µg/ml cholesterol, HDL almost completely abolished the inhibitory effects of LDLox and PLA_2 - LDL (up to 250 µg/ml cholesterol each). Lysolecithin, which is discussed to be responsible for the atherogenic properties of modified LDL, also inhibited stimulation of soluble guanylyl cyclase when applied in concentrations detectable in our LDLox preparations. Similar to modified LDL, the inhibitory effect of lysolecithin was also antagonized by HDL (200 µg/ml pcholesterol) as characterized by a shift of the respective IC_{50}-value from 61 µM to 861 µM.
These data support the hypothesis that lysolecithin contributes to the inhibitory effect of modified forms of LDL on smooth muscle relaxation. Furthermore, the protective effect of HDL against desensitization of soluble guanylyl cyclase by modified LDL provides one possible explanation for the anti-atherogenic properties of HDL.

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EVIDENCE FOR MULTIPLE ENDOTHELIN/SARAFOTOXIN RECEPTORS MEDIATING CONTRACTION OF PIG CORONARY ARTERY

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The four isopeptides, endothelin-1 (ET-1), endothelin-3 (ET-3), sarafotoxin 6b (S6b) and sarafotoxin 6c (S6c), were used to characterize the endothelin/sarafotoxin receptors mediating contraction of pig isolated coronary artery. Pig hearts were obtained from the slaughterhouse. Rings from the left circumflex coronary artery were dissected out, de-endothelialized by rubbing and suspended in organ baths for measurements of isometric changes in tension. They were equilibrated, stimulated by prostaglandin $F_{2\alpha}$, then washed before one of the peptides was added. Cumulative concentration-response curves were constructed. ET-1, ET-3, S6b and S6c elicited concentration-dependent contractions of pig coronary artery. S6c behaved as a partial agonist compared to the other peptides (about 30 % the efficacy of ET-1). The curves to ET-1, S6b and S6c were monophasic, with mean EC_{50} values of 6.7, 14.8 and 1.6 nmol/l, respectively. ET-3 produced a biphasic concentration-response curve, which could be resolved into a high affinity (EC_{50} 9.6 nmol/l, 37 % of the total effect) and a low affinity component (EC_{50} 0.32 µmol/l, 63 %). In the presence of a maximally effective concentration of S6c (0.3 µmol/l), the curves to ET-1 and to S6b were not shifted to the right, whereas the curve to ET-3 appeared now monophasic, the high affinity component being abolished and the low affinity component remaining. It is concluded that at least two endothelin/sarafotoxin receptors mediate contraction of pig coronary artery. One receptor shows the rank order of agonist potencies described for the ET_A receptor (ET-1 > S6b > ET-3) with no effect of S6c. Another receptor, at which ET-3 is more potent than at the former, is stimulated potently by S6c but not by ET-1 and S6b. The latter receptor is different from the non-isopeptide-selective ET_B receptor.

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INFLUENCE OF A HIGH GLUCOSE CONCENTRATION ON THE RESPONSES TO DIFFERENT VASOCONSTRICTORS IN RAT AORTIC RINGS

K.L. Kam

In connection with a programme on the influence of experimental diabetes on cardiovascular responses to drugs we investigated whether the high blood glucose level and/or the accompanying hyperosmolarity as such may influence the in vitro contractile behaviour of blood vessels isolated from non-diabetic rats. Rat thoracic aortic rings were suspended in three media, containing 5.5 mM glucose (control), 30.5 mM glucose (high glucose and hyperosmolar) or 5.5 mM glucose and 25 mM saccharose (hyperosmolar), respectively. The concentrations of all other ions were kept constant. The characteristics of the cumulative concentration-response curves (pD_2 and E_{max}), are listed in the table.

	5.5 mM glucose pD_2 (E_{max})	30.5 mM glucose pD_2 (E_{max})	5.5 mM glucose + 25 mM saccharose pD_2 (E_{max})
noradrenaline	7.56 ± 0.085 (1.15 ± 0.067)	7.40 ± 0.111 *** (0.94 ± 0.095 ***)	7.10 ± 0.086 *,** (1.21 ± 0.090 **)
serotonin	6.34 ± 0.081 (1.12 ± 0.024)	6.29 ± 0.039 (1.33 ± 0.101)	6.29 ± 0.060 (1.21 ± 0.092)
angiotensin II	8.74 ± 0.085 (1.03 ± 0.092)	8.16 ± 0.081 * (0.76 ± 0.075)	8.31 ± 0.091 * (1.03 ± 0.128)
K^+	1.68 ± 0.026 (1.40 ± 0.065)	1.69 ± 0.031 (1.10 ± 0.057 *,***)	1.65 ± 0.005 (1.30 ± 0.063 **)
Ca^{2+}	3.40 ± 0.078 (1.56 ± 0.081)	3.63 ± 0.051 * (1.13 ± 0.081 *)	3.61 ± 0.050 * (1.39 ± 0.093)
U 46619	6.91 ± 0.025 (1.68 ± 0.052)	6.97 ± 0.045 (1.65 ± 0.078 ***)	7.02 ± 0.017 * (1.45 ± 0.055 *,**)

mean pD_2-values ± SEM (n=5-8), (E_{max}: maximal force of contraction (g) ± SEM)
*: $p<0.05$ control, **: $p<0.05$ high glucose, ***: $p<0.05$ hyperosmolar

Although statistically significant changes were observed in the relative positions of the concentration-response curves and the attained maximal forces of contraction, we were unable to demonstrate a distinct pattern in the alterations of the various constrictor effects at different glucose levels. The differences obtained in the various media are very small. These findings suggest that potential changes in contractile behaviour in isolated vessels from diabetic animals cannot be attributed to high glucose levels or hyperosmolarity, but rather reflect vascular changes associated with the diabetic state.

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CIRCADIAN PHASE-DEPENDENT EFFECTS OF DOXAZOSIN ON BLOOD PRESSURE AND HEART RATE OF THE RAT

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Effects of the α_1-selective adrenoceptor antagonist doxazosin [DOXA] on circadian rhythms in blood pressure [BP] and heart rate [HR] were determined in male Wistar-Kyoto rats using a telemetric system (Dataquest III^R). Rats were synchronised to a 12:12h light:dark cycle (L:7.00-19.00h). Sensors for BP, HR and motility [MA] were implanted i.p. with catheder tips in abdominal aorta. Circadian control values (saline) were recorded from 3 weeks after operation and dose-dependent (0.1, 0.3, 1 mg/kg i.p.) effects of DOXA were studied after drug injection at either 7.00h or at 19.00h. Significant circadian rhythms in BP, HR and MA were found with maxima in the dark span (D). Effects of DOXA were calculated as net effects to circadian control values (AUC_{cont} - AUC_{DOXA}). Mean values for systolic and diastolic BP and HR are compiled in the table.

DOXA (mg/kg)	Difference to Control [AUC 0-9h] Blood Pressure SBP [mm Hg·h]	DBP	Heart Rate [beats/min·h]
Light Phase			
0.1	- 50 ± 14	- 25 ± 10	+112 ± 17
0.3	- 47 ± 14	- 14 ± 9	+134 ± 49
1.0	- 66 ± 20	- 20 ± 15	+218 ± 25
Dark Phase			
0.1	- 81 ± 34	- 46 ± 30	+ 83 ± 67
0.3	- 96 ± 30	- 61 ± 26	+ 72 ± 50
1.0	-128 ± 19	- 60 ± 11	+147 ± 59

Results indicate that the degree in hemodynamic effects of DOXA were dependent on time of drug injection. Decrease in BP was more pronounced in D than in L, whereas the increase in HR on DOXA was more pronounced in L.

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THE CARDIOVASCULAR EFFECTS OF TALIPEXOLE ARE ANTAGONIZED BY METOCLOPRAMIDE BUT NOT BY YOHIMBINE

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The cardiovascular interactions of the adrenergic antagonists yohimbine (α_2) and prazosin (α_1), and the dopamine antagonists metoclopramide (central and peripheral D_2), domperidone (peripheral D_2) and SCH 23390 (R(+)-8-Chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol HCl) (D_1) with the selective α_2-adrenergic and D_2-dopaminergic agonist talipexole (B-HT 920) and the α_2-adrenoceptor agonist clonidine were investigated in anaesthetized rabbits after i.v. administration.

Both talipexole (0.03 - 0.1 mg/kg) and clonidine (0.01 - 0.03 mg/kg) induced dose-dependently hypotension and bradycardia. Talipexole had a shorter duration of action than clonidine.

Of the antagonists, yohimbine (3 mg/kg), prazosin (0.01 - 0.1 mg/kg), domperidone (3 mg/kg), and SCH 23390 (1 mg/kg) had hypotensive effects. Yohimbine, prazosin and SCH 23390 also exerted slight bradycardic effects.

The hypotensive effect of talipexole (0.03 mg/kg) was antagonized by pretreatment with metoclopramide (3 mg/kg) and domperidone (0.3 - 3 mg/kg), but not with yohimbine (3 mg/kg), prazosin (0.1 mg/kg) and SCH 23390 (1 mg/kg). The bradycardic effect of talipexole could only be antagonized by metoclopramide (3 mg/kg).

The hypotensive and bradycardic effects of clonidine (0.03 mg/kg) could best be antagonized by yohimbine (0.3 - 3 mg/kg). Less pronounced antagonism was observed after pretreatment with metoclopramide (1 - 10 mg/kg), and only slight reductions after prazosin (0.03 - 0.1 mg/kg) and SCH 23390 (1 mg/kg).

We conclude that, in anaesthetized rabbits after i.v. administration, talipexole, unlike the centrally acting α_2-adrenoceptor agonist clonidine, lowers blood pressure by peripheral, and heart rate by central D_2-dopaminergic mechanisms.

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ANTIHYPERTENSIVE EFFECTS AND MECHANISMS OF DIHYDROERGOCRISTINE IN SPONTANEOUSLY HYPERTENSIVE RATS.

W. Häuser

The combination of the ergot alcaloid dihydroergocristine (DHEC) with the diuretic clopamide and the rauwolfia alcaloid reserpine is wide spread used in the treatment of hypertension. Although the antihypertensive mechanisms of reserpine and clopamide are well documented, only a few data exist about the action and the mechanism of DHEC. Therefore it was of interest to study the cardiovascular effects of DHEC in rats and to differentiate its mode of action. - Spontaneously hypertensive rats (SHR) were orally treated for 21 days with increasing doses of DHEC (1 - 10 mg/kg/day) by gavage. Blood pressure was measured plethysmographically before (chronic effects) and two hours after drug administration (acute effects). Blood pressure (BP) and heart rate (HR) were also determined in anaesthetised and pithed SHR during i.v. bolus injections of DHEC (1 - 1000 µg/kg). In the latter, the dose response effects were investigated subsequently to the blockade of α_1- and α_2-adrenoceptors by using phentolamine (50 µg/min), prazosin (50 µg/kg) and idazoxan (30 µg/kg/min) i.v. In another group of pithed SHR the sympathetic outflow was induced by preganglionic electrical stimulation of the spinal cord during DHEC infusion and BP, HR and stimulation dependent catecholamines (noradrenaline [NA], adrenaline [A]) were measured by HPLC and ELCD in blood samples obtained from a carotid artery. LVP_{max}, HR, dp/dt_{max} and coronary flow were determined in Langendorff heart-preparations of SHR under constant pressure conditions. - DHEC lowered dose dependently BP of conscious and anaesthetised SHR. However, in pithed SHR BP was dose dependently increased. HR did not change significantly. During phentolamine and idazoxan but not prazosin BP response to DHEC was significantly diminished in pithed SHR. BP of spinal cord stimulated SHR decreased also dose dependently when DHEC was administered. NA outflow increased only after the dose of 100 µg/kg/min DHEC. LVP_{max}, dp/dt_{max} and coronary flow increased in a dose dependent pattern. - It is concluded that DHEC lowered BP in part by a clonidine like action. However, other receptors like dopamine- and serotonine-receptors may also be involved.

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TIME COURSE OF β_1- AND α_1-ADRENOCEPTOR BLOCKING ACTIVITIES AFTER ORAL ADMINISTRATION OF CARVEDILOL TO HEALTHY VOLUNTEERS

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To profile the extent and duration of β_1- and α_1-antagonistic activity of carvedilol in man different oral doses of carvedilol (12.5 (A), 25 (B), 50 (C) and 100 mg (D)) were compared with 40 mg (E) of propranolol given to 12 healthy male volunteers (placebo-controlled, randomized cross-over design).
Carvedilol inhibits dose-dependently the increase in heart rate during bicycle ergometry (placebo:145, A:140, B:133, C:130, D:122, E:126 beats/min) 2 h after drug administration whereas there was no effect on resting heart rate. The corresponding average β_1-adrenoceptor occupancies amounted to A:36, B:53, C:72, D:82, E:61 %; occupancies of α_1-adrenoceptors were smaller with A:4, B:23, C:37, D:58 % (examined with radioreceptor-assays (RRA) using plasma samples). The time course of reduction of exercise tachycardia at any dose given was in close correlation to the β_1-adrenoceptor occupancies for both drugs.
The results from the enantiospecific determination of the plasma concentrations of carvedilol as well as from the determination of effect equivalents ($n \cdot K_i$) using the β_1- and α_1-RRA indicated that plasma concentrations at any time point and AUC's were proportional to the doses applied.
In conclusion, carvedilol acts as a combined ß-/α_1-antagonist after oral administration in man with a β_1/α_1-ratio of about 3.5 with respect to the drug concentrations expressed as effect equivalents at the respective adrenoceptor. Therefore, the duration of action mediated via α_1-adrenoceptors might be shorter than that mediated via β_1-adrenoceptors. The results from the β_1-RRA measurements suggest that one or several active metabolites contribute to the ß-antagonistic effects of carvedilol in vivo.

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THE PAF-ANTAGONISTS WEB 2086 AND WEB 2170 IMPROVE ENDOTOXIN- AND PAF-INDUCED DISTURBANCES IN LEUKOCYTE RHEOLOGY.

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Septic shock is a frequent complication of acute systemic infections and it is also known that the platelet activating factor (PAF) plays an important role as a mediator of the septic shock (LEFER AM (1989) Circulatory Shock 27:3-12). In this study the endotoxin- and PAF-induced shock was used as an experimental model to test the effect of the specific PAF-antagonists **WEB 2086** (3-[4-(2 Chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-2-yl]-1-(4-morpholinyl)-1-propanone) and **WEB 2170** (8-(R,S)-6-(2 Chlorophenyl)-8,9-di-hydro-1-methyl-8-[(4-morpholinyl)carbonyl]-4H,7H-cyclopenta[4,5]thieno[3,2-f][1,2,3]triazolo[4,3-a][1,4]diazepine). In anesthetized rats the influence on systemic blood pressure, and especially microcirculatory parameters (calculated microflow and velocity ratio of leukocytes) was evaluated in the rat cremaster muscle. The leukocytes were divided into four classes: fast leukocytes, fast rollers, slow rollers and sticker. The test procedure was divided into a preparatory control period, followed by bolus injection of saline or the drugs, and endotoxin (E. coli 0111:B4) with 30 mg/kg i.v. or PAF with 0.5 µg/kg i.v. and a final follow-up phase of 180 minutes.

After the application of the PAF-antagonists no cardiovascular effects were observed. Within the follow-up phase the rats pretreated with **WEB 2086** and **WEB 2170** showed a restored blood pressure and microflow and also the rheologic properties of the leukocytes were improved.

	fast leukocytes	fast rollers	slow rollers	sticker
NaCl-Control	76.0 [%]	16.2	4.8	3.0
Endotoxin 30 mg/kg (ET)	46.1 [%]	16.5	19.4	18.0
WEB 2086 1 mg/kg + ET	72.2 [%]	16.9	7.3	3.6
WEB 2170 1 mg/kg + ET	66.0 [%]	15.9	10.4	7.7
PAF 0.5 µg/kg	29.0 [%]	14.6	40.2	16.2
WEB 2086 1 mg/kg + PAF	66.9 [%]	5.4	21.9	5.8
WEB 2170 1 mg/kg + PAF	67.8 [%]	16.2	12.3	3.7

The anti-shock effect produced by **WEB 2086** and **WEB 2170** is based on their action on the microcirculation.

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COMPARATIVE EFFECTS OF HEPARIN AND THE THROMBIN INHIBITOR ARGATROBAN ON SARUPLASE (R-SCU-PA)-INDUCED FEMORAL ARTERY THROMBOLYSIS IN RABBITS

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Anticoagulants can improve the efficacy of thrombolytic agents. Direct inhibitors of thrombin may be superior to heparin. Therefore, the effects of heparin (150 U/kg + 100 U/kg·hr; 5.3-fold PTT-prolongation) and the thrombin inhibitor argatroban (1 mg/kg + 3 mg/kg·hr; 2.3-fold PTT-prolongation) have been compared in respect to enhancement of thrombolysis with saruplase (r-scu-PA). In rabbits, totally occlusive thrombi have been induced in femoral arteries by local clotting of autologous blood mixed with thrombin. Infusion of 3 to 12 mg/kg saruplase resulted in dose-dependent recanalization in this model. For the combination studies with heparin or argatroban, saruplase was administered as i.v.-infusion over 60 min of 3 mg/kg (threshold dose) or given as i.v.-bolus injection of 6 mg/kg.

Treatment	Time to reperfusion [min]	Incidence of reperfusion	Incidence of reocclusion	Patency rate at 120 min
Saruplase 3 mg/kg, 60 min infusion	42 ± 5	3/6	2/3	1/6
+ Heparin	39 ± 7	6/6	3/6	3/6
+ Aragatroban	26 ± 5	6/6	0/6	6/6*
Saruplase 6 mg/kg, bolus	15 ± 3	5/6	4/5	1/6
+ Heparin	8 ± 3	4/6	0/4	4/6
+ Aragtroban	8 ± 3	6/6	0/6	6/6*

* $p < 0.05$ vs saruplase alone

The height of the reperfusion blood flow (as % of baseline) for infused (3 mg/kg) or bolus-injected (6 mg/kg) saruplase was 22 ± 12 % resp. 24 ± 16 %. The magnitude of the restored blood flow was increased to 49 ± 18 % resp. 71 ± 13 % ($p < 0.05$) by heparin and to 84 ± 10 % resp. 80 ± 16 % (both $p < 0.05$) by argatroban. Thus, despite less PTT-prolongation argatroban enhanced saruplase-induced thrombolysis more effectively than heparin in rabbit femoral artery thrombosis.

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TRANSGENIC RATS HABOURING THE HUMAN RENIN OR ANGIOTENSINOGEN GENE

K. Zeh, J. Wagner, A. Wystrychowski, J.-B. Michel, K. Murakami and D. Ganten

The renin-angiotensin system (RAS) is one of the most important regulators of electrolyte homeostasis and blood pressure. In order to generate a new model for the human RAS we have generated transgenic rats harbouring either the entire human renin or human angiotensinogen gene under the control of their own promoters. At present there are two lines with the human renin and four lines with the human angiotensinogen gene available. The highest expression of the transgenes could be detected for human renin in the kidney and for human angiotensinogen in the liver but positive signals were also found in various other tissues. The plasma values of active human renin were 4.8 ± 1.1 pg/ml and 266 ± 0.7 pg/ml. The corresponding human prorenin levels amounted to 125 ± 0.7 pg/ml and 6600 ± 290 pg/ml. Human angiotensinogen concentrations ranged from 125 µg/ml up to 5000 µg/ml depending on the line. Despite this high levels of the human transgene products the blood pressure in all transgenic rats was normal as compared to controls. However, infusion of human renin in transgenic rats harbouring the human angiotensinogen gene induced a marked hypertensive response which could be completley blocked by the primate specific renin inhibitor RO 425892 (Hypertension 1991;18:22-31). The blood pressure elevation following rat renin infusion was not influenced by RO 425892 but by the angiotensin II receptor antagonist DUP 753 (*Losartan*).
These data demonstrate that the new transgenic rat lines can serve as a model for studying the human renin-angiotensin system in vivo. Furthermore, new pharmacological compounds like human specific renin inhibitors can be tested in these animals.

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ATENOLOL AND DUP753 INHIBIT THE INCREASE IN PLASMA ANGIOTENSINOGEN LEVEL AFTER NEPHRECTOMY.

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Objective: Plasma angiotensinogen (Agen) levels increase up to 10-fold in the rat 24h after bilateral nephrectomy. This increase is mainly caused by an increased hepatic synthesis and secretion of Agen. Although a number of factors are known to influence Agen synthesis, the signal for this increase is still unknown. Glucocorticoids from the adrenals and angiotensin II (AngII) have shown to stimulate the Agen synthesis in hepatocytes *in vivo* and *in vitro*. However it is still an unresolved question whether both hormones participate in this increase. In addition there is a persisting controversy concerning the existence and contribution of a "nephrectomy factor".

Design and Methods: The time course of plasma Agen- and AngI-levels and of plasma renin concentration was investigated in conscious rat after nephrectomy and nephrectomy plus adrenalectomy at 0, 1, 2, 4, 8, 12 and 24h after surgery. Thirty min prior surgery the animals have been treated with atenolol, 12mg/kg in order to inhibit renin secretion during surgery and DuP753, 10mg/kg I.V. for an inhibition of the AT1 receptor, respectively.

Results: In the absence of the adrenals nephrectomy triggers a slight but significant increase in plasma Agen levels indicating that a renal factor may be involved. This effect could completely be inhibited after pretreatment with atenolol and DuP753, respectively. The increase in plasma Agen levels after nephrectomy was significantly lower in presence that in the absence of atenolol. After pretreatment with the AngII antagonist DuP753 the Agen values after nephrectomy were close to those of the control rats. After nephrectomy AngI levels decrease by 30% and persisted after 4 to 24h. They were somewhat higher than after nephrectomy plus adrenalectomy (NS). In all animals atenolol caused a significant decline of plasma AngI levels up to 80%. In presence of DuP753 there was a statistically significant difference in the AngI values between nephrectomized and nephrectomized plus adrenalectomized rats.

Conclusion: These data suggest that after nephrectomy the increase in plasma Agen is caused by renal renin, that is released during surgery. Subsequently AngII is generated. AngII triggers the Agen synthesis in the liver directly. Simultaneously AngII stimulate glucocorticoid release from the adrenals that has shown to be the most potent stimulus of the Agen synthesis. In the absence of the adrenals there is only a slight increase in Agen synthesis after nephrectomy that is caused by the direct action of AngII.

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ISCHEMIA-INDUCED RENAL ANGIOTENSINOGEN, RENIN AND CONVERTING ENZYME mRNA EXPRESSION IN SPONTANEOUSLY HYPERTENSIVE (SHR) AND NORMOTENSIVE WISTAR-KYOTO RATS (WKY). R. Rettig, M. Büch, and M. Paul

When transplanted to normotensive recipients, kidneys from SHR but not WKY donors induced posttransplantation hypertension. The underlying mechanisms are currently unknown. The intrarenal renin angiotensin system (RAS) is a major regulatory mechanism for electrolyte and blood pressure control. To investigate the differential responses of the intrarenal RAS to an ischemic insult in SHR and WKY, we subjected kidneys from both species to 1 h of cold ischemia which was similar to that during transplantation (kidney flushed with ice-cold Euro-Collins solution through the renal artery followed by external cooling) while they remained in situ. Kidneys were removed and processed for determination of angiotensinogen (A), renin (R) and converting enzyme (CE) mRNA by Northern blot analysis immediately at the end of cold ischemia periods (SHR n=8, WKY n=9) and 24 h after cold ischemia (SHR n=5, WKY n=5). ß-Actin mRNA levels were used as reference points. Basal concentrations of R-mRNA were significantly lower in SHR vs. WKY kidneys. Basal A-mRNA and CE-mRNA were not different between the two strains. Immediately after cold ischemia A-mRNA and R-mRNA were significantly stimulated in SHR and WKY kidneys while CE-mRNA was unchanged. 24 h after cold ischemia A-mRNA and R-mRNA had returend to baseline levels, whereas CE-mRNA was significantly stimulated in kidneys from both strains. Our data indicate that the RASs in SHR and WKY kidneys respond similarly to cold ischemia, although basal R-mRNA levels were suppressed in SHR vs. WKY. In addition, our results suggest that A and R may be involved in the acute regulatory response to cold ischemia in the rat kidney, whereas CE appears to be associated with long-term events such as restructering and repair.

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ANGIOTENSINOGEN-mRNA STABILIZATION BY ANGIOTENSIN II

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We have previously shown that the vasoactive peptide angiotensin II (ANGII) stimulates the synthesis and secretion of angiotensinogen (Ao) in freshly isolated hepatocytes. In the present study we have focused on the intracellular mechanisms by which ANGII increases the concentrations of angiotensinogen mRNA.

First we have examined whether ANGII directly modulates the transcription rate for Ao mRNA in isolated nuclei from ANGII (90 nM) pretreated hepatocytes. In this system ANGII pretreatment failed to influence the transcription rate of ^{32}P-UTP-labelled Ao mRNA as determined by dot blot or liquid hybridization techniques. In contrast, in hepatocytes in which the de novo transcription is blocked by actinomycin D (2 µg/ml) or 5,6 dichlorobenzimidazole riboside (DRB, 50 µg/ml) and also in a ^{3}H-uridine pulse and chase system, ANGII increased the half life of Ao mRNA about 2.5 fold. We were further able to isolate two proteins from a cytosolic preparation of hepatocytes, which showed binding to a non-translated 3'fragment of Ao mRNA. The sequence for the 3'fragment was restricted from the vector pRAG 16 which contains the complete sequence for angiotensinogen and was cloned into the expression vector pGEM5 Zf+. Binding analysis were done by gel mobility shift and crosslinking assays. Binding of both proteins was specific, because they were competed by the cold 3'tail but not by a renin mRNA fragment of similar size. Molecular weights (60 000, 18 000) and isoelectric points (4-5) of the binding proteins correlate with two phosphorylated proteins which occur in cells exposed to ANGII and other compounds transiently decreasing cAMP. Phosphorylation patterns were determined in ^{32}P-orthophosphate loaded hepatocytes. The cytosol of these cells were subjected to 2D electrophoresis for autoradiographic evaluation.It remains to be clarified whether the 3'tail binding proteins are indeed identical to those phosphorylated in response to a transient decrease in cAMP.

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STRUCTURAL AND FUNCTIONAL VASCULAR ALTERATIONS FOLLOWING HIGH- AND LOW-DOSE ACE INHIBITOR TREATMENT IN SHR

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We investigated structural alterations in mesenteric arteries (media and wall thickness, media/lumen and wall/lumen ratios, number of smooth muscle cell layers) and functional changes in aortic preparations of SHR treated in utero and up to 20 weeks of age (prevention study) and of adult 16 week old SHR treated for 16 weeks (regression study) with the ACE inhibitor ramipril (0.01 and 1 mg/kg/day p.o.). Control animals received vehicle (H_2O). Low- and high-dose treatment with ramipril inhibited vascular ACE activity in vivo, as demonstrated by the inhibition of aortic vasoconstrictor responses to angiotensin I but not to angiotensin II, and in vitro (fluorometric assay of ACE activity). Early onset treatment with high-dose (1 mg/kg/day) ramipril prevented the development of hypertension and vascular hypertrophy, increased aortic vasodilatory responses to bradykinin (BK) (10^{-7}-10^{-5}M) and acetylcholine (ACh) (10^{-8}-10^{-6}M) and decreased vasoconstrictor responses to noradrenaline (NA) (10^{-8}M). Treatment of adult SHR for 16 weeks with 1 mg/kg/day ramipril normalized blood pressure (BP) and had similar effects on vascular function, but did not affect vascular hypertrophy. Low-dose (0.01 mg/kg/day) ramipril, although having no effect on BP and vascular structure, significantly decreased the vasoconstrictor responses to NA in both the prevention and the regression study, and increased the vasodilatory responses to BK and ACh in the regression study.

In conclusion, mesenteric vascular hypertrophy could be prevented by early onset high-dose treatment with the ACE inhibitor but not once hypertrophy was established. However, the ACE inhibitor improved vascular function under all treatment conditions independently of its effect on vascular hypertrophy.

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PASSAGE OF VARIOUS CONVERTING-ENZYME INHIBITORS THROUGH THE ENDOTHELIUM.

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The specific actions of converting enzyme inhibitors (CEI) are mainly due to the inhibition of the enzymes on the endothelium or some other tissues. CEI diminish the biosynthesis of Ang II and the degradation of bradykinin, both peptides are held to be involved in the antihypertensive and cardio-protective actions. The various unspecific effects of Captopril (C), Enalapril (E) and Ramipril (R) on inhibition of monoamine oxidase and blockade of noradrenaline uptake (Blöchl 1990, Inauguraldissertation) were discussed to be dependent on the lipophilicity of the 3 CEI used. For the latter effects it is required that CEI pass the endothelial barrier and even penetrate the cell membrane. Therefore the aim of our study was to reinvestigate the lipophilicity of various CEI and to study their passage through the endothelium. - Captopril, Enalapril, Ramipril and the respective diacides (Enalaprilat [EP], Ramiprilat [RP]) were dissolved in water and transferred to an octanol/water mixture. The octanol/water distribution coefficient was determined by HPLC (UV detection at 214 nm) and a C-18 reversed phase column at different pH values (detection limit: 1 -10 μg) varying from pH 1 to pH 12. Endothelial cells from bovine aorta were cultured for 3 to 10 passages and transferred to polycarbonate filters (Costar). C, E, R and their respective diacides (500 μM) were applied to the luminal side of the endothelium or to a control filter. The concentrations of the CEI were measured time dependently on both sides of the filter. The integrity of the endothelium was controlled by using albumin marked with trypan blue. - At the physiological pH of 7.4 the octanol/water distribution coefficient of the CEI was for C: 0.01, E: 0, EP: 0, R: 0.25 and RP: 0. All 5 substances could be detected 5 min after application at the basal side of the endothelium. While the initial velocity of passage was not significantly influenced by the endothelium, the cell layer diminished the passage rate of the various CEI between 1 and 4 hours of incubation in the following order: C = E < EP = R < RP.- The passage of the various converting-enzyme inhibitors seems to be dependent on the molecular weight and not on the lipophilicity.

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CHRONIC BLOCKADE OF BRADYKININ B_2-RECEPTORS ATTENUATES THE ANTIHYPERTENSIVE EFFECT OF ACE INHIBITOR IN 2K1C HYPERTENSIVE RATS

G. Bao, and P. Gohlke

Angiotensin converting enzyme (ACE) is identical to kininase II, which catabolizes bradykinin (BK) to its inactive fragments. It has been postulated that the antihypertensive effect of ACE inhibitors (ACEI) is partly due to a potentiation of endogenous BK. We investigated the effect of chronic BK B_2-receptor blockade on the antihypertensive action of the ACEI ramipril by using the new potent and long-acting BK B_2-receptor antagonist Hoe140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK). In a first approach, 2-kidney 1 clip (2K1C) hypertensive Wistar rats were pretreated with ramipril (1 mg/kg/d, p.o.) for 4 weeks to reach normotensive levels (group 1 and 2; n= 12 and 14). In a second approach the experiment was performed in untreated 2K1C hypertensive Wistar rats (group 3 and 4; n=10 and 11). All animals were treated with ramipril (1 mg/kg/d, p.o.) for 6 weeks. Group 2 and 4 were additionally treated with Hoe140 (500 ug/kg/d), group 1 and 3 with physiological saline subcutaneously via osmotic minipumps. Mean arterial blood pressure (MAP) was measured before as well as 3 and 6 weeks after the beginning of the treatment. In group 1 and 2, plasma catecholamines were determined at the end of the experiment. Results (mean±SEM):

Group	Treatment	MAP (mmHg)			Adrenaline	Noradrenaline
		before	3 weeks	6 weeks	(pg/ml)	
1	ACEI+Vehicle	112.0±6.5	110.3±4.9	103.7±5.0	131.3±14.2	71.5±5.5
2	ACEI+Hoe140	115.3±4.6	123.8±3.3*	121.3±2.9*	151.4±9.0 ns	102.6±20.8 ns
3	ACEI+Vehicle	180.1±8.1	115.7±4.5	105.4±4.8		
4	ACEI+Hoe140	180.3±4.1	131.8±2.6*	124.4±4.2*	*p<0.05	
					ns: not significant	

Our results demonstrate for the first time that the chronic antihypertensive action of an ACEI can be attenuated by BK receptor blockade. This effect was not due to a BK-like agonistic action of Hoe140 on catecholamine release. Thus, our data support the hypothesis, that potentiation of endogenous BK contributes to the chronic antihypertensive action of ACEI.

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ACE-INHIBITORS MODULATE SYMPATHETIC ACTIVITY IN MYOCARDIUM M. Reinhardt and C.Hoffmann

Presynaptic Angiotensin II-receptors at sympathetic nerve fibres are pharmakologically identified. Ang.I and Ang.II stimulate sympathetic nerves to release norepinephrine and additionally reduce coronary flow in isolated perfused rat hearts. We have shown, that the SH-containing ACE-inhibitor Captopril is able to suppress these effects. There is evidence, that the SH-group is partially responsible for this ability. To eliminate this SH-group dependent effect we studied the effects of the non SH-containing ACE-inhibitor Moexiprilat ($2x10^{-7}$M) on isolated at constant pressure perfused rat hearts. Histofluorescent visualisation was induced by glyoxylic acid according De La Torre and morphometric quantification of intraneuronal catecholamines was performed by a digitalized picture analyzing system.

[%of control]	Ang.I 10^{-8}M	Moex, $2x10^{-7}$M	Ang.I+Moex, 10^{-8}M + $2x10^{-7}$M
Area of intraaxonal catecholamines in myocardium	73%	99%	83%
Effluate from 25. to 45. min	51%	100%	60%

This data shows that Moexiprilat was able to suppress the vascular and neuronal reaction to Ang.I in myocardium. These effects do not dependent on SH-groups.

Lit.: K. Addicks et al.:Captopril influences sympathetic activity in myocardium, J-Mol-Cell-Cardiol 23, Suppl. V, S. 19/44 (1991).

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EFFECTS OF THE ANGIOTENSIN II-ANTAGONIST DUP 753 AND THE CONVERTING-ENZYME INHIBITOR RAMIPRIL ON THE ^{3}H-NORADRENALINE RELEASE OF LEFT GUINEA PIG ATRIA.

L. Sieroslawski and P. Dominiak

In previous studies concerning the sympathetic outflow during converting-enzyme inhibition (CEI) controversial results have been reported. Depending on the different species and methods used in such experiments the catecholamine release during CEI was found to be decreased, unchanged or even increased. Since the converting-enzyme is rather unspecific, some other endogenous peptides such as kinins or Ang I could contribute to the above mentioned controversial results. Therefore it was of interest to investigate the actions of the high specific non-peptidic Ang II-antagonist DUP 753 on tritiated noradrenaline release in guinea pig atria and to compare the results with those of CEI. - Left guinea pig atria were used for the experiments and kept in an organ bath containing 20 ml of Kreb's solution (95 % O_2, 5 % CO_2). Two hours prior to the experiments ^{3}H-noradrenaline (10 μCi) was added to the organ bath. Platinium electrodes were placed on either side of the atrium for field stimulation. Each square wave field pulse was of 2 ms duration, 30 V and 0.5 Hz delivered from a HSE-T-stimulator (Hugo Sachs, Hugstetten). Three field stimulations were applied for 5 min. After each stimulation period 1 ml was kept from the organ bath to determine the release of ^{3}H-noradrenaline using a liquid scintillation counter. Two subsequent stimulation periods (S2/S1) served as control. The test substances, Ang I (10^{-6} - 10^{-5} m) Ang II (10^{-8} - 10^{-5} m), DUP 753 (10^{-8} - 10^{-5} m) and ramipril (10^{-6} - 10^{-5} m) were added to the organ bath 10 min prior to the 3rd field stimulation (S3). The ^{3}H-noradrenaline release after administration of the different substances was calculated by S3/S1. The experiments were performed with and without uptake-1 inhibition by cocaine and desipramine. - As could expected, Ang I and II significantly increased the ^{3}H-noradrenaline release. DUP 753 significantly reduced tritiated NA and antagonized the actions of Ang II. The NA releasing effects of Ang I were influenced by DUP 753 in a similar pattern. Ramipril slightly increased the ^{3}H-NA release when cocaine and desipramine were added to the organ bath. However, without uptake-1 inhibition the NA release remained unchange. - It is concluded that the specific Ang II-antagonist DUP 753 inhibits the ^{3}H-NA release by presynaptic Ang II receptor blockade whereas the effects of CEI on NA outflow were blunted by some other unspecific actions of ramipril such as inhibition of the uptake-1 and metabolism of catecholamines (Dominiak, Blöchl 1991, Basic Res. Cardiol.).

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ADRENERGIC AND MUSCARINIC RECEPTOR CHARACTERISTICS IN MESENTERIC ARTERIES OF SHR AND WKY OF DIFFERENT AGE.

M.G.C. Hendriks.

We investigated possible hypertension-induced changes in the characteristics of vascular adrenergic and muscarinic receptors in isolated perfused mesenteric vascular bed preparations from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats, respectively. We also assessed whether these changes were different in animals of 18 versus 28 weeks of age. Methoxamine (α_1-agonist) and metacholine (muscarinic agonist) induced concentration-dependent vasoconstriction and vasodilation, respectively. In order to quantify the vasodilator effect of MCh the preparations were precontracted with methoxamine. Results are shown in table 1 (means ± S.E.M).

table 1. Characteristics (EC_{50}, E_{max} and slope) of the concentration response curves of methoxamine and metacholine in the isolated mesenteric vascular beds from SHR and WKY, respectively. Mean arterial blood pressure (MAP) of the donor rats is also shown

	MAP (mmHg)	EC_{50}	E_{max} (mmHg)	(slope)
WKY 18 weeks	98.4±2.5			
methoxamine		5.1±0.8 µM	155.2±8.2	2.8±0.6
metacholine		44±0.6 nM	23.1±4.5	1.1±0.2
WKY 28 weeks	112.5±8.5			
methoxamine		6.0±0.5 µM	138.3±1.7	2.1±0.2
metacholine		0.3±0.9 µM	26.7±4.9	0.9±0.1
SHR 18 weeks	184.1±2.7			
methoxamine		3.8±0.3 µM	250.2±16.4	3.5±0.5
metacholine		41±0.9 nM	20.6±1.6	1.2±0.3
SHR 28 weeks	192.4±2.6			
methoxamine		4.3±0.5 µM	220.2±14.4	3.9±0.3
metacholine		0.3±0.8 µM	30.0±4.3	0.9±0.1

As to be expected for methoxamine, the mesenteric preparations taken from both 18 and 28 week old SHR yielded significantly ($p<0.05$) stronger maximal responses and steeper slopes of the curves than preparations taken from control animals. Age *per se* did not change these values. Several authors have claimed an altered response to muscarinic agonists in conduit arteries from SHR. We could not detect differences in the concentration response-curves of metacholine between the age-matched groups. However, the sensitivity to muscarinic receptor stimulation decreased age-dependently in both groups, which may contribute to the elevation of the MAP observed in older animals.

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Hyperreactivity to vasoconstrictors in ischemic rat hindlimb and the effect of angiotensin-I-converting enzyme (ACE) inhibitors and DuP753.

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To investigate the role of local renin-angiotensin systems in the compensatory mechanisms following chronic ischemia of skeletal muscles, functional responses to angiotensin I (AI), II (AII) and phenylephrine (PHE) and the influences of treatment with the ACE-inhibitors, captopril (CAP) or zabiciprilate (ZAB), or the AT_1-antagonist DuP753 were investigated in control and chronically ischemic animals. Ischemia was induced in spontaneously hypertensive rats by partial occlusion of the left common iliac artery and complete occlusion of the left iliolumbar artery, whereafter treatment was started (CAP: 500 µg/kg/h; ZAB: 25 µg/kg/h; DuP753: 625 µg/kg/h). In a perfusion model the hindlimbs of control and treated and untreated ischemic animals (4 weeks) were perfused with an isooncotic Krebs-Ringer solution and perfusion pressure and flows in both common iliac arteries were measured. The effects of injections of AI, AII and PHE on resistances were determined. Chronic ischemia reduced left flow to 5-75% of contralateral flow. In control animals ED_{50} and the maximal increase in resistance (ΔR_{max}; mmHg.min/ml) were comparable in both hindlimbs for all vasoconstrictors. In contrast, ΔR_{max} for AI, AII and PHE increased significantly (* $p < 0.05$) in the severely ischemic hindlimb (flow < 40%), whereas ED_{50} was not influenced.

	ΔR_{max} left		ΔR_{max} right	
	control	ischemia	control	ischemia
AI	11.1±1.16	44.0±9.41*	13.2±2.31	7.4±0.82
AII	12.1±1.56	42.3±12.9*	8.5±0.94	7.9±1.00
PHE	224.7±66	761.7±416	181.4±28.3	146.2±34

CAP and ZAB did not affect AII responses and ΔR_{max} of AI, but increased ED_{50} of AI of both hindlimbs slightly (2 fold). DuP753 increased ED_{50} of both AI and AII more than 300 fold; furthermore, the increase in ΔR_{max} to PHE in ischemic hindlimbs was similar to control. In conclusion, severe ischemia causes a non-specific vascular hyperreactivity in ischemic hindlimbs. This hyperreactivity is abolished by DuP753. Therefore, the AT_1-receptor seems to be involved in hyperreactivity of the vascular bed following chronic ischemia.

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THE ANGIOTENSIN II EFFECT ON THE SPONTANEOUS PHASIC ACTIVITY OF RAT PORTAL VEIN IS MEDIATED BY AT_1 RECEPTORS

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In the present study, the functional angiotensin II (AII) receptors mediating the potentiation of spontaneous phasic myogenic activity of rat portal vein to AII were classified using the AT_1 receptor antagonist DuP753 and the AT_2 receptor antagonist PD123177 (Timmermans et al., TIPS **12**, 55-62). The angiotensin II antagonism of BIBS39 (4'-[(2-n-butyl-6-cyclohexyl-aminocarbonylamino-benzimidazole-1-yl)-methyl] biphenyl-2-carboxylic acid and BIBS222 (2-n-butyl-1-[4-(6-carboxy-2,5-dichlor-benzoylamino))-benzyl]-6-N-(methylaminocarbonyl)-N-pentylamino-benzimidazole), two new nonpeptide angiotensin II-receptor antagonists with less subtype selectivity, was also examined.

At an extracellular Ca^{++} concentration of 0.9 mmol/l and a KCl concentration of 4.0 mmol/l, AII, when administered in a cumulative manner ($3x10^{-10}$ to $3x10^{-6}$ M), caused concentration-dependent increases in the spontaneous phasic contraction and a decrease in contraction frequency of longitudinal smooth muscle of rat portal vein. The maximal increase in phasic contraction induced by AII was 14.8 ± 0.9 mN (at $3x10^{-6}$ M). The contraction frequency was maximally reduced to 36.6% of control values (at $3x10^{-6}$ M) (n=8).

DuP 753 concentration-dependently shifted the concentration-phasic contraction response curve of AII to the right without reducing of the maximal response to AII. A pA_2 value of 8.60 ± 0.05 and slope of 0.91 ± 0.06 were obtained, suggesting a competitive functional AT_1 receptor antagonism. By contrast PD123177, at 10^{-6} or even 10^{-5} M, caused no significant change of the phasic myogenic response to AII, indicating the absence of functional AT_2 receptor involvment.

BIBS39 and BIBS222, two new potent nonpeptide AII-receptor antagonists also caused parallel rightward shifts of the concentration-phasic contraction response curve to AII without altering the maximal response to AII, yielding pA_2 values of 8.63 ± 0.06, 8.59 ± 0.04 and slopes of 0.87 ± 0.002, 0.95 ± 0.01, respectively.

In conclusion, the functional receptors mediating the enhancement of spontaneous phasic activity by AII in rat portal vein belong to the AT_1 receptor subtype population.

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ANGIOTENSIN II-1 RECEPTOR BLOCKADE IN EXPERIMENTAL HEART FAILURE IN RATS

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In rats with heart failure due to myocardial infarction (MI), early captopril (CAP) treatment (1-21 days after MI) decreases cardiac function whereas late (21-35 days) treatment restores cardiac function to normal values. In the present study we administered losartan (LOS; DuP753), an angiotensin II-1 (AT-1) receptor antagonist, during the same early and late periods to investigate involvement of this receptor in the responses to CAP. MI was induced by left coronary artery ligation. Animals were treated with saline or LOS (15 mg/kg•day; Alzet osmotic minipump). In pilot-studies this dose caused a rightward shift of the angiotensin II (AII) dose-pressor response curve by a factor of 35, as compared to a 17-fold shift of the AI dose-response curve by an effective dose (12 mg/kg•day) of CAP. A week before the end of the treatment, animals were instrumented for measurement of blood pressure (MAP), central venous pressure (CVP) and cardiac output (CO). At the end of the treatment we measured MAP, CVP and CO at rest and upon rapid volume loading ($CO_{VL,max}$) with 12 ml warm Ringer's solution. In sham animals, CO and $CO_{VL,max}$ are 94±4 and 154±4 ml/min, resp. (mean±SEM; n=10). In saline-treated MI animals, the corresponding values were 74±3 and 107±5 ml/min (n=12; 35 days). Neither early nor late LOS treatment had any effect on cardiac function (late treatment: CO: 78±5, $CO_{VL,max}$: 118±9 ml/min; n=6), although both treatments effectively reduced MAP (late treatment: sham:100±3; saline: 92±6; LOS: 78±4 mmHg). We conclude that the AT-1 receptor is not involved in the effects of captopril on cardiac function following MI in rats.

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CHARACTERIZATION OF BIBS 39 AND BIBS 222, TWO NEW ANGIOTENSIN II ANTAGONISTS, IN RECEPTOR BINDING AND AUTORADIOGRAPHIC STUDIES. M. Entzeroth, C. Karg, D. Lefèvre and U. Schmid

BIBS 39, 4'-[(2-n-butyl-6-cyclohexylureido-benzimidazol-1-yl)methyl]-biphenyl-2-carbonic acid trifluoracetate, and BIBS 222, 2-n-butyl-1-[4(2,5-dichlor-6-carboxybenzoylamido)-benzyl]-6-(N-methylaminocarbonyl)pentylamino-benzimidazole) are two recently developed angiotensin II antagonists, which only 17- and 37-fold, respectively, discriminate between AT_1 and AT_2 receptors as determined in radioligand binding studies. The K_i values for AT_1 binding sites in rat lung were 29 ± 7 nM and 20 ± 7nM, respectively. The K_i values for AT_2 binding sites in adrenal medulla were 480 ± 110 nM and 739 ± 170 nM, respectively. Both compounds did not interfere with other receptor systems tested in sub-micromolar concentrations. In addition, we investigated the interaction of BIBS 39 with angiotensin II receptors in rat adrenal and kidney using autoradiographic techniques. In rat adrenal 10 μM BIBS 39 displaced ^{125}I-Sar,Ile-angiotensin II binding in both zona glomerulosa and medulla while the AT_1 selective antagonist DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)bi-phenyl-4-yl)-methyl]imidazole, 10 μM) only reduced radioligand binding in the zona glomerulosa. The AT_2 selective antagonist PD 123.177 (1-(4-amino-3-methyl-benzyl)-5-diphenylacetyl-4,5,6,7-tetra-hydro-imidazole[4,5]pyridine-6-carboxylic acid, 10 μM) inhibited radioligand binding only in the medulla. In rat kidney slices ^{125}I-Sar,Ile-angiotensin II specifically interacted with binding sites in renal medulla and at the glomeruli. No difference was observed in the inhibition of radioligand binding between BIBS 39 and DuP 753. At a concentration of 10 μM both compounds completely abolished the specific binding. In contrast, at 10 μM PD 123.177 failed to inhibit specific ^{125}I-Sar,Ile-angiotensin II binding to rat kidney slices.
In conclusion, BIBS 39 and BIBS 222 are potent and selective angiotensin II antagonists. BIBS 39 interacts with both AT_1 and AT_2 receptor subtypes in rat adrenal as well as AT_1 receptors in rat kidney. No evidence for the presence of AT_2 receptors in rat kidney was obtained from autoradiographic studies.

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DIFFERENTIAL EFFECTS OF THE NONPEPTIDE ANGIOTENSIN II (AII)-ANTAGONIST EXP3174 AND CAPTOPRIL ON FUNCTIONAL PARAMETERS IN ISOLATED RAT KIDNEY.
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The effects of the AII antagonist EXP3174 (2-n-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl) methyl] imidazole-5-carboxylic acid) and captopril (on renal perfusate flow (RPF), urinary flow (UF) and glomerular filtration rate (GFR) were investigated in the isolated rat kidney perfused at constant pressure (105 mm Hg), in the absence and presence of furosemide. Administration of EXP3174 (0.01, 0.1 and 1 μM) in the absence of furosemide resulted in a concentration-dependent increase in RPF to 104, 109 and 116% of the control value, respectively ($p<0.05$; n=6). UF increased likewise to 103, 114 and 127%, respectively ($p<0.05$), whereas GFR remained unchanged. In contrast, no significant increase in RPF and UF was observed during administration of captopril (10 μM) in the absence of furosemide and GFR decreased to 91% of the control value ($p<0.05$; n=8). Continuous infusion of furosemide (10 μM) increased UF significantly ($p<0.01$; n=7) and consistently to about 135% whereas RPF and GFR remained unchanged. Additional administration of EXP3174 (0.1 and 1 μM) in the presence of furosemide resulted in an increase in RPF to 106 and 112% ($p<0.05$; n=8) and a further increase in UF to 148 and 163% ($p<0.05$), respectively, whereas GFR did not change significantly. In contrast, captopril (0.1 and 1 μM), in the presence of furosemide, had no effect on RPF and UF, whereas GFR decreased significantly to 91% ($p<0.05$; n=8).
These results demonstrate that the AII antagonist EXP3174, in contrast to captopril, exerts direct effects on renal function in the isolated organ and reveals potentiation of furosemide-induced diuresis, possibly by specific interaction with receptors on afferent/efferent arterioles and/or on proximal tubular cells.

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THE TRULY ISOLATED MUCOSA OF GUINEA-PIG DISTAL COLON IS NOT SENSITIVE TO TETRODOTOXIN (TTX) G. Sprakties and P. Kaufmann

Two in vitro preparations of guinea-pig distal colonic mucosa were characterized in their responses to carbachol, an inductor of electrogenic Cl secretion. Tissues were scraped using a razor blade to isolate the epithelium or used as full-thickness preparations. As could be shown by light microscopy, the scraped preparation (S) was practically devoid of submucosal tissue. In voltage-clamp experiments, the two preparations responded to carbachol ($2*10^{-5}$ mol/l, serosal side) in a comparable, but not identical manner. After sharp increases in short-circuit current (I_{sc}) to 16.7±1.1 (S) or 10.3±1.2 μmol/cm^{2}h (native preparation, N), the I_{sc} decreased exponentially. In S, it returned to pre-treatment values within 60 min. In N, spontaneous I_{sc} significantly exceeded that found in S; responses to carbachol were smaller, but still visible after a 60 min period. In sum, the same amount of current (AUC, 4.1±0.4 μmol/cm^2) was produced in S and N. Atropin (10^{-5} mol/l, serosal bath) totally blocked the effects of carbachol. After a 30 min preincubation of N with TTX (10^{-6} mol/l, serosal side) the high spontaneous I_{sc} values and the total amount of carbachol-inducible I_{sc} were significantly reduced; in S, no influence of TTX could be detected. TMB8 ($2*10^{-4}$ mol/l, serosal side), an intracellular Ca-channel blocker, lowered spontaneous I_{sc} in N and reduced the amount of inducible I_{sc} in S and N. In N, this effect was due to an accelerated decline of I_{sc} after an unchanged peak increase. In S, the decrease was caused by a drastic drop of peak stimulation with no significant change after a 60 min exposure. Our data show that drug effects on in vitro preparations may be considerable modified by submucosal structures. The scraped tissue preparation allows direct assessment of the responses of epithelial cells to experimental treatment.

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EFFECTS OF PGE_2, MISOPROSTOL, NOCLOPROST AND RIOPROSTIL ON PROTEIN AND RNA PRODUCTION OF ISOLATED GASTRIC MUCOUS CELLS
A. Osterwald, H.-K. Heim, A. Oestmann and K.-Fr. Sewing

Introduction: E-type prostaglandins are known to enhance weight and thickness of the gastric mucosa "in vivo". To examine possible underlying effects at the cellular level we investigated the influence of the E-type prostaglandins PGE_2, misoprostol, nocloprost and rioprostil on protein and RNA production of isolated gastric mucous cells. Methods: Gastric mucous cells were isolated from pig gastric fundic mucosa (pronase/collagenase) and enriched (>70%) by counterflow centrifugation. Incorporation of [^{3}H]L-leucine and [^{3}H]uridine into cellular acid-precipitable material was used as a probe for protein and RNA synthesis, respectively, of cells incubated for up to 20 h in Dulbecco's modified Eagle's medium without (control) or with the prostaglandins (100 pmol/l to 10 μmol/l). Results: Significant effects of PGE_2 (1 μmol/l) on RNA and protein synthesis of gastric mucous cells were detectable from 4 and 8 h, respectively, up to 20 h of incubation. Stimulation by PGE_2 after 20 h (incubation time used in all further experiments) was concentration-dependent with EC_{50}-values of 9 and 38 nmol/l, respectively. The other tested E-type prostaglandins also resulted in concentration-dependent stimulations, the rank order of potency being PGE_2 > misoprostol > nocloprost > rioprostil for both measured parameters. Conclusions: Our results indicate that direct stimulatory effects of E-type prostaglandins on protein and RNA synthesis of gastric mucous cells are involved in their stimulation of weight and thickness of the gastric mucosa "in vivo".

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STIMULATION OF ENTEROCYTE PROTEIN KINASE C BY LAXATIVES IN VITRO A.Schirgi-Degen and E.Beubler

Phorbolesters, the active principle of Croton oil, a strong cathartic, are known to stimulate protein kinase C (PKC) directly.
Activation of PKC induces intestinal ion and fluid secretion in vivo and in vitro, possibly via stimulation of prostaglandin formation, effects, that are elicited by laxatives in a similar way.
We tested the effect of the phorbolester 4-ß-phorbol 12-myristate 13-acetate (PMA), the laxatives deoxycholic acid (DCA), deacetyl-bisacodyl (dBIS), ricinoleic acid (RA), and the C_{18}-fatty-acids oleic acid (OA) and stearic acid (StA) on PKC-activity in a preparation of lysed rat enterocytes.
Rat jejunal epithelial cells were lysed and mixed with the substances to be tested in a L-α-phosphatidylserine and Triton-X-100 containing solution. Buffers and PKC-specific peptide were taken from the "Protein kinase C enzyme assay system" (Amersham, RPN77). The solutions were incubated with [^{32}P]ATP, phosphorylation activity was determined and expressed as pmoles phosphate transferred per minute. PMA dose-dependently (2, 20 and 200 µg/ml)stimulated PKC-activity from 1.6 to 5.1 pmol/min. DCA, dBIS and RA (2, 20 and 200 µg/ml)stimulated PKC-activity from about 0.1 to 0.6 pmol/min in a dose-dependent manner. OA (200 µg/ml) elicited a slight PKC-activation (0.2 pmol/min), StA (200 µg/ml) inhibited phosphorylation activity significantly.
The effects of RA, OA and StA obviously run in parallel concerning PKC-activation, fluid secretion and prostaglandin E_2-release, RA beeing most effective in stimulating PKC-activity, inducing secretion and PGE_2-formation. StA is an inhibitor of PKC-activity and fluid secretion, and has no effect on PGE_2-formation, OA takes a mid position concerning these three parameters.
The laxatives tested are about ten times less active than PMA concerning PKC-activation, but PMA on the other hand is also ten to fifty times more potent in inducing secretion and PGE_2-formation.
We conclude, that the laxatives tested are direct stimulators of PKC, thus inducing PGE_2-formation via activation of phospholipase A_2, which finally results in fluid secretion.

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ROLE OF NITRIC OXIDE IN GASTRIC MUCOSAL HYPERAEMIA DUE TO ACID BACK-DIFFUSION P. Holzer and Irmgard Th. Lippe

Acid back-diffusion through a disrupted gastric mucosal barrier leads to gastric mucosal hyperaemia which in the rat stomach is mediated by a neural reflex. The mediators of this vasodilator response have not yet been explored except that a participation of acetylcholine and histamine has been ruled out. Since there is evidence that endothelium-derived nitric oxide (NO) is involved in the regulation of gastric mucosal blood flow (MBF), the possible participation of NO in the gastric mucosal hyperaemic response to acid back-diffusion was examined.

Acid back-diffusion was elicited by perfusion of the stomach of anaesthetized rats with dilute ethanol in 0.15 N HCl, and MBF was measured by the hydrogen gas clearance technique.

N^G-nitro-L-arginine methyl ester (L-NAME; 13 and 43 μmol kg^{-1} i.v.), an inhibitor of NO formation, increased mean arterial blood pressure (MAP) in a dose-dependent manner. Whilst basal MBF was hardly changed, the hyperaemia caused by gastric perfusion with dilute ethanol in acid was dose-dependently depressed by L-NAME. The loss of H^+ ions from the gastric lumen, an indirect measure of acid back-diffusion, was significantly enhanced by 43 μmol kg^{-1} L-NAME. In contrast, D-NAME (13 and 43 μmol kg^{-1}) was without effect on MAP, basal and stimulated MBF, and acid back-diffusion. Infusion of L-arginine (120 μmol kg^{-1} min^{-1} i.v.) led to a partial, but significant, reversal of the effects of L-NAME on MAP and hyperaemia due to acid back-diffusion.

These findings indicate that NO plays an important mediator role in the gastric mucosal hyperaemia due to acid back-diffusion.

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ARE POTASSIUM COMPETING PROTON PUMP INHIBITORS SUBSTRATES OF CYTOCHROME P450 DEPENDENT ENZYMES ?
W.A.Simon

The H,K-ATPase acting as proton pump in the apical membrane of the parietal cell belongs to the E1E2 class of ATPases which execute the vectorial counter-transport of hydrogen and potassium ions. Potassium binds to the E2P form at the extracytosolic face of the enzyme with high affinity. This in turn induces the dephosphorylation of the H,K-ATPase. The blockade of the K-binding leads to inhibition of the proton pump. This is found with substituted imidazopyridines (SIPs) like SCH28080 (8-benzyloxy-3-cyanomethyl-2-methylimidazo-[1.2-a]pyridine) which are shown to be competitive inhibitors of the K-binding site. The SIPs are potent inhibitors of acid secretion in vitro as well as in vivo. However, after oral administration in dog, the bioavailability of the compounds is quite low. The suspicion was that the SIPs undergo rapid biotransformation in the liver which leads to inactivation of the compounds. This was confirmed with SCH28080 and its 3-methyl and 3-hydroxymethyl derivatives. The debenzylation at position 8 was also identified as additional possible metabolic pathway of the SIPs. SIPs with different substituents at position 3 were compared in respect to their inhibitory potency in isolated gastric glands and their affinity to cytochrome P450 measured as 7-ethoxycoumarin dealkylase activity.
The following conclusions can be drawn:
1. Substituents at 3-position are crucial for interaction with the H,K-ATPase.
2. The SIPs may be substrates and inhibitors of cytochrome P450 as well. Substituents at 3-position seem to be the main target for cytochrome P450 attack.
3. Bulky substituents at 3-position reduce markedly H,K-ATPase inhibition.

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PGE_2 LARGELY SUBSTITUTES FOR CA^{2+} AS A COFACTOR OF HCO_3^- IN TIGHTENING GUINEA-PIG DUODENAL MUCOSA H.J. Macherey

20 mmol/l HCO_3^- decrease electrical tissue conductance (G_t) of guinea-pig duodenal mucosa by a Ca^{2+}-requiring mechanism. Along with G_t, permeabilities of Na^+,Cl^-, and mannitol are reduced (Macherey, Naunyn-Schmiedeberg's Arch. Pharmacol. 343:R76,1991). The nature of the Ca^{2+} requirement was further studied, using stripped preparations mounted in Ussing-type chambers and short-circuited continuously. In concentration-response experiments, 1.2 mmol/l Ca^{2+} shifted the EC_{50} for HCO_3^- from ≈35 to ≈15 mmol/l, without major changes in the maximum decrease in G_t (8-10 mS/cm^2) at 60-80 mmol/l HCO_3^-. Addition of 1.2 mmol/l Ca^{2+} to nominally Ca^{2+}-free HCO_3^- Ringer's (20 mmol/l) produced a G_t drop from 24.8±1.8 to 16.1±1.1 mS/cm^2 (n=12) within 40 min. This decrease was mimicked by serosal addition of PGE_2 (10^{-6} mol/l), G_t falling from 24.8±2.5 to 17.6±1.6 mS/cm^2 (n=11). As detailed previously (Macherey and Petersen, Gastroenterology 97:1448-1460,1989), availability of prostaglandins, added to the bath in the presence of the cyclooxygenase inhibitor indomethacin or synthesized within the tissue, is a prerequisite for the G_t reduction by HCO_3^-. Pretreatment with the cyclooxygenase inhibitors meclofenamate or diclofenac ($3x10^{-4}$ mol/l) was able to reduce by ≈45% the G_t effect of 1.2 mmol/l Ca^{2+}. Our data suggest that Ca^{2+} serves to maintain the activity of phospholipase A_2 to supply arachidonic acid for prostaglandin synthesis. In a semilog plot, a linear decrease in G_t (20 mmol/l HCO_3^- present) was found at bath concentrations of Ca^{2+} in a range from 36 µmol/l - 1.2 mmol/l. Hence, in the presence of HCO_3^-, Ca^{2+} regulation of permeability would fall into the physiological range of extracellular Ca^{2+} concentrations.

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BIPHASIC STIMULATION BY BRADYKININ OF ELECTROGENIC HCO_3 SECRETION IN GALLBLADDER EPITHELIUM K.-U. Petersen and F. Höfken

In guinea-pig gallbladder epithelium, cAMP converts electroneutral secretion of HCO_3 into an electrogenic process. Bradykinin (BK) has been reported to elicit electrogenic HCO_3 secretion (Baird and Margolius, J. Pharmacol. Exp. Ther. 248:268, 1989), an effect we have substantiated and further analyzed. Voltage clamp and pH-stat techniques were used for in vitro determination of short-circuit current (I_{sc}) and unidirectional HCO_3 fluxes (J_{ms}, absorptive, J_{sm}, secretory direction), respectively. Serosal addition of BK produced concentration-dependent increases in I_{sc} (lumen negative). At 30 µmol/l, I_{sc} rose, at steady state, by ≈2.8 µmol/cm²h (n=4), preceded by a transient increase to ≈3.4 µmol/cm²h. In pH-stat experiments, J_{sm} and I_{sc} rose from 2.9±0.4 to 3.6±0.5 and from 0.3±0.04 to 1.9±0.1 µmol/cm²h, respectively (n=4). There was a small though significant decrease in J_{ms} from 0.8±0.1 to 0.7±0.1 µmol/cm²h (n=7). Thus net HCO_3 secretion still exceeded I_{sc}, indicating an incomplete conversion of electroneutral secretion, similar to effects of low concentrations of PGE_1 (Petersen, unpublished). Pretreatment with the cyclooxygenase inhibitor indomethacin (10 µmol/l) prevented the I_{sc} response to BK. With the phospholipase A_2 inhibitor, mepacrine (100 µmol/l), peak response was more than halved and steady-state increase was missing. Complete peak suppression was achieved by combined pretreatment with 5 mmol/l $BaCl_2$ (both sides) and mepacrine. The intracellular Ca antagonist, TMB8 (200 µmol/l), alone or in combination with mepacrine, forestalled the entire peak response. When added alone, TMB8 permitted, after a considerable delay, a slowly rising I_{sc} response. In conclusion, BK causes a partial conversion of electroneutral into electrogenic HCO_3 secretion. This effect seems to be mediated by a moderate increase in prostaglandin synthesis. Mostly during the early phase, conductive anion exit at the apical membrane may be boosted by opening of K channels, secondary to intracellular release of Ca.

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The use of isolated perfused duodenal segments to quantitate the transfer of ^{59}Fe from the duodenal tisue into the serosal absorbate

K. Schümann, W. Forth

In Fe-deficiency intestinal Fe absorption is increased. The mechanisms of the underlying absorption process is not well understood. Isolated duodenal segments from normal and iron-deficient rats were perfused with ^{59}Fe-NTA(1:2)containing Tyrode solution in vitro (1-500 µmol/l; Fisher-Parsons-method) in order to load the segments with ^{59}Fe. When the ^{59}Fe-uptake and ^{59}Fe-absorption process had reached a steady state (loading period) ^{59}Fe absorption rates and ^{59}Fe content in the mucosa were determined. The segments were then flushed and transfered into a second perfusion apparatus, in which they were perfused under identical conditions (mobilizing period). The perfusion medium either contained no additives, or unlabeled Fe-NTA and NTA were offered in concentrations corresponding to those used during the loading period. Under these conditions transient ^{59}Fe was mobilized to different degrees from the segments. Its cumulative appearance in the serosal absorbate corresponded to the serosal transfer step. It fitted first order rate kinetics and could be mathematically described ($M = A(1-e^{kt})$. Thus, extent (asymptote A) and velocity (rate constante k) of the ^{59}Fe transfer from the tissue into the serosal absorbate could be calculated.

When unlabelled Fe-NTA was offered the ^{59}Fe fraction availabel for serosal transfer was increased because the intermediary iron pool was mobilized to a greater extent. In iron-deficiency the availabel ^{59}Fe fraction in the tissue was enlarged because the mucosal ^{59}Fe uptake increased while the ^{59}Fe content of the segments after the mobilization period was unaltered. The rate constants for the ^{59}Fe transfer to the serosal side were not influenced by changes in the iron state or in the perfusion medium during the mobilizing period.

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INTERACTION OF COLLOIDAL BISMUTH SUBCITRATE WITH ENZYMATIC AND NON-ENZYMATIC SULFHYDRYL-GROUPS. W. Beil, S. Bierbaum

Colloidal bismuth subcitrate (CBS) precipitates in the stomach at low pH as bismuth oxychloride and bismuth citrate. Animal studies have shown that the bioavailability of bismuth is increased in the presence of SH containing compounds (cysteine, penicillamine), indicating that SH containing compounds form water soluble complexes with bismuth. To investigate how CBS reacts with SH groups we studied the reaction pathway of CBS with the dithiol dithiothreitol (DTT), the monothiol reduced glutathione (GSH), and the thiol enzymes H^+/K^+-ATPase (which possesses 4 free reactive SH groups/mol enzyme) and papain (which possesses one SH group/mol enzyme).

Methods: The number of free thiol groups was determined with Ellman reagent (DTNB). H^+/K^+-ATPase activity in purified intact, inside-out gastric membrane vesicles was measured by the release of P_i from ATP, H^+/K^+-ATPase-mediated H^+ transport was monitored with the pH-sensitive dye acridine orange. Papain activity was determined with the substrate N-benzoyl-arginine-p-nitroaniline.

Results: CBS reduced the number of free SH groups of DTT in a concentration dependent manner, but not of GSH. CBS inhibited H^+/K^+-ATPase activity (IC_{50}: 23 µmol/l) and H^+/K^+-ATPase-mediated proton transport but did not affect papain activity. The mercaptan dithioerythritol (DTE) (100 µmol/l) totally prevented CBS (50 µmol/l)-induced inhibition of H^+/K^+-ATPase-mediated proton transport. In contrast, the protective action of GSH (100 µmol/l) on H^+/K^+-ATPase activity was transient, i.e. it faded with increasing incubation time. DTE (1 mmol/l) fully restored inhibited (50 µmol/l CBS) enzyme activity, whereas GSH (1 mmol/l) did it incompletely.

Conclusions: CBS is a thiol reagent. CBS forms stable complexes with dithiols and reacts transiently with monothiols.

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THE SOMATOSTATIN ANALOGUE SMS 201-995 (OCTREOTIDE, SANDOSTATIN®) IS A POTENT INHIBITOR OF BETHANECHOL-STIMULATED PEPSINOGEN SECRETION IN GASTRIC FISTULA DOGS

Th. Buhl, A.W. Dunbar

Besides acid, pepsin is an important aggressive factor in gastric juice which rapidly induces peptic lesions of the gastric or duodenal mucosa if aggressive factors outweigh mucosal protection. Therefore, inhibition of pepsinogen secretion might be an alternative approach to suppressing acid secretion for the treatment of peptic ulcer disease. Animal *(1)* and human *(2)* studies have shown that somatostatin-14 when administered by continuous infusion potently inhibits acid and pepsinogen secretion. But, as pepsinogen and pepsin are chemically stable only in an acidic environment, it was always a problem to quantify pepsinogen secretion under conditions where acid secretion is largely or completely inhibited. Therefore, we set up and validated a model that allowed us to quantify pepsinogen secretion almost independently from simultaneous effects on acid secretion in gastric fistula dogs *in vivo*. Stomachs were continuously perfused with hydrochloric acid (0.02 N, 2 ml/min). Acid perfusion kept gastric pH < 4 during the whole experiment and did not affect basal or bethanechol-stimulated pepsinogen secretion. In this model we characterized the effect of the long-acting somatostatin analogue SMS 201-995 and of the H_2 antagonist cimetidine on pepsinogen secretion. SMS 201-995 (s.c.) completely and dose-dependently inhibited bethanechol-stimulated (60 µg/kg s.c.) pepsinogen secretion with an ID_{50} (dose leading to a 50 % inhibition) of 50 ng/kg. Under these same conditions, cimetidine did not significantly inhibit pepsinogen secretion in doses up to 30 mg/kg (p.o.). In ***conclusion***, our data show that SMS 201-995 is an extremely potent inhibitor of pepsinogen secretion in gastric fistula dogs *in vivo*, whereas the H_2 antagonist cimetidine has no effect on this parameter which is independent of its inhibitory effect on gastric acid secretion.

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THE EFFECTS OF 5-AMINOSALICYLIC ACID, SULFASALAZINE AND ZILEUTON IN A NEW MODEL OF INFLAMMATORY BOWEL DISEASES

K. Auler and N. Selve

Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis represent chronic inflammations of the gastrointestinal tract of unknown etiology involving immunologic events. Research on the etiopathogenesis of IBD have been hampered by the paucity of reproducible and relevant animal models of chronic intestinal inflammation. A new model of IBD was developed by realising the following 3 step concept, using a defined hapten:
1. specific hypersensitivity by active immunisation
2. local inflammation by local challenge
3. chronification by chronic application of the immunogen.
The hapten chosen was TNBS (2,4,4-trinitrobenzenesulfonic acid). Rats were sensitised by intradermal injection of 0.8 % TNBS in 0.05 ml Freund's complete adjuvant with 1 mg/ml ovalbumine once for 3 consecutive days, and boostered on day 18. This treatment induced a long lasting (6 month) delayed type of immunologic reaction to TNBS. Pre-immunised animals received intrajejunal challenge with 0.08 % TNBS in 0.2 ml saline/rat via an implanted catheter for 21 consecutive days. This treatment induced chronic inflammation of the distal small intestine characterised by intense hyperaemia, oedema and gut wall thickening as assessed by macroscopic scoring and weighing a defined part of the dissected intestine. To prove the relevance of this model animals were orally treated with clinically relevant drugs, 5-aminosalicylic acid (5-ASA) and sulfasalazine (SS). 5-ASA, SS and the newly developed 5-lipoxygenase inhibitor zileuton dose dependently improved enteritis score and reduced gut wall thickening. These results indicate similarities to human inflammatory bowel diseases. So this model may be a suitable new animal model and a useful tool for testing new drugs of IBD.

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NUTRITIONAL REGULATION OF GLUCOKINASE AND GLUT-2 GLUCOSE TRANSPORTER GENE EXPRESSION IN PANCREATIC ISLETS AND LIVER FROM RATS

M. Tiedge and S. Lenzen

Glucokinase, a high K_m glucose phosphorylating enzyme, is the signal recognition enzyme for glucose-induced insulin secretion. Glucose enters the B-cell of the pancreas through the high K_m glucose transporter GLUT-2 via facilitated diffusion. The aim of this study was to investigate the effect of fasting and refeeding on the expression of the glucokinase and GLUT-2 gene in pancreatic islets compared with liver. Wistar rats were fed a copper-deficient and D-penicillamine-substituted diet to reduce the portion of the exocrine pancreas. This model permitted the measurement of glucokinase and GLUT-2 gene expression in mRNA fractions of total pancreas tissue. Glucokinase mRNA is expressed in pancreatic islet tissue in a high molecular 4.4 kb and a previously described 2.8 kb form. A fasting period of two days reduced the 2.8 kb form to less than 50 % of the fed control. Refeeding for 4 hours reconstituted the low molecular form to the level of the fed control animal with a 2.8 kb and a 2.4 kb transcript which could be a product of mRNA splicing. The 4.4 kb transcript of glucokinase was drastically reduced during refeeding. The GLUT-2 glucose transporter gene expression was identically regulated under different nutritional states in pancreatic islets and liver with a 50 % decrease after fasting and a slow reconstitution after refeeding. These results demonstrate the complex regulation of glucokinase gene expression in pancreatic islets compared with liver and may reflect the function of a signal recognition enzyme for glucose-induced insulin secretion.

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INHIBITION OF PROTEIN PHOSPHATASES OF RINm5F CELLS BY OKADAIC ACID *R.O. Heurich and H.P.T. Ammon*

Protein phosphatases are important antagonists of protein kinases in a variety of cell functions and are suggested to be related to the regulation of calcium channel activity. To evaluate a possible role of protein phosphatases in insulin release, we examined the effect of okadaic acid on phosphorylase *a* phosphatase activity and insulin secretion using RINm5F cells. Okadaic acid is a specific inhibitor of type 1 and type 2A protein phosphatases. Phosphorylase *a* phosphatase activity was determined by measuring the release of radioactive inorganic phosphate from [^{32}P] - labeled phosphorylase *a* in homogenates of RINm5F cells. The specific activity of phosphorylase *a* phosphatase was dependent on the dilution of the homgenate before the assay and ranged from 0.33 $\pm$ 0.08 mU/mg to 3.79 $\pm$ 1.31 mU/mg (n = 3, SD).
Okadaic acid inhibited phosphorylase *a* phosphatase activity in non-stimultated RINm5F cells in a concentration dependent manner. Complete inhibition was achieved with 10^{-6} M okadaic acid, the IC_{50} being 10^{-8} M.
Addition of okadaic acid to intact RINm5F cells in the same concentration range did not affect basal or KCl (25 mM) - stimulated release of insulin in 60 minute batch incubations.
Our data indicate that RINm5F cells contain phosphorylase *a* phosphatase activity, which can be inhibited by okadaic acid. However, okadaic acid did not affect insulin release. Wether the ineffectiveness of okadaic acid on insulin secretion is due to the fact that protein phosphatases 1 and 2A are not related to the mechanism of insulin release or to other reasons, including poor permeability of okadaic acid through the cell membrane, remains to be established.

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EFFECT OF PHORBOL ESTER TPA ON KINETICS OF cAMP RELEASE FROM RINm5F CELLS. E.M. Mosbacher

Release of cAMP from the intracellular to the extracellular space has been suggested to correlate with the intracellular levels of cAMP. Phorbol esters - activators of protein kinase C - have been shown to interfere with the adenylate cyclase / cAMP system.
In RINm5F cells forskolin (100 µM) increased cAMP-levels, the maximum was achieved at 20 minutes and thereafter remained nearly constant. Release of cAMP into the medium was approximately linear over a period of 60 minutes, being 0.6 pmol / mg protein per min. Addition of the phorbol ester TPA (100 nM) to the incubation medium diminished release of cAMP (40 %) in response to forskolin without affecting intracellular cAMP levels. TPA was without effect on basal release of cAMP.
In RINm5F cells preincubated with TPA (100 nM) for 24 hours - a precedure, known to cause depletion of PKC - addition of TPA to the incubation medium over a period of 60 minutes again did not affect basal and forskolin-mediated increase of intracellular cAMP. However, the 24 hours preincubation of RINm5F cells with TPA abolished the inhibitory effect of short-term incubation with TPA on cAMP release.
The data suggest that activation of PKC by TPA is involved in the diminished cAMP release during forskolin-induced cAMP production.

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ACTIVIN A: ITS EFFECTS ON RAT PANCREATIC ISLETS AND THE POSSIBLE MECHANISMS OF ACTION INVOLVED

E.J. Verspohl*, M.A. Wahl

Activin A is a 26 kD polypeptide which stimulates FSH (follicle-stimulating hormone). In rat pancreatic islets 10 nM activin A increased insulin secretion at either 3.0, 8.3 or 16.7 mM glucose from 111 to 144, 318 to 584, and 536 to 932 μU/5 islets x 90 min, respectively ($p<0.05$). The effect on insulin release was concentration dependent; significant increases were obvious at both 1 and 10 nM activin A. $^{86}Rb^+$ efflux as an indicator of potassium channel activity was inhibited at 3.0 mM glucose by 10 nM activin A. The effect on insulin release was paralleled by an effect on $^{45}Ca^{2+}$ net uptake except at 3.0 mM glucose. 10 nM activin A were effective in elevating Ins-1,4,5-P_3 content (0.398 vs. 0.227 pmoles/100 islets at 16.7 mM glucose). It decreased glucagon secretion at 3.0, had no effect at 8.3 and increased glucagon secretion at 16.7 mM glucose. The data indicate (1) that activin A elevates insulin release at 3.0 mM glucose by affecting potassium channel activity without significantly influencing Ca^{2+} channels, at 8.3 and 16.7 mM glucose without further inhibiting $^{86}Rb^+$ efflux but increasing $^{45}Ca^{2+}$ uptake. (2) Accumulation of Ins-1,4,5-P_3 is involved at least at high glucose concentrations. (3) Glucagon release is modulated in a specific manner thus contributing to the enormous insulinotropic effect of the peptide at a high glucose concentration but marginal effect at a low glucose concentration.

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THE EFFECT OF THE PROTONOPHORE NIGERICIN ON THE INOSITOL 1,4,5 - TRISPHOSPHATE - SENSITIVE CALCIUM STORE IN RAT INSULINOMA CELLS

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In several types of cells, for example exocrine pancreas (Thevenod et al. 1989, J.Membr.Biol.109, 173-186) or parotid gland, calcium uptake into the inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium store depends on an inside to outside-proton gradient maintained by a vacuolar-type proton pump. To investigate whether this might apply to endocrine cells, too, we have examined the effect of protonophores and bafilomycin, an inhibitor of vacuolar proton pumps, on calcium uptake by non-mitochondrial calcium stores in saponin-permeabilized RINm5F cells. The potassium proton exchanging protonophore nigericin (1-10 μM) inhibited calcium uptake by and released calcium from nonmitochondrial and IP_3-sensitive calcium stores in these cells, suggesting the involvement of a proton gradient in calcium sequestration. However, neither bafilomycin nor NH_4Cl, which dissipates proton gradients, had any effect on nonmitochondrial calcium uptake. This rules out an involvement of a vacuolar proton pump in calcium uptake under the experimental conditions as well as a strict dependence of nonmitochondrial calcium uptake in RINm5F cells on a proton gradient. Nonmitochondrial calcium uptake was completely inhibited by vanadate. It is concluded that the intracellular, IP_3-sensitive calcium store in RINm5F cells is filled by a calcium pump, which might operate as a calcium proton exchanger (similar to plasma membrane calcium pumps), nigericin producing an alkalinization of this store, thereby depleting the proton supply for the action of such a calcium pump.

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ROLE OF THE ADRENAL GLAND IN THE HYPERTENSIVE TRANSGENIC RATS TGR(MREN2)27

M. Sander, B. Djavidani, M. Bader and J. Peters

Transgenic rats with the murine ren2d gene are a monogenetic model for hypertension characterized by high plasma prorenin, low plasma renin, angiotensin I and II. The transgene is highly expressed in extrarenal tissue, especially in the adrenal gland. Measurement of the steroid excretion pattern revealed significantly higher levels for deoxycorticosterone (DOC), corticosterone, 18-OH corticosterone (18-OH B) in young (6-15 weeks) heterozygous animals than in SD-rats. The elevated steroid excretion is paralleled by the development of hypertension. Four week treatment with spironolactone (50 mg/kg bw) did not lower blood pressure neither in young nor in adult animals. Plasma 18-OH B and aldosterone is only slightly elevated in heterozygous rats, in contrast homozygous animals have 5 respectively 9 fold higher 18-OH B and aldosterone levels than SD-rats. These results provide evidence for the dependence of plasma 18-OH B and aldosterone on the number of copies of the transgene. ACTH stimulation led to a significant increase in DOC and corticosterone excretion compared to SD-rats. Plasma prorenin and angiotensin I markedly increased in transgenic animals due to the ACTH injection. In contrast angiotensin I decreased in SD-rats. Dexamethasone was capable of suppressing hypertension development in the TGR (control: 223 mmHg, 25 μg: 203 mmHg, 100 μg 165 mmHg) being paralled by a decrease in plasma prorenin (control: 392 ng ANGI/min*h, 25 μg: 245 ng, 100 μg: 184 ng).
These data indicate that not only mineralocorticoids but possibly a combination of different perhaps undefined ACTH dependent steroids are involved in hypertension development of TGR(mRen2)27. The transgene in the adrenal gland seems to be regulated by ACTH. TGR(mRen2)27 are a suitable model to study the interaction between an intraadrenal RAS and adrenal steroid production.

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ADRENERGIC RECEPTOR AGONISTS AND ANTAGONISTS AFFECT ANDROGEN PRODUCTION IN CULTURED MOUSE LEYDIG CELLS.

F.W. Jekat and M.L. Meisel

Adrenergic receptor agonists and antagonists are clinically well established and widely used as drugs. Thus a possible impact on androgen production is of distinct interest. The effects of oxymetazoline, norfenefrine, dopamine, etilefrine, isoprenaline, terbutaline, propranolol, nadolol and atenolol were studied in an in-vitro model using a primary culture of mouse testicular interstitial cells.
A procedure described by van Damme was modified and crude cells were mechanically dispersed by shaking and the suspension was filtered through a nylon gauze. After centrifugation at 80g for 10 minutes the cells were preincubated for one hour, washed and resuspended, and then the test substances as well as LH (luteinizing hormone) were added for a total incubation time of 3 hours. After that period the samples were diluted, shock frozen and stored deep frozen for subsequent determination of testosterone by radioimmunoassay.
In these in-vitro experiments none of the substances enhanced the stimulating effect of LH. Norfenefrine, etilefrine, terbutaline, atenolol and nadolol showed no or only minor effects on the LH-stimulus on testosterone-production. Oxymetazoline, dopamine, isoprenaline and propranolol revealed a clear and dose-dependent reduction of the LH induced testosterone synthesis.
The obtained data suggest that under experimental conditions some of the substances act on testicular tissue, perhaps by inhibition of early steps of androgen synthesis.

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STIMULATION WITH THYROID STIMULATING HORMONE OR THYROID STIMULATING ANTIBODIES IS REDUCED BY EXTRACTS FROM LYCOPUS EUROPAEUS

M. Lücke, H. Winterhoff

Extracts from Lycopus species are used for the treatment of moderate forms of hyperthyroidism. Antithyrotropic activity of these extracts has been demonstrated, but intrathyroidal effects are not yet confirmed. Thyroid stimulating antibodies (TSAb) are responsible for development of hyperthyroidism and can be isolated from sera of patients with Graves'-disease.
Our studies should clarify now, whether the TSH- or TSAb-caused increase in cAMP-accumulation and iodine uptake of a continuous thyroid cell line (FRTL 5) could be diminished by Lycopus europaeus-extract.
For these investigations FRTL 5-cells were cultured according to the methods of Ambesi-Impiombato. After incubation with TSH, TSAb, Lyc. eur.-extract or mixtures of these, cAMP-accumulation in medium and iodine uptake of the cells were measured.
TSH-effects on cAMP-accumulation and iodine uptake were inhibited in a similiar dose-dependent matter. On the contrary cAMP-accumulation was reduced and iodine uptake was increased by the simultaneous administration of TSAb and Lycopus extracts.
These results indicate, that Lycopus inhibits not only the receptor binding (as previously described) but has also an intracellular point of attack.

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MECHANSIM OF ERYTHROPOIETIN-INDUCED HYPERTENSION: IMPORTANCE OF TRANSMEMBRANE CALCIUM INFLUX.

M. Tepel, M. Neusser, K.H. Rahn, W. Zidek

Hypertension is one of the most important side effects during the therapy of renal anemia with recombinant human erythropoietin (EPO). To evaluate the mechansims by which EPO causes hypertension the effect of EPO on microdissected renal resistance vessels from rats was determined. EPO had direct vasopressor effects on resistance vessels in a calcium containing suspension. Using 20 U/ml EPO the active renal vessel wall tension was 0.43 $\pm$ 0.02 mN (mean $\pm$ SEM, n=8). The effect of EPO was abolished in a calcium-free suspension.
Cytosolic free calcium concentration was measured in cultured vascular smooth muscle cells from normotensive rats using the fluorescent dye fura2. The resting cytosolic free calcium concentration was 85.0 $\pm$ 7.8 nM (mean $\pm$ SEM, n=28). Addition of 100 U/ml EPO increased cytosolic free calcium concentration by 22.1 $\pm$ 25.8 nM within 100 sec. EPO did not increase cytosolic free calcium concentration in a calcium free suspension. EPO dose-dependently increased angiotensin II induced rise of cytosolic free calcium in vascular smooth muscle cells. At saturation doses of 250 U/ml EPO cytosolic free calcium concentration was increased by 67.4%.
In addition the cytosolic free calcium concentration was measured in intact human blood platelets. The resting cytosolic free calcium concentration was 59.8 $\pm$ 7.0 nM (mean $\pm$ SEM, n=25). EPO dose-dependently elevated thrombin-induced changes of cytosolic free calcium in platelets. At saturation doses of 250 U/ml EPO cytosolic free calcium concentration was elevated by 51.8%. The apparent K_D of EPO out of these experiments was 1.1 nM.
It is concluded that EPO causes its vasopressor effects by transmembrane calcium influx and thereby elevating cytosolic free calcium concentration. In addition EPO amplifies the effects of synergistic vasopressor hormones.

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MAST CELL DEGRANULATION ENHANCES EVOKED NEUROTRANSMITTER OVERFLOW ON THE RAT ISOLATED HEART

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Mast cell degranulation can be activated by immunological or non-immunological stimuli and leads to the release of various mediators such as histamine (HIS). We examined the interaction between stimuli-induced exocytosis of HIS-containing cells and the nerve stimulation evoked noradrenaline (NA) or ^{14}C-acetylcholine (ACH) release in the rat isolated perfused heart with intact extrinsic sympathetic or vagal innervation. In the perfusates, HIS was determined fluorometrically after precolumn derivatization and HPLC-separation, NA by HPLC-coupled electrochemical detection and ACH by liquid scintillation spectroscopy after preloading the heart with ^{14}C-choline. Upon perfusion with the mast cell degranulating agent compound 48/80 (48/80), HIS overflow increased about tenfold compared to basal overflow, the basal NA or ACH overflow remained unaffected; the stimulation (270 pulses at 3 Hz)-evoked NA overflow was enhanced by 34.2 ± 4.7 %, (n=4). This increase was abolished in the simultaneous presence of the HIS-antagonists mepyramine (MEP, 10^{-6}M, H_1), cimetidine (CIM, 10^{-5}M, H_2) and thioperamide (THIO, 10^{-7}M, H_3); it remained unaffected when MEP, CIM or THIO were administered separately. Exogenous HIS (10^{-6}M) or the H_3-selective agonist R-(-)-α-methylhistamine (10^{-6}M) inhibited the evoked NA overflow marginally (-11.6 ± 3.2 %, n=4; -19.6 ± 8.1 %, n=4); the inhibition by HIS was abolished by THIO. Vagus nerve stimulation (720 pulses at 10 Hz)-induced ^{14}C-overflow was enhanced by 76.5 ± 6.5 % (n=3) upon perfusion with 48/80. This facilitation was unaffected by MEP, but further increased to 169.8 ± 18.3 % (n=4) when CIM was present. Exogenous HIS had no effect on ACH release. Neither sympathetic nor vagal nerve stimulation nor the H_1-, H_2- or H_3-antagonists affected basal or 48/80-induced HIS release. Degranulation of HIS-containing cells in rat heart by 48/80 is accompanied by an increase of evoked NA and ACH overflow. Whether other mediators besides HIS are involved in a prejunctional influence on autonomic nerves remains subject of further investigations.

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EXPRESSION OF ATRIAL NATRIURETIC PEPTIDE IN CULTURED MOUSE MACROPHAGES.

A. M. Vollmar and E. Partaanen

Exposure of rats to glucocorticoids or to X-rays causes a striking increase of ANP-expression in the acutely involuted thymus. Thymic macrophages, which are in a highly activated state of phagocytosis, have been identified to be responsible for this stimulated ANP-production. Thus, a link between ANP-expression and the activation of phagocytotic cells has been suggested. To generalize this proposal, the aim of the present study was, firstly, to investigate whether other phagocytes, such as bone marrow derived macrophages and peritoneal macrophages also synthesize ANP. Secondly, whether in vitro activation of the macrophages, e.g. by incubation with bacterial lipopolysaccharides, induces an alteration of ANP expression. By means of Northern Blot analysis, HPLC analysis of cell extracts as well as by immunocytochemical techniques the following results were obtained:
1. Mouse peritoneal macrophages and mouse bone marrow macrophages express the 950 base ANP-specific mRNA. In addition, the transcription product, ANP 1-126, was detected after HPLC separation of acidic extracts (about 500 fmol IR-ANP/ 10^8 cells) as well as by immunocytochemistry.
2. Peptone-elicited peritoneal macrophages display an approximately 2-fold higher amount of ANP-transcript as compared to resident macrophages of the peritoneum. Furthermore, activation of bone marrow macrophages by incubation with bacterial lipopolysaccharides (10μg/ml, E.choli 055:B5) resulted in a time-dependent increase of ANP-gene expression (maximum after 24 h, about 3-fold).
In conclusion, ANP represents a constituent of macrophages derived both from peritoneum as well as from bone marrow, and the degree of its expression seems indeed to be linked to the status of activation of the cells.

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GLUCOCORTICOIDS INHIBIT THE TNFα-INDUCED ACTIVITY OF THE INTRACELLULAR PHOSPHOLIPASE A_2. M. Goppelt-Struebe

Glucocorticoids have long been known to be effective inhibitors of prostanoid synthesis, especially in inflamed tissues. The molecular mechanism of the inhibition seemed to be evident, when lipocortins where thought to be the mediators of glucocorticoid effects on prostanoid synthesis. Now, as this hypothesis is rejected by most investigators, the molecular mechanism is undefined again.
We could show earlier in macrophages and monocytic cells that the two key enzymes of prostanoid synthesis, phospholipase A_2 and cyclooxygenase, both are inhibited by glucocorticoids.
We were now interested, which type of phospholipase A_2 was inhibited by glucocorticoids. As a modell system, we used the human carcinoma cell line HEp-2. In these cells we characterized a high molecular weight phospholipase A_2, which was activated by micromolar concentrations of calcium. The enzyme was found primarily in the cytosolic fraction, but associated with the cellular membranes in a calcium-dependent way. In contrast to the secreted phospholipases A_2, this type of enzyme showed a preference for the cleavage of polyunsaturated fatty acids.
When the cells were stimulated for 14 h with TNFα, one of the potent inflammatory cytokines, the activity of phospholipase A_2 was induced in a concentration-dependent manner. So far, TNFα had only been shown to activate the secreted phospholipase A_2.
When the cells were incubated with dexamethasone, the activity was decreased. There was a moderate effect on the activity in control cells. The induction of phospholipase A_2 activity by TNFα, however, was completely suppressed. This suggests that glucocorticoids most effectively inhibit the liberation of arachidonic acid and subsequent synthesis of prostanoids in activated cells.
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MACROPHAGES ARE ACTIVATED FOR SYNTHESIS OF TUMOR NECROSIS FACTOR (TNF-α) BY ANTIPROTOZOAL AGENT PENTAMIDINE S. Heidenreich, J. Crout, C. Mohr*, and D. Gemsa*

In immunocompromised patients, Pneumocystis carinii (Pc) pneumonia is a frequent, life-threatening opportunistic infection. Inhalation or systemic application of the antimicrobial agent pentamidine (PE) has been shown to be a very efficient therapy or prophylactic treatment against Pc infection. The mode of action of PE against Pc protozoa, however, is still unclear.
In the present study, we investigated whether PE affected cellular defence mechanisms of peritoneal and alveolar macrophages (MØ) in-vitro. For that, antimicrobial effector molecules TNF-α as well as reactive oxygen and nitrogen intermediates were measured in MØ culture supernatents. PE (1 μg/ml) activated MØ for enhanced TNF-α synthesis (17.2 ± 4.1 ng/ml vs. 4.9 ± 1.8 ng/ml without PE) and potentiated TNF-α release in MØ that were prestimulated with suboptimal doses of IFN-γ plus LPS. H_2O_2 release was not affected by PE, whereas NO_2^- synthesis was enhanced in IFN-γ/LPS prestimulated MØ by PE. On a molecular level we could demonstrate by northern blot analysis, that PE increased TNF-α production by post-transcriptional processes. By immunofluorescence staining of vital Pc protozoa we found, that PE and TNF-α similarly inhibited adhesion of Pc protozoa on alveolar MØ, indicating that MØ derived soluable factors might limit Pc survival.
These data suggest, that PE activates MØ for enhanced TNF-α synthesis that might contribute to defend Pc infection.

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GM-CSF IS INVOLVED IN LPS HEPATOTOXICITY

Gisa Tiegs and Bernd Matiba

LPS-induced TNF production of monocytes is known to be potentiated by GM-CSF (granulocyte monocyte colony stimulating factor) *in vitro* (Cannistra et al. 1988, Blood 71, 672). The present study describes GM-CSF effects on LPS-induced TNF release *in vivo* and *ex vivo* and correlates these effects with LPS-induced hepatotoxicity.
For *ex vivo* experiments, male Wistar rats were pretreated s.c. with 20μg GM-CSF *per* animal 24h before isolation of alveolar macrophages, bone marrow macrophages and peritoneal macrophages. Within these cells, TNF release into the supernatants was significantly enhanced 4h and 14h after *ex vivo* incubation with 10μg/ml *Salmonella abortus equi* LPS as compared to cells of control animals.
For induction of hepatitis, male NMRI mice were sensitized by 700mg/kg galactosamine i.p. and challenged by simultaneous i.p. injection of 5μg/kg LPS. Liver injury was assessed by determination of transaminases 8h after challenge, *in vivo* TNF-release was determined 90 min after LPS administration. Pretreatment of mice with 50μg/kg GM-CSF i.v. between 1h up to 24h prior to challenge led to a dramatic increase of LPS-induced serum TNF levels. Lethality was increased from 17% to 80% within 8h. Furthermore, GM-CSF pretreatment induced hepatitis after subtoxic LPS challenge (0.2μg/kg) in galactosamine-sensitized mice. The final conclusion that GM-CSF is involved in LPS-induced hepatotoxicity was demonstrated by the lack of induction of hepatitis and reduction of TNF release in LPS-challenged mice which had been pretreated with polyclonal rabbit anti mouse GM-CSF antibodies.

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INHIBITION OF THE CONCANAVALIN A AND LIPOPOLYSACCHARIDE INDUCED LYMPHOCYTE PROLIFERATION BY MURINE BONE MARROW DERIVED MACROPHAGES
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The concanavalin A (Con A) or lipopolysaccharide (Lps) induced as well as the spontaneous proliferation of spleen lymphocytes was inhibited by coculturing the cells with murine bone marrow derived macrophages (BMDM). The inhibitory effect was found to be dependent on the maturation stage of BMDM reflected by macrophages of the phenotype BM 8 appearing during cultivation and the number of BMDM added to the lymphocytes. The spontaneous proliferation was inhibited by adding 20% of BMDM cultured for 7 days. The LPS induced response was strongly suppressed with increasing proportions of BMDM either cultured for 2, 3 or 7 days. By contrast the Con A induced lymphocyte proliferation was inhibited depending on the maturation stage of BMDM e.g. by 2-20% BMDM of day 7, by 10-20% BMDM of day 3 but only by at least 20% BMDM of day 2. Addition of culture supernatant of BMDM of day 7 to the lymphocytes only slightly decreased the Con A induced but strongly enhanced the spontaneous proliferation. The Lps induced proliferation was differently affected. 10-20% supernatant increased, 40-80% decreased the proliferative activity. When lymphocyte-BMDM cell-cell contact was prevented using Teflon membranes of 0,4 um pore size BMDM only slightly inhibited the proliferation. These results show that BMDM differently influence the spontaneous and mitogen induced lymphoproliferation. They also demonstrate that the inhibitory effect of BMDM on the lymphocyte proliferation is only partially mediated by secretory products of macrophages but apparently requires cell-cell contact between both cell types.

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PARTICIPATION OF LEUKOTRIENE D_4 IN ENDOTOXIN-INDUCED CYTOTOXICITY IN LIVER CELL CULTURES

T. Hartung

In a cellular coculture system with hepatocytes and Kupffer cells from rats (Biochem. Pharmacol. 42 (1991), 1129-1135) the possible involvement of leukotrienes in mediating lipopolysaccharide (endotoxin)-inducible cell injury to hepatocytes was investigated. Seventeen different inhibitors of peptido-leukotriene synthesis significantly reduced endotoxin-inducible cytotoxicity. Protection was found using either antibodies directed against peptido-leukotrienes or two leukotriene D_4 receptor antagonists. Upon endotoxin stimulus, isolated Kupffer cells secreted a compound coeluting with leukotriene D_4 on high pressure liquid chromatography. Addition of 0.1μg/ml LTD_4 but not of LTC_4 to the cell cultures enhanced cell death induced by suboptimal amounts of endotoxin by 80%. The glutamyl transpeptidase inhibitor acivicine (AT125) but no inhibitor of LTD_4 degradation was protective in-vitro. Kupffer cells secreted tumor necrosis factor upon stimulation by endotoxin. Inhibition of LTD_4 synthesis or action also attenuated formation of TNF-α. Addition of LTD_4 enhanced LPS-induced release of TNF-α. It is concluded that LTD_4 represents an autocrine stimulus of LPS-induced secretion of TNF-α by Kupffer cells.

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RECONSTITUTION OF PROLIFERATION CAPACITY OF GSH-DEPLETED MOUSE SPLEEN LYMPHOCYTES BY DIFFERENT THIOLS

F. Gantner and A. Wendel

Glutathione-depleted lymphocytes (GSH level <40% compared to controls) are unable to respond to Concanavalin A-induced (Con A) proliferation (Hamilos and Wedner 1985, J. Immunol. 135, 2740). In order to explore the glutathione selectivity of this phenomenon, we examined the effect of several thiol compounds on reconstitution of lymphocyte proliferation after GSH-depletion.
Intracellular glutathione was depleted by the GSH-S-transferase-substrate 1-chlor-2,4-dinitrobenzene (CDNB) [5μM] or the synthesis inhibitor buthionine sulfoximine (BSO) [200μM], or by a combination of both agents. Freshly prepared mouse spleen lymphocytes were polyclonally stimulated with Con A [5μg/ml] and incubated at 37° C for 48h in culture medium containing different concentrations of the tested thiol compound.
The results demonstrate that various compounds containing a free SH-group restored Con A-induced proliferation after GSH-depletion, even when *de novo* synthesis of GSH was blocked. No correlation between intracellular GSH or thiol concentration and the effects of reconstitution was observed. $NaBH_4$-reduced bovine serum albumine (BSA) also resulted in reconstitution of proliferation of the cells.
We conclude from these results extracellular cell surface thiols are the signal that allows lymphocyte proliferation.

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THALIDOMIDE DERIVATIVES ALTER THE EXPRESSION OF LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGENS IN MARMOSET BLOOD.

Reinhard Neubert, Ana Cristina Nogueira, and Diether Neubert

We have found that thalidomide (Thd) and its highly teratogenic derivative EM12 alter the epitope pattern on lymphocytes of marmosets after oral doses of 5-50 mg/kg body wt daily. Here, additional data are presented of studies in which these substances and the non-teratogenic derivative supidimide were given orally to marmosets. Lymphocyte function-associated antigens were analysed on the surface of lymphocytes and monocytes with commercially available anti-human monoclonal antibodies and flow cytometry (FACScan) at the end of the 10-day treatment and 23 days thereafter (data: M ± SD):

	Control	EM12 10mg/kg	EM12 50mg/kg	Supidimide 100mg/kg	Thalidomide 100mg/kg
n =	4	4	2	4	4
			% lymphocytes		
CD2	**71.78** ± 5.3	**28.34** ± 6.5*	**20.39** ± 0.5*	**46.10** ± 21§	**52.58** ± 14*
	78.10 ± 3.5	*44.51 ± 4.2**	*30.81 ± 8.0**	*77.55 ± 2.7*	*75.34 ± 2.3*
CD11a (LFA-1α)	**77.40** ± 3.6	**33.09** ± 9.4*	**20.75** ± 0.8*	**36.78** ± 20*	**35.63** ± 16*
	83.96 ± 1.6	*61.40 ± 14**	*33.30 ± 2.5**	*71.87 ± 16*	*73.59 ± 3.1*
CD18 (LFA-1β)	**74.18** ± 4.8	**32.07** ± 8.7*	**17.00** ± 1.4*	**21.70** ± 15*	**14.00** ± 9.0*
	76.63 ± 2.3	*66.25 ± 8.9**	*54.59 ± 4.1**	*80.63 ± 4.4*	*76.97 ± 5.5*

* $p < 0.05$; § no response in one marmoset, for 3 remaining: $p < 0.05$
bold: after 10 days of treatment *italic: 23 days after the end of treatment*

All the Thd derivatives tested reduced the expression of lymphocyte function-associated antigens, but with different potencies (EM12 being the most potent derivative), and the effect was rather persistent. As a result $CD4^+CD2^-$ $CD11a^-$ and $CD8^+CD2^-CD11a^-$ cells appear, which are virtually absent under normal conditions. Expression of CD11a and CD11c was also found to be reduced on monocytes. CD2 as well as the integrins CD11a and CD11c are known to be essential as adhesion factors for many cell-cell interactions.
Although tempting to assume, it has still to be elucidated whether the alterations described are causal for any of the various pharmacologic or toxic effects of Thd (e.g. suppression of graft-vs-host disease or teratogenicity).

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EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON THE DEVELOPMENT OF FETAL RAT THYMUSES IN AN ORGAN CULTURE SYSTEM.

M. Foerster and H.-J. Merker

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent substance in the group of ploychlorinated dibenzo-p-dioxins. Studies in various animal systems indicated that TCDD produces profound and diverse effects on the immune system, including thymic atrophy at rather high doses.

In order to obtain information on direct thymus toxicity of TCDD in fetal rats, we have tested this compound in an organ culture system. Lobes of 17-day-old rat fetuses were cultivated at the medium/air interphase for six days. TCDD was added to the medium in concentrations ranging from 0.1 ng/ml to 10 ng/ml.

The investigations showed an influence of TCDD on the proliferation of the lymphatic cells. The reduction of the number of thymocytes was significant at concentrations of 0.3 ng/ml. Results of flow cytometry showed the relative decrease in the number of $CD4^+CD8^+$ and $CD4^+CD8^-$ cells, whereas percentages of $CD4^-CD8^-$ cells were increased. Alterations in the flourescence intensity of the CD5-marker indicated the inhibition of the maturation of the thymocytes. Light and electron microscopical investigations showed that lymphatic cells were affected as well as the epithelial network.

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MOLSIDOMIN - AN ANTI-INFLAMMATORY SUBSTANCE

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NO/EDRF has been associated with a proinflammatory activity, increased capillary permeability and edema formation (Chander et al., J Pharm Pharmacol 40:745, 1988). On the other hand protective effects of NO and NO-donating substances have been reported under hypoxic and ischemic conditions (Saito, Jap J Clin Exp Med 51:243, 1974; Bassenge & Mulsch, J Cardiovasc Pharmacol 14:S23, 1989). The obvious discrepancy of these statements stimulated us to test the effect of molsidomin, a NO-donating substance, on the development of edema and hyperalgesia as induced by carrageenin injection in the rat paw. The extent of edema and hyperalgesia were measured by the methods of Winter et al. (Proc Soc Expt Biol Med 111:544,1962) and Randall & Selitto (Arch Int Pharmacodyn 61:409,1957), respectively.

Molsidomin reduced edema and raised pain-threshold in a dose dependent manner, when applied intraperitoneally (i.p.) 1 hour later than Carrageenin. Dosages between 0.3 and 30mg/kg were necessary, the highest dose reduced edema formation bei 80% and elevated pain-threshold 8 fold. The combination of molsidomin (3mg/kg and 20 mg/kg i.p.) with indometacin (0,5 mg/kg and 2 mg/kg i.p.) caused retardation of the onset and reduction of the anti-inflammatory response to molsidomin.

Molsidomin ist without doubt a hemodynamic active drug, but nevertheless the reported finding concerning the rat paw edema and hyperalgesia can not be solely explained by changes in the microcirculation. Since NO has been shown to be tissue protective, it is speculated that molsidomin preserves the tissue under conditions of inflammatory stimuli.

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EFFECT OF S-ADENOSYLMETHIONINE AND DICLOFENAC-NA ON FIBRONECTIN (FN) AND KERATAN SULFATE (KS) SYNTHESIS BY CULTURED CANINE ARTICULAR CARTILAGE CHONDROCYTES

J. Steinmeyer[1], N. Burton-Wurster[2] and G. Lust[2]

Several studies disclosed that osteoarthritic cartilage of several species contain increased amounts of FN. Since articular chondrocytes are potential target for drugs which can influence the integrity of cartilage, we investigated the effects of diclofenac-Na and S-adenosylmethionine sulfate p-toluenesulphonate (SAMe) on FN, total protein and DNA synthesis as well as on extra domain A containing FN (ED-A FN) and KS content in chondrocyte culture. - Chondrocytes were isolated from adult canine articular cartilage and cultured in Ham F 12 media supplemented with 10% FN depleted fetal bovine serum. At the initial seeding diclofenac-Na (10^{-7}, 10^{-6} or 10^{-5} mol/L) and SAMe (10^{-6}, 10^{-5} or 10^{-4} mol/L) was added to the chondrocyte cultures. After days culture media were harvested, the cells fed, the same amount of drugs added and cultured for another 3 days. On day 6 chondrocytes were labeled with ^{35}S-methionine or ^{3}H-thymidine. The contents of FN, ED-A FN and KS were determined with ELISA. - SAMe decreased dose dependently the synthesis of FN as well as the content of FN and KS of chondrocyte cultures. At the highest concentration of this drug tested, data suggest that cell viability was impaired as assessed by loss of DNA and protein synthesis and the release of lactate dehydrogenase into the incubation media. Our study confirm that diclofenac-Na does not affect the anabolic activity of chondrocytes as do some other nonsteroidal anti-inflammatory drugs.

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IMMUNOHISTOCHEMICAL LOCALIZATION OF ARTICULAR CARTILAGE PROTEOGLYCAN AND LINK PROTEIN IN SITU USING MONOCLONAL ANTIBODIES AND LECTIN-BINDING METHODS

S. Hoedt-Schmidt and J. McClure*

Lectin-binding techniques have been established to characterize cellular as well as extracellular glycoconjugates of the cartilage matrix in situ and may thus be used to visualize the localization and distribution of proteoglycan aggregates. In the present study monoclonal antibodies raised against chondroitin sulfate and keratan sulfate containing proteoglycans and link proteins were applied on bovine articular cartilage derived from the metacarpophalangeal joints in order to validate lectin-binding patterns. An experimental degradation of the cartilage was performed by incubation with chondroitinase ABC and keratanase, enzymes known to remove chondroitin sulfate and keratan sulfate side chains from the proteoglycan aggregate respectively. Lectin-binding patterns of normal and degraded tissue were determined using several lectins with different carbohydrate specificity (e.g. wheat germ agglutinin, Ulex europeus agglutinin I, soybean agglutinin, peanut agglutinin). In our experiments the resulting lectin-binding patterns of articular cartilage glycoconjugates cannot be directly related to the specific binding of monoclonal antibodies to certain epitopes on proteoglycans but reveal a more complex association to matrix macromolecules. In degraded cartilage lectins show binding patterns distinct for certain degradative processes in cartilage matrix. These immunohistochemical procedures provide a considerable potential in diagnosis both for identifying particular disease states and for monitoring the effects of drug therapy on a particular pathologic condition.

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IMMUNOTOXICOLOGICAL EFFECTS OF CONSTITUENTS OF LEAVES FROM GINKGO BILOBA L. ON THE POPLITEAL LYMPH NODE REACTION IN MICE.

E. Koch, S.S. Chatterjee and H. Jaggy*

Allergic reactions due to skin contact with different parts of the ancient tree Ginkgo biloba L. have repeatedly been reported. Alkylphenols like bilobol and ginkgolic acids have been suggested as causative agents. Different types of ginkgo leaf extracts are widely employed for the therapy of circulatory disorders. Since the above mentioned constituents are also present in the leaves, potential contact-sensitizing and other immunological hazards of such preparations have to be carefully controlled. Thus, we have attempted to standardize simple immunotoxicological models suitable for the detection and eventually the elimination of such compounds. In the present communication some experimental observations made with an alcoholic extract and its fractions using the popliteal lymph node reaction (PLR) in the mouse will be described. This assay has been shown to detect compounds with contact sensitizing as well as autoimmune-inducing properties. Subplantar injection (2 mg) of an alcoholic extract from ginkgo leaves caused a significant lymph node reaction. PLR-active compounds in this extract could be enriched by serial extraction with heptane and ethylacetate. Analysis of the heptane fraction revealed the presence of high concentrations of ginkgolic acids. In further studies it could be shown that ginkgolic acids evoke a dose-dependent (62,5 to 500 µg/paw) enlargement of the popliteal lymph node. In the ethylacetate fraction other substances which until now had not been suspected to possess immune-disregulating effects were detected. These experiments establish that leaf extracts from Ginkgo biloba L. contain constituents with potential immunotoxicological properties underlining the need for adequate production procedures to eliminate compounds with adverse effects.

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EXPERIMENTAL ASTHMA IN NON ANAESTHETIZED GUINEA PIGS: EFFECTS OF CETERIZINE AND REPROTEROL ON THE HISTAMINE INDUCED BRONCHOCONSTRICTION

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Conventionally the measurement of broncho-constriction (BC) is carried out invasively in anaesthetized animals. There are certain disadvantages as the time for experiment is limited and the results may be influenced by the effects of the anaesthetic drugs and by the diminished physiological reflexes. Recently Hutson et al. (Am Rev Respir Dis 137, 548, 1988) described a non-invasive method which permitted the repeated measurment of BC in conscious animals. The BC is calculated from records of pressure and maximal inspiratory air flow using a body plethysmograph: the specific airway conductance (SGaw) is the reciprocal of the product of thoracic gas volume and airway resistance.

To improve the reproducibility of the results we modified the apparatus to ensure airtightness without causing uncontrolled nasal obstruction (n=94). For final validation of the system 10 animals were measured 15 times over a period of 10 days. No significant variations could be found. Maximal deviation was 6.4 ±1.8 %. Neither an increase of respiratory frequency by 23.5% (±4.7%) nor exposure to nebulised 0.9% NaCl affected the SGaw. Challenge (30 sec) with nebulised 0.05% and 0.3% histamine solutions exerted a significant, dose dependent decrease of SGaw at 1, 3 and 5 mins.

Animals given Ceterizine (2mg/kg i.p.) 60 mins before histamine challenge (0.05%; 45 sec.) showed only a very small SGaw decrease of -10.5±4.8% and 9.3±3.1% for 3 mins after challenge. This amounted to an almost total inhibition of the histamine induced BC.

Reproterol (6µg/kg i.p.) given 30 mins before histamine challenge increased the SGaw by 11.5±8.1% to 27.4±7.3% indicating a slight bronchodilation. The histamine induced BC however was initially unaffected and was inhibited significantly only from 5 mins after challenge.

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THROMBOXANE MEDIATES LPS-INDUCED DETERIORATION OF THE ISOLATED PERFUSED RAT LUNG.

Stefan Uhlig.

Administration of lipopolysaccharide (LPS) to experimental animals causes symptoms similar to those observed in the human adult respiratory distress syndrome (ARDS). We showed previously that LPS provokes a pathophysiological reaction of the isolated and blood-free perfused rat lung (IPL) [1]. Here we present evidence that these effects of LPS are mediated by thromboxane.

Isolated rat lungs were prepared from female Wistar rats (200-250g) and perfused with Krebs-Ringer bicarbonate buffer (pH 7.35) containing 2% albumin, 3% HEPES and 0.1% glucose. The role of thromboxane in the LPS-induced deterioration of the IPL was studied by the following experiments: i) The effects of LPS (0.5 mg) on the IPL, e.g. fall in lung compliance, increase in lung resistance and formation of lung edema, were dose-dependently prevented by the cyclooxygenase inhibitor acetyl salicylic acid and the thromboxane receptor antagonist BM13177 ([4-[2-(phenylsulfonyl)amino]ethyl]phenoxy acetic acid). ii) After the addition of LPS the concentration of the stable metabolites of thromboxane and prostacyclin increased in the perfusate. iii) Direct administration of the stable thromboxane agonist U46619 produced effects similar to LPS.

The data show that endogenously produced thromboxane plays an important role in mediating the effects of LPS on the IPL.

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[1] S. Uhlig. Naunyn-Schmiedebergs's Arch. Pharmacol. 343 (1991) R87 (Suppl.), A345.

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NEW PHOSPHOLIPID-ANALOGOUS PAF ANTAGONISTS WITH MAST CELL (MC) DEGRANULATION-INHIBITING PROPERTIES

R. Grupe[1], T. Ziska[1] and H.-P. Kertscher[2]

We showed earlier that PAF antagonists (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)-(1,2,4)triazolo(4,3-a)(1,4)diazepine-2-yl)-1-(4-morpholinyl)-1-propanone, WEB 2086, the terpene-ginkgolide B, BN 52021, and triazolam) inhibit the degranulation of activated peritoneal rat mast cells (pRMC) in vitro, but the inhibition does not occur via PAF receptors, and the concentration for that action is considerably higher than the concentration for PAF-antagonistic activity and therefore not therapeutically relevant.

Substances with both properties must be very effective as antiallergic/antiasthmatic drugs, if both properties were identically intensive. By us synthetized, new phospholipid-analogous derivatives with a 2-n-propylpropane-1,3-diol basic structure and with differently heterocyclic head groups are such substances. Their PAF-antagonistic activity was investigated using the method of the PAF-induced aggregation of human platelets in vitro, their MC degranulation-inhibiting activity using pRMC activated by protamine (calcium mobilization from the cell's internal pool of calcium) or A 23187 (influence on calcium-influx). The most active substances of that group (picolinium derivatives) inhibit the platelet aggregation with an $IC_{50} \sim 3 \cdot 10^{-6}$ mol/l (IC_{50} of the WEB 2086 under identical conditions $\sim 3 \cdot 10^{-8}$ mol/l), the protamine-induced release of histamine from pRMC with an $IC_{50} \sim 2 \cdot 10^{-6}$ mol/l, but considerably weaker - or not - the A 23187-induced degranulation of pRMC.

This implies that these phospholipid-analogous PAF antagonists are MC degranulation inhibitors, which act on the calcium mobilization from the cell's internal pool.

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THE REDUCED PLATELET AGGREGABILITY BY ANP IS MOST PROBABLY MEDIATED BY REDUCTION OF SODIUM-INFLUXES VIA THE Na^+/H^+-EXCHANGER

R. Pella

Since the demonstration of receptors for Atrial Natriuretic Peptide (ANP) on platelets the function is not yet clear. In a previous report we showed that platelet aggregation can be inhibited by ANP ex vivo. We speculated that ANP modulates platelet aggregability in vivo, especially in the heart. Now we tried to elucidate the mechanism of this ANP-action. ANP acts at least on two different types of receptors. One is coupled to the particulate guanylate cyclase (pGC) and cGMP is the second messenger of ANP in most tissues. Elevated cGMP-levels by ANP would block platelet aggregability. Therefore we measured cGMP and found up to 1 µM ANP no elevated cGMP-contents. This result is corresponding to the denied existence of particulate guanylate cyclase (J. Pharm. Exp. Ther. 1987;226: 180-186). The function of the other type of receptor is up to this day not clear. Ion-channels, probably via G-proteins could be the target. Therefore, we performed inflow experiments with Ca^{2+}, Na^+ and Rb^+. The Ca^{2+}-mobilization is intimately involved in platelet aggregability. The Rb^+-inflow was not changed, but the basal and by GDPßS or monensine stimulated Na^+-inflow was significantly inhibited. The basal Ca^{2+}-inflow was not lowered, but the inflow by calimycin was reduced. From this data we conclude, that like in rabbit-aorta the Na^+/H^+-antiporter is the target of the second type of the ANP-receptor. A G-protein seems to be involved in the signal transduction. The Ca^{2+}-mobilization could then be reduced by a feedback mechanism via the Ca^{2+}/Na^+-exchanger. This would lead to the reduced platelet aggregability.

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COAGULATION-INDUCED LEUKOTRIENE-FORMATION BY HUMAN MONOCYTES. EFFECTS OF PLATELETS. I. Weide and Th. Simmet

We have previously shown that contact activation of the intrinsic coagulation cascade in whole human blood in vitro not only triggers generation of cyclooxygenase-derived thromboxane (TX) B_2 but also activates the 5-lipoxygenase pathway leading to cysteinyl-leukotriene (cysteinyl-LT) formation. By supplementation of whole human blood with autologous mononuclear cells or purified peripheral monocytes we were able to demonstrate that the process of clotting activates the 5-lipoxygenase in monocytes but apparently not in polymorphonuclear neutrophils. When peripheral monocytes purified over Percoll gradients were incubated in recalcified autologous platelet-rich plasma, a time-dependent formation of immunoreactive cysteinyl-LT and LTB_4 could be observed. By contrast, upon incubation in recalcified autologous platelet-poor plasma no cysteinyl-LT, but still a time-dependent formation of LTB_4 was found. However, in the absence of platelets (3 x 10^8/ml), i.e. in recalcified platelet-poor plasma, significantly less LTB_4 was produced by the purified monocytes suggesting a stimulating effect of platelets in this setting. Indeed, the stimulatory effect observed was dependent on the platelet number. In order to elucidate whether the thrombin-activated platelets may feed substrate arachidonic acid (AA) to the monocytes, ^{3}H-AA-labeled platelets were coincubated with monocytes in recalcified platelet-poor plasma. Upon reverse phase HPLC large amounts of label coeluted with the retention time of 12-hydroxyeicosatetraenoic acid and putatively with that of 12,20-dihydroxyeicosatetraenoic acid, but only unlabeled cysteinyl-LT could be detected. In contrast to experiments performed in recalcified plasma, monocytes did not produce any detectable amounts of LT when incubated in parallel in Hank's balanced salt solution. Thus, coagulation-mediated leukotriene formation by human monocytes is further stimulated by the presence of platelets, an effect not due to a potential substrate transfer.

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CONTRACTILE EFFECT OF THROMBIN ON PORCINE PULMONARY ARTERIES
E. Brüggener, E. Glusa and M. Paintz

The proteolytic enzyme thrombin catalyzes the conversion of fibrinogen to fibrin, but it is also a potent activator of a variety of cellular mediated events. In isolated vessels thrombin may induce a biphasic response, i.e. relaxation and/or contraction depending on the endothelium, the type of vessels and the species studied. This paper deals with the contractile response of pulmonary arteries to thrombin and the changes in phosphatidyl inositol metabolism.
Thrombin (0.1 - 10.0 IU/ml) caused a slowly increasing concentration-dependent contraction which was sustained after repeated washouts. In the presence of intact endothelium the contractile response was significantly lower than those in deendothelialized arteries. The thrombin (3 IU/ml)-induced contractions were comparable to those induced by $PGF_{2\alpha}$ (3µmol/l). Exposure of the vessels to calcium-free medium or verapamil reduced markedly the thrombin-mediated contractions. The vasoconstrictor effect of thrombin was due to its proteolytic activity and was inhibited by the tight-binding inhibitor hirudin and by synthetic competitive inhibitors of the benzamidine type in a concentration-dependent manner. In order to measure changes in the level of inositol phosphates the vessels were preincubated with ^{3}H myoinositol for 6 hrs; after addition of thrombin (10 IU/ml) the reaction was stopped by freezing. The concentration of inositol phosphates increased; the level of IP_3 reached a plateau after 2 min.
Thus, thrombin generated during thrombotic and haemorrhagic processes may modify the vascular tone.

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IN VITRO AND IN VIVO EVALUATION OF NEUTRAL BORON CONTAINING THROMBIN INHIBITORS. R. Metternich, C. Ehrhardt and C. Tapparelli.

Peptidic boronic acid derivatives have proven to be very potent inhibitors of serine proteases with boroarginine being particularly potent thrombin inhibitors (Kettner et al., J.Biol. Chem. 265,18289,1990; Tapparelli et al., Thromb. Haemostas. 65,774,1991). We synthesized boronic acid derivatives in which the guanidino group in P-1 which significantly contributes to the inhibitor affinity to the thrombin active site (Asp-189) was replaced by a methoxy group. These compounds, e.g. SDZ 216-744 (Z-D-Phe-Pro-boroMpg, Mpg=methoxypropylglycine) inhibited thrombin by a fast and reversible binding mechanism with K_i= 8.9 nM. In comparison to the boroarginine derivatives, SDZ 216-744 displayed higher selectivity for thrombin over trypsin (K_i= 1.1 µM) and plasmin (K_i= 15.7 µM), wherein the nature of the N-terminal protecting group profoundly affected the selectivity degree.
Prolongation of TT and APTT were observed in a dose-dependent manner in human plasma with elevation of TT and APTT in the micromolar (5 µM) concentration range. In a thrombin-dependent in vitro aggregation assay of human platelets, SDZ 216-744 inhibited aggregation with a IC_{50}= 85 nM. When tested in a thrombin-dependent platelet accumulation model in the rat by the Automated Isotope Monitoring System (AIMS), SDZ 216-744 dose-dependently (0.3 to 3 mg/kg) inhibited the thrombin effect upon i.v. administration of the inhibitor.
The substitution of the charged guanidino group in the P-1 side chain by the neutral methoxy group resulted in potent and highly selective thrombin inhibitors with an interesting pharmacological profile in vitro as well as in vivo models.

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COMPARATIVE STUDIES OF r-HIRUDIN AND HEPARIN ON ARTERIAL REOCCLUSION AFTER EXPERIMENTAL THROMBOLYSIS WITH STREPTOKINASE AND r-tPA B. Kaiser

The effect of r-hirudin (rH) and unfractionated heparin (UH) on reocclusion rate after lysis of an arterial thrombus was studied in rabbits and rats. In the rabbit femoral artery or the rat abdominal aorta arterial thrombi were formed as result of endothelial damage either by an internal balloon catheter or by three external vessel clamps and following stasis for 1h. In the abdominal aorta of rats an additional copper coil was placed within the vessel lumen. The occlusive arterial thrombi formed were removed by fibrinolytic therapy with either streptokinase in rabbits or r-tPA in rats. Both thrombus formation and thrombolysis were followed by measuring either the surface temperature of the vessel with a thermistor or the arterial blood flow with a flow meter. Whereas in control animals reocclusion of the arteries after thrombolysis was observed within a relatively short period of time, s.c. injection of rH and UH reduced the incidence of reocclusion in dependence on the dose administered or prolonged the time until reocclusion occurred. Comparing rH and UH, on a weight basis rH was more effective in preventing reocclusion processes than UH. Whereas rH at a dose of 4 mg/kg s.c. in rabbits and 10 mg/kg s.c. in rats prevented reocclusion of the arterial vessels by newly formed thrombi within an observation period of 4 h after finishing the infusion of the thrombolytics, for UH about 2-3 times higher doses were required to achieve the same effectiveness.

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HIRUDIN CONSUMPTION RESULTING FROM SNAKE VENOM-INDUCED ACTIVATION OF PROTHROMBIN IN RATS

G. Nowak, E. Bucha, and *J. Meier

Bleeding after overdosages of hirudin or its impaired renal elimination in case of nephropathies represents the most undesirable side effect of this tight-binding selective thrombin inhibitor from medicinal leeches or its recombinant variants. So far, no efficient hirudin antidote has been found. In this context, meizothrombin, a stable intermediate product of the Echis carinatus-induced prothrombin-thrombin conversion was studied for its suitability as hirudin antidote. The meizothrombin formation was recorded in highly diluted human plasma by a chromogenic substrate method. Hirudin is able to completely inhibit the formation of meizothrombin in a dose-dependent manner.

In vivo, bilateral nephrectomy in Wistar rats of either sex was followed by administration of hirudin (1 mg/kg). One hour later, the hirudin blood level remained constant at 3.5 - 4.2 µg/ml. An E. carinatus venom dose of 50 µg/kg x h was infused 180 min after hirudin administration. After starting the infusion the hirudin blood level dropped sharply reaching significantly reduced values of 2.2 µg/ml. Platelet count and fibrinogen level in plasma remained unchanged in all experiments.

Hence follows that ecarin, the purified protease from E. carinatus represents a remarkably efficient hirudin antidote.

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EFFECT OF SUPERSULPHATED LOW MOLECULAR WEIGHT HEPARIN ON FIBRINOLYSIS H.-P. Klöcking

A supersulphated low molecular weight heparin (SSH; MW 3500 D) produced by Iketon, Milano, was tested for fibrinolytic activity in vitro and in vivo. SSH developed profibrinolytic activity on the basis of tissue-type plasminogen activator (t-PA) release during perfusion of isolated organ preparations (pig ear) and after application in experimental animals (rats). An acute increase of t-PA by 200 % was caused by 10 µmol SSH/l perfusion solution in the isolated perfused pig ear. A statistically significant increase in maximum t-PA release was achieved by the i.v. administration of 5 and 10 mg SSH/kg in rats. The application of 5 mg SSH/kg and 10 mg SSH/kg enhanced the acute t-PA release by 7.9 ± 2.0 and 6.6 ± 2.4 IU/ml plasma, resp. The plasminogen activator inhibitor (PAI-1) activity was abolished after the administration of 5 and 10 mg SSH/kg, resp. An additional effect of SSH was found to be caused by activation of the intrinsic pathway of the fibrinolytic system. SSH enhanced the lytic activity of resuspended euglobin fractions after preincubation of plasma with SSH in vitro. At concentrations of 0.1 µmol/l and 1 µmol SSH/l caused an increase in fibrinolytic activity by about 33 % and 77 %, resp. This activity is inhibited by amiloride, an inhibitor of urokinase. It is concluded that SSH activates both the extrinsic and intrinsic pathway of the fibrinolytic system.

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CONTRIBUTION OF THE KIDNEYS TO THE CATABOLISM OF THE NOVEL RECOMBINANT PLASMINOGEN ACTIVATOR BM 06.022 IN RATS U. Martin, D. Gärtner, and K. Strein

The novel recombinant plasminogen activator BM 06.022 is an unglycosylated deletion variant of human t-PA consisting of the kringle 2 and protease domains. Recent experiments indicated that the uptake of BM 06.022 in rat liver was significantly lower than that of wild type t-PA. The purpose of the present study was to evaluate whether the kidneys contributed to the catabolism of BM 06.022. Recombinant t-PA (alteplase) or BM 06.022 was administered as a single i.v. bolus injection of 200 kU/kg to anesthetized rats of either sex with bilateral nephrectomy or sham surgery (n = 6/group). At serial intervals blood samples were taken on citrate to measure plasma concentration of t-PA like activity in an indirect spectrophotometric assay. In rats with sham surgery BM 06.022 demonstrated a 5-fold higher ($p<0.05$) area under the curve (AUC)-value than alteplase (549 ± 49 vs. 106 ± 18 $IU \cdot h \cdot ml^{-1}$; mean ± SEM). In rats with bilateral nephrectomy, the AUC of BM 06.022 was 2.1-fold higher ($p< 0.05$) than in rats with sham surgery (1130 ± 107 vs. 549 ± 42 $IU \cdot h \cdot ml^{-1}$). Bilateral nephrectomy did not significantly change the AUC after alteplase compared with sham surgery (115 ± 20 vs. 106 ± 18 $IU \cdot h \cdot ml^{-1}$). These data suggest that the kidneys contribute significantly to the catabolism of BM 06.022-activity in rat plasma in contrast to alteplase.

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IS THE ANTINOCICEPTIVE EFFECT OF PROTEIN C MEDIATED BY ß-ADRENOCEPTOR STIMULATION? L. Pichler and H.P. Schwarz

The analgetic effect of protein C (PC)-concentrate observed in patients with warfarin-induced skin necrosis was confirmed by Schwarz et al. (Blood 76 (Suppl.1), 520 a, 1990) in animal experiments by demonstration of a dose-dependent antinociception in a carrageenan rat paw edema model. Using the same model (Arch. Int. Pharmacodyn. 111, 409, 1957) we investigated the interaction between the adrenergic system and PC. Parameter measured was the pain threshold as the amount of pressure in mmHg required to induce a struggle reaction when applied to the paw 3 h after intraplantar (i.pl.) injection of carrageenan. The α-adrenoceptor blocking agent phentolamin (5 mg/kg i.v.) did not affect the antinociceptive effect of 800 IE/kg PC i.v., whereas the ß-adrenoceptor blocking drugs propranolol and pindolol antagonized this effect in a dose-dependent manner. ß-adrenoceptor agonists mimicked the antinociceptive effect of PC both when given locally (0.5 mg orciprenaline i.pl.) or systemically (3 or 5 mg/kg fenoterol s.c.). In chemically "sympathectomized" rats (reserpine + α-methyl-p-tyrosine) the antinociceptive effect of PC as well as its antagonism by ß-adrenoceptor blockers could also be shown. Based on these in vivo experiments we assumed direct stimulation of postsynaptic ß-adrenoceptors as the mode of action of PC but this was not confirmed by in vitro experiments: in the guinea pig tracheal strip PC only slightly decreased the histamine-induced contracture and this was not antagonized by pindolol. In the isolated rat uterus PC completely abolished the rhythmic contractions as elicited by oxytocin. However, the same effect was seen with the sodium citrate containing PC-buffer solution. Dialysis of both substances abolished this effect in the uterus. Therefore the antagonism of ß-adrenoceptor blocking drugs towards the antinociceptive effect of PC in vivo is most likely functional in nature.

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NEURO-EPITHELIAL INTERACTIONS IN THE ISOLATED RAT TRACHEA: EFFECTS OF POTASSIUM CHANNEL OPENERS AND AGONISTS AT ß-RECEPTORS

I. Wessler and D. Pohan

Potassium channel openers and agonists at ß-receptors can effectively reduce the muscular tone within the airways by a postsynaptic site of action. In addition, an inhibitory action on the release of spasmogenic transmitters (e.g. acetylcholine) has been postulated. Recently, the release of newly-synthesized [^{3}H]acetylcholine (ACh) elicited from the isolated rat trachea by stimulation of the innervating parasympathetic nerves (recurrent laryngeal nerves) has been measured [Wessler et al. (1991) this journal **344**:403-411]; the present experiments were addressed to investigate the effects of cromakalim (CRO), lemakalim (LEM) and isoprenaline on evoked ACh release.

Isolated rat tracheae were opened longitudinally by cutting the cartilage or remained as intact tubes; in some experiments the epithelium was removed mechanically from intact or opened tracheae. After labeling (incubation with 5 µCi [^{3}H]choline) release of ACh was elicited by two periods (S_1, S_2) of electrical nerve stimulation (four or six 20 s stimulations at 15 Hz with 10 s intervals between the trains). The potassium channel openers or isoprenaline were added 15 min before S_2 and their effects are estimated by a comparison of the release of ACh evoked by S_2 and S_1, respectively (S_2/S_1 ratio).

Neither CRO nor LEM (1 - 10 µM) reduced evoked ACh release in opened trachea. However, in intact tracheae (epithelium present) 0.1 µM LEM and 1 µM CRO reduced evoked ACh release by 40 and 50%, respectively. CRO mediated a concentration-dependent inhibition, whereas LEM, at concentrations higher than 0.1 µM, lost its inhibitory action. The presynaptic inhibitory effect of CRO and LEM was prevented by 0.1 µM glibenclamide and was also abolished, when the epithelium of intact tracheae was removed. Likewise, isoprenaline (0.1 µM) significantly inhibited (40%) evoked ACh release only in the presence of the epithelium.

In conclusion the present experiments suggest that potassium channel openers and agonists at ß-receptors inhibit ACh release in the airways by an indirect, epithelium-dependent mechanism. Opening of the trachea may change the microenvironment within the epithelium and intramural neurones allowing a rapid exchange of epithelium-dependent mechanisms with the incubation medium.

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PARADOXICAL FACILITATORY EFFECT OF HEXAMETHONIUM ON NEURALLY-EVOKED CONTRACTIONS IN THE ISOLATED RAT TRACHEA

T. Reinheimer

Preganglionic stimulation of the recurrent laryngeal nerves (PNS) or transmural stimulation (TS) of isolated rat tracheae suspended in a zig-zag fashion were performed, to investigate neurally-evoked contractions in epithelium-denuded or epithelium-intact tracheae.

Tracheae together with the innervating recurrent laryngeal nerves were removed from rats and opened longitudinally by cutting the cartilage. The nerves were placed in a platinum electrode and the tracheae were fixed horizontally to record the contraction of the tracheal muscle. In additional experiments, the opened tracheae were cut in a zig-zag fashion and placed in an organ bath parallel to two platinium electrodes that allowed transmural stimulation. The epithelium was removed mechanically by means of a pipe cleaner.

Tetrodotoxin (1 µM) and scopolamine (0.1 µM) abolished the contractions elicited by PNS irrespective of the presence of the epithelium; tubocurarine (TC) and hexamethonium (C6) in a concentration-dependent manner (10 - 1000 µM) reduced contractions by about 80%. However, in epithelium-denuded tracheae C6 tended to increase smooth muscle contractions evoked by PNS, although evoked [^{3}H]-acetylcholine release was reduced by 70%. Scopolamine (0.1 µM) abolished TS-induced contractions, whereas C6 enhanced TS-induced contractions by about 200% and 300% in epithelium-containing or epithelium-denuded tracheae, respectively. The C6-induced increase in contractions was abolished by scopolamine. This observation excludes a facilitatory effect of C6 on the release of other spasmogenic transmitters.

In epithelium-denuded tracheae C6 (300 µM) did not shift the concentration-response curves for applied oxotremorine or acetylcholine to the left or increased the maximal contraction amplitude, i.e. C6 did not increase smooth muscle contractility. In addition, C6 did not inhibit the activity of the enzyme acetylcholinesterase, i.e. may not have increased the biophase concentration of released acetylcholine. A sensitizing effect at the postsynaptic muscarine receptors can be excluded as well as a channel blocking effect at the smooth muscle fibres, because both facilitatory effects should have become evident also with applied acetylcholine or oxotremorine. So far, the paradoxical effect of C6 is not understood but the co-release of an unknown modulator has to be considered.

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ON THE MUSCARINIC RECEPTOR SUBTYPE SELECTIVITY OF CLOZAPINE AND THE QUESTION OF THE RECEPTOR TYPE IN THE RABBIT IRIS SPHINCTER

I.T. Bognar, C. Beinhauer, H.Fuder

In binding studies on cloned muscarinic receptors clozapine (CLZ) shows a 15-fold selectivity for m_1 over m_2, and an about 5-fold preference for m_1 over m_3, m_4 and m_5 receptors (Bolden et al. 1991, Eur J Pharmacol 192:205). An M_1 selectivity of CLZ might be the reason for the low tendency of this atypical neuroleptic to cause extrapyramidal disturbances. Here we determined functional affinity constants for CLZ as an antagonist of the inhibition by McN-A-343 of the twitch contraction in the rabbit vas deferens (RVD), of the inhibition by methacholine (MCH) of the stimulation-evoked ^{3}H-noradrenaline release from the rat iris (RI) and of the MCH-induced contraction on the guinea-pig ileum (GPI). In addition, we determined the potency of CLZ and of the (R)- and (S)-enantiomers of the M_1-selective, antiparkinsonian agent, trihexyphenidyl (THP) as antagonists of the MCH-induced contraction of the rabbit iris sphincter (RIS).

ANTAGONIST	TISSUE	pA_2 (95% conf. limits)	SLOPE ± SD
clozapine	RVD (M_1)	7.34 (7.07 - 7.63)	1.13 ± 0.08
	RI (M_2)	7.26 ± 0.20[a]	- - -
	GPI (M_3)	7.58 (7.45 - 7.65)	0.99 ± 0.03
	RIS ($M_?$)	5.60 (5.52 - 5.72)	1.52 ± 0.09*
(R)-THP	RIS	7.23 (7.03 - 7.49)	1.22 ± 0.12
(S)-THP	RIS	5.34 (4.93 - 6.64)	0.82 ± 0.20

[a] $-\log K_B$ (± SE) = $-\log$ [antagonist] + $\log$ (DR-1)

CLZ failed to differentiate between functional M_1, M_2 and M_3 receptors, and was about 100-fold less potent at the receptors in the RIS. Likewise, pA_2 values of (R)- and (S)-THP in the RIS were 1-3 log units lower than the affinity constants at cloned m_1-m_5 receptors (Dörje et al. 1991, JPET 256:727). Thus, muscarinic receptors in the RIS differ from the pharmacological M_1-M_3 classes, and from m_4 and m_5 receptors. Our results do not confirm an M_1-selectivity of CLZ. Whether this points towards differences between central and peripheral M_1 receptors or merely reflects a lack of correlation between binding and functional data remains open.

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INHIBITORY AND EXCITATORY MUSCARINIC AUTORECEPTORS MODULATING THE RELEASE OF ACETYLCHOLINE (ACh) IN CHICKEN HEART

G. Brehm and R. Lindmar

The release of ^{3}H-ACh from the perfused chicken heart was evoked by field stimulation at 5 Hz. In the absence of cholinesterase (ChE) inhibition, various muscarinic receptor antagonists enhanced ACh release showing the following order of potency: atropine > 4-DAMP > sila-hexocyclium > pirenzepine (Pz) = hexahydro-sila-difenidol > AF-DX 116. Comparison with known pA_2-values for M1 receptors is compatible with the inhibitory muscarinic autoreceptor being M1. These observations support a previous result (Jeck et al., Brit. J. Pharmacol. 93: 357-366, 1988). - ChE inhibition by DFP or tacrine enhanced the basal overflow of unlabelled ACh that was further elevated by 5 nM (±)-telenzepine (Tz) or by 50 nM Pz up to levels that were equivalent to the release evoked by high frequency electrical stimulation. This ACh release observed in the presence of ChE inhibition plus Tz or Pz had the following characteristics: it was maintained for at least one hour and was highly dependent upon the presence of choline in the medium; it was calcium-dependent, TTX-sensitive, resistant to (+)-tubocurarine and was blocked by high concentrations of atropine (1 uM) or Tz (1 uM). - It is concluded that the release of ACh from the cardiac parasympathetic neuron of the chicken heart is modulated by a presynaptic inhibitory M1-receptor and by an unknown excitatory M-receptor. The excitatory receptor is unlike M1, as it shows relatively low affinities to Tz and Pz, is activated by ChE inhibition and appears to trigger postganglionic firing in the parasympathetic nerve.

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MUSCARINIC AND PURINERGIC INHIBITION OF NORADRENALINE RELEASE EVOKED BY FIELD STIMULATION OF RABBIT ISOLATED VAS DEFERENS

U. Grimm

Field stimulation of the rabbit vas deferens results in a biphasic contraction. ATP triggers the initial twitch response, while noradrenaline (NA) is responsible for the slow second phase (G. J. Trachte, EJP 164: 425, 1989). This study describes the muscarinic and purinergic modulation of NA overflow evoked by field stimulation (HPLC analysis). Vasa deferentia were incubated in Krebs solution (1.8 mM Ca^{2+}) containing (μM) cocaine (10), corticosterone (10) and rauwolscine (1).
At **4 Hz** (1 ms, for 30 s), the NA overflow was unaffected by the muscarinic agonists 4-Cl-McN-A-343 [4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide] and (±)-methacholine (MCH).
At **1 Hz** (1 ms, for 30 s), the A1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.03μM) increased the evoked NA overflow by 150.5 ± 52.6 %, while the A2 antagonist 4-amino-8-chloro-1-phenyl(1,2,4)triazolo (4,3-a)quinoxaline mesylate (CP-66.713-27, 0.3 μM) did not change the evoked overflow. Thus, the release of NA was under a tonic inhibition by endogenous adenosine activating a prejunctional A1 receptor. At 1 Hz (DPCPX, 0.03 μM present throughout) 4-Cl-McN-A-343 or MCH (0.1 - 10 μM) suppressed the evoked release (EC_{50} = 0.3 and 0.8 μM, respectively). Atropine (0.03 μM) and pirenzepine (0.1 μM), when present alone, failed to affect the evoked overflow, but shifted the MCH and 4-Cl-McN-A-343 concentration-inhibition curve to the right without suppressing the maximum. The calculated apparent -log K_B values were 9.8 ± 0.1 for atropine (n=4; MCH as agonist) and 7.4 ± 0.1 for pirenzepine (n=4; 4-Cl-McN-A-343 as agonist). These results clearly indicate the muscarinic nature of the prejunctional inhibitory effects of 4-Cl-McN-A-343 and MCH. Furthermore, it was found that the ATP-blocking agent pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10 μM; Lambrecht et al., this meeting) enhanced the NA overflow, indicating that endogenous ATP inhibited NA release. Thus, experiments to further characterize the muscarinic receptor subtype involved in inhibition of NA release will be carried out in the presence of 10 μM PPADS.

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PPADS SELECTIVELY BLOCKS P_2-PURINOCEPTOR-MEDIATED RESPONSES IN RABBIT VAS DEFERENS

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Electrical stimulation of the rabbit vas deferens (RVD) results in a biphasic contraction. ATP triggers the initial twitch response, while noradrenaline (NA) is responsible for the slow second phase (G.J. Trachte, EJP 164:425, 1989). This study describes the effect of PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) on ATP-, α,ß-methylene ATP-(α,ß-mATP) and NA-induced responses in RVD. Vasa deferentia of New Zealand white rabbits were incubated at 31°C in a modified Krebs solution (1.0 mM Ca^{2+}) containing 1 μM yohimbine. The preparations were stimulated electrically for 30 s with trains of rectangular pulses (0.05 ms, 4 Hz, 40 V) at intervals of 15 min.
PPADS (10 μM) selectively abolished the first component of the neurogenic response. Prazosin (1 μM) was able to antagonize completely the slow second phase of the contraction, but scarcely affected the first phase. PPADS dose-dependently (1-5 μM) blocked the prazosin (1 μM)-resistant component of the response (pIC_{50} = 5.83 ± 0.15; n = 4). PPADS was a slowly-equilibrating antagonist, and the tissue took 30-90 min to regain its control twitch height by washing. Responses to exogenous α,ß-mATP (0.3-10 μM) were monophasic contractions. PPADS (1-10 μM) itself did not change the mechanical tension, but antagonized contractions to α,ß-mATP in a pseudoirreversible manner. The calculated pK_B of PPADS, derived from a double-reciprocal regression, was 6.34 ± 0.07 (n = 3-5). PPADS (100 μM) had no significant effect on responses of RVD to exogenous NA (α_1-receptors), of guinea-pig ileum to arecaidine propargyl ester (APE; M_2-receptors) and histamine (H_1-receptors) as well as of guinea-pig atria to APE (M_2-receptors) and adenosine (A_1-receptors). In addition, PPADS (3-10 μM) significantly enhanced the NA overflow in RVD evoked by field stimulation at 1 Hz (U. Grimm, this symposium).
In conclusion, PPADS selectively blocks ATP- and α,ß-mATP-induced effects at pre- and postjunctional sites in RVD. It may provide a starting point for the development of selective P_2-antagonists and a novel probe to study cotransmission in autonomically innervated tissues.

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DISTINCTIVE RESPONSE OF THE RAT ISOLATED PORTAL VEIN TO MUSCARINIC STIMULATION

M. Pfaffendorf

The rat isolated portal vein displays spontaneous myogenic activity. When stimulated by carbachol the amplitude of the rhythmic contractions increases and, at higher doses a concentration dependent contracture develops. We compared the ability of several muscarinic agonists to induce this biphasic response.
Portal veins of male Wistar rats (350-400 g) were mounted longitudinally with a load of 10 mN in thermostatically controlled organ baths (37° C). The preparations were suspended in a Tyrode's solution (in mmol/l: NaCl 124, KCl 4.0, $MgCl_2$ 1.1, $NaHCO_3$ 24.9, Na_2HPO_4 0.42, $CaCl_2$ 0.9, glucose 5.5) and gassed with 95% O_2 and 5% CO_2. Isometric contractile activity was measured via force displacement transducers. After a one hour period of equilibration cumulative concentration-response curves for the muscarinic agonists were constructed. The following agonists were used (E_{max}-values (in mN) of the increase of the amplitude and the basal tone are given in brackets; Mean ± S.E.M., n=6; -- indicates no response): arecoline (16.1 ± 1.03, --), aceclidine (15.5 ± 1.75, 11.7 ± 1.33), bethanechol (15.4 ± 1.75, 11.5 ± 1.43), acetylcholine (15.3 ± 2.75, 11.6 ± 1.36), oxotremorine (15.2 ± 1.22, --), muscarine (13.6 ± 1.56, 16.4 ± 1.67), carbachol (12.5 ± 0.54, 14.3 ± 0.53), metacholine (12.3 ± 0.57, 11.0 ± 1.75), McN 343 (7.1 ± 0.93, --), and pilocarpine (7.1 ± 1.00, --), respectively. Acetylcholine was used in the presence of 0.5 μmol/l physostigmine. Furthermore, we used propylbenzylcholine mustard which is known to block muscarinic receptors irreversibly. After reducing the number of receptors with several concentrations of this compound, we could demonstrate a large receptor reserve for the effect of carbachol (CCh) on the increase of the amplitude whereas almost no receptor reserve was established for the CCh-induced contracture.
From these data we conclude that the biphasic response of the rat isolated portal vein to muscarinic stimulation can be used to discriminate between 'full' and 'partial' muscarinic agonists.

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PHARMACOLOGICAL CHARACTERIZATION OF MUSCARINE RECEPTORS INVOLVED IN THE ENDOTHELIUM-MEDIATED RELAXATION OF GUINEA-PIG CORONARY ARTERIES

G.Stroman

Muscarine receptors are involved in endothelium-mediated vascular relaxation. The phenomenon shows remarkable species differences and depends upon the region of the vascular tree, from which the test vessel is obtained. In order to investigate the conditions in resistance vessels and to obtain information on the type of muscarine receptors involved, small coronary arteries of the guinea pig were used and the potencies of a series of muscarine receptor antagonists in reducing the acetylcholine-induced relaxation were tested. The vessels were mounted in a set up according to Mulvany & Halpern (Circ Res 41:19, 1976), and stimulated by high potassium solutions. Carbachol (CCh) and arecaidine-propargyl-ester (APE) were used as agonists. Both drugs caused a dose-dependent and almost complete relaxation (ED_{50} CCh: 1.4 x 10^{-7} and APE: 4.6 x 10^{-8}M).
pA_2-values were estimated from Arunlakshana-Schild plots using four concentrations of atropine, 4-DAMP (4-diphenyl-acetoxy-N-methyl-piperidine), AFDX-116 (11-[(2-[(diethylamino)methyl]-1-piperidinyl)-acetyl]-5,11-dihydro-6H-pyrido (2,3-b)(1.4) benzodiazepine-6-one), pirenzepine, HHSiD (hexahydro-sila-diphenidol) and are presented in Table 1.

	agonist CCh		agonist APE	
	pA	slope	pA	slope
atropine	9,32	0,96	9,61	0,85
pirenzepine	7,19	1,00	7,70	0,75
HHSiD	8,33	1,06	8,58	0,99
4-DAMP	9,67	0,88	9,64	0,85
AFDX-116	6,15	0,79	6,26	0,91

The relative potencies of the antagonists were found independent of the agonist investigated. A comparison of the presented pA_2-values with results from literature for other vascular preparations and for other muscarine receptor mediated responses suggests the predominance of a M_3-receptor subtype at the endothelium of the guinea-pig coronary artery. This suggestion is in accordance with the interpretation of findings in the rabbit ear artery (Duckles et al., J Pharmacol exp Therap 253:608, 1990) and in feline cerebral arteries (Dauphin et al., Eur J Pharmacol 178:203, 1990) concerning the M-receptor subtype involved in endothelium mediated relaxation.
The generous gift of HHSiD by Dr. G. Lambrecht is gratefully acknowledged.
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CHARACTERIZATION OF m3 MUSCARINIC ACETYLCHOLINE RECEPTOR-INDUCED STIMULATION OF PHOSPHOLIPASE D

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We have recently reported that the human muscarinic acetylcholine receptor (mAChR) subtypes m1 and m3 are coupled to phosphoinositide-specific phospholipase C (PI-PLC) as well as to phospholipase D (PLD). A human embryonic kidney (HEK) cell line transfected with, and stably expressing the m3 mAChR subtype was used to further characterize the mechanism(s) which couple the receptor to PLD. PLD activity was assayed in [^{3}H]oleic acid prelabelled cells by measuring the formation of ^{3}H-labelled phosphatidylethanol (PEt) which is, in the presence of ethanol, the product of the transphosphatidylation reaction catalyzed by PLD. PI-PLC activity was assayed in [^{3}H]inositol prelabelled cells by measuring the formation of ^{3}H-labelled inositol phosphates. The muscarinic agonist carbachol (CCh, 1mM) and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA, 0.1µM) maximally stimulated PLD activity and PEt formation. Removal of extracellular calcium caused a slight inhibition (20%) of CCh-induced PEt-formation whereas the PMA-induced PEt formation was not affected. After stimulation with CCh in the presence of ethanol PEt was formed within 30 seconds and reached a plateau after 1-2 minutes suggesting that PLD was no longer activated after two minutes of stimulation by CCh. In contrast, PMA induced a linear increase in PEt for at least 30 minutes. The protein kinase inhibitor staurosporine effectively inhibited the PMA-induced PEt formation, maximal inhibition (about 80%) was obtained at 0.1 µM staurosporine. The CCh-mediated formation of PEt was not affected by this concentration of staurosporine suggesting that activation of PKC is not required for mAChR-induced stimulation of PLD. PLD activity and PEt formation in HEK cells could also be stimulated by AlF_4^- which may indicate that G-protein(s) are involved in the regulation of PLD.

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COMPARISON BETWEEN THE STABILIZING EFFECTS OF W84 ON [^{3}H]NMS-BINDING IN CARDIAC MEMBRANES AND IN BEATING ATRIA FROM GUINEA PIG HEARTS

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W84 is hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)-ammonium bromide]. In guinea pig atria, W84 combined with 10^{-7}M N-methyl-scopolamine (NMS) acts overadditively antimuscarinic in concentrations >10^{-5}M. In guinea pig cardiac membranes, W84 retards the dissociation of [^{3}H]NMS ($t_{1/2}$ = 4min) from the M_2-receptors (3mM $MgHPO_4$; 50mM Tris, pH 7.3, 37°C). The rate of [^{3}H]NMS-dissociation is reduced to 50% of the control value already at EC_{50} = 1x10^{-6}M. The aim of the present study was to evaluate the potency of W84 to stabilize [^{3}H]NMS-receptor-complexes in intact cardiac preparations. A method was applied which allowed to measure a complete time-course of [^{3}H]NMS-release in one preparation.

Beating guinea pig left atria (Tyrode's solution with 1.8mM Ca^{2+}, 2.7mM K^+, 32°C; 3Hz) were loaded with [^{3}H]NMS (10^{-8}M) for 60 min. The tissue/medium ratio amounted to 4.5. To define nonspecific [^{3}H]NMS-uptake, atria were loaded in the presence of 10^{-4}M unlabelled NMS; the tissue/medium ratio was 1.2. The release of [^{3}H]NMS was studied by incubating the contracting preparations successively in batteries of oxygenated miniature (2ml) organ baths containing 10^{-4}M unlabelled NMS. W84 was either applied to both the uptake- and the washout vials or to the washout vials only. When applied to the uptake vials, W84 inhibited specific [^{3}H]NMS uptake with an IC_{50} of about 5x10^{-8}M. The dissociation of specifically bound [^{3}H]NMS was calculated by subtracting the release of nonspecifically bound [^{3}H]NMS from the release of the totally bound [^{3}H]NMS. The dissociation of [^{3}H]NMS was monophasic with a half-life time of $t_{1/2}$ = 5min. W84 reduced the rate of [^{3}H]NMS-dissociation concentration-dependently; a reduction by 50% was obtained at EC_{50} = 1x10^{-5}M, independent of the mode of W84-application. Thus, the potency of W84 to retard [^{3}H]NMS-dissociation is about tenfold weaker in intact atria than in cardiac membranes. The concentration range for the retarding effect in intact atria corresponds well with the W84-concentrations inducing overadditive antimuscarinic actions.

The stabilizing effect of allosteric modulators on antagonist-cholinoceptor-complexes is commonly measured in membrane-suspensions; however, to assess the biological significance of this effect, measurements in intact preparations seem indispensable.

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THE STABILIZING EFFECT OF W84 ON THE BINDING OF THE M_2-SELECTIVE ANTAGONIST [^{3}H]AF-DX 384 IS WEAK COMPARED WITH THE W84 EFFECT ON [^{3}H]NMS BINDING

C.-M. Staschen and C. Tränkle

W84 is hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)-ammoniumbromide]. In isolated guinea pig atria combinations of the hexamethonium-derivative W84 and a number of nonselective muscarinic antagonists (e.g. N-methylscopolamine (NMS)) act considerably stronger anticholinergic than expected from the effects of the individual compounds. The M_2-subtype selective antagonist AF-DX 384 failed to produce an overadditive action in combination with W84 (Andresen I & Mohr K (1991) Naunyn-Schmiedeberg's Arch Pharmacol 344:R79). W84 strongly stabilizes [^{3}H]NMS binding to M-receptors. Aim of the present study was to evaluate the effect of W84 on [^{3}H]AF-DX 384 binding to M_2-receptors in comparison with the effect on [^{3}H]NMS binding. Radioligand-binding was measured using suspensions of guinea pig cardiac membranes (3 mM $MgHPO_4$, 50 mM Tris, pH 7.3, 23°C). The K_D values were 11 nM for [^{3}H]AF-DX 384 and 0.9 nM for [^{3}H]NMS. W84 inhibited the binding of [^{3}H]AF-DX 384 (2nM) and [^{3}H]NMS (0.5nM) binding concentration-dependently with IC_{50} values of 0.29 µM and 9 µM, respectively. Dissociation of both radioligands was visualized by addition of 10^{-4}M atropine and proceeded monophasically. W84 concentration-dependently retarded [^{3}H]AF-DX 384 dissociation (control $t_{\frac{1}{2}}$ = 4 min). A plot of k_{-1} versus W84-concentration yielded the dose-response curve for the retarding action on the dissociation. A 50% reduction was obtained at EC_{50} = 30 µM. [^{3}H]NMS dissociation (control $t_{\frac{1}{2}}$ = 20 min) was almost completely inhibited at high W84 concentrations, half maximal suppression was achieved at EC_{50} = 0.4 µM.

In conclusion, W84 has a considerably weaker stabilizing effect on [^{3}H]AF-DX 384 than on [^{3}H]NMS M_2-receptor complexes. It is tempting to speculate that the special characteristics of the interaction of [^{3}H]AF-DX 384 with M_2-receptors on which the M_2-selectivity is based, hinder W84 from exerting its stabilizing effect on the dissociation of this antagonist.

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THE GLUCOCORTICOID-INDUCED INHIBITION OF CHOLINE ESTERASE ACTIVITY AND THE IMPORTANCE FOR DRUG INTERACTIONS

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Glucocorticoids (GC) are able to inhibit plasma cholinesterase (PChE) in different species. Because of the frequency of GC-treatment and the importance of PChE for the hydrolysis of drugs, dose- and time relationships of this effect and the importance for drug interactions were investigated in a joint experimental and clinical study.
Dexamethasone (D) or prednisolone (P) were applied in rats for 7 days once / day (p.o., i.p. resp.). The activity of PChE (pH-state-technique) and the time of respiratory-arrest caused by 0.8 mg/kg succinylbischoline (Suc) were measured. D (0.5 - 5.0 mg/kg and P (2.5 - 5.0 mg/kg) caused in rats (male and female) a dose-dependent inhibition of PChE (between 13 - 75 %) which returned to normal within 8 days. This effect was stronger after oral administration as compared to the i.p. one. D was more potent than P; sex differences were observed. The time of respiratory-arrest caused by Suc was prolonged significantly up to 124 % (2.5 mg/kg P) and 169 % (0.5 mg/kg D) after GC-treatment.
In GC-treated patients (1 - 3 mg/kg/day P; time of treatment 9 - 18 days) a strong reduction of PChE to 50 - 30 % of normal values was measured. Sex differences were not observed.
It must be concluded from these results that the inhibition of PChE induced by therapeutic doses of GC influences the effects of drugs which are hydrolysed by PChE.

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EFFECTS OF ATROPINE AND TWO OXIMES ON ACETYLCHOLINESTERASE IN SEVERAL TYPES OF MUSCLE IN SOMAN POISONED MICE

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Conventional therapy of organophosphate poisoning with atropine (A) and presently used oximes is insufficient after soman (S), mainly because of lacking oxime efficacy. Searching for an alternative the efficacy of the new oxime 1-((((4-aminocarbonyl)pyridino)methyl)-2,4-bis)(hydroxyimino)methyl) pyridinium dimethanesulfonate (HLö 7) was checked in soman poisoned mice and compared to the efficacy of 1-(((4-(aminocarbonyl-pyridinio)methoxy)methyl)-2-(hydroxyimino)methyl)dichloride (HI 6). 7 groups of 6 white male NMRI mice each, were built: group 1 (control) received saline 0.9% (0.01 ml/g), group 2: S 0.1 mg/kg s.c., group 3: S + A 10 mg/kg i.p. , group 4: S + A + HI 6 10 µmol/kg i.p., group 5: S + A + HI 6 150 µmol/kg i.p., group 6: S + A + HLö 7 10µmol/kg i.p. and group 7: S + A + HLö 7 150 µmol/kg i.p. After a period of 90 min samples of M soleus (SOL), M extensor digitorum longus (EDL) and diaphragma (DIA) were homogenized and acetylcholinesterase activity was measured photometrically (Ellman et al., 1961).

Group	NaCl	S	S+A	S+A+ HI6 10	S+A+H I6 150	S+A+ HLö 7 10	S+A+H Lö 7 150
SOL (U/l) SD±	1547 250	42 111	350 298	226 38	378 172	796 245	1527 354
EDL (U/l) SD±	2876 668	273 267	546 462	626 228	1160 495	1502 190	2430 483
DIA (U/l) SD±	3059 314	870 311	910 315	804 422	1946 365	2277 217	3284 756

There was a significantly higher reactivation of acetylcholinesterase by HLö 7 (150 µmol/kg) compared to HLö 7 (10) and HI 6 (10 and 150 µmol/kg) in all muscles after inhibition by soman. HLö 7 seems to be at least equally effective as HI 6 concerning reactivation of AChE in several types of muscle.

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CHROMAFFIN CELLS ACCUMULATE TETANUS TOXIN AND ACTIVATE IT BY REDUCTIVE CLEAVAGE

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Tetanus toxin is produced by clostridial bacteria as a single-chain protein. It is cleaved by limited proteolysis into a heavy (HC) and a light chain (LC) which remain connected by one disulfide bond. The LC contains the intrinsic activity whereas the HC binds to gangliosides, which is a prerequisite for the cellular uptake of the toxin. Though chromaffin cells lack specific gangliosides it is possible to render the cells sensitive for the toxin by preincubating them with exogenous gangliosides or by permeabilizing their plasma membrane by subjecting the cells to an electric field. In both cases the toxin enters the cytoplasm as can be shown by tetanus toxin bound to 10 nm gold particles. Cells pretreated with gangliosides display labelling of the plasma membrane as well. After 48 hours the label is associated with the Golgi complex indicating that the elimination of the toxin or its fragments begins at a relatively early stage. Radioiodinated toxin introduced into chromaffin cells by electroporation is progressively split into its LC and HC and further degraded into smaller fragments. The LC survives the HC. The reductive cleavage of the sulphur bonds of the toxin indicates an enzymatic activity present in chromaffin cells that is responsible for the activation of the di-chain form. Since chromaffin cells, like neurons, contain thioredoxin-reductase which is able to reduce the toxin, this enzyme is assumed to play a major part in the pathogenesis of tetanus. Reductive cleavage, however, may not really separate both chains from each other, because the protoxin is 10 times more potent than the isolated LC when applied to electroporated chromaffin cells. Thus, the HC somehow contributes to the action of the LC. Not only chromaffin cells are sensitive to tetanus toxin, but they also possess the enzymatic equipment to activate it. They represent a suitable model in the study of the pathogenesis of tetanus.

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AMEZINIUM AND 1-METHYL-4-PHENYLPYRIDINIUM (MPP^+): TOOLS FOR THE STUDY OF CARRIER-MEDIATED AMINE UPTAKE INTO CHROMAFFIN GRANULES

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Amezinium is accumulated in sympathetically innervated tissue and released from it by nerve stimulation (Steppeler and Starke 1982: Biochem Pharmacol 31: 1075-1080). Therefore, we examined whether amezinium is transported by the carrier into ghosts, i.e. into catecholamine (CA) depleted membrane vesicles prepared from bovine adrenomedullary chromaffin granules. In the presence of ATP-Mg^{2+}, incubated ghosts (30°C; pH 7.4) showed a reserpine-sensitive net uptake of CA (K_m 8 µmol/l; V_{max} 20 nmol/[mg protein · min]) during the first 3 min of incubation. In the presence of amezinium (30-300 µmol/l) and ATP-Mg^+, incubated ghosts showed a reserpine-sensitve uptake of amezinium (K_m 181 µmol/l; V_{max} 4 nmol/[mg prot. · min]), which was competitively inhibited by CA. Furthermore, uptake studies with the neurotoxic agent MPP^+ (5.5 - 132 µmol/l) revealed a reserpine-sensitive uptake into ghosts (K_m 24 µmol/l; V_{max} 8 nmol/[mg protein·min]). The K_m of uptake of amezinium and of MPP^+ agreed with K_i, found after inhibition of uptake of CA into ghosts by the agents. The reserpine-insensitive uptake of CA, amezinium and MPP^+ showed the same linear dependence on the external concentration; it corresponded to an increment of uptake of 2 nmol/[mg protein · min] per 100 µmol/l external concentration. This is a high rate of membrane diffusion with regard to the positive charge of the agents. Moreover, the uptake of amezinium and MPP^+ contradicts the hypothesis of transport of the uncharged form of CA by the granular carrier, which was deduced from the pH dependence of K_m of CA uptake, and the abolition of pH dependence, when the uncharged form of CA was considered. Determinations of K_m and V_{max} of MPP^+ at various pH gradients and at various external pH should help to find out, whether pH dependence of K_m is caused by competition between protons and positively charged substrates for binding to ionizable groups of the carrier.

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The influence of the intracellular ion concentration on the desipramine-sensitive ^{3}H-noradrenaline uptake (uptake$_1$)

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Uptake$_1$ is well known to depend on the extracellular concentrations of sodium and chloride. Little is known about the influence of intracellular ion concentrations on uptake$_1$. Hence, it was of interest to investigate the dependence of kinetic constants of uptake$_1$ on the intracellular concentration of protons, sodium, and chloride. Clonal PC12 cells and isolated membrane vesicles from PC12 cells were choosen as experimental model. Initial rates of ^{3}H-noradrenaline (^{3}H-NA) uptake were determined. Non-specific uptake was determined in the presence of 1 µmol/l Desipramine.

Extracellular acidosis (pH 6.9) present during 1 min of incubation with ^{3}H-NA did not affect kinetic constants. However, prolonged extracellular acidosis (pH 6.9; present during 25 min of preincubation and 1 min of incubation; controls at pH 7.4) increased K_m from 910 (95% confidence limits; 580; 1500) to 2200 (1500; 3200) nmol/l ($p<0.01$, n=6) and V_{max} from 100 ± 13 to 150 ± 20 pmol mg^{-1} min^{-1} ($p<0.05$, n=6). In isolated membrane vesicles the increase of the intracellular proton concentration from pH 8.3 to pH 6.5 increased V_{max} 3-fold ($p<0.001$, n=6-9) and decreased K_m 2.3-fold ($p<0.05$, n=6-9).

Although uptake$_1$ is absolutely dependent on the concentration of extracellular chloride, the increase of intravesicular chloride from 2 mmol/l to 50 mmol/l failed to affect the kinetics of uptake$_1$. However, an increase of intravesicular sodium from 0 mmol/l to 50 mmol/l increased the K_m from 140 (70, 250) nmol/l to 450 (290, 650) nmol/l ($p<0.001$, n=6), but let the V_{max} unchanged.

It is concluded that uptake$_1$ markedly depends on the intracellular concentration of protons and sodium.

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Cyanine-derived dyes as a novel class of potent inhibitors of extraneuronal noradrenaline transport (uptake$_2$)

H. Russ, W. Engel* and J. Sonna

While highly potent inhibitors for the desipramine-sensitive neuronal transport mechanism of noradrenaline (uptake$_1$) are widely used, there is a lack of potent inhibitors for the extraneuronal mechanism (uptake$_2$). The recent development of an experimental model for uptake$_2$ which is based on the clonal cell line Caki-1 (Schömig and Schönfeld, Naunyn Schmiedeberg's Arch Pharmacol 1990, 341:404-410) facilitates the search for more potent inhibitors of uptake$_2$ markedly.

(1) Confluent monolayers of Caki-1 cells were incubated for 15 min at 37°C with 100 nmol/l ^{3}H-noradrenaline in the absence and presence of various substances. Cationic quinoline compounds derived from the dye cyanine inhibited uptake$_2$. Especially, certain derivatives of the isocyanine and pseudoisocyanine group as 1,1'-diethyl-2,2'-cyanine and the newly synthesized 1-methyl-1'-isopropyl-2,4'-cyanine and 1,1'-diisopropyl-2,4'-cyanine were found to be highly potent inhibitors of uptake$_2$, exhibiting IC$_{50}$'s of 16 (95% confidence interval: 8, 28), 62 (41, 89), and 14 (5, 26) nmol/l, respectively (n=5). Under these conditions, the most potent inhibitor of uptake$_2$ known so far, corticosterone, was about ten times less potent.

(2) In the perfused rat heart (Langendorff technique) and in the isolated incubated rabbit aorta, 1-methyl-1'-isopropyl-2,4'-cyanine inhibited the initial rate of ^{3}H-noradrenaline (10 nmol/l) transport via uptake$_2$ with high potency, the IC$_{50}$'s being 100 (70, 140) and 740 (318, 1280) nmol/l, respectively (n=4). Corticosterone inhibits uptake$_2$ in the perfused rat heart about ten times less potently.

(3) Uptake$_1$ is hardly influenced by the cyanine-derived dyes. In PC12 cells 3 µmol/l 1,1'-diethyl-2,2'-cyanine inhibited uptake$_1$ by only 14.7 ±2.3% (n=6).

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SIMILARITIES BETWEEN THE EXTRANEURONAL TRANSPORT SYSTEM FOR NORADRENALINE AND THE RENAL TRANSPORT SYSTEM FOR ORGANIC CATIONS

Kathrin Keppler and E. Schömig

Recent findings indicate a close relationship between the corticosterone-sensitive extraneuronal transport system for noradrenaline (uptake$_2$) and the renal transport system for organic cations (RTOC) (Schömig and Schönfeld 1990, Naunyn Schmiedeberg's Arch Pharmacol 341:404-410). Hence, it was of interest to investigate RTOC in more detail. Clonal LLC-PK1 cells (pig kidney, proximal tubule) were grown either on plastic dishes to measure inwardly directed transport of ^{14}C-tetraethylammonium (^{14}C-TEA) through the apical cell membrane or on filters to measure transepithelial transport of ^{14}C-TEA. The rate of outward transport through the apical membrane was calculated from the transepithelial (basolateral to apical directed) transport rate and from the intracellular concentration of ^{14}C-TEA. Inhibitors of transport were present on the apical side only. Non-specific transport was determined in the presence of 10 µmol/l cyanine863 which is a known inhibitor of RTOC.

The results suggest two transporters being involved in ^{14}C-TEA transport through the apical membrane of LLC-PK1 cells. Both inward and outward transport were sensitive to cyanine863, the IC$_{50}$'s being 99 (95% confidence interval: 64, 153) and 140 (50, 230) nmol/l, respectively (n=7). However, only inward transport of ^{14}C-TEA through the apical membrane was sensitive to the uptake$_2$ inhibitor corticosterone (300 nmol/l), which reduced inward transport markedly (46 ± 5 % of control; p<0.001; n=6), but failed to inhibit outward transport (96 ± 12 %; n=6).

Inhibition by various compounds of inward transport through the apical membrane of ^{14}C-TEA was investigated. There was a positive correlation between the IC$_{50}$'s for the inhibition of ^{14}C-TEA transport and the IC$_{50}$'s for the inhibition of uptake$_2$ (r=0.626; n=14; p<0.05). The potent uptake$_2$ inhibitor 1,1'-diethyl-2,2'-cyanine turned out also to inhibit RTOC very potently, the IC$_{50}$ being as low as 6.1 (3.8, 9.6) nmol/l (n=6). Inward transport of ^{14}C-TEA at the apical membrane of LLC-PK1 cells is mediated by a mechanism which is very similar to uptake$_2$.

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THE ROLE OF CATECHOL-O-METHYLTRANSFERASE (COMT) IN THE CLEARANCE OF NORADRENALINE (NA) FROM AND THE APPEARANCE OF DIHYDROXYPHENYLGLYCOL (DOPEG) IN PLASMA

T. Halbrügge, J. Ludwig and K.-H. Graefe

Rabbits pretreated with reserpine (1 mg/kg 20 h and 0.5 mg/kg 2 h prior to the experiment) and anaesthetized with alfadolone plus alfaxalone were infused with NA (0.4 - 1.5 nmol kg^{-1} min^{-1}), and steady-state increases in the plasma levels of NA and DOPEG (ΔDOPEG) were determined both before and after blockade of uptake$_1$ by desipramine (2 mg/kg). At the end of each experiment, animals also received a DOPEG infusion (1.2 nmol kg^{-1} min^{-1}) to measure the plasma clearance of DOPEG. Experiments were carried out under control conditions or 90 min after commencement of treatment with the COMT inhibitor 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone (Ro 40-7592; 3 mg/kg followed by 1.5 mg/kg every 30 min). Besides the assessment of the plasma clearances of NA (CL$_{NA}$) and DOPEG (CL$_{DOPEG}$), the results served to determine the clearance index of neuronally formed DOPEG (CLI$_{DOPEG}$) from the ratio of "neuronal DOPEG formation rate to ΔDOPEG". The neuronal DOPEG formation rate was assumed to be identical with the steady-state rate of neuronal NA uptake (i.e., the fraction of CL$_{NA}$ due to uptake$_1$ times the NA infusion rate). The results given below are geometric means (95% confidence limits) of 7 experiments each.

Under control conditions, CL$_{NA}$ was 83.6 (71.2; 98.1) ml kg^{-1} min^{-1} and the fraction of CL$_{NA}$ brought about by uptake$_1$ 0.437 (0.369; 0.517). Neither the former nor the latter was affected by COMT inhibition [87.7 (82.0; 93.8) ml kg^{-1} min^{-1}; 0.443 (0.375; 0.525)]. However, COMT inhibition reduced CL$_{DOPEG}$ from 47.6 (38.2; 59.2) to 30.2 (26.0; 35.1) ml kg^{-1} min^{-1} (P < 0.01) and the CLI$_{DOPEG}$ from 95.3 (76.0; 119.5) to 29.9 (24.0; 37.3) ml kg^{-1} min^{-1} (P < 0.001).

Hence, COMT did not contribute to the removal of NA from plasma by neuronal and non-neuronal processes. By contrast, O-methylation is responsible for 37% of the DOPEG removal from plasma and fully accounts for the DOPEG removal (50%) taking place on the way from its site of neuronal formation to plasma.

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EFFECT OF CATECHOL-O-METHYLTRANSFERASE (COMT) INHIBITION ON THE IN-VIVO KINETICS OF DIHYDROXYPHENYLGLYCOL (DOPEG), 3-METHOXY-4-HYDROXY-PHENYLGLYCOL (MOPEG) AND DIHYDROXYPHENYLALANINE (DOPA)

B. Friedgen

In anaesthetized rabbits (alfadolone + alfaxalone) COMT inhibition was achieved by administration of Ro 40-7592 (3,4-dihydroxy-4'-methyl-5-nitrobenzophenone) at a dose of 3 mg/kg followed by 1.5 mg/kg every 30 min. This dose was found to be maximally effective in that it reduced the plasma level of endogenous MOPEG by 70% and increased that of endogenous DOPEG by 340%; it did not change significantly the levels of endogenous noradrenaline and DOPA (n = 4).

Constant rate infusions of DOPEG and DOPA (2.5 nmol kg^{-1} min^{-1} each) or MOPEG (5 nmol kg^{-1} min^{-1}) were given to rabbits with COMT being either intact or inhibited by treatment with Ro 40-7592. The plasma levels of infused DOPEG, DOPA and MOPEG were followed during and after the end of infusion. Plasma clearances (Cl) of DOPEG, DOPA and MOPEG were determined from the ratio of "infusion rate/steady-state increase in plasma level". Elimination half-lifes ($t_{1/2}$) were derived from the terminal decline of arterial plasma levels after cessation of infusion and steady-state volumes of distribution (V_{ss}) estimated from "A_{ss}/steady-state increase in plasma level" (with A_{ss} being the amount of compound in body at steady state). The results were as follows:

		Cl (ml kg^{-1}min^{-1})	V_{ss} (ml/kg)	$t_{1/2}$ (min)
DOPEG	Control	44.1 ± 2.5	89.0 ± 11.8	4.8 ± 0.9
	Ro 40-7592	25.8 ± 2.0**	476.3 ± 57.1**	31.0 ± 3.3**
DOPA	Control	37.6 ± 2.3	135.5 ± 20.0	4.9 ± 0.7
	Ro 40-7592	37.5 ± 3.0	253.5 ± 46.7*	13.0 ± 3.5*
MOPEG	Control	29.9 ± 3.2	768.8 ± 66.0	53.7 ± 4.5

Given are means ± SEM of 6 experiments each. COMT inhibition did not affect the kinetics of infused MOPEG. * P < 0.05, ** P < 0.001 for effects of COMT inhibition when compared with controls.

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BIOPHASE CONCENTRATIONS OF NORADRENALINE AT PRESYNAPTIC α_2-ADRENOCEPTORS DURING ELECTRICAL FIELD STIMULATION OF BRAIN SLICES

C. Allgaier and G. Hertting

The present experiments were designed to obtain an estimation of the local concentrations of noradrenaline (NA) existing at presynaptic α_2-adrenoceptors during electrical field stimulation of brain slices. They were based on the assumption that the concentration of released NA at the α_2-adrenoceptors exerting a certain autoinhibition should be equal to the concentration of exogenous NA causing the same inhibition under conditions in which any influence of the released transmitter is excluded.
Slices of rabbit and rat hippocampus and cortex, labelled with [^{3}H]NA, were superfused in the presence of an inhibitor of re-uptake and subjected to electrical field stimulation. Autoinhibition was avoided by stimulating the tissue with 4 pulses/100 Hz. Exogenous NA diminished the evoked [^{3}H]NA release in a concentration-dependent manner. When electrical stimulation was performed with trains of 36 pulses at 0.1, 0.3, or 3 Hz, in the absence or the presence of an uptake inhibitor, the α_2-adrenoceptor antagonist yohimbine (1 or 10 μmol/l) enhanced the evoked NA release in a manner which was dependent on the frequency of stimulation and on blockade of the re-uptake mechanism. The facilitatory effects of yohimbine reflected an autoinhibition which was between 53% (36 pulses/0.1 Hz, no uptake inhibitor) and 85% (36 pulses/3 Hz, uptake inhibitor present) in rabbit and between 16% (36 pulses/0.3 Hz, no uptake inhibitor) and 71% (36 pulses/3 Hz, uptake inhibitor present) in rat brain slices. The corresponding estimated biophase concentrations of NA were between 15 and 23.1 nmol/l or 6.1 and 18.6 nmol/l in the rabbit and between 32.5 and 74.5 nmol/l or 5.1 and 51.6 nmol/l in the rat (in the presence or the absence of an uptake inhibitor, respectively). The observed frequency dependence of the effect of re-uptake blockade on the calculated biophase concentrations of NA would be compatible with the idea of a dependence of the effectiveness of the re-uptake mechanism on the firing rate of the neurone in being more effective at lower frequencies. Moreover, the strikingly low biophase concentrations of NA suggest a lateral inhibition of release.

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PERTUSSIS TOXIN PRETREATMENT REVERSES α_2-ADRENOCEPTOR-MEDIATED INHIBITION OF Ca^{2+} CURRENTS AND OF NORADRENALINE RELEASE IN CHICK SYMPATHETIC NEURONS

E.A.Singer, S.Boehm, S.Huck and H.Drobny

Sympathetic neurons were derived from twelve day old chick embryos and kept in culture for one (electrophysiology) or five (superfusion experiments) days. One half of each tissue culture preparation was exposed to 100 ng/ml pertussin toxin (PTX) for 24 hours.
Electrophysiology: Experiments were carried out at room temperature using the whole-cell configuration of the patch-clamp technique. Ca^{2+} currents were induced from a holding potential of -80 mV by a 150 ms depolarizing voltage step to 0mV at intervals of 20 s. The amplitudes of the Ca^{2+} currents in the absence and presence of the α-adrenoceptor agonists noradrenaline (NA, 1 μmol/l), clonidine (CL, 10 μmol/l) or (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline (UK 14304, 10 μmol/l) were compared.
Superfusion experiments: Cells were labelled with ^{3}H-NA (40 Ci/mmol, 0.05 μmol/l, 37°C, 60 min), superfused with physiological salt solution at 25°C, and the superfusate was collected in 4-min fractions. Electrical field stimulation was applied after 72 (S_1) and 96 (S_2) min of superfusion using 36 monophasic rectangular pulses (0.5 ms, 40 V/cm, 60 mA) delivered at 3 Hz. The α-adrenoceptor agonists NA (in the presence of 10 μmol/l cocaine), CL and UK 14304 were added 16 min before S_2 at the concentrations indicated above. Drug effects were calculated as S_2/S_1 ratios.
All three agonists significantly reduced the amplitude of the Ca^{2+} current, measured 10 ms after the start of the depolarization, as well as the stimulation-evoked overflow of tritium. By contrast, the α-adrenoceptor agonists did not exert any effect in PTX-treated cells. Furthermore, a small increase in the electrically evoked overflow of radioactivity was observed in PTX treated cells.

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RECEPTOR RESERVE AT α_2-ADRENORECEPTORS IN RAT BRAIN CORTEX.

E. Agneter

Male Sprague-Dawley rats were pretreated either with the irreversible α_2-adrenoceptor antagonist EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, 1.6 mg/kg i.p) or with saline (1 ml/kg i.p.) and killed 24, 48, 96, 192, 336, or 744 hr later. Slices of of parieto-occipital cortex were prepared, labelled with (-)-[3H]noradrenaline (0.125 μmol/l, 30 min, 37°C) and superfused with physiological salt solution in the presence of 1 μmol/l desipramine (0.7 ml/min). After a washout period of 30 min the collection of 4 min fractions was commenced. Electrical field-stimulation was applied at the beginning of the 2nd, 9th, 16th and 23rd fraction (S1-S4; monophasic rectangular pulses, 2ms, 12V/cm, 18mA, 4 pulses delivered at 100 Hz). α_2-Adrenoceptor agonists were added at increasing concentrations 20 min before S2, S3 and S4. Cumulative concentration-response curves were generated for 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline (UK 14304), clonidine and noradrenaline in slices of control and EEDQ treated animals.
The fraction of receptors remaining active (q) and the agonist dissociation constant (K_a) were determined for each agonist at each time point according to the method of Furchgott and Bursztyn (1967, Ann NY Acad Sci 144:882-899).
The amount of tritium overflow at S1 was not different in slices of control and EEDQ treated animals. The table shows q-values and maximal inhibition of tritium overflow at the different post treatment intervals.

	24hr	48hr	96hr	192hr	336hr	744hr
q (%)	4.1	7.8	16.5	28.1	42.9	67.5
UK 14304	50.8	67.1	81.6	95.2	100.0	
clonidine	26.3	39.7	66.1	85.4	90.4	100.0
noradrenaline	n.d.	73.5	100.0			

The calculated time needed to resynthesize 50 % of the receptors was 352 hr. The K_a values calculated for a given agonist were comparable at all timepoints used for calculation. The mean values were 155.3 nM, 71.6 nM and 1202.7 nM for UK14304, clonidine and noradrenaline, respectively.

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PRESYNAPTIC α_2-AUTORECEPTORS IN RAT ATRIA AND SUBMAXILLARY GLANDS: A NON-α_{2A}, NON-α_{2B} SUBTYPE

A. U. Trendelenburg and N. Limberger

The presynaptic α-autoreceptors in rat atria have been postulated to represent the α_1 or α_{2B}, and those in rat submaxillary glands (sg) the α_{2B} or α_{2D} subtype. In order to examine these suggestions, pieces of atria and sg were preincubated with ^{3}H-noradrenaline and then superfused in presence of desipramine 1 μM and corticosterone 10 μM. Four periods of electrical stimulation were applied, each period consisting of 10 trains of 6 pulses/100 Hz that were delivered at intervals of 30 s. Cumulative concentration-response curves for the α-adrenoceptor agonists 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK14304) and methoxamine were determined. - UK14304 inhibited the overflow of tritium with an EC_{50} of about 6 nM in either tissue. α-Adrenoceptor antagonists, given at one or two concentrations, shifted the concentration-response curve to the right. From these shifts the following affinity constants (pA_2) were calculated. Atria: rauwolscine (concentration used 0.1 μM) 8.2; 2-(2,6-dimethoxyphenoxyethyl-)aminoethyl-1,4-benzodioxane (WB4101; 0.3 μM) 7.5; 2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL44408; 1 μM) 7.4; prazosin (0.1 and 1 μM) 6.8; 6-chloro-9-(3-methyl-2-butenyl)oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine (SKF104078; 1 μM) 6.6; 1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine (BRL41992; 1 μM) 6.5; corynanthine (3 μM) 6.1. sg: rauwolscine 7.8; BRL44408 7.7; BRL41992 7.2; WB4101 7.0; SKF104078 6.5; prazosin 6.1; corynanthine 5.7. - Methoxamine reduced the evoked overflow of tritium with an EC_{50} of about 9 μM in either tissue. Rauwolscine antagonized the effect of methoxamine with a potency similar to that observed against UK14304, and the same was true for prazosin. - The results show that the α-autoreceptors in rat atria and sg are not α_1 but α_2. In terms of the proposed α_2-subclassification into $\alpha_{2A\text{-}D}$, the α_{2B} subtype is excluded by the finding that BRL44408 was 3-8 times more potent than BRL41992, whereas at the prototype α_{2B} binding site, i.e. in rat lung, BRL41992 has 130 times higher affinity than BRL44408 (Young et al., Eur J Pharmacol 168:381-386, 1989). In addition, WB4101 was 5-8 times more potent than prazosin, whereas in rat lung prazosin has slightly higher affinity than WB4101 (Simonneaux et al., Molecular Pharmacol 40:235-241, 1991). They probably also do not belong to the α_{2A} subtype since at the prototype α_{2A} binding site, i.e. in human platelets, BRL44408 has 60 times higher affinity than BRL41992 (Young et al. 1989) and WB4101 has 330 times higher affinity than prazosin (Simonneaux et al. 1991) - much greater differences than observed in the present experiments. The antagonist affinities of the autoreceptors agree best with either the α_{2C} or the α_{2D} subtype. The different antagonist rank orders of potencies in the two tissues do not exclude the possibility that the α_2 subtypes differ in the two tissues.

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INTERACTION BETWEEN α_2-ADRENOCEPTORS, OPIOID μ-RECEPTORS AND GALANIN-RECEPTORS OF RAT LOCUS COERULEUS NEURONES

J. Sevcik and E. Schöffel

The measurement of [^{3}H]noradrenaline release from brain slices proved an interaction between presynaptic receptors for various cotransmitters (Illes et al., Ann. N.Y. Acad. Sci., 604:197, 1990). We investigated, whether a similar interaction occurs also between receptors situated at the somata of noradrenergic locus coeruleus (LC) neurones, in which histological findings indicate the presence of the possible peptide cotransmitters [Met^5]enkephalin and galanin. The spontaneous firing of LC cells was measured in a midpontine slice preparation of the rat brain. Noradrenaline, [Met^5]enkephalin and galanin depressed the discharge of action potentials. Since the α_2-adrenoceptor antagonist rauwolscine, but not the α_1-adrenoceptor antagonist prazosin counteracted the effect of noradrenaline, it may be assumed that noradrenaline activates only α_2-receptors. Similarly, the strong antagonism between the preferential opioid μ-antagonist naloxone and [Met^5]enkephalin, in conjunction with the weak antagonism between the preferential opioid δ-antagonist ICI 174864 (N,N-bisallyl-Tyr-Aib-Aib-Phe-Leu-OH) and [Met^5]enkephalin (Williams and North, Mol. Pharmacol., 26:489, 1984) suggest the sole existence of μ-receptors. Neither rauwolscine nor naloxone altered the firing on their own. Rauwolscine potentiated the effect of [Met^5]enkephalin, but not the action of galanin. The effect of [Met^5]enkephalin was unchanged by preincubation with noradrenaline. Furthermore, [Met^5]enkephalin or noradrenaline produced the same inhibition, both in the presence and absence of galanin. In conclusion, among α_2-adrenoceptors, opioid μ-receptors and galanin-receptors, only the first two types appear to interact with each other.

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INHIBITORY PRESYNAPTIC IMIDAZOLE RECEPTORS ON NORADRENERGIC NERVES IN CENTRAL AND PERIPHERAL TISSUES OF RAT AND RABBIT

G.J.Molderings

Imidazole (I) recognition sites, recently identified in central and peripheral tissues of various species, may play a role in regulation of blood pressure. In slices and synaptosomes from the rat brain, in the rat vena cava and aorta and in the rabbit aorta preincubated with [^{3}H]noradrenaline (NA) und subsequently superfused with uptake$_1$-, uptake$_2$- and ß-adrenoceptor blockers, respectively, the involvement of I-receptors in modulation of NA release was investigated. To investigate α_2-adrenoceptor-independent inhibition of [^{3}H]NA release, the presynaptic α_2-adrenoceptors were blocked either by phenoxybenzamine (PBA) or rauwolscine. In slices and synaptosomes from the frontal cortex of the rat brain, NA, clonidine and moxonidine inhibited the release of [^{3}H]NA by stimulating presynaptic α_2-adrenoceptors only. In the rat medulla oblongata, α_2-adrenoceptors as well as an α_2-adrenoceptor-independent mechanism were involved in the inhibitory effect of clonidine and moxonidine on [^{3}H]NA release, since it was partly resistant to PBA pretreatment. The sympathetic nerve terminals in the rat vena cava and aorta are endowed with presynaptic inhibitory α_2-adrenoceptors. In addition, an α_2-adrenoceptor-independent (PBA-resistant) mechanism, which can be activated by cirazoline, clonidine, moxonidine and BDF 6143 (4-chloro-2-(2-imidazolin-2-ylamino)-isoindoline), seems to be operative at µmolar concentrations. After blockade of α_2-autoreceptors, the evoked ^{3}H overflow from rabbit aorta was inhibited by various imidazolines and guanidines. The rank order of potencies fits well to the pharmacological properties of the I-receptors described for the rabbit pulmonary artery, but not for those of I recognition sites in other tissues.

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MODIFICATION OF CATECHOLAMINE RELEASE IN THE LOCUS COERULEUS BY EXPERIMENTALLY INDUCED BLOOD PRESSURE CHANGES

N. Singewald

To investigate the role of catecholaminergic neurons of the locus coeruleus in central cardiovascular control, the locus coeruleus of the anaesthetized cat was bilaterally superfused with artificial cerebrospinal fluid through push-pull cannulae. The superfusion rate was 150µl/min. The release rates of endogenous noradrenaline and dopamine were determined radioenzymatically in the superfusates, collected in time periods of 3 min. Arterial blood pressure and heart rate were recorded in the femoral artery. Experimentally induced blood pressure changes were elicited by intravenous administrations of drugs, controlled haemorrhage, or blood injection.

Rises in blood pressure elicited by infusion of noradrenaline (5µg/kg/min, i.v.) for 3 min or by hypervolaemia (30% of total blood volume) were associated with a decrease in the release of noradrenaline in the locus coeruleus. The release of dopamine was not influenced.

A fall of blood pressure caused by i.v. administration of nitroprusside (injection: 5µg/kg, infusion: 8µg/kg/min) or chlorisondamine (3mg/kg) did not modify the release rates of noradrenaline or dopamine. Hypotension elicited by a controlled haemorrhage (15% of total blood volume) enhanced the release of noradrenaline drastically, while the release of dopamine was not modified.

The results indicate that pressor responses due either to vasoconstriction or to hypervolaemia inhibit the release of noradrenaline in the locus coeruleus. Decreases in blood pressure enhance the release of noradrenaline if they are elicited by hypovolaemia only; a vasodilatation caused by nitroprusside or chlorisondamine is ineffective, even if the drugs lead to a very profound depressor response. Hence, the activity of noradrenergic neurons of the locus coeruleus is influenced by impulses from aortic and cardiopulmonary baroreceptors. Noradrenergic neurons of the locus coeruleus seem to be involved in central cardiovascular control, pointing to a possible hypertensive function in counteracting blood pressure changes.

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ANGIOTENSIN II-INDUCED VASOPRESSIN RELEASE: MEDIATION BY α1-ADRENOCEPTORS IN THE SUPRAOPTIC NUCLEUS

Qadri, F., Maas, K., Culman, J.

The vasopressin (AVP) producing supraoptic nucleus (SON) receives afferents from angiotensin II (ANG II) receptor containing circumventricular organs (e.g. the subfornical organ) as well as catecholaminergic inputs arising from the brain stem. In the present study we investigated in conscious rats whether the ANG II-induced AVP release involves a catecholaminergic pathway in the SON. In the first experimental protocol we used the microdialysis technique to study the effect of stimulation of periventricular ANG II receptors on the release of catecholamines in the SON. ANG II applied intracerebroventricularly (icv) at doses between 100 pg - 100 ng induced a selective, dose-dependent noradrenaline (NA) release, which was sensitive to icv pretreatment with the ANG II-AT$_1$ receptor antagonist Losartan (DuP 753/MK 954). In the second experimental protocol, we measured the ANG II (icv)-induced AVP release after adrenergic receptor blockade or stimulation in the SON. The α1-adrenoceptor antagonist prazosin (0.7 nmol) injected bilaterally into the SON reduced the ANG II (100 ng, icv)-induced AVP release by 50%, while the α2-, ß1-, ß2-antagonists, idazoxan, atenolol and ICI 118551, respectively, had no effect. Microinjections of low doses of NA (1 - 10 nmol) into the SON produced a dose-dependent increase in AVP release, whereas higher doses (30 - 100 nmol) of the same drug were inhibitory. The stimulatory effect of NA in the SON was mimicked by the application of the α1-agonist methoxamine (1 - 5 nmol) into the SON, which also had inhibitory effects at higher doses (10 - 30 nmol). Neither the α2-agonist, clonidine, nor the ß1- or ß2-adrenoceptor agonists, dobutamine or salbutamol, injected into the SON, affected the AVP release.

Our data are the first in vivo characterization of the pathway engaged by central ANG II to release AVP via the SON. ANG II acting on periventricular ANG II-AT$_1$ receptors induces the release of NA in the SON to selectively stimulate α1-adrenoceptors on vasopressinergic neurons, thereby causing these cells to secrete AVP into the circulation.

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SARALASIN INHIBITS PERIPHERAL SYMPAPTHETIC ACTIVITY

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The effect of the Angiotensin II antagonist Saralasin on peripheral sympathetic nerve-activity, vascular smooth muscle tone and the discussed local renin-angiotensin-system was studied on isolated perfused rat hearts. Intraaxonal catecholamines were measured in shock-frozen right ventricle tissue by fluorescence-microscopy with picture-analysing-system. Also the coronary flow, frequency and pressure were measured.

	control	AII (10^{-8}M)	Sar ($10^{-6,5}$M)	AII+Sar (10^{-8}M/$10^{-6,5}$M)
intraaxonal Catecholamines area in %	1,77	1,41	1,85	1,67
Flow in 35min.	249	102	270	196 ml

The peptide AII, added to the perfusate induces a release of catecholamines and a decrease of coronary flow, both are reducted by simultanously added Saralasin. Saralasin only causes an increase of these parameters, thus effects of endogenous AII cannot be excluded.Resuming these facts, it is to conclude that Saralasin is a potent AII-antagonist, influencing the AII receptors, which are located on vessels and on the sympathetic nerve membrane.

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EFFECT OF THE DA_2-RECEPTOR AGONIST QUINPIROLE ON RENAL SYMPATHETIC NERVE ACTIVITY AND RENAL NORADRENALINE SPILLOVER IN ANAESTHETIZED RABBITS

Bela Szabo and Dietmar Crass

The effect of intravenous quinpirole on blood pressure, heart rate, renal sympathetic nerve activity, the renal noradrenaline spillover rate (calculated from renal blood flow, arterial and renal venous concentrations of endogenous noradrenaline and the fractional renal extraction of ^{3}H-noradrenaline) and the renal vascular resistance was studied in anaesthetized rabbits (alfadolone + alfaxalone).

Quinpirole 100 $\mu g\ kg^{-1}$ + 5 $\mu g\ kg^{-1}\ min^{-1}$ lowered blood pressure and increased renal nerve activity, the latter through the baroreflex. In spite of the enhanced activity of the renal nerves, renal noradrenaline spillover was reduced, indicating an inhibition of transmitter release in the kidney. Renal vascular resistance was also decreased; the decrease persisted in animals with surgically denervated kidneys. Quinpirole did not influence resting heart rate but completely abolished the reflex tachycardia during infusion of sodium nitroprusside. Effects of quinpirole on blood pressure, renal nerve activity, renal spillover of noradrenaline and renal vascular resistance were antagonized by the DA_2-receptor-selective antagonist domperidone (1 mg kg^{-1} + 0.2 mg $kg^{-1}\ h^{-1}$).

This is the first direct measurement of the effect of a DA_2-receptor agonist, in vivo, on the release of noradrenaline from a peripheral organ with uninterrupted sympathetic impulse traffic. The results demonstrate that activation of DA_2-receptors, probably at terminal axons of postganglionic sympathetic neurones, reduces transmitter release per unit nerve activity. However, the fall in renal vascular resistance was not, or only partly, due to the decrease of the release of noradrenaline. Presynaptic inhibition in the heart prevented the reflex cardioacceleration during hypotension.

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ACTIVATION OF α-ADRENOCEPTORS OF THE α_{2A}-SUBTYPE INHIBITS NORADRENALINE RELEASE IN RAT KIDNEY: NO EVIDENCE FOR INHIBITORY α_{2B}-ADRENOCEPTORS.

C. Bohmann, P. Schollmeyer

The aim of the present study was to test whether the α_{2A}- or the α_{2B}-adrenoceptor subtype mediates inhibition of noradrenaline release in rat isolated kidney. Kidneys were incubated with ^{3}H-noradrenaline. The stimulation-induced (S-I) outflow of radioactivity was taken as an index of noradrenaline release. There were 6 short trains of stimulation (S_1-S_6), each at 100 Hz for only 60 ms, to avoid autoinhibition. None of the α-adrenoceptor antagonist used (see below) enhanced S-I outflow of radioactivity. The α_2-adrenoceptor agonist clonidine (non-selective for α_{2A} and α_{2B}) induced a maximal inhibition of the S-I outflow of 90 % (EC50=7.8 nM). The following α-adrenoceptor antagonists shifted the concentration response curve to the right: Idazoxan (0.1 μM, non-selective for α_{2A} and α_{2B}) - pA_2=8.3; 2-[2H-(1-methyl-1,3-dihydroiso-indole)methyl]-4,5-di-hydroimidazole (BRL 44408, 0.3 μM, α_{2A}-selective) - pA_2=7.7; 1,2,-dimethyl-2,3,9, 13b-tetrahydro-1H-dibenzo[c,f]-imidazo[1,5-a]azepine (BRL 41992, 0.3 μM, α_{2B}-selective) - pA_2=7.0; prazosin (0.3 μM; α_{2B}-selective) - pA_2=6.8; imiloxan (0.3 μM, α_{2B}-selective) - pA_2=6.7. In kidneys, which were pretreated with the irreversible α-adrenoceptor antagonist phenoxybenzamine (PBZ, 1 μM) for 40 min, clonidine failed to inhibit S-I outflow of radioactivity. However, when α_{2A}- or α_{2B}-adrenoceptors were protected during the PBZ pretreatment by either BRL 44408 (0.3 μM) or by prazosin (1 μM), clonidine again induced a maximal inhibition of 70% and 56%, respectively. In the experiments, in which α-adrenoceptors had been protected by prazosin during the PBZ treatment, the concentration response curve for clonidine was again shifted more potently to the right by BRL 44408 (0.3 μM) with a pA_2 of 7.3 than by imiloxan (3 μM) with a pA_2 of 6.5. The rank order of affinities for the α-adrenoceptor antagonists (BRL 44408 > BRL 41992 > prazosin and imiloxan) suggests that prejunctional inhibitory α-adrenoceptors in rat kidney are of the α_{2A}-subtype. Even after elimination of α_{2A}-adrenoceptors by PBZ, functional α_{2B}-adrenoceptors (BRL 44408 > imiloxan) could not be detected.

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EFFECTS OF CARMOXIROLE (EMD 45609) ON NEUROTRANSMISSION IN HUMAN KIDNEY SLICES AND RAT ISOLATED KIDNEY.

L.C. Rump

The effect of the classical dopamine D_2-receptor agonist quinpirole and EMD 45609, a drug with high affinity to (D_2)-receptors (Haase et al. 1991, Naunyn-Schmiedeberg's Arch Pharmacol, 343: 588-594), on the stimulation induced (S-I) outflow of radioactivity from human kidney slices and rat isolated kidneys preincubated with ^{3}H-noradrenaline was investigated. There were two stimulation periods (S_1 and S_2) at a frequency of 5 Hz in human kidney slices and of 1 Hz in rat isolated kidneys. In human kidney slices quinpirole (1 μM) and in rat kidney quinpirole (0.3 μM) inhibited S-I outflow of radioactivity. In both tissues the inhibitory effect of quinpirole was blocked by the D_2-receptor antagonist (-)-sulpiride (10 μM) but not by the non-selective α-adrenoceptor antagonist phentolamine (1 μM). Phentolamine but not sulpiride blocked the inhibitory effect of the α_2-adrenoceptor agonist UK 14304 (0.1 μM) in human kidney. Phentolamine (1 μM) by itself facilitated S-I outflow of radioactivity in human kidney to a lesser extent than in rat kidney. In human and rat kidney EMD 45609 (0.003 μM) had no effect on the S-I outflow of radioactivity. EMD 45609 (0.03 μM) had no effect on the S-I outflow of radioactivity in rat kidney but in human kidney inhibited S-I outflow, an effect blocked by sulpiride (10 μM) but unaltered by either the D_1-receptor antagonist R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH 23390, 1 μM) or by phentolamine. EMD 45609 (0.3 μM) had no effect on the S-I outflow of radioactivity in human kidney but facilitated S-I outflow in rat kidney. In the presence of phentolamine EMD 45609 (0.03 and 0.3 μM) inhibited S-I outflow in both tissues. The inhibitory effect of EMD 45609 (0.3 μM) in the presence of phentolamine in rat kidney was blocked by sulpiride (10 μM). At a lower stimulation frequency of 0.3 Hz EMD 45609 (0.03 μM) inhibited S-I outflow even in the absence of α-adrenoceptor blockade by phentolamine, an effect again blocked by sulpiride (10 μM). The data suggest that EMD 45609 activates inhibitory prejunctional D_2-receptors in human kidney slices and rat isolated kidney. However, this effect is masked by a simultaneous blockade of inhibitory α-autoreceptors in rat and to a lesser extent also in human kidney.

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EP_3 RECEPTOR-MEDIATED INHIBITION OF NORADRENALINE RELEASE FROM THE POSTGANGLIONIC SYMPATHETIC NERVE FIBRES OF THE RAT VENA CAVA

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Rat vena cava segments were preincubated with ^{3}H-noradrenaline and superfused with physiological salt solution containing desipramine and corticosterone. The electrically (0.66 Hz) evoked tritium overflow was inhibited by prostaglandin E_2 (PGE_2) in the absence and presence of rauwolscine 1 µmol/l; the maximum extent of inhibition amounted to about 80 % under either condition and the pIC_{40} value was 7.49 and 7.61, respectively. All subsequent experiments were carried out in the presence of rauwolscine 1 µmol/l, which, by itself, increased the evoked overflow by 277 %. The effect of PGE_2 on the evoked overflow was mimicked by prostaglandin E_1 (pIC_{40} 7.65), the EP_1 / EP_3 agonist sulprostone (7.85), the EP_2 / EP_3 agonist misoprostol (7.97) and, although less so, the IP / EP_1 agonist iloprost (5.87). The EP_1 antagonist AH 6809 10 µmol/l (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) had no effect by itself on the evoked overflow nor did it affect the concentration-response curve of PGE_2. Indometacin 3 µmol/l also failed to affect both the evoked overflow and its inhibition by PGE_2. Previous exposure of the vena cava segments to N-ethylmaleimide 30 µmol/l for 45 min, which, by itself, increased the basal tritium efflux (by 84 %) and decreased the evoked tritium overflow (by 56 %), tended to attenuate the inhibitory effect of PGE_2. The present results suggest that the sympathetic nerve fibres supplying the rat vena cava are endowed with inhibitory presynaptic EP_3 receptors, which do not appear to interact with the presynaptic α_2-autoreceptors and to be activated by endogenously formed prostaglandins of the E series. The possibility has to be considered that the EP_3 receptors under study may be coupled to a G protein.

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CHARACTERIZATION OF MUSCARINE RECEPTORS INVOLVED IN THE MODULATION OF ENDOGENOUS NORADRENALINE (NA) RELEASE FROM GUINEA-PIG ISOLATED TRACHEA.

C. Hey and K. Racké

The impulse-induced release of endogenous NA from the isolated rat trachea is inhibited via muscarine receptors [Racké et al. (1991) Br J Pharmacol 103:1213]. There is evidence that these presynaptic muscarine receptors belong to the M_2 subtype [Brunn et al. (1991) this journal 344:R74]. On the other hand, based on functional observations Pendry and Maclagan [Br J Pharmacol (1991) 103:1165] suggested that the muscarine receptors on sympathetic nerve endings in the guinea-pig trachea may belong to the M_3 subtype. These discrepancies could reflect species differences between rats and guinea-pigs. However, conclusions about muscarinic modulation of NA release based on the airways smooth muscle response are complicated by the fact that M_3 receptors mediate contraction of the airways smooth muscle. Therefore, in the present study the muscarinic modulation of NA release in the guinea-pig trachea was studied in experiments in which the release of endogenous NA was measured directely.

Guinea-pig isolated tracheae were incubated in 1.7 ml Krebs-HEPES solution (containing yohimbine, desipramine and tyrosine) and the release of endogenous NA was determined. Two periods of electrical field stimulation (S1, S2; 540 pulses at 3 Hz) were carried out. Oxotremorine was added 10 min before S2, antagonists were present from the onset of incubation.

In the absence of test drugs, S1 evoked the release of 37 pmol/g NA, corresponding to about 3.5 % of the respective tissue NA content. The muscarine receptor agonist oxotremorine concentration-dependently inhibited the evoked NA release, by 92 % at 1 µmol/l, and an IC_{50} of 40 nmol/l was calculated. The M_2 selective muscarine receptor antagonist methoctramine (1 µmol/l) caused a marked shift to the right of the concentration response curve of oxotremorine and an apparent pA_2 value of 7.6 was calculated. On the other hand, in the presence of the M_3 selective muscarine receptor antagonist p-fluoro-hexahydrosiladifenidol (1 µmol/l) the concentration response curve of oxotremorine was only marginally shifted to the right and an apparent pA_2 value of 5.9 was calculated.

In conclusion, noradrenergic nerve endings in the guinea-pig trachea are endowed with inhibitory muscarine receptors of the M_2 subtype.

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IN VIVO MODULATION OF THE HISTAMINE RELEASE IN THE HYPOTHALAMUS BY DOPAMINERGIC NEURONS

M.Heistracher, A.Philippu

The hypothalamus possesses a relatively high density of catecholaminergic and histaminergic neurons. Recently, we reported that the release of histamine from its neurons is modulated by noradrenergic neurons (Naunyn-Schmiedebergs's Arch.Pharmacol.344:183-186,1991). The influence of dopamine receptor agonists and antagonists on the release of endogenous histamine in the hypothalamus was now investigated.

In anaesthetized rats, a guide cannula with its mandrin was stereotaxically inserted into the posterior hypothalamus. Some days after operation, the mandrin was replaced by a push-pull cannula and the hypothalamus of the conscious rat was superfused with artificial CSF at a rate of 30 µl/min. The superfusate was continuously collected in time periods of 10 min. Drugs were dissolved in CSF. Superfusions with drugs also lasted for 10 min. Histamine was determined radioenzymatically in the superfusate.

The spontaneous release rate of histamine was 6.9±0.4 (fmol/min; mean value ± SEM, N=27). Hypothalamic superfusion with dopamine (10 µmol/l) enhanced slightly but significantly the release rate of histamine. Superfusion with the same concentration of apomorphine (dopamine receptor agonist) also tended to enhance the release of histamine. Hypothalamic superfusion with the D2 receptor antagonist (-)-sulpiride (20 µmol/l) inhibited the release rate of histamine. On the other hand, superfusion with the D1 antagonist SKF 83566 (20 µmol/l) enhanced the histamine release .

The results suggest that in the hypothalamus dopaminergic neurons modulate the release of histamine from histaminergic neurons.

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PULSE-TO-PULSE CHANGES IN THE CALCIUM SOURCES FOR NEUROGENIC ADRENERGIC CONTRACTIONS OF RAT VAS DEFERENS

R. Bültmann and K. Starke

Twitches of the rat vas deferens (RVD) evoked by single or low frequency electric pulses are mediated by noradrenaline and ATP. Blockade of Ca channels by nifedipine (NIF) has widely been used to suppress the purinergic component of such twitches. To examine whether the adrenergic component also is partly NIF-sensitive, the ATP component was blocked by suramin 300 µM.

Stimulation by twin pulses 3 s apart elicited separate twitches of which the second was smaller than the first one. Both twitches were reduced by NIF 0.01-10 µM, the second much more markedly (by 73% maximally) than the first one (by 14% maximally). In the presence of rauwolscine 0.1 µM, or when the second pulse was replaced by two high-frequency pulses (100 Hz), the second twitch was similar in amplitude to the first. Nevertheless, twitch 2 was still reduced more markedly by NIF than twitch 1. NIF also suppressed the second twitch to a greater extent than the first one when the pulse interval was 5-60 instead of 3 s, but the inhibition by NIF decreased with increasing pulse interval. The effect of a functional removal of intracellular Ca stores by ryanodine 20 µM was also studied in RVD stimulated by twin pulses 5-60 s apart. Both twitches were reduced by ryanodine, but in this case the inhibition of twitch 1 was far greater (by 98%) than the inhibition of twitch 2 (by 76%) so that twitch 2 exceeded twitch 1. In contrast to the effect of NIF, the effect of ryanodine *increased* with the interval between the twin pulses. Stimulation of the RVD with single pulses of increasing current strength (30-100 mA) elicited increasing twitch contractions. NIF 1 µM attenuated the smaller responses to a greater extent than the larger responses. Ryanodine 20 µM almost abolished the single pulse-evoked twitches. All responses were abolished by prazosin 0.3 µM.

The results demonstrate a NIF-sensitive component in the adrenergic response of the RVD to single and widely spaced pulses. Ca influx is particularly important for small twitches and seems to cooperate with intracellular Ca release in triggering contraction. Ca sources change from pulse 1 to pulse 2, in that Ca influx plays a greater role for twitch 2. This change has two reasons: the smaller amplitude of twitch 2 (due to α_2-autoinhibition), and an as yet undefined process through which smooth muscle Ca channels seem to become more sensitive to activation for several seconds after a wave of α_1-adrenoceptor activation.

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NORADRENALINE INCREASES Ca^{2+} IN CULTURED PORCINE AORTIC SMOOTH MUSCLE CELLS: INVOLVEMENT OF α_1-, α_2- AND ß-ADRENOCEPTORS. W. Erdbrügger, H.-J. Bauch

Since smooth muscle contraction involves increases in intracellular Ca^{2+}, we have investigated the effects of noradrenaline on the free intracellular Ca^{2+} concentration in cultured porcine aortic smooth muscle cells (assessed as fluorescence of Fura-2 loaded cells) and determined the adrenoceptor subtype involved using selectice agonists and antagonists. Noradrenaline concentration-dependently increased intracellular Ca^{2+} ($EC_{50} \approx 40$ nM); 1 µM noradrenaline was used in all further experiments. The noradrenaline-stimulated Ca^{2+} increase was inhibited almost completely by 10 µM phentolamine or 1 µM yohimbine and by 32% and 17% by 100 nM prazosin and 10 µM propranolol, respectively. Conversely, the α_2-selective agonist α-methyl-noradrenaline, the α_1-selective agonist phenylephrine and the ß-selective agonist isoprenaline were 105%, 21% and 10% as effective as noradrenaline, respectively. Whereas chelation of extracellular Ca^{2+} did not significantly inhibit the Ca^{2+} increase in response to noradrenaline or to α-methyl-noradrenaline, it attenuated the response to phenylephrine by 47%. Similarly, 100 nM nifedipine did not attenuate the Ca^{2+} response to noradrenaline. Pre-treatment of cultured cells with 100 ng/ml pertussis toxin for 24 h inhibited the Ca^{2+} response to noradrenaline, phenylephrine, α-methyl-noradrenaline and isoprenaline completely. We conclude that α_1-, α_2- and ß-adrenoceptors are involved in the noradrenaline-stimulated Ca^{2+} increases in cultured porcine aortic smooth muscle cells with a dominating contribution from the α_2-subtype.

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PHARMACOMECHANICAL COUPLING OF α_1-ADRENOCEPTORS IN THE ISOLATED RAT PORTAL VEIN.

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The isolated rat portal vein (RPV) is a good example of a vascular smooth muscle preparation which spontaneously generates phasic myogenic contractions. Receptor stimulation with excitatory neurotransmitters is known to increase mechanical activity of the RPV, but the underlying Ca^{2+}-mobilizing mechanisms are largely unknown.

To gain more insight in the pharmacomechanical coupling of α_1-adrenoceptors in the RPV, we studied the mechanical response to the addition of phenylephrine (PE, 100 µM), after treatment with different concentrations of nifedipine (0.3 nM - 0.1 µM) and cromakalim (10 nM -10 µM), and after different periods (0 - 6 min) of incubation in a nominally Ca^{+}-free Tyrode's solution. Since stimulation of α_1-adrenoceptors in the RPV with high concentrations of full agonists is known to cause a sustained depolarization of the smooth muscle cells, we also studied the mechanical response to depolarization with high K^{+}-solution (50 mM) under the same circumstances as those for PE. This allowed us to compare mechanical effects mediated by pure voltage-regulated Ca^{2+}-mobilization and those induced by receptor-regulated Ca^{2+}-mobilization which also partly involves the stimulation of voltage-regulated Ca^{2+}-mobilization in the RPV. The results show that the early phasic and sustained components of the K^{+} and PE induced contraction are differentially sensitive towards nifedipine and cromakalim. As compared to the sustained components, the early phasic components of both contractions, which are associated with an initial burst of action potential discharge, are relatively sensitive to cromakalim and rather insensitive to nifedipine and utilize a more slowly exchangeable Ca^{2+}-pool for contraction. This slowly exchangeable Ca^{2+}-pool, however, is already depleted after 6 min in a nominally Ca^{2+}-free Tyrode's solution.

In conclusion, the results suggest that in the RPV coupling of the α_1-adrenoceptor to its mechanical effector mechanism involves different modes of Ca^{2+}-mobilization, which can be pharmacologically separated by nifedipine and cromakalim.

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SDZ NVI 085 DISCRIMINATES BETWEEN RAT VAS DEFERENS ALPHA$_{1A}$- AND RAT AORTA ALPHA$_{1B}$-ADRENOCEPTORS

M. Eltze, and R. Boer

The anticataplectic effect of SDZ NVI 085* has been associated with its ability to selectively stimulate α_{1A}-adrenoceptors. Evidence from functional studies suggests that α_{1A}-adrenoceptors mediate contraction of prostatic portions of rat vas deferens (RVD) to noradrenaline (NA), whereas rat aorta (RA) contains primarily α_{1B}-adrenoceptors which show different affinities for various antagonists (pA_2):

	RVD	RA	Ratio pA_2 (RVD:RA)
5-Methyl-urapidil	9.10	7.03	117
Ipsapirone	7.94	5.90	110
Wy-49.051**	7.56	5.59	100
Flesinoxan	6.99	5.48	30
Urapidil	7.52	6.16	23
5-Formyl-urapidil	7.92	6.55	23
Benoxathian	9.35	8.14	18
WB 4101***	9.56	8.53	11
Spiperone	7.63	7.82	0.6

Contractions of RVD to SDZ NVI 085 (pD_2 = 5.5, i.a.= 0.42) were sensitive to 5-methyl-urapidil and isradipine but resistant to chlorethylclonidine, whereas SDZ NVI 085 was inactive in RA. The clear differences in sensitivity to these agents and the antagonist affinities in both tissues further support the suggestion that primarily α_{1A}- and α_{1B}-adrenoceptors are functionally involved in contraction to NA in RVD and RA, respectively.

*(-)-(4aR,10aR)-3,4,4a,10,10a-hexahydro-6-methoxy-4-methyl-9-methylthio-2H-naphth[2,3,-b]-1,4-oxazine (Sandoz); **7-[3-[4-(diphenylmethoxy)-1-piperidinyl]propyl]-3,7-dihydro-1,3-dimethyl-1H-pyrine-2,6-dione (Wyeth-Ayerst); ***rac-2-(2,6-dimethoxphenoxyethyl)-aminomethyl-1,4-benzodioxane.

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COMPUTER-ASSISTED VIDEOMICROSCOPY: α_{1A}-ADRENOCEPTORS MEDIATE TONIC CONTRACTION TO NORADRENALINE IN GUINEA-PIG ILEUM SUBMUCOSAL ARTERIOLES E. Bungardt, U. Moser, and E. Mutschler

Noradrenaline (NA) causes a biphasic contraction in isolated guinea-pig ileum submucosal arterioles (GPSA), the tonic phase of which can be abolished by α_1-antagonists. This study was designed to further characterize the α_1-adrenoceptor subtype involved using a set of selective $\alpha_{1A/B}$-antagonists/$5HT_{1A}$-agonists. The preparation consisted of arteriolar trees embedded in a thin connective tissue sheath. This was pinned out on the base of a small organ bath (0.3ml) and continuously flowed with gassed Tyrode solution at 32°C. Arteriolar diameter was monitored with the Diamtrack®-system; outside diameter of arterioles examined in this study ranged from 40-70 µm. Vessels were preconstricted with 10µM NA. Antagonists were then applied in a cumulative fashion. -log IC_{50} values for vasodilation derived from semi-logarithmic dose-response curves were:

Antagonist	-log IC_{50} ± S.E.M. (GPSA)	
Urapidil	6.56 ± 0.04	n=8
5-Methyl-urapidil	8.00 ± 0.05	n=11
Ipsapirone	6.95 ± 0.05	n=8
WB 4101*	8.55 ± 0.06	n=10

-log IC_{50} values (GPSA) were compared with antagonist potencies (pK_i, pA_2) obtained in radioligand binding studies and in functional experiments. Urapidil was used as reference antagonist (D. Mackay, TIPS 12:181, 1991). No correlation was found between -log IC_{50}'s (GPSA) and pK_i values at $5\text{-}HT_{1A}$ (pig cortex; r=0.474) and α_{1B} (rat cortex; r=0.739) binding sites. In contrast, -log IC_{50}'s (GPSA) were significantly correlated with pK_i values at α_{1A} (rat cortex) binding sites (r=1.000), pA_2 values at rat vas deferens (r=0.999) and -log IC_{50} values found in rat kidney (r=0.989)(M. Eltze et al., EJP 202:33, 1991). In conclusion, tonic contraction to NA in guinea-pig ileum submucosal arterioles appears to be mediated by adrenoceptors of the α_{1A}-subtype.

* (±)-2-[2.6-dimethoxyphenoxyethyl]aminomethyl-1.4-benzodioxane

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INTRINSIC SYMPATHOMIMETIC ACTIVITY (ISA) OF THE ADRENOCEPTOR ANTAGONISTS CELIPROLOL, DILEVALOL, AND PINDOLOL

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INTRODUCTION: ISA is one of the extraordinary properties of some ß-blockers. It may prevent negative chronotropic and inotropic effects during therapeutic use of ß-blockers, but it is a matter of debate whether lack of cardioprotection evidenced for some ß-blockers in epidemiological studies is due to their ISA. The aim of this study was to quantify the ISA on the $ß_1$- and $ß_2$-receptor sites since it has been claimed for dilevalol (**D**) and celiprolol (**C**) that they have specific $ß_2$-ISA. **METHOD:** Isolated organs from rats were used. For the $ß_1$-ISA increase in the rate of spontaneous beating right atria and for $ß_2$-ISA the relaxation of K^+ ($3.6*10^{-4}$ M)-precontracted uteri were used. Concentration-response curves (**CRC**) were established for the test compounds. Additionally, it was investigated in the uterus preparation whether the CRC of test compounds could be shifted in a parallel manner by preincubation with the $ß_2$-selective Adrenoceptor-antagonist ICI 118551 (10^{-6}M). **RESULTS:** The data in the table give the maximum response. At maximum, isoprenaline (**ISO**) increased heart rate by 188 b.p.m., and it relaxed the rat uteri by 92%. These values were set for 100%.

Model	Rat atrium ($ß_1$-ISA)		Rat uterus ($ß_2$-ISA)	
Drug	Conc. (M)	% increase	Conc. (M)	Relaxation (%)
Isoprenaline	10^{-7}	=100	10^{-7}	=100
Celiprolol	10^{-5}	20	10^{-6}	93
Dilevalol	10^{-5}	0	10^{-6}	94
Pindolol	10^{-6}	6	10^{-6}	71
Propranolol	10^{-4}	<0	10^{-6}	0

As expected, propranolol had neither $ß_1$-ISA nor $ß_2$-ISA. ICI 188551 was able to shift the CRC of ISO, C, D, and pindolol in a parallel manner, but to a different degree to the right, suggesting that relaxing mechanisms other than $ß_2$-stimulation may contribute to the effect on this preparation. All test compounds (including ISO) reacted more sensitively on the $ß_2$-receptor site. Remarkably, - in contrast to C - D exerted ISA exclusively on the $ß_2$-site.

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(-)-(125 I)-IODOCYANOPINDOLOL BINDING ON EPIDERMAL MEMBRANES OBTAINED FROM HUMAN SPLIT THICKNESS SKIN GRAFTS

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Permanent epidermal keratinocyte cell cultures express high densities of ß-adrenoceptors as shown by radioligand binding studies (Steinkraus et al., Arch. Dermatol. Res. 283: 328-332, 1991). We demonstrate a method to directly measure ß-adrenergic binding characteristics in membrane preparations obtained from left overs of split-thickness skin grafts (0.3 mm).

Membranes were sequentially homogenized and centrifuged in order to obtain pure epidermal fragments which were analysed ultrastructurally. Saturation experiments with the non-selective ß-adrenoceptor antagonist (-)-(125 I)-iodocyanopindolol (ICYP, 6-8 different concentrations ranging from 5 to 150 pmol/l) used as radioligand showed saturable specific binding isotherms. Scatchard transformation of the data revealed a high affinity binding of ICYP to a single class of ß-adrenoceptors. The total ß-adrenoceptor density was found to be 80.0±10.0 fmol/mg protein (mean±SEM, n=8). The equilibrium dissociation constant (KD) was 8.0±0.9 pmol/l. Displacement curves for different ß-adrenoceptor agonists as well as ß-adrenoceptor antagonists were best fitted to a monophasic sigmoid. The rank order of potency for the ß-agonists was isoprenaline> adrenaline > noradrenaline. The IC-50 values (nmol/l) for the ß-antagonists were: ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, ß2-selective) 14.7; propranolol (non-selective) 24.8; CGP-12177 (4-(3-tertiarybutylamino-2-hydroxy-propoxy)-benzimidazole-2-on, non-selective) 28.9; bisoprolol (ß1-selective) 1500; CGP-20712A (1-(2-(3-carbamoyl-4-hydroxy)phenoxyethylamino)-3-(4-(1-methyl-4-tri-fluoromethyl-2-imidazolyl)-phenoxyl)-2-propanol methanesulfonate, ß1-selective) 8990.

It is concluded that human epidermis contains a high density of ß2-adrenoceptors. ß-adrenoceptor binding characteristics in the investigated epidermal membranes are very similar to those observed in human epidermal keratinocyte cell cultures or other human tissues.

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KETOTIFEN ATTENUATES TERBUTALINE-INDUCED DESENSITIZATION OF β-ADRENOCEPTOR-MEDIATED PHYSIOLOGICAL IN VIVO EFFECTS IN HUMANS.

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The use of β-adrenoceptor (AR) agonists in long-term treatment of asthma or chronic heart failure is of limited value as β-AR desensitization develops. The antiallergic drug ketotifen (KET) prevents desensitization of rat and human pulmonary β-AR as well as down-regulation of human lymphocyte $β_2$-AR caused by chronic β-AR treatment. We studied whether KET may also prevent β-AR agonist-induced desensitization of β-AR mediated physiological in vivo effects in humans. 9 Healthy volunteers were treated for 14d with 3x5mg/d terbutaline (TER) with or without KET (2mg, then 2x1 mg/d); before and after TER-treatment bicycle exercise-induced tachycardia ($β_1$-AR mediated) and isoprenaline-infusion (3.5;7;17.5;35 and 70ng/kg/min for 5 min each)-evoked increase in systolic blood pressure ($β_1$-AR mediated), decrease in diastolic blood pressure and increase in plasma noradrenaline ($β_2$-AR mediated), and tachycardia ($β_1$- and $β_2$-AR mediated) were assessed. TER-treatment desensitized all $β_2$-AR mediated effects but not $β_1$-AR mediated effects; concomitant KET-administration markedly attenuated this TER-induced $β_2$-AR desensitization. We conclude that KET does not only prevent β-AR agonist-induced down-regulation of β-AR number but also β-AR agonist-induced blunting of β-AR mediated physiological in vivo effects. Thus, concomitant KET-administration to β-AR agonist treatment might prevent, or at least delay, development of tolerance and hence may improve efficacy of treatment.

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THE ß$_2$-ADRENOCEPTOR AFFINITY IS IMPAIRED IN PATIENTS WITH ACUTE ALLERGIC RHINITIS.

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An altered ß-adrenergic regulation is discussed as a pathogenetic factor in atopic diseases. In this study the ß$_2$-adrenoceptors on peripheral leukocytes of 7 patients with hayfever were evaluated during pollen season and compared to 7 healthy controls. Leukocytes were harvested by Macrodex sedimentation and adjusted to a concentration of $5.0x10^6$ cells/ml. ß$_2$-adrenoceptor density (Bmax) and affinity (Kd) were determined in a radio-receptor assay with 12 concentrations of ^{125}Iodocyanopindolol (1.0-150 pmol/l). The intracellular formation of cyclic adenosine monophosphate (cAMP) was tested in dose-response curves with 5 concentrations of isoproterenol (10^{-8}-10^{-4} mol/l) yielding basal (BW) and maximal cAMP content (Emax) as well as the isoproterenol concentration necessary to achieve half maximal stimulation (EC_{50}).

Bmax was only slightly (1607±94 vs. 1863±248 sites/cell), receptor affinity however significantly decreased in hayfever patients (6.4±1.0 vs. 3.4±0.6 pmol/l; p<0.05). Both BW (4.9±0.8 vs. 8.4±1.3 pmol/10^6 cells; p<0.05) and plasma cAMP concentrations (25.7±1.2 vs. 31.3±1.6 nmol/l; p<0.05) were lower in the atopic patients. The formation of cAMP could be stimulated in all cells. However, Emax expressed as a percentage of BW, was reduced (117±3% vs. 145±8%; p<0.01), EC_{50} was increased (1089±669 vs. 71±51 nmol/l; p<0.005) in allergic rhinitis.

It is concluded that in allergic rhinitis ß$_2$-adrenoceptor affinity as well as stimulation of cAMP are impaired. In comparison with a previous study done outside the pollen season [Naunyn-Schmiedeberg's Arch. Pharmacol. 1991, 343, R 99] these alterations were now more pronounced.

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AGONIST-INDUCED β-ADRENOCEPTOR DOWN-REGULATION IN SK-N-MC CELLS AND LYMPHOCYTES: NO CHANGE IN G_i-PROTEIN α-SUBUNITS

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Evidence has accumulated that chronic activation of cardiac β-adrenoceptors (AR) is not only accompanied by a decrease in β-AR number but also by an increase in cardiac G_i-protein α-subunits. To study whether this is a general phenomen we investigated a) in vitro the effects of 24 h incubation of SK-N-MC cells (homogeneous class of β_1-AR) with 10μM isoprenaline (ISO) and b) in vivo in 8 healthy volunteers in circulating lymphocytes (homogeneous class of β_2-AR) the effects of 2 weeks treatment with terbutaline (TER, 3x5 mg/day) on β-AR number and on the amount of G_i-protein α-subunits. β-AR number was assessed by [^{125}I]-iodocyanopindolol (ICYP) binding, the amount of SK-N-MC cell and lymphocyte G_i-protein α-subunits by pertussis toxin(PTX)-catalyzed [^{32}P]-ADP-ribosylation. In vitro SK-N-MC cell β-AR number(91.0±8.2 fmol ICYP bound/mg protein, n=7) was decreased after 24 h ISO-incubation by 53±2%; the amount of SK-N-MC cell PTX-substrates, however, was not affected by the ISO-incubation (control: 1739±130 fmol/mg protein, after ISO: 1703±140 fmol/mg protein). In vivo TER-treatment caused a significant decrease in lymphocyte β_2-AR density (control: 1079±136 ICYP binding sites/cell; after 2 weeks TER: 649±75 ICYP binding sites/cell, n=8, $P<0.05$); lymphocyte PTX-substrates, however,were not changed by the TER-treatment (control: 5282±731 fmol/mg protein, after TER: 4676±508 fmol/mg protein). The fact the neither an agonist-induced decrease in the number of β_1-AR (SK-N-MC cells) nor in β_2-AR (lymphocytes) is accompanied by changes in G_i-protein α-subunits does not support the view that the increase in cardiac G_i-protein α-subunits following long-term activation of cardiac β-AR is a general phenomenon but rather indicates that this may be a tissue-specific effect.

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CHARACTERIZATION OF THE ß-ADRENOCEPTOR ON HUMAN LYMPHOCYTES BY DOSE-RESPONSE CURVES FOR THE STIMULATION OF cAMP

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Peripheral mononuclear leukocytes (pMNL) express ß-adrenoceptors that have been classified by radioligand binding studies as $ß_2$-adrenoceptors (Brodde et al 1981: Life Sci 29, 2189). This study complements this classification by a functional criterion. The stimulation of the intracellular formation of cyclic adenosine monophosphate (cAMP) was tested by incubating intact pMNL for 10 min in the presence of 10^{-4} mol/l isobutylmethylxanthine (IBMX) with 24 concentrations (2.5 x 10^{-10} - 1 x 10^{-2} mol/l) of α-adrenergic agonists (xylometazoline, oxymetazolin), dopamine, unselective ß-adrenergic agonists (noradrenaline, adrenaline, isoprenaline), $ß_2$-selective agonists (salbutamol, terbutaline, fenoterol), and the new long-acting $ß_2$-selective agonist formoterol (2.5 x 10^{-12} - 1 x 10^{-4} mol/l). A dose-response curve was fitted by non-linear regression analysis to the data obtained with pMNL from at least three healthy male volunteers / each substance yielding the basal cAMP content (E_0), its maximal increase (E_m), and the concentration needed to achieve half-maximal stimulation (EC_{50}). The α-adrenergic agonists were not effective, noradrenaline was only effective in very high concentrations (lg EC_{50}: -2.67). The stimulation by ß-adrenergic agents proceeded in two steps. In the first step the following sequence was observed (lg EC_{50}): Adrenaline (-5.64) ≤ terbutaline (-5.68) < isoprenaline (-6.73) < salbutamol (-7.52) ≤ fenoterol (-7.63) < formoterol (-12.17). E_m was largest for terbutaline (275 % of E_0) and fenoterol (237 % of E_0) and lowest for salbutamol (130 % of E_0) and formoterol (116 % of E_0). In the concentration range of 2.5 x 10^{-10} - 2.5 x 10^{-9} mol/l isoprenaline and adrenaline an increase in the cAMP content may be observed that decreases again to basal values. The results functionally classify the ß-adrenoceptor on pMNL as $ß_2$-adrenoceptor. There is no evidence for a functionally active $ß_1$-adrenoceptor. The decreasing stimulation in the (physiologically important) low concentration range suggests the inhibiting action of $α_2$-adrenoceptors. Adrenaline is suggested to be the physiological ligand for adrenoceptors on pMNL. The varying degree of stimulation (E_m) after incubation for 10 minutes probably reflects the different onset of action of the various ß-sympathomimetic agents.

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COUPLING OF $ß_1$- AND $ß_2$-ADRENOCEPTORS TO ADENYLATE CYCLASE OF BOVINE ENDOTHELIAL CELLS

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$ß_2$-adrenoceptors ($ß_2$AR) are involved in the regulation of endothelial cell permeability. It is unclear until now if $ß_1$AR also coexist in endothelial cells, and if $ß_1$AR are coupled to the adenylate cyclase (AC). Endothelial cells from bovine aorta (BAEC) were disaggregated with collagenase and cultured on polycarbonate filters to study the permeability of cell monolayers and in flasks (75 cm^2) for the preparation of membranes. The permeability of monocellular endothelial layers was determined by the exchange of fluorescence labelled dextrans (4 - 150 kDa). In cell membranes using ^{125}J-(-)-pindolol as radioligand and CGP 20,712 A {1-[2(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol} as $ß_1$AR- and ICI 118,551 {erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol} as $ß_2$AR-selective antagonists we found that both $ß_1$AR and $ß_2$AR coexist in a proportion of 13 : 87%. AC-activity was assayed using α-^{32}P-ATP as substrate and ^{3}H-cAMP as recovery marker. AC-activity was stimulated with formoterol (FOR), (-)-isoprenaline and (-)-noradrenaline in the absence and presence of CGP 20,712 A or ICI 118,551 maximally up to 6-fold of basal activity. The results show that both $ß_1$AR (up to 40 % of v_{max}) and $ß_2$AR (< 80% of v_{max}) are involved in the stimulation of AC with the catecholamines, whereas FOR exclusively stimulated AC-activity through $ß_2$AR. FOR also reduced the permeability of BAEC monolayers up to 60% with concentrations ≈ 100-fold lower than those necessary to stimulate $ß_2$AR coupled AC indicating the existence of spare receptors.

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INVOLVEMENT OF TRAPIDIL-SENSITIVE PHOSPHODIESTERASE INHIBITION IN THE REGULATION OF CARDIAC PROTEIN PHOSPHORYLATION AND CONTRACTILITY

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The involvement of a cAMP-sensitive protein phosphorylation (P) of phospholamban (PLB), 15kD-protein (15kDP) and troponin I (TNI) in the regulation of cardiac contractility was investigated in the isolated perfused rat heart exposed to trapidil (T; Rocornal^R). T produced a dose-dependent positive inotropic effect (max. stimulation at 0.1mM T) of 67% of the control level. This was accompanied by an increase of cAMP (pmol/mg prot.) from 2.5±0.3 (n=7) to 10.4±1.0 (n=5) as well as by a change in the cAMP-dependent protein kinase activity ratio (-cAMP/+cAMP) from 0.12±0.06 (n=6) in the control myocardium to 0.29±0.02 (n=5) in the T-treated hearts, resp.. In consequence of this T-initiated changes there was a dose-dependent in vivo increase of the P-state of PLB, 15kDP and TNI. 0.1 mM T increased the P-state (pmol/mg fraction prot.) of PLB by 41±3 (n=7), 15kDP by 3.3±0.3 (n=5) and TNI by 22±2 (n=7), resp.. The increased sensitivity in P of PLB and 15kDP relative to TNI indicates that T inhibits PDE activity preferentially in the compartment of the sarcoplasmic reticulum. As an expression of nonspecific PDE-inhibition an enhanced cGMP-level was only observed at T >0.1mM. In conclusion, influence of T on the contractile activity of the heart via cAMP-regulated P might be the rational basis of its efficacy in the treatment of heart diseases connected with changes in ß-adrenergic response.

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VASCULAR PRE-PREFUSION MARKEDLY AFFECTS THE MODULATION BY NICOTINE AND MUSCARINE RECEPTORS OF INTESTINAL SEROTONIN RELEASE STUDIED IN USSING CHAMBERS.

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In the intestine serotonin (5-HT) is present in enterochromaffin cells (ECs) of the mucosa. ECs are bipolar cells and can secrete 5-HT at the luminar as well as interstitial side. In the present experiments a possible differential release of 5-HT from the mucosal and serosal side of the small intestine was studied by the use of Ussing techniques.

Segments of the rabbit small intestine were incubated in Krebs-HEPES solution in Ussing chambers allowing the separate determination of mucosal and serosal outflow of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA). Test drugs were added to both sides. In some experiments, arterial perfusion of the small intestine via the superior mesenteric artery was performed in situ for 20 min prior to the dissection of the tissue.

After 50 min of incubation *in vitro*, the spontaneous outflow of 5-HT and 5-HIAA (as pmol/cm^2/10 min) from pre-perfused segments amounted to 1.5 ± 0.14 and 30 ± 2.5 at the serosal side (n=36) and to 8 ± 0.8 and 67 ± 6.9 at the mucosal side (n=17). The outflow of 5-HT and 5-HIAA from non-perfused tissue was not significantly different. In contrast, pre-perfusion markedly affected the response to muscarine and nicotine receptor agonists. In pre-perfused tissue, oxotremorine concentration dependently increased the serosal outflow of 5-HT, maximally 30-fold at 10 µmol/l, whereas in non-perfused tissue the stimulatory effects of oxotremorine were less consistent and less pronounced (maximal increases 3-8-fold). Similar differences were also observed when nicotine (100 µmol/l) was applied. In pre-perfused tissue, nicotine caused a clear cut and transient increase (by 150 %) of the serosal outflow of 5-HT, whereas in non-perfused tissue the effect of nicotine was inconsistent and markedly less pronounced (maximal increase 40 %). Neither nicotine nor oxotremorine significantly affected the outflow of 5-HT at the mucosal side.

In conclusion, activation of muscarine or nicotine receptors selectively stimulates the release of 5-HT at the interstitial side of the ECs. The present experiments also indicate that arterial pre-perfusion of intestinal segments (possibly by washing out inhibitory mediators) markedly affects the magnitude of the response of 5-HT release to different stimuli.

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5-HT EFFECTS ON ISOLATED STRIPS FROM GUINEA PIG STOMACH

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Both, stimulation and inhibition of gastric motility has been observed after administration of 5-HT to various species. Direct effects on stomach tissue and/or modulation of the activity of endocrine glands or the autonomic nervous system could be involved in such complex effects. We used a nerve/muscle preparation from the guinea pig stomach to analyze the local effects of 5-HT on stomach motility and the 5-HT receptor subtypes possibly involved therein. After removal of the mucosa, strips from the circular muscle of guinea pig corpus and fundus were set up in an organ bath in Krebs solution under an initial tension of 1 g and were stimulated intermittently (electrical field stimulation (EFS), 40 V, 0.1 ms, 1 Hz, for 30 s at 5 min intervals). Non-cumulative concentration response curves were recorded. 5-HT augmented EFS-induced contractions in strips from the fundus (pD_2 9.1) and in the corpus (pD_2 9.6). Contractions were increased by 300-400 %. In strips from the corpus, the 5-HT effect was insensitive to 10^{-7} M ICS 205-930 (ICS; tropisetron) but was antagonized by the 5-HT_4 receptor antagonist SDZ 205-557 (10^{-7} M) [SDZ; 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino) ethyl ester]. ICS and SDZ had no effect on EFS-induced contractions. The effect of 5-HT was mimicked by the 5-HT_3 receptor agonist 2-methyl-5-HT (pD_2 5.6) and by metoclopramide (pD_2 7.2) and renzapride (pD_2 7.9). The effect of 2-methyl-5-HT was sensitive to blockade by ICS, whereas the effects of renzapride and metoclopramide (partly) were inhibited by SDZ. Agonists at 5-$HT_{1A/D}$ receptors (5-carboxamidotryptamine) or 5-$HT_{1C/2}$ receptors (α-methyl-5-HT) inhibited contractions. It is concluded that, despite the presence of 5-HT_3 receptors, the stimulatory effects of 5-HT on guinea pig stomach strips are predominatly mediated by 5-HT_4 receptors.

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EFFECTS OF THE 5-HT_4 RECEPTOR AGONIST BIMU8 ON RELEASE OF ACETYLCHOLINE (ACh) AND 5-HYDROXYTRYPTAMINE (5-HT) FROM GUINEA PIG ILEUM

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BIMU8 is a novel 5-HT_4 agonist (Dumuis et al., this journal 343: 245, 1991) which enhances electrically evoked twitch contractions and peristalsis in the guinea pig ileum (Tonini et al., Pharmacol Res 24: 5, 1991). We have studied the effects of BIMU8 on release of [^{3}H]ACh from myenteric plexus longitudinal muscle strips preincubated with [^{3}H]choline, and on release of 5-HT from enterochromaffin cells of the vascularly perfused ileum (Schwörer et al., Neuroscience 21: 297, 1987).

Basal release of [^{3}H]ACh: BIMU8 caused a concentration-dependent increase in basal release (EC_{50}: 26 nmol/l) which was abolished by tetrodotoxin (1 µmol/l) or by removal of extracellular calcium. Ondansetron (1 µmol/l) did not affect the stimulatory response to BIMU8. Tropisetron (1 µmol/l) and the 5-HT_4 receptor antagonist DAU 6285 (1 µmol/l) (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct 3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate) shifted the concentration-response curve for BIMU8 to the right yielding pK_B values of 6.9 (tropisetron) and 7.5 (DAU 6285).

Evoked release of [^{3}H]ACh: Strips were stimulated at 0.1 Hz. BIMU8 increased the [^{3}H]ACh release evoked by submaximal electrical stimulation (voltage gradient between the electrodes: 5-6 V/cm) (EC_{50}: 5 nmol/l). DAU 6285 (1 µmol/l) alone did not affect the evoked [^{3}H]ACh release but competitively antagonized the effects of BIMU8 on release (pK_B: 6.8).

Basal release of 5-HT: The release was inhibited by BIMU8 (EC_{50}: 7 nmol/l) but was not changed by 1 µmol/l DAU 6285.

Conclusion: BIMU8 is a potent agonist at 5-HT_4 receptors in guinea pig ileum, and causes an increase in both basal and evoked ACh release, and a reduction in 5-HT release.

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ACTIONS OF THE NOVEL 5-HT_3 ANTAGONISTS MDL 73147EF AND MDL 74156 ON NG108-15 NEUROBLASTOMA CELLS

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In radioligand binding experiments, MDL 73147EF (1*H*-indole-3-carboxylic acid, trans-octahydro-3-oxo-2,6-methano-2*H*-quinolizin-8-yl ester, methanesulfonate) and MDL 74156 (1*H*-indole-3-carboxylic acid, trans-octahydro-3-hydroxy-2,6-methano-2*H*-quinolizin-8-yl ester) displaced [^{3}H]GR65630 (3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-$^3H_3$-1*H*-indol-3-yl)-1-propanone) from 5-hydroxytryptamine$_3$ (5-HT_3) binding sites on membranes prepared from NG108-15 neuroblastoma-glioma cells. The competition curves were monophasic indicating a single binding site. The estimated dissociation constants (K_I) were 20.03 ± 6.58 and 0.44 ± 0.18 nM, respectively (means ± SEM, n=6 and 9). 5-HT (10-50 µM) pressure-ejected from a micropipette onto single NG108-15 cells voltage-clamped at -50 mV, elicited a transient inward membrane current characteristic for the activation of 5-HT_3 receptors. The 5-HT current was blocked in a reversible, concentration-dependent manner following a 5 min bath application of MDL 73147EF or MDL 74156EF (the methanesulfonate salt of MDL 74156). The concentrations required to half block the 5-HT response (IC_{50}) were approximately 3.8 and 0.1 nM respectively. It is concluded that both compounds are potent and reversible antagonists at 5-HT_3 receptors in this neuroblastoma cell line.

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INHIBITION BY ANAESTHETICS AND STEROIDS OF 5-HT_3 RECEPTOR-MEDIATED INFLUX OF ^{14}C-GUANIDINIUM IONS IN NEUROBLASTOMA CELLS

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The ^{14}C-guanidinium ion is a useful tool for measuring cation fluxes through sodium channels (Reith, Eur J Pharmacol 188:33, 1990) or the cation channel of the 5-hydroxytryptamine$_3$ (5-HT_3) receptor (Reiser and Hamprecht, Eur J Pharmacol 145:273, 1988; Barann et al., Naunyn-Schmiedeberg's Arch Pharmacol 344 (Suppl): R36, 1991). Here the influence of some anaesthetics and steroids on the 5-HT-mediated ^{14}C-guanidinium ion influx was studied on the 5-HT_3-receptor of N1E-115 mouse neuroblastoma cells.

N1E-115 cells were cultured essentially as described by Hoyer and Neijt (Mol Pharmacol 33:303, 1987). After removal of the culture medium cells were preincubated (at 37°C for 15 min) in a Ca^{++}- and Na^+-free buffer (Na^+ replaced by choline$^+$) and, where indicated, in the presence of an anaesthetic or a steroid. The cells were then incubated for 2 min in the same buffer which now additionally contained ^{14}C-guanidinium hydrochloride (5 µmol/l) and 100 µmol/l 5-HT. Cells were then washed with cold buffer, dissolved in triton X-100 (0.1%) and the ^{14}C-content of the cells was determined.

The ^{14}C-guanidinium influx elicited by 5-HT was inhibited by the anaesthetics with the following rank order of potencies: propofol = etomidate > alfaxalone = methohexital > ketamine ≥ thiopental. The IC_{50}-values were about 8 and 115 µmol/l for propofol and thiopental, respectively.

Five additionally tested steroids caused also inhibition of 5-HT-mediated ^{14}C-guanidinium influx with the following rank order of potencies: 17ß-estradiol > progesteron = allotetrahydrodeoxycorticosterone > testerosterone > dexamethasone. The IC_{50} for 17ß-estradiol was 3-4 µmol/l.

In agreement with earlier data (Göthert et al., Arch Int Pharmacodyn Ther 242:196, 1979) this study shows that the cation channel of the 5-HT_3 receptor is a target for anaesthetics. In addition, steroids also have a fast inhibitory effect on the ligand-gated 5-HT_3-receptor.

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REPLACING THE 2-*METHYL* IMIDAZOLE NUCLEUS OF THE 5-HT_3 ANTAGONIST ONDANSETRON BY A 2-*AMINO* IMIDAZOLE MOIETY DOES *NOT* AFFECT THE AFFINITY AT ILEAL 5-HT_3 RECEPTORS

W.L. Heil, S. Elz*, H. Pertz and W. Schunack

Structure-activity studies have been carried out in the field of the selective 5-HT_3 antagonist ondansetron (GR 38032). Thus, a series of new analogues was synthesized and the affinities for 5-HT_3, M_3 and H_1 receptors were estimated in isolated quiescent guinea-pig ileal tissues. One compound (racemic HW 13§), characterized by a 2-*amino* imidazole instead of a 2-*methyl* imidazole ring, equals ondansetron at ileal 5-HT_3 receptors with a pK_B value in the range of 0.1 µM, and in addition has a similar affinity profile at other neurotransmitter receptors (Tab. 1). However, the activity of HW 13 in those assays where ondansetron is more potent, *e. g.* at the vagus nerve or in 5-HT_3 binding experiments, remains to be determined. In the series of analogues the affinity is clearly dependent on the imidazole substitution pattern and the distance between the carbonyl group and the basic heterocycle.

Tab. 1. Selected affinity data (mean $pK_B \pm$ s.e.m.)

receptor	assay	HW 13§ (racemic)	Ondansetron (racemic) this study	literature data
5-HT_3	guinea-pig ileum	6.93 ± 0.06[a] (slope 0.98)	7.01 ± 0.06[a] (slope 0.87)	6.95[a,b] (slope 0.88)
M_3	guinea-pig ileum	4.91 ± 0.05	4.72 ± 0.06	–
H_1	guinea-pig ileum	5.15 ± 0.11	4.35 ± 0.07	<5.0[c]
5-HT_2	rat tail artery	5.39 ± 0.04	4.66 ± 0.05	4.6[c,d]
H_2	guinea-pig atrium	4.54 ± 0.11	not determ.	5.5[c]
β	guinea-pig atrium	4.57 ± 0.13	4.53 ± 0.05	<4.5[c,e]

[a] pA_2 from *Schild* regression. [b] *Eglen* et al., Br. J. Pharmacol. (1990) 101: 513-520. [c] *Butler* et al., Br. J. Pharmacol. (1988) 94: 397-412. [d] Rabbit aorta. [e] Rat atrium.

§ (±)-1.2.3.9-Tetrahydro-3-[(2-amino-1*H*-imidazol-1-yl)methyl]-9-methyl-4*H*-carbazol-4-one

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ANTAGONIST EFFECTS OF RITANSERIN IN THREE IN VIVO MODELS OF 5-HT_{1C} RECEPTOR FUNCTION

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We investigated ritanserin in three putative models for central 5-HT_{1C} function. MK-212 (1-(2-chlor-6-pyrazinyl)-piperazine), a mixed 5-HT_{1C}/5-HT_2 receptor agonist and mCPP (m-chlorophenylpiperazine), a mixed 5-HT_{1C}/5-HT_{1B} agonist, induced dose-dependent hypophagia, hypolocomotion and ACTH release in rats. From dose-effect curves high but not supramaximal doses of MK-212 were selected to test the inhibitory effect of ritanserin.

MK-212 (1 mg/kg ip, 30 min before decapitation) significantly increased plasma ACTH levels (200 ± 25 ng/ml, n=16; controls: 30 ± 4, n=16). Ritanserin (1 mg/kg, 60 min prior to MK-212) produced a 50%-, ritanserin (3 mg/kg) a nearly 100% inhibition of the MK-212-stimulated effect.

In rats that were starved for 48 h, MK-212 (0.5 mg/kg sc, -5 min) strongly inhibited consumption during a 45 min feeding period. Control food-intake: 4-6 g; after MK-212 approximately 1 g. Ritanserin (1 mg/kg, -100 min, sc) almost restored food-intake to control level, however it also produced some hyperphagia when given alone.

mCPP-induced hypolocomotion has been described as a 5-HT_{1C} model (Kennett and Curzon, 1988, Br J Pharmacol 94, 137-147). Locomotion was quantified during 15 min in an Optovarimex cage. Saline-injected rats interrupted light beams 800-1000 times; after mCPP (3 mg/kg, -5 min, sc) this was reduced to about 100. Similar results were obtained with MK-212 (1 mg/kg, -5 min, sc). Ritanserin (1 mg/kg, -60 and -100 min, sc) did not inhibit hypolocomotion induced by either MK-212 or mCPP. When tested alone, ritanserin did not alter spontaneous locomotion (at -100 min) or slightly suppressed it (in one experiment at -60 min). Higher doses of ritanserin (3-16 mg/kg) also did not produce significant inhibition of hypolocotion, whereas clearcut inhibition was observed after mianserin. Experiments with other 5-HT antagonists are required to validate the model.

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FUNCTIONAL RESPONSES OF 5-HT_{1c} RECEPTORS EXPRESSED IN A9 CELLS

H.W.G.M. Boddeke, B. Hoffman*, J.M. Palacios**

The effects of a series of 5-HT receptor ligands upon A9 fibroblast cells transfected with 5-HT_{1c} receptors were investigated. Application of 5-HT (10^{-8} - 10^{-7}M) to cells voltage clamped at -50 mV induced a transient outward current response of mainly potassium ions. This effect was blocked by addition of BAPTA (5mM) to the patch pipette solution. At similar concentrations 5-HT induced transient calcium responses which were observed using fura-2. Agonist-stimulated calcium transients were quantified and compared with radio-ligand binding data (pK = pEC_{50} values (agonists) or pK_B values (antagonists), Efficacy = % of the maximal effect produced by 5-HT, binding affinity = pK_D)

Compound	Calcium pK	Eff.	Affinity pK_D
5-HT	7.32 ± 0.08	100	6.17 ± 0.05
MCPP[1]	6.89 ± 0.11	89 ± 4	6.16 ± 0.07
TFMPP[2]	6.67 ± 0.09	53 ± 4	6.31 ± 0.08
MK212[3]	6.32 ± 0.13	92 ± 6	5.24 ± 0.03
DOI[4]	7.43 ± 0.09	87 ± 5	6.78 ± 0.12
Ketanserin	6.61 ± 0.12	0	7.20 ± 0.11
Mianserin	7.93 ± 0/10	0	8.11 ± 0.09

[1] *m-chlorophenylpiperazine* [2] *trifluoromethylphenylpiperazine*
[3] *1-(2-chloro-6-piperazinyl)-piperazin-acetate* [4] *(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane*

From the data we conclude that 5-HT_{1c} receptor activation in A9 cells induces a transient calcium increase. Stimulation of 5-HT_{1c} receptors activates calcium dependent potassium and to a lesser extent chloride conductances. The pharmacology of the calcium transients and radioligand binding is similar to that of 5-HT_{1c} receptors in other systems.

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Effect of naftopidil an α1-adrenoceptor antagonistic and 5-HT1A-agonistic compound on the adrenaline and noradrenaline levels in rat plasma

M. Schwahn and H. O. Borbe

Increasing evidence appears that 5-HT1A-receptors are involved in the regulation of vascular resistance by inhibition of preganglionic sympathetic neurons and by activation of postganglionic vagal neurons. Antihypertensive drugs like naftopidil and urapidil possess comparable affinity for α1-adrenoceptors and 5-HT1A-receptors. Prazosin (α1-antagonist) and 8-OH-DPAT (5-HT1A-agonist) are compounds with high selectivity for either receptors.The aim of the present study was to investigate the influence of these compounds on adrenaline (A) and noradrenaline (NA) levels in rat plasma.

Male Sprague-Dawley rats were sacrificed 5 min after i.v.-administration of the test compounds or vehicle and A- and NA-levels in plasma were determined by an internally standardized HPLC-assay with electrochemical detection. Significance was calculated by a t-test on the basis of the daily experiments.

8-OH-DPAT (1 mg/kg) increased A-levels, whereas NA-levels remained unchanged; prazosin (1 mg/kg) leads to a decrease in A and NA was increased. Urapidil (10 mg/kg) slightly decreases A and showed a trend to increase NA. Naftopidil (2.5 and 10 mg/kg) leads dose-dependently to a pronounced decrease in A and NA.

	A-level (ng/ml)			NA-level (ng/ml)		
Control	8.76 ±	0.34		6.56 ±	0.38	
8-OH-DPAT (1)	9.73 ±	0.45	p < 0.01	5.71 ±	0.42	n.s.
Prazosin (1)	6.32 ±	0.47	p < 0.01	8.88 ±	0.57	p < 0.01
Urapidil (10)	6.05 ±	0.48	p < 0.001	8.19 ±	1.07	n.s.
Naftopidil (2.5)	7.01 ±	0.72	p < 0.07	3.95 ±	0.39	p < 0.01
Naftopidil (10)	4.43 ±	0.40	p < 0.0001	3.03 ±	0.25	p < 0.0001

n.s. = not significant

In conclusion, despite similarities in the receptor binding properties of naftopidil and urapidil (IC50 α1/5-HT1A: 235/108 and 800/42 nmol/l) both compounds differently influence A- and NA-levels: urapidil reveals similar effects like prazosin, whereas naftopidil seems to possess additional properties that lead to a decrease in both A- and NA-levels.

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5-HT1A-AGONISTIC PROPERTIES OF DIFFERENT ANTIHYPERTENSIVE DRUGS AS FOR INSTANCE NAFTOPIDIL

P. Metzenauer and R. Dedecke

Stimulation of different serotonin subreceptors seems to induce various hemodynamic effects like bradycardia or hypotension. In anesthetized cats of either sex, the 5-HT1A-agonistic compound 8-OH-DPAT produced a decrease in blood pressure and heart rate. Dose dependently from 1 to 10 µg/kg the intravenous bolus caused a fall in diastolic blood pressure in the range of 10 ± 5 to 51 ± 6 mm Hg. The 5-HT1A-antagonistic compound spiroxatrine completely abolished the hypotensive property of 8-OH-DPAT. In addition, different antihypertensive drugs with multifactorial mode of action were tested in the same model before and after treatment with spiroxatrine. Urapidil (1 mg/kg) decreased blood pressure by 31 ± 4 mm Hg, while after spiroxatrine no hypotension could be observed. Naftopidil (0.3 mg/kg) reduced blood pressure by 41 ± 13 mm Hg. This hypotensive effect was diminished after spiroxatrine. Carvedilol (0.3 mg/kg) didn't show differences in hypotension before and after spiroxatrine. Antihypertensive drugs like prazosin (0.03 mg/kg) and verapamil (0.1 mg/kg) or vasodilators like nitroglycerin (0.03 mg/kg) also caused a fall in arterial blood pressure, but their hypotensive reaction was not influenced by pretreatment with spiroxatrine. The hemodynamic results correspond very well with binding studies; these antihypertensive drugs also interact with other receptors like alpha- and/or beta-receptors, with the calcium channel or with the NO-system, but with a different pattern and different affinities.

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AGONIST/ANTAGONIST INTERACTIONS WITH 5-HT_{1A} RECEPTORS IN TRANSFECTED HELA CELLS:

D. Hoyer

We studied the interaction of 5-HT receptor ligands with human 5-HT_{1A} receptors transfected into HeLa cells. Agonist-stimulated calcium transients were measured using fura 2 in HA 7 and HA 6 cells expressing ≈ 500 and 3000 fmol receptors/mg protein and compared with 5-HT_{1A} receptor-mediated inhibition of adenylate cyclase in calf hippocampus (see Table: pK = pEC_{50} values (agonists) or pK_B values (antagonists), Efficacy= % of maximal effect produced by 5-HT).

Drug	Hippocampus		HA 6 cells		HA 7 cells	
	pK	Eff.	pK	Eff.	pK	Eff.
5-HT	7.83	100	7.11	100	6.01	100
5-CT	8.59	108	8.05	92	6.71	93
8-OH-DPAT	8.22	96	7.73	97	6.52	106
Flesinoxan	7.68	106	7.14	105	6.22	96
Ipsapirone	7.48	77	6.22	40	7.84	0
Buspirone	7.32	77	6.22	40	7.84	0
Spiroxatrine	7.75	91	6.62	54	8.16	0
MDL 73005	7.32	92	6.11	82	7.52	0
NAN 190	8.60	0	8.23	0	8.42	0
Pindolol	7.87	0	7.57	0	7.74	0
Methiothepin	7.73	0	N.T.		8.23	0

Compounds such as flesinoxan or 5-CT (full agonists), pindolol or NAN 190 (antagonists) displayed no change in intrinsic activity whichever the model, although the apparent potency varied significantly. By contrast, ipsapirone, buspirone, spiroxatrine or MDL 73005 displayed from nearly full agonism to silent antagonism depending on the second messenger and/or conditions. Since G-protein-coupled receptor can activate a variety of different effectors, the intrinsic activity and apparent potency of a drug at the same receptor may vary quite dramatically depending on the functional model. (5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-N,N-dipropyl-amino-tetraline; MDL 73005, 8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl-methylamino)ethyl]-8-azaspiro[4,5]decane-7,9-dione; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-phthal-imido)butyl]piperazine).

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INTRINSIC ACTIVITIES OF IPSAPIRONE AND BUSPIRONE AT THE RAT DOPAMINE D_2-RECEPTOR

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The nonbenzodiazepine anxiolytics ipsapirone (IPS) and buspirone (BUS) have, besides their affinity for the 5-HT_{1A} receptor, also affinity for the dopamine D_2-receptor. While IPS binds with high affinity at the 5-HT_{1A} receptor site (K_i=2.6±0.6 nM), it has only moderate affinity for the D_2-receptor (K_i=175±20 nM). In contrast, BUS has comparable affinities for the 5-HT_{1A} (K_i=9±2 nM) and the D_2-receptor (K_i=22±3 nM). So far, there are little informations about the intrinsic activities of these two compounds at the D_2-receptor. Using [^{3}H]-DA prelabelled rat striatal slices for measuring intrinsic activity at the nerve terminal dopamine autoreceptors, BUS increased the electrically evoked dopamine release, indicating that BUS has antagonistic properties at these receptor sites. In contrast, IPS was without an effect in this test. For further characterization, the mouse fibroblast cell line LZR-1 was used to measure signalling via the rat D_2-receptor. This cell line is transfected with an eucaryotic expression vector containing the gene of the rat D_2-receptor. Incubation of the cells with forskolin increased the cyclic-AMP level in LZR-1 cells by direct stimulation of the adenylyl cyclase. Quinpirole (a D_2-receptor agonist) decreased the cyclic-AMP concentration in forskolin pretreated LZR-1 cells. The D_2-receptor mediated inhibition of adenylyl cyclase by 10 µM quinpirole could be partially antagonized by BUS and IPS. The antagonistic properties of BUS were weak compared to sulpiride (a well characterized D_2-receptor antagonist). The effect of IPS was even weaker than that of BUS. Neither addition of BUS nor IPS to forskolin prestimulated LZR-1 cells had an effect on the level of cAMP, excluding the possibility of an agonistic activity of these both compounds. Therefore, one has to conclude that BUS and IPS are weak antagonists at the D_2-receptor, with IPS being less active than BUS.

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PROPHYLAXIS AND TREATMENT OF HALOTHANE-INDUCED MALIGNANT HYPERTHERMIA: COMPARISON OF DANTROLENE AND SEROTONIN ANTAGONISTS

C. Gerdes and A. Richter

Malignant hyperthermia (MH) is a hypermetabolic syndrome in skeletal muscle of genetically susceptible humans and pigs. The immediate cause is a loss of intracellular calcium regulation. MH-triggering substances are volatile anaesthetics, depolarizing muscle relaxants and agonists at serotonin (5-HT)$_2$-receptors, e.g. 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Löscher et al., Naunyn-Schmiedeberg's Arch. Pharmacol. 341: 483-493, 1990). In pigs, MH can also be induced by physical and psychological stress. MH can effectively be prevented and reversed by dantrolene, a peripheral muscle relaxant known to re-establish intracellular calcium control. There are contradictory reports about the effectiveness of 5-HT$_2$-antagonists in the prevention of halothane-induced MH in pigs (Ooms et al., in Ruckebusch et al.: Veterinary Pharmacology and Toxicology, MTP, Boston, 1983, pp. 263-281; Ørding et al., Acta Anaesthesiol. Scand. 30: 7-9, 1986).
This study compared the effects of dantrolene and the 5-HT$_2$-antagonist ritanserin on halothane-induced MH. MH-susceptible pigs were anaesthetized by azaperone/metomidate and artificially ventilated. MH was induced by adding 3 % halothane for 15 minutes to the inhaled volume. Dantrolene (3,5 mg/kg) or ritanserin (1-2 mg/kg) were administered intravenously either 10 minutes prior to the halothane-application or at the end of the halothane period.
Untreated controls developed a fulminant MH with rigidity, a sudden rise in body temperature and acidosis and died within 30 to 60 minutes after starting halothane inhalation. Dantrolene both prevented and interrupted MH leading to the survival of all dantrolene-treated animals, whereas ritanserin could not prevent death of the animals.
In further experiments, it was studied if the effect of ritanserin on halothane-induced MH could be altered by changes in preanaesthetic medication or the concentration of halothane used to trigger MH.

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PARTIAL CLONING OF A RECEPTOR GENE FROM NEUROBLASTOMA X GLIOMA (NG108CC15) CELLS WHICH IS DOWNREGULATED UPON TREATMENT WITH D-ALA-D-LEU-ENKEPHALIN

M. Kouba, M. Vanetti, and V. Höllt

Guanine nucleotide-binding protein (G-protein)-coupled receptors share sequence homology among each other. Based on this observation, degenerate primers corresponding to consensus sequences of the third and sixth transmembrane segments have successfully been used for amplification of DNA sequences of novel receptors by the polymerase-chain-reaction (PCR) technique (Libert et al. Science 244, 569-572 (1989)). Using this strategy, we amplified DNA derived from mRNA of NG 108CC15 cells. Following PCR, DNA fragments of about 400 bases were cloned and sequenced. Four sequences with homology to G-protein-coupled receptors were found (561-8, 561-39; 561-45; 561-51). One of them (561-45) was structurally identical with rat endothelin 1A receptor; the other sequences contained fragments of putative novel receptors. These clones were used as probes for hybridization to RNA blots obtained from NG108CC15 hybrid cells, as well as from the parent cells (N18TG2; C6-BU-1). The probes hybridized to mRNA species of a molecular size between 2 and 8 kb. Clone 561-51 hybridized to a 2.1 kb mRNA species in Northern blots obtained from NG108CC15 cells The hybridization signal to the 2.1 kb mRNA species was much less intense when the RNA was isolated from NT18TG2 cells and undetectable with RNA from C6-BU-1 cells. Northern blot analysis carried out on RNA from NG108CC15 cells treated with 10 μM D-ala-D-leu-enkephalin (DADLE) for 24 hours revealed a decrease of the hybridization signal of probe 561-51 to the 2.1 mRNA species as compared to that of untreated control cells. In contrast, the signals with clones 561-8 and 561-39 which hybridized to various mRNA species in Northern blots derived from NG108CC15 cells (2.4, 4.2 and 8.0 kb) did not show any specific decrease when the cells were treated with DADLE for 24 hours.
The specific distribution and the downregulation of the 2.1 kb mRNA by opioids are in line with the hypothesis that the 2.1 kb mRNA (which hybridizes with clone 561-51) encodes the delta-opiate receptor in the NG108CC15 cells. Presently, cDNA and genomic libraries are being screened to islolate full length clones and to demonstrate opioid receptor binding by expression in COS-cells.

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FUNCTIONAL ALTERATIONS IN THE RAT BRAIN AFTER SELECTIVE ACTIVATION OF μ OPIOID RECEPTORS

A. Ableitner

Intracerebroventricular (i.c.v.) administration of the selective μ-opioid agonist (D-ala^2-N-methyl-Phe^4-Gly-ol) enkephalin (DAMGO) produces various behavioral responses in the rat which include antinociception, a modulation of motor activity, profound effects on motivational processes and electroencephalographic epileptiform actions.
In order to obtain information on the neuroanatomical areas affected by μ-opioid receptor activation, and which might be functionally involved in these actions, changes in local cerebral glucose utilization, which served as a measure of neuronal activity, were determined after i.c.v. administration of DAMGO. 0.5 μg of the agonist produced profound increases in glucose utilization in the visual cortex (30%), in limbic regions, such as the hippocampus (up to 170%), the lateral septum (30%) and the lateral habenula (30%) and in motor regions, such as the globus pallidus (50%) and the substantia nigra (40%). In addition, glucose utilization was increased in the dorsal raphe (30%) as well as the lateral and dorsal part of the central gray (40%).
This regional pattern of changes is assumed to underlie the various behaviors observed after i.c.v. administration of DAMGO. The strong alterations in the hippocampus, furthermore, may indicate a prominent role of μ-opioid receptors in the modulation of hippocampal excitability.

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PHARMACOLOGICAL CHARACTERISTICS OF THE PERIPHERALLY-SELECTIVE k-OPIOID AGONISTS EMD 60400 AND ICI 204448

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There is good evidence that opiates can suppress inflammatory hyperalgesia via peripheral mechanisms. EMD 60400 (N-methyl-N-[(1S)-1-phenyl-2-((3-S)-3-hydroxypyrrolidine-1-yl)-ethyl]-2-aminophenylacetamide) is a k agonist designed to have limited access to the CNS. We have compared the pharmacological properties of EMD 60400 with those of the peripherally-selective k agonist ICI 204448 ((R,S)-N-[2-(N-methyl-3,4-dichlorophenylacetamido)-2-(3-carboxyphenyl)-ethyl]-pyrrolidine HCl) and the centrally-active k agonist ICI 197067 ((2S)-N-[2-(N-methyl-3,4-dichlorphenylacetamido)-3-methylbutyl]-pyrrolidine). EMD 60400 binds to k receptors in the guinea pig cerebellum with a K_i of 1 nmol/l and inhibits electrically-evoked contractions of the rabbit vas deferens with an IC_{50} of 10 nmol/l. Results were similar for ICI 204448 and 197067, as were the binding affinities for μ and δ receptors, which were at least 100 x weaker than for k receptors. CNS concentrations in the mouse after 1 mg/kg s.c. were near or below the limits of detection (2 pg/mg) for EMD 60400 and ICI 204448, respectively, while ICI 197067 was found at over 80 x this level. EMD 60400 had antinociceptive effects, with ID_{50} values of 0.23, 0.22 and 0.47 mg/kg s.c. in the rat and mouse writhing tests and mouse formalin test, respectively. ICI 204448 was 10-50 x less, and ICI 197067 3-6 x more active than EMD 60400 in these models. Hyperalgesic pressure pain (carrageenan) and neurogenic plasma extravasation in the skin were inhibited by EMD 60400 in rats with ID_{50} values of 0.0001 mg/kg s.c. and 0.17 mg/kg i.v., respectively. ICI 204448 and 197067 were ≈ 7300 x and 90 x less active than EMD 60400 in the pressure pain test, respectively, and both were ≈ 8 x less effective than EMD 60400 against neurogenic extravasation (despite the much better ability of ICI 197067 to cross the blood-brain barrier). EMD 60400 is a potent and selective k agonist which is a promising candidate for development against inflammatory hyperalgesia since the known side-effects of centrally-active k agonists (sedation, dysphoria, etc) can be avoided.

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PAIN-RELATED RESPONSES OBTAINED FROM THE HUMAN NASAL MUCOSA

H.G. Kraetsch, and T. Hummel

It has already been shown that after painful stimulation of the nasal mucosa with carbon dioxide pain-related responses can be obtained which are thought to be summated receptor potentials from chemosensitive nociceptors. The aim of this study was to test the reliability of this technique of assessment of peripheral nociceptive function in a large panel of volunteers (n=29). Artifact-free responses to all of the three concentrations of carbon dioxide could be analysed in 65% of the subjects with a maximum negative amplitude approximately 1.1 s after stimulus onset. This negativity was related to both the stimulus concentration and the intensity estimates of the painful sensations. In addition, psychophysical measures and recordings of chemosomatosensory evoked potentials (CSSEP) revealed a close relation to the stimulus concentration.
Thus, it was demonstrated that this technique allows the simultaneous, non-invasive, measurement of the human processing of nociceptive information at several different levels (mucosal potential, chemosomatosensory evoked potentials, psychophysical responses).

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NOVEL TOOLS TO STUDY NEUROPEPTIDE Y (NPY) RECEPTOR SUBTYPES. I. ENDOGENOUS AND SYNTHETIC PEPTIDE YY (PYY) ANALOGS

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PYY has 66% homology with NPY and potently activates most NPY receptors. NPY receptor subtypes are frequently distinguished by C-terminal fragments such as NPY_{13-36} or NPY_{18-36} or by [Pro^{34}]-substituted NPY analogs. We have recently detected an endogenous long C-terminal fragment of PYY, PYY_{3-36} in extracts of human colon and have also synthesized a [Pro^{34}]-substituted PYY analog, [Pro^{34}]PYY. The potency of these to PYY analogs was compared in known model systems of Y_1- and Y_2-like NPY receptors. In radioligand competition binding experiments [Pro^{34}]PYY had high affinity for Y_1-like [^{125}I]NPY binding sites in intact SK-N-MC and HEL cells with pK_i-values of 7.82±0.23 and 8.24±0.12, respectively. In contrast, PYY_{3-36} in concentrations up to 1 μM did not compete for NPY binding sites in either cell line. In binding studies at the Y_2-like [^{125}I]NPY receptors in porcine splenic membranes PYY_{3-36} competed for [^{125}I]NPY binding with high affinity (pK_i=10.13±0.31), whereas [Pro^{34}]PYY had a 1000-fold lower affinity (pK_i=7.30±0.14). In functional studies, [Pro^{34}]PYY mobilized intracellular Ca^{++} in SK-N-MC (pEC_{50}=8.49±0.05) and in HEL cells (pEC_{50}=8.24±0.06), whereas PYY_{3-36} was ineffective in concentrations up to 1 μM. Our data indicate that the endogenous PYY_{3-36} despite its length is highly selective for Y_2-like NPY receptors whereas the synthetic [Pro^{34}]PYY has a similar selectivity for Y_1-like NPY receptors as [Pro^{34}]NPY.

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NOVEL TOOLS TO STUDY NEUROPEPTIDE Y (NPY) RECEPTOR SUBTYPES. II. CENTRALLY TRUNCATED NPY ANALOGS

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Present attempts to define NPY receptor subtypes rely on a single class of chemicals, i.e. C-terminal analogs of NPY. We have tested the subtype-selectivity of a novel class of compounds, centrally truncated NPY analogs where the PP-fold had been substituted by ε-aminocaproic acid (Aca), in Y_1-like (Ca^{2+} mobilization in HEL-cells, blood pressure increase in pithed rats) and Y_2-like model systems ([^{125}I]NPY binding to rabbit kidney, presynaptic inhibition of contractile responses in rat vas deferens). All centrally truncated NPY analogs were poor agonists in the blood pressure assay and lacked agonistic effects in HEL-cells. Some of these analogs (e.g. NPY-(1-4)-Aca-25-36)) also lacked antagonistic effects in HEL-cells suggesting very low affinity for the Y_1-receptor subtype. Other analogs (e.g. [Ala^{28}]-NPY(1-4)-Aca-(25-36)) shifted the NPY concentration-response curve in HEL-cells to the right in a parallel manner (pK_b of 6.3) suggesting lack of intrinsic efficacy despite of moderate affinity at the Y_1-like subtype. On the other hand, some centrally truncated NPY analogs (e.g. NPY(1-4)-Aca-25-36)) had high affinity for [^{125}I]NPY binding sites in rabbit kidney and were high potency agonists in rat vas deferens suggesting high intrinsic efficacy and Y_2-selectivity. We conclude that centrally truncated NPY analogs confirm the concept of Y_1- and Y_2-subtypes of NPY receptors and discriminate subtypes by affinity and efficacy. Selectivity and efficacy, however, vary considerably within this class.

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EFFECTS OF NPY AND SELECTIVE ANALOGS IN THE ISOLATED PERFUSED RABBIT EAR

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Postjunctional effects of neuropeptide Y (NPY) on vascular smooth muscle are twofold. A direct vasocontractile response of NPY has been reported for certain bloodvessels. However of greater importance seems to be the ability of NPY to potentiate the effects of several vasoconstricting agents e.g. noradrenaline. In the present study we investigated the effects of NPY, the Y1-selective agonist [Leu^{31},Pro^{34}]NPY as well as the Y2-selective agonists NPY(13-36) and NPY(18-36) in the isolated perfused rabbit ear. New Zealand rabbits of either sex (3 kg) were killed and the ears were rapidly removed. The rabbit ear vasculature was perfused at a rate of 3.5 ml/min with a modified Krebs-Ringer solution containing the H_1-antagonist mepyramine (1 μM). The basal perfusion pressure of 5-10 mmHg was raised up to approximately 50 mm Hg by adding noradrenaline to the perfusion medium (0.5-1.0 nM). In separate experiments noradrenaline and mepyramine were omitted from the perfusion medium. Dose-response curves to the agonists were constructed by bolus injection of the drugs into the perfusion solution. At the basal perfusion pressure NPY elicited a dose-dependent (1-10 nM) increase in perfusion pressure. The maximum response was approximately 220 mmHg. However, this response was almost completely abolished by 1 μM mepyramine. Similar results were obtained with the histamine liberator compound 48/80. When vascular tone had been increased to approximately 50 mmHg, NPY induced a dose-dependent increase in perfusion pressure which was histamine independent. The maximum response was 100-120 mmHg and the -log dose (mol) of the ED50 for NPY was 9.91 ± 0.18 (n = 8). -Log ED50 values for [Leu^{31},Pro^{34}]NPY, NPY(18-36) and NPY(13-36) were 10.36 ± 0.23 (n = 5), 8.15 ± 0.15 (n = 11) and 7.94 ± 0.20 (n = 5), respectively.
In conclusion, NPY and the Y1-selective agonist (Leu^{31},Pro^{34}]NPY are equipotent whereas the Y2-selective C-terminal fragments were 162-263-fold less potent to induce vasoconstriction in preparations in which vascular tone was increased with noradrenaline. These results indicate the involvement of Y1-receptors. In addition NPY can also produce a direct vasoconstriction, however this is due to the release of histamine.

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CHARACTERIZATION OF THE BOMBESIN RECEPTOR SUBTYPE IN GUINEA-PIG ILEUM LONGITUDINAL MUSCLE PREPARATIONS

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The amphibian peptide bombesin has been reported to exert powerful stimulatory effects in many mammalian smooth muscle preparations *in vitro*. The mechanism of the contractile effects of bombesin (BBS), the structurally related mammalian peptides gastrin-releasing peptide$_{14-27}$ (GRP$_{14-27}$) and neuromedin B (NMB) were examined in longitudinal muscle preparations from guinea-pig ileum (LM-GPI). All three peptides evoked tonic contractions of the LM-GPI in a concentration-dependent manner. NMB (EC_{50}: 7.3±0.1) was equally potent as BBS (EC_{50}: 7.5±0.1), however, (GRP$_{14-27}$) exhibited a significantly lower potency (EC_{50}: 6.9±0.1).

The susceptibility of the contractile activity to atropin (unsurmountable inhibition) clearly proved the involvement of a cholinergic transmission. Likewise, administration of the sodium channel blocker tetrodotoxin prevented the formation of BBS-induced smooth muscle contractions. Hexamethonium, mecamylamine, mepyramine, methysergide, and the $GABA_A$ receptor antagonist bicuculline did not affect the contractile activity. Putative peptidergic BBS receptor antagonists of different classes were applied for further characterization of the receptor subtype involved. Results obtained with a D-amino acid substituted analogue of substance P, a reduced peptide bond analogue of bombesin, and [Des Met^{27}] gastrin-releasing peptide analogues will be discussed.

This study provides evidence that the bombesin receptor of the guinea-pig ileum longitudinal muscle preparation is located on myenteric neurons and represents a member of the neuromedin B-preferring subtype family.

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EFFECTS OF PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE (PACAP) AND VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) ON HUMAN COLONIC MOTILITY

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PACAP is a novel brain-gut peptide of the glucagon/secretin/VIP family. The two molecular forms PACAP-27 and PACAP-38 show high homology to VIP. PACAP-immunoreactive nerve fibers and nerve cell bodies in intramural ganglia are present in the human gastrointestinal tract. We studied the effects of PACAP peptides and VIP in vitro on the spontaneous contractility of the longitudinal muscle of human colonic sigma. Muscle strips of macroscopically normal appearing colonic sigma were cut in the longitudinal axis and suspended isometrically in a 3 ml organ bath containing oxygenated Krebs solution. PACAP peptides were synthesized as alpha-amides using the Fmoc solid-phase strategy and purified by HPLC.
The spontaneous phasic contractions of the longitudinal muscle were abolished by verapamil (10 µM) and not significantly affected by tetrodotoxin (TTX, 1 µM). PACAP-27, PACAP-38 and VIP reduced concentration-dependently the phasic contractions (IC_{50} of PACAP peptides: 130 nM, IC_{50} of VIP: 470 nM). The effects of PACAP peptides and VIP were preserved in the presence of TTX. Apamin (30 nM), a specific blocker of Ca^{2+}-dependent potassium channels, reduced only the inhibitory effects of the PACAP peptides (1 µM) by about 80 %. In the presence of tetraethylammonium (0.3 mM) only the inhibitory effect of VIP (1 µM) was attenuated by about 70 %. In conclusion, PACAP and VIP activate specific receptors coupled to different potassium channels.

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POSITIVE INOTROPIC EFFECT OF CENTRALLY ACTING ANGIOTENSIN II

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The aim of this study was to investigate whether a positive inotropic effect contributes to the blood pressure increases in response to periventricular angiotensin II (ANGII) receptor stimulation. Male Wistar rats were chronically instrumented with an intracerebroventricular (icv) cannula, catheters in the femoral artery and vein, jugular vein and a thermistor probe in the ascending aorta for measurement of cardiac output. In conscious rats arterial blood pressure (BP), heart rate (HR), cardiac output (CO), total peripheral resistance (TPR=BP/CO), stroke volume (SV=CO/HR) and cardiac power (CP=BP*CO) were recorded before and after an icv bolus injection of 100ng ANGII. The experiments were performed under control conditions (C) and were compared to those during peripheral V1-vasopressin receptor blockade (V) (n=7; 5µg β-Mercapto-β,β-cyclopentamethylene-propionyl-O-methyl-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly amide i.v.), α-1-adrenergic receptor blockade (A) (n=8; 0.5mg prazosin i.v.), and β_1-adrenergic receptor blockade (B) (n=6; 4mg atenolol i.v.). The responses to ANGII are shown in the following table (means ± SEM, *:p<0.05):

	C	V	A	V+A	B	
BP	+26±2 *	+15±2,5 *	+27±3,6 *	+6±1,1 *	+22±2,4 *	[mmHg]
HR	-33±8 *	+19±13	-64±9 *	-21±7 *	-31±21	[bpm]
CO	-10±2 *	+10±6	-5±6	+16±3 *	-10±7	[ml/min]
TPR	+0,33±0.04 *	+0,07±0,04	+0,24±0,03 *	-0,03±0,01	+0,37±0,1 *	[mmHg*min/ml]
SV	-4±6	+10±15	+25±10 *	+51±5 *	+31±48	[µl]
CP	+4,5±0,8 *	+6,4±1,9 *	+6,4±1,9 *	+4,3±0,7 *	+2,9±2,3	[mWatt]

During C the increase in BP was mediated by an increase in TPR and CP. In contrast to all other groups, the ANGII-induced increase in CP was not observed during B, and HR, CO and SV increased under V. These findings support our conclusion that centrally acting ANGII induces a positive inotropic effect that is at least partly due to an increase in cardiac sympathetic tone. The ANGII-induced increase in HR, CO and SV during V also supports this hypothesis.

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RP 69758, A NOVEL CHOLECYSTOKININ B-TYPE AND GASTRIN ANTAGONIST.

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Cholecystokinin recognizes two different binding sites termed CCK-A and CCK-B receptors. Brain CCK-B receptors have a similar binding profile as gastrin receptors in the stomach. Understanding the respective role of receptor subtypes in the physiological functions of CCK requires the availability of potent, selective and easy to handle antagonists. Here we describe the properties of RP 69758 (3-{3-[N-(N-methyl N-phenyl-carbamoylmethyl) N-phenyl-carbamoylmethyl] ureido} phenylacetic acid), a member of a new class of CCK-B/gastrin antagonists. This compound displaces [^{3}H]pCCK-8 from guinea-pig cortex membranes and [^{125}I]-Leu^{15}-gastrin-17 from gastric gland preparations with nanomolar affinity (pK_i=8.19 ± 0.07 and 8.44 ± 0.04, respectively). Moreover, RP 69758 displays a high selectivity over pancreatic CCK-A receptors (pK_i= 5.85 ± 0.04) and does not interact with other classical neurotransmitter or neuropeptide receptors. In rat hippocampal slices in vitro, a model in which CCK-8 increases neuronal firing by acting on CCK-B receptors, RP 69758 dose-dependently inhibits the excitatory effects of CCK-8 with a half maximal effective concentration of 1.4 10^{-7}M. Finally, RP 69758 (0.1-30 mg/kg i.v.) dose-dependently reduces basal acid secretion in the Shay rat model. RP 69758 appears therefore as a useful tool in exploring the functional roles of CCK-B/gastrin receptor mediated processes.

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IN VIVO ANALYSIS OF DOPAMINE AND SEROTONIN NEUROTRANSMISSION AFTER PERIPHERALLY ADMINISTERED CCK-B RECEPTOR AGONIST CCK-4

Th. Barth, R. Sohr and H. Fink

A subtype of cholecystokinin (CCK-) receptors occuring only in the brain is the CCK-B receptor. CCK-4 exhibits a high affinity for the CCK-B receptor and has a lower affinity for the peripherally and centrally occuring CCK-A receptor.
In several studies CCK-4 has been shown to induce behavioral effects.
Furtheron, it has been demonstrated in neurochemical, electrophysiological and behavioral experiments that CCK-8, an agonist at both CCK-A and CCK-B receptors interacts with the central dopamine (DA) and serotonin (5-HT) system.

This study was designed to investigate the role of CCK-B receptors in the interaction of CCK with DA and 5-HT systems.

Changes of extracellular levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were assessed by *in vivo* microdialysis in anesthetized rats. The effect of CCK-4 (0.4, 2.0 and 10.0 µg/kg i.p.) was investigated in the medial prefrontal cortex because of its rich DA, 5-HT and CCK innervation.

CCK-4 in the dose of 10.0 µg/kg i.p. failed to influence the DA level, but decreased the DOPAC level to approximately 85 %, the HVA level to 60 % and the 5-HIAA level to 80 % of control (0.9% NaCl) values.

It is suggested that CCK-B receptor-induced effects are mediated or accompanied by changes of DA transmission.

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ISOLATION OF A SYNAPTIC PLASMA MEMBRANE VESICLE FRACTION FROM PIG BRAIN CORTEX

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We have previously reported on an isolation procedure for synaptosomes from pig brain cortex using a discontinuous Ficoll gradient (Kliem et al.:Naunyn-Schmiedeberg's Arch. Pharmacol. 341: R 95, 1990). We now describe a method for the isolation of synaptic plasma membrane vesicle fraction from this highly purified synaptosomal fraction. All steps of this procedure were carried out at 4° C. The synaptosomal fraction was resuspended in a 0.01 M Tris-HCl-buffer (pH 8.5) and lysed for 15 min. For separation a layer of 10% (w/w) Ficoll/buffer (comprising 0.25 M sucrose, 10 mM L-histidine, 0.5 mM K+-EDTA, pH 7.2) at the bottom of a centrifuge tube was overlaid with buffer and the lysate was placed upon the buffer surface. After centrifugation at 52800 x g for 45 min the fraction that had formed at the interface was collected, resuspended in the sucrose containing buffer (1:3), and centrifuged at 105000 x g for 45 min. This pellet and the pellet obtained from the first centrifugation step were examined by electron microscopic and biochemical methods. – Electron microscopy showed that the fraction found on top of 10% Ficoll consisted of the synaptic plasma membrane vesicle fraction. By contrast, the pellet from the first centrifugation step contained predominantly small synaptic mitochondria. The biochemical markers for the synaptic plasma membrane fraction (alcaline phosphatase, Na+/K+-ATPase) and synaptic mitochondria (different types of monoamine oxidases, cytochrome c oxidase, succinate dehydrogenase) displayed the patterns typical of these structures and testified to the high degree of purity of the individual fractions.

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ROLE OF GLUCOCORTICOIDS IN THE CHOLINERGIC LESION IN RAT HIPPOCAMPUS INDUCED BY ETHYLCHOLINE AZIRIDINIUM (AF64A)

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The hippocampus is the primary target tissue for glucocorticoids in the brain. Exposure to glucocorticoids has been shown to potentiate hippocampal damage induced by various noxious insults [Sapolsky RM (1987) TINS 10:346]. The purpose of the present study was to find out whether short-term exposure to glucocorticoids in doses equivalent to a major stress reaction aggrevates the cholinergic lesion in rat hippocampus induced by ethylcholine aziridinium (AF64A) and to which extent endogenous glucocorticoids are involved. Male Sprague- Dawley rats (280-370g) received bilateral stereotaxic infusions of AF64A (1 nmol) or vehicle into each lateral ventricle. Corticosterone (10 mg in sesame oil) or sesame oil was injected s.c. 24 h and 15 min before and 24 h after AF64A. Bilateral adrenalectomy or sham-operation was performed through dorsal incisions 7 days before AF64A. In both experiments rats were killed 7 days after AF64A. Short-term exposure to corticosterone resulted in a potentiation of the AF64A induced cholinergic damage in the hippocampus (dissected in CA1, CA3 and dentate gyrus). The following changes in choline acetyltransferase (ChAT)-activity (nmol/h/mg tissue + SEM) were observed (*p < 0.05; **p < 0.01 vs sesame oil/AF64A):

	(n)	CA1	CA3	dent.gyrus
sesame oil/vehicle	(6)	6.37±0.18	4.83±0.09	6.59±0.26
corticosterone/vehicle	(6)	6.63±0.11	5.02±0.09	6.59±0.09
sesame oil/AF64A	(5)	3.52±0.39	1.94±0.32	3.53±0.28
corticosterone/AF64A	(6)	2.82±0.12*	1.69±0.24	2.64±0.17**

In contrast, after adrenalectomy the AF64A-induced reduction in ChAT activity was prevented in all subregions of the hippocampus. Supplementation with corticosterone in adrenalectomized rats restored the neurotoxicity of AF64A.
The data indicate that glucocorticoids play a critical role in the toxicity of AF64A on the septohippocampal cholinergic pathway, even under physiological conditions.

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GRAFTS OF FETAL NEURONS INTO THE DENERVATED HIPPOCAMPUS: NEUROCHEMICAL AND BEHAVIOURAL EFFECTS.

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Female rats sustained lesions of the septohippocampal pathways and, 14 days later, received intrahippocampal suspension grafts prepared from the medial septum (rich in cholinergic neurons), from the dorsal raphe (rich in serotonergic neurons) or from both regions together. Lesion-only and sham-operated rats served as controls. Between 6,5 and 8,5 months after grafting, all rats were tested for home cage activity and radial maze performance. One month later, their hippocampi were prepared for determination of **(i)** choline acetyltransferase **(ChAT)** activity, **(ii)** high affinity synaptosomal uptake of ^{3}H-choline **(HACU)** and ^{3}H-serotonin **(HASU)**, and **(iii)** serotonin concentration **([5-HT])**. The lesions increased locomotor activity, impaired radial maze performances and decreased ChAT activity, HACU, HASU and [5-HT]. Neither type of graft produced any significant behavioural effect. Septal grafts increased ChAT activity and HACU, but had no effect on HASU and [5-HT]. Raphe grafts overcompensated HASU, [5-HT] and slightly increased ChAT activity and HACU. Grafts from both regions induced mixed effects, *i.e.*, on all neurochemical variables. These results suggest that intrahippocampal grafts may foster a neurotransmitter-specific recovery in the denervated hippocampus which depends on the neurochemical identity of the grafted cells. However, separate or combined attenuation of the neurochemical deficits induced by septohippocampal lesions is not sufficient to induce behavioural recovery.

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EFFECTS OF CHOLINE DEFICIENCY ON CHOLINE AVAILABILITY IN THE BRAIN

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The brain is dependent on the supply of free choline from the arterial blood in order to support the synthesis of acetylcholine and of choline-containing phospholipids. The present study is concerned with the effects of dietary choline deficiency on the availability of choline in the brain. Choline was measured by an HPLC-based procedure in arterial and venous blood entering resp. leaving the brain and in CSF. Choline metabolites were determined in whole brain homogenate using a newly developed procedure which involves the conversion of metabolites to and measurement as free choline. Dietary choline deficiency lowered the arterial plasma choline level from 10.6 ± 0.9 µM to 4.9 ± 0.4 µM after two weeks, 7.9 ± 0.7 µM after six weeks and 6.7 ± 0.2 µM after six months of diet. Concomitantly, the arterio-venous difference of choline across the brain was reduced from -3.0 ± 0.4 µM to -1.9 ± 0.2, -2.0 ± 0.7 and -1.8 ± 0.3 µM indicating a reduced net release of choline from the brain under these conditions. CSF choline levels were well preserved: 5.9 ± 0.3 µM in control animals, and 4.8 ± 0.2, 4.5 ± 0.3 and 6.0 ± 0.4 µM in the choline-deficient animals. The levels of choline-containing metabolites in the brain were also unchanged during the first six weeks of choline deficiency, with the exception of glycerophosphocholine which was slightly (by 18%) but significantly ($p<0.05$) increased. The present data indicate a relatively well preserved choline homeostasis in the brain under the conditions of dietary choline deficiency.

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REGULATION OF BRAIN EXTRACELLULAR CHOLINE STUDIED BY MICRODIALYSIS

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Previous studies from our laboratory have shown that the choline concentration in the CSF of rats (taken from the cisterna magna) was increased after acute or chronic administration of choline chloride, but there seemed to be a ceiling value of 15-20 µM which was not overcome even by large doses. The aim of the present study was to monitor changes of the concentration of choline in the brain extracellular space and to investigate possible pharmacological means to elevate brain choline levels. - Concentric microdialysis probes (O.D. 0.24mm) were placed into the ventral hippocampus of male Wistar rats, and choline efflux was monitored by HPLC. Pentobarbital anaesthesia (60-80 mg/kg i.p.) did not change the basal choline efflux (1.8 ± 0.2 pmol/min), whereas urethane anaesthesia (1.25 g/kg i.p.) increased choline efflux by 70%. This finding agrees with published results showing that urethane enhanced the CSF choline concentration. - Injection of 6 and 20 mg/kg choline chloride (i.p.) to awake animals led to small increases in choline efflux (+ 7% and + 47% after 10 min, respectively), whereas 60 mg/kg raised choline efflux by 130% which is equivalent to the above mentioned ceiling values obtained from CSF. One hour after choline injection the choline levels were almost back to basal values. - It has recently been shown that nicotinamide inhibits choline transport from CSF into blood. In our studies, 10 mmol/kg s.c. elevated choline efflux by 100% lasting from 0.5 to 4 hours after administration. - The present results suggest that extracellular brain choline is elevated by supplementation of large doses of choline and, possibly more effectively, by blocking the outward transport of choline.

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INHIBITION OF EXCITATORY AMINO ACID (EAA)-INDUCED NORADRENALINE (NA) RELEASE BY GENERAL ANAESTHETICS

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Previous studies (Fink and Göthert, 1990, Eur J Pharmacol 191:225-229) revealed that the N-methyl-D-aspartate (NMDA)-induced NA release in the rat brain cortex is inhibited by aliphatic alcohols in proportion to their lipophilicity. The aim of the present study was to investigate whether general anaesthetics also inhibit the EAA-induced NA release in the rat brain cortex. Rat brain cortex slices preincubated with ^{3}H-NA were superfused with Krebs-Henseleit solution (Mg^{2+}-free in experiments with NMDA). The ^{3}H overflow from the slices was stimulated for 2 min either with 300µmol/l NMDA, or 1mmol/l kainate or 20mmol/l KCl (isoosmolar replacement of NaCl). Anaesthetic agents were present from 20 min before stimulation until the end of superfusion. The NMDA-evoked ^{3}H overflow was concentration-dependently inhibited by ketamine, methohexital, thiopental, propofol, etomidat, diethyl ether, halothane, enflurane, isoflurane. Ketamine inhibited the NMDA-induced ^{3}H overflow much more potently (IC_{50} = 0.92µmol/l) than the kainate (IC_{50} = 1.1mmol/l)-or KCl (IC_{50} = 0.7mmol/l)-induced ^{3}H overflow. Methohexital was also slightly more potent in inhibiting the NMDA-induced ^{3}H overflow (IC_{50} = 18.6µmol/l) than the kainate (IC_{50} = 74µmol/l)- or KCl (IC_{50} = 130µmol/l)-induced ^{3}H overflow. However, propofol did not exhibit different inhibitory potencies on NMDA-, kainate-, or KCl-induced ^{3}H overflow. Diethyl ether only inhibited the NMDA-evoked ^{3}H overflow but had no effect on the kainate- or KCl-induced ^{3}H overflow. Except for ketamine the inhibitory potency of the general anaesthetics on the NMDA-evoked ^{3}H overflow was correlated to their membrane/buffer (m/b) partition coefficient ($r=0.88$; $p<0.0001$). It is concluded that, as a rule, the effect of general anaesthetics on the responses to NMDA or non-NMDA receptor activation may be due to hydrophobic interaction with the receptor protein or the plasma membrane lipid bilayer. In contrast, the about 1000 times higher potency of ketamine in inhibiting the response to NMDA than predictable from its m/b partition coefficient is compatible with its specific action via the PCP binding site within the NMDA-gated ion channel.

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MEMANTINE LEVELS AND DOPAMINE RELEASE IN STRIATUM AND PREFRONTAL CORTEX IN THE RAT

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Memantine (1,3-dimethyl-5-aminoadamantane-HCL) has been characterized as an uncompetitive antagonist of the N-metyl-D-aspartate (NMDA) receptor (Kornhuber et al., 1989, Eur. J. Pharmacol. 166: 589-592, 1989). The main therapeutic potential of this compound is in the field of dementia and spasticity. Increasing evidence suggest that NMDA-antagonists act in an indirect manner on dopaminergic systems. Therefore, we have examined the pharmacokinetics of memantine after i.p. administration in different brain areas and simultaneously the release of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

DA and its metabolites as well as memantine were measured in the striatum and prefrontal cortex in anesthetized rats by microdialysis and high-performance liquid chromatography with electrochemical detection. Memantine (5-20 mg) caused a dose-dependent increase in DA release in the striatum. A significant effect occured 40 and 60 min postinjection up to 44 % over basal levels. A similar enhancement of DA release could be observed in the prefrontal cortex. The output of DOPAC and HVA was also dose-dependently enhanced. These effects were of later onset and longer duration than for DA. For all dosages, and in both brain areas, memantine could be detected over the whole time course. The C_{max} values were reached within 60 min, thereafter memantine showed a very flat slope with a half life of between 4-8 h.

Thus, although the main effect of this compound is the modulation of the fast glutamatergic transmission, memantine also showed a significant effect on the release of DA.

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BOTH NON-COMPETITIVE AND COMPETITIVE NMDA RECEPTOR ANTAGONISTS INCREASE DOPAMINE AND SEROTONIN TURNOVER IN SEVERAL BRAIN REGIONS OF RATS

R. Annies and W. Lõscher

MK-801 (dizocilpine) and other non-competitive antagonists of N-methyl-D-aspartate (NMDA) receptors induce in rats a characteristic behavioural syndrome, including hyperlocomotion, lateral head weaving, circling and ataxia. At least part of these behaviours are thought to be mediated through interactions with the dopaminergic system. However, more recent studies indicated that interactions with serotonin metabolism might also be involved. Since similar behaviours as those induced by MK-801 are also observed after high doses of competitive NMDA receptor antagonists, such as CGP 37849 (DL-[E] -2-amino-4-methyl-5-phosphono-3-pentenoic acid), we compared the effects of MK-801 and CGP 37849 on dopamine and serotonin metabolism in rats. After handling habituation, rats were injected i.p. with either MK-801 (0.3 mg/kg) or CGP 37849 (30 mg/kg) and all behaviours induced by these treatments were recorded. Both drugs induced similar behavioural alterations at these doses. At time of maximum behavioural effects, monoamines and their metabolites were determined by HPLC with electrochemical detection in 14 brain regions. MK-801 and CGP 37849 increased the turnover of dopamine and serotonin in several brain regions, although differences were found between both drugs with respect to the extent and localization of the respective changes. With both treatments, dopamine turnover increased in cerebral cortex and N. accumbens. Furthermore, both drugs induced increases in serotonin turnover in cerebral cortex and the striatum. The neurochemical data indicate that not only effects on dopaminergic transmission but also on serotonergic transmission may be involved in the pharmacodynamic actions of NMDA receptor antagonists.

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THE VASOPRESSIN (AVP) RELEASE FOLLOWING ANGIOTENSIN II (ANG II) IS MEDIATED THROUGH ALPHA-ADRENERGIC RECEPTORS IN THE PARAVENTRICULAR NUCLEUS (PVN).

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ANG II is a powerful releaser of AVP from hypothalamic nuclei through the pituitary gland into the blood. We investigated in vivo, whether this ANG II- induced AVP release involves a hypothalamic catecholaminergic pathway as has been suggested by previous in vitro studies. Using the microdialysis technique in conscious rats, we first studied the effect of stimulation of periventricular ANG II receptors on the release of catecholamines in the paraventricular nucleus (PVN). ANG II injected intracerebroventricularly (icv) at doses between 100pg-100ng caused a dose-dependent and selective noradrenaline (NA) increase.This effect was abolished by icv pretreatment with the ANG II-AT 1 receptor antagonist Losartan (DuP 753/MK-954). We then investigated, whether the ANG II-induced AVP release into the blood is mediated by adrenergic receptor stimulation in the PVN. For this purpose we measured the ANG II-induced AVP release after α- and ß-adrenergic receptor blockade in the PVN in conscious rats. The α_1- and α_2-adrenoceptor antagonists, prazosin (0.7nmol), idaxozan (4nmol) and rauwolcine (5nmol), injected bilaterally into the PVN significantly reduced the ANG II (100ng, icv)-induced AVP release into the blood. Pretreatment via the same route with the $ß_1$- and $ß_2$-adrenoceptor antagonist, atenolol (4nmol) and ICI 118551 (0.96nmol) had no effect. The injections of NA (3-100nmol) and of ANG II (100pg-100ng) into the PVN increased plasma AVP dose-dependently, but the increase following ANG II into the PVN was smaller than after icv injection. Pretreatment with Losartan (5, 10ug), injected bilaterally into the PVN, partially inhibited the ANG II (icv)-induced AVP release.
Our results show for the first time in vivo a release of NA in the PVN following stimulation of periventricular ANG II-AT 1 receptors. Further, they provide evidence that NA in the PVN acts as a transmitter to release ANG II by acting on α-receptors in the PVN, which need to be further characterized. Finally, our findings also suggest that ANG II-AT 1 receptors in the PVN itself contribute to the ANG II-induced AVP release.

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ENDOGENOUS NORADRENALINE (NA) INHIBITS THE RELEASE OF SEROTONIN (5-HT) IN HUMAN NEOCORTEX

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Since both NA and 5-HT are involved in the pathophysiology of CNS disorders like cyclothymia, it is of interest to investigate also the presynaptic linkage between these neuronal systems. Human neocortex slices, prepared from neurosurgically removed tissue, were incubated with (^{3}H)-5-HT, then superfused and twice stimulated electrically. The evoked (^{3}H)-overflow, assumed to represent 5-HT release, was concentration dependently inhibited by the NA uptake inhibitor (+)oxaprotiline. In the presence of the 5-HT receptor antagonist metitepine, the α_2-adrenoceptor antagonist (and 5-HT receptor agonist) rauwolscine disinhibited the evoked 5-HT release. The α-adrenoceptor antagonist phentolamine had no effect. In the additional presence of (+)oxaprotiline, however, an enhancement of 5-HT release due to phentolamine was observed. Göthert et al., 1983 (this journal, 322: 121-128), have shown that superfusion at 17°C instead of 37°C diminished the release of 5-HT and enhanced the release of NA from rat neocortex slices. Similar conditions existed in our experiments performed with human neocortex slices. While the α_2-adrenoceptor antagonist idazoxane was ineffective at 37°C, it significantly disinhibited 5-HT release in the presence of an increased biophase concentration of NA due to hypothermia. This is in accordance with the partial antagonistic property of idazoxane. Clinically, these findings of an innervation of the α_2-heteroreceptors on 5-HT terminals in the human neocortex indicate that thymoleptic treatment, which enhances NA activity, affects also the serotonergic system.

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EFFECTS OF DOPAMINERGIC DRUGS ON K^+-STIMULATED RELEASE OF GLUTAMATE IN THE STRIATUM OF NON-ANESTHETIZED RATS - STUDIES USING MICRODIALYSIS

S.Dietze and K.Kuschinsky

The present experiments were performed in order to study the effects of dopaminergic drugs on the K^+-stimulated release of glutamate (GLU) in the striatum of non-anesthetized, freely moving rats. K^+-stimulation was applied in order to increase the neuronal fraction of extracellular GLU which was determined by using microdialysis in combination with HPLC and fluorometric detection. The microdialysis probe was perfused with Ringer's solution containing a reduced Ca^{2+} concentration: Na^+: 148 mM; K^+: 4 mM; Ca^{2+}: 1,2 mM for baseline conditions and Na^+: 52 mM; K^+: 100 mM; Ca^{2+}: 1.2 mM for K^+-stimulation. This stimulation was performed twice: first in the absence and then in the presence of the drugs which were administered systemically (s.c.).
Neither stimulation of D-1 dopamine receptors by the selective D-1 agonist SKF 38393 [2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine hydrochloride](15 mg/kg), alone or in combination with the D-2 receptor antagonist sulpiride (150 mg/kg) or by the combination of the mixed D-1/D-2 agonist apomorphine (1 mg/kg) with sulpiride significantly influenced K^+-induced GLU release. The D-2 receptor agonists talipexole (B-HT 920; 50µg/kg) or quinpirole (0.5 mg/kg) also did not produce significant effects. The results suggest that the actions of dopamine receptor agonists on GLU release are less pronounced than might be expected from the literature.

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CHANGES OF GLUTAMATERGIC SYNAPTIC PROCESSES AFTER PENTYLENETETRAZOL(PTZ)-KINDLING OF RATS

H.Schröder and B.Lössner

Rats were kindled to three class 5 seizures by repeated application of PTZ (45 mg/kg, ip., 10 times for 20 days). After one stimulus-free week, (i)the active uptake of ^{3}H-D-aspartate into crude hippocampal synaptosomes, (ii)the influence of L-glutamate on ^{3}H-D-aspartate release from hippocampal slices and (iii)the specific ^{3}H-L-glutamate binding to crude synaptic membranes of kindled rats were measured.

The active ^{3}H-D-aspartate uptake into hippocampal synaptosomes as well as the K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices was not altered after PTZ kindling. In contrast, the L-glutamate induced increase of the K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices of kindled rats was consistently greater than that from slices of saline treated controls.

PTZ kindling resulted in a significant increase of specific ^{3}H-L-glutamate binding in the hippocampus. Kinetic studies indicated that the enhanced binding to hippocampal membranes from kindled rats reflects changes in the density of binding sites, but not in the affinity. Since single application of convulsive doses of PTZ did not alter the glutamate binding, kindling-induced augmentation of glutamate receptor density is assumed to be a correlate of plastic changes linked to the development of enhanced excitability of the hippocampus.

This study provides evidence that enhanced glutamatergic activity occurs in PTZ kindling and may be involved in the common permanent effects of kindling.

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IN VITRO AND IN VIVO STUDIES ON MUSCARINIC RECEPTOR PLASTICITY IN INTACT MOUSE BRAIN CELLS: ONLY A SPECIFIC FRACTION OF MUSCARINIC CHOLINERGIC RECEPTORS IS SUSCEPTIBLE TO RECEPTOR REGULATION

L. Stoll*

The phenomena of down-regulation of muscarinic cholinergic receptors (mACHRs) induced by chronic overstimulation with agonists or acetylcholineesterase inhibitors, resp. antagonist-induced upregulation has been demonstrated in numerous studies either in vivo (using different species) or in vitro (usually using clonal cell lines of nervous origin as a model). An alternative technique, which allows both in vivo and comparative in vitro studies is the use of mechanically dissociated intact brain cells obtained from adult mice.

Incubation of the dissociated neurons of mouse brain in vitro with carbachol (1 mmol/l) at 37°C resulted in a reduction of mACHR concentration by about 30% as measured by the specific binding of tritiated N-methyl-scopolamine (^{3}H-NMS). The same reduction of mACHR density was found after in vivo treatment with diisopropyl-fluorophosphate (DFP) (1 mg/kg/i.p.) for 6 days. Moreover, when mice were pretreated with DFP no further reduction of mACHR concentration was observed in subsequent carbachol-induced down-regulation experiments in vitro.

Alternatively, in vivo pretreatment with scopolamine (10 mg/kg/i.p.) for 10 days enhanced the mACHR density about 15%. However, subsequent in vitro down-regulation decreased the enhanced density of mACHRs to about the same level as found in saline or DFP treated animals.

Further experiments using ^{3}H-Pirenzepine as radioligand show that the specific fraction of mACHRs which is susceptible to carbachol-induced down-regulation consists to a great extent of M_1-subtype (about 75% of down-regulated receptors). But, also other subtypes of mACHR e.g. M_2 and M_3 are involved.

Our findings are consistent with the assumption that only a specific fraction of central mACHRs is available for bidirectional regulation.

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FUNCTIONALLY DIFFERENT PURINERGIC P2-RECEPTOR MEDIATE PROSTANOID SYNTHESIS AND PHOSHATIDYL-INOSITOL BREAKDOWN IN CULTURED ASTROCYTES

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Astroglial cells in primary culture are able to synthesize large amounts of prostanoids upon stimulation with the calcium ionophor A23187 (1μM), suggesting the involvement of a Ca^{2+}-dependent-phospholipase (PL) A_2 in this process. When added simultaneously with subthreshold doses of A23187 diacylglycerol (DAG)-mimetic phorbol-esters synergistically stimulate prostanoid formation, showing that a cooperation between PLA_2 and PLC might exist. On the other hand, stimulation of astroglial receptors with 0.1mM noradrenaline (NA), 1mM carbachol (CCh) or 1mM adenosine triphosphate (ATP) resulted in enhanced phosphatidylinositol (PI) breakdown. Among the three agonists tested, however, only ATP (1mM) was able to induce prostanoid formation. There was a rapid transient increase of IP_3 liberation upon ATP-stimulation (most probably via a P_{2Y} receptor), while NA and CCh caused only IP_1 and IP_2 formation. Thus, simultaneous increase of free DAG and of IP_3 via PIP_2-specific PLC seemed to be necessary for the induction of astroglial prostanoid synthesis. The phorbol ester TPA inhibited concentration-dependently (1nM-10μM) the ATP-induced IP-liberation, but simultaneously stimulated the synthesis of PGD_2. Furthermore, the PLC-inhibitor neomycine (0.1mM) inhibited the ATP-induced (but not the NA- or CCh-induced) PI-breakdown whithout affecting ATP-induced prostanoid formation.

These results show that P_2-receptor-mediated prostanoid formation may occur independently of ATP-induced PI-hydrolysis in astrocytes. This suggests the existence of functionally distinct purinergic receptors on these cells. One of them might be coupled to PLA_2 regulating arachidonic acid release and prostanoid formation, the other one to PLC producing IP_3 and DAG. DAG formed by the latter seems to be capable to amplify some of the steps of the PLA_2 pathway, which leads to prostanoid synthesis in astrocytes.

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AGE-DEPENDENT CHANGES IN RECEPTOR-MEDIATED AND DEPOLARIZATION-INDUCED PHOSPHATIDYLINOSITOL-TURNOVER IN MOUSE BRAIN

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Alterations of neuronal signal-transduction during the course of aging may contribute to age-related disturbances of brain function. The phosphoinositide-phospholipase-C (PI-PLC)-system represents an important pathway of central neurotransmission, where hydrolysis of phosphoinositides leads to the formation of two second messengers (IP_3 and/or diacylglycerol). We examined possible effects of aging on receptor-G-protein-mediated and depolarization-induced PI-hydrolysis in dissociated neurons from young (3 mon.) and aged (20-22 mon.) NMRI mice. Contrary to our preliminary findings, using a small group of aged animals (Stoll et al., Neurobiol. Aging 1992), stimulation of the muscarinic cholinergic receptor by full (carbachol, oxotremorine-M) and partial (RS 86 (2-ethyl-8-methyl-2,8-diazospiro-4,5-decan-1,3-dion hydrobromide), pilocarpine) revealed no general age-related changes of inositol-monophosphate (IP_1)-accumulation. Similarly, noradrenaline and serotonin-mediated PI-hydrolysis (also mediated by receptors coupled to PLC by G-proteins) was not changed in aged mice. However, IP_1-accumulation induced by direct stimulation of G-proteins by fluoride was increased in aged animals. Depolarization-induced PI-hydrolysis which activates PLC by increasing intracellular calcium (Ca_i), revealed a similar increased PI-turnover in aged animals. This effect was similar in the absence (EGTA 0.5mmol/l) and in the presence ($CaCl_2$ 1mmol/l) of extracellular calcium. We interprete these findings by well known disturbances of calcium-homeostasis in aged animals, resulting in increased Ca_i, which differentially affects receptor-mediated and depolarization-induced PI-turnover. (Chandler et al.,Ann.N.Y.Acad.Sci. 1989). These alterations may represent one of the pathological mechanisms by which increased intracellular calcium affects central neurotransmission in aged animals.

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BETA-ADRENOCEPTOR SUBTYPES IN CEREBELLUM AND HIPPOCAMPUS OF ALZHEIMER PATIENTS AND NON-DEMENTED CONTROLS

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Previously we reported on a significant decrease in basal and stimulated adenylate cyclase activity in postmortem hippocampi of Alzheimer patients [AD]. The aim of this study was to investigate whether this finding was paralled by similar changes in beta-adrenoceptor [ß-AR] distribution including subtypes. The investigation was performed in hippocampi [HIP] and cerebella [CER] - the latter being not affected by histopathological changes - of 8 AD/SDAT and 10 non-demented controls [CO]. Total ß-AR was determined in saturation experiments with the hydrophilic, non-selective antagonist ^{3}H-CGP 12177 (0.04-4 nM), using carteolol (10 µM) for non-specific binding. $ß_1$- and $ß_2$-AR subtypes were determined in competition experiments with 1.3 nM ^{3}H-CGP 12177 and the $ß_1$-selective antagonist CGP 20712A ($3x10^{-11}$-10^{-3} M). All data were analysed by non-linear fitting. Data (mean ± SD) are compiled in the table.

	Bmax (fmol/mg)	Kd (nM)	$ß_1$-AR (%)	$ß_2$-AR (%)
HIP CO	38.8 ± 12.7	0.30 ± 0.27	24.6	75.4
HIP AD	31.5 ± 8.3	0.16 ± 0.07	28.1	71.9
CER CO	22.1 ± 4.7	0.41 ± 0.36	18.7	81.3
CER AD	18.8 ± 5.5	0.28 ± 0.19	17.3	82.7

Thus, in contrast to the reduced basal, isoprenaline-, Gpp(NH)p-, forskolin-stimulated adenylate cyclase activity in AD reported previously (Brain Res 540:229-236,1991) no significant differences between CO and AD were found in total ß-AR number, affinity nor in ß-AR subtype populations in postmortem brain areas affected (HIP) or non-affected (CER) by the disease.

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THYROTROPIN-RELEASING HORMONE (TRH) AND VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) MODULATE SYNAPTIC TRANSMISSION IN NEOCORTICAL NEURONS

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Current research suggests that TRH and VIP are involved in synaptic transmission and modulation in local circuits in the CNS. In the present study, pyramidal cells were recorded intracellularly employing standard techniques in an *in vitro* slice preparation of the neocortex of the adult rat. All cells included in this sample (n=48) were located in lamina II/III and had action potentials >90 mV and resting membrane potentials ≤-70 mV. The synaptic responses were elicited by electrical stimulation (0.2 Hz; 0.1 ms) in lamina V - VI. The peptides (0.1- 50.0 µM) were added to the perfusion medium.

VIP increased the excitability of pyramidal cells, evidenced by an increase in action potential firing during a current pulse, without changing the input resistance, membrane potential or rectifying properties (n=34). At comparable concentrations, the late component of the excitatory postsynaptic potential (l-EPSP) was increased in amplitude and duration. Furthermore, the conductance of the early component of the inhibitory synaptic potential ($IPSP_A$) was enhanced.

Also TRH enhanced direct excitability and increased excitatory postsynaptic potentials without marked effects on membrane potential or input resistance (n=14). Unlike VIP, TRH also markedly increased the early component of the EPSPs. It has been shown that the l-EPSP is mediated through the activation of NMDA receptors.

In neurons of the cerebral cortex, VIP synergistically acts with noradrenaline (NA) to enhance cAMP formation. The long-term enhancement of the l-EPSP by VIP resembles the effects observed after β-adrenergic activation of these neurons. A synergistic action of VIP and NA via a common metabolic link through an elevation of intracellular cAMP has been suggested from biochemical studies.

The present results are evidence in favour of a peptidergic modulation of neuronal excitability in neocortical structures which might act together with β-adrenergic mechanisms in fine tuning the local circuits. Since both neuropeptides affect synaptic components as well as direct neuronal excitability, they may enable neurons to function in a more plastic manner to heterosynaptic activation.

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APPEARANCE OF AN OUTWARD POTASSIUM CURRENT IN RAT MICROGLIA AFTER PRETREATMENT WITH LIPOPOLYSACCHARIDE

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In the central nervous system, tissue damage or infection appears to elicit the transformation of resident microglial cells into macrophages, which play an important role in inflammation and immunity (Jordan and Thomas, Brain Res. Rev., 13:165, 1988). Bacterial lipopolysaccharide (LPS) exerts both stimulatory and suppressive effects on different aspects of macrophage activation. While in microglial cells the only voltage-dependent membrane current found under whole-cell clamp conditions was a hyperpolarization-evoked inwardly directed potassium current (Kettenmann et al., J. Neurosci. Res., 26:278, 1990), a 24 h preincubation of these cells with LPS 100 ng/ml led to the appearance of a depolarization-induced outward current. This current was abolished by external 4-aminopyridine 1 mmol/l, charybdotoxin 100 nmol/l and quinine 1 mmol/l, as well as by internal Cs^+ 150 mmol/l. The outward current was reduced by external tetraethylammonium 10 mmol/l, but was insensitive to Ba^{2+} 5 mmol/l and Cs^+ 1 mmol/l. When tail currents were determined with a pre-pulse protocol and in the presence of external Cs^+ 1 mmol/l to block inward currents, the reversal potential was close to the potassium equilibrium potential. The current showed time-, but not voltage-dependent inactivation in the presence of tetraethylammonium 10 mmol/l; this was more prominent when instead of the whole-cell recording the patches were nystatin-permeabilized. The outward current was stable when the depolarizing steps were applied at a frequency of 0.125 Hz, but declined markedly at 1 Hz. When the preincubation with LPS 100 ng/ml was prolonged from 24 to 72 h, the outward current disappeared. We suggest that after LPS-treatment of microglia the outward current is due to the opening of previously undetected potassium channels, which are difficult to classify into the known types, but are similar to those found in peripheral macrophages.

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NERVE GROWTH FACTOR RECEPTORS IN RAT BASAL FOREBRAIN AFTER TRANSIENT CEREBRAL ISCHEMIA

Th. Beck and A. Wree

Survival of basal forebrain neurons depends on Nerve Growth Factor (NGF). These NGF-receptor (NGFr) bearing cells, receive trophic support from target cells in the hippocampus, where they take up and retrogradely transport NGF. Only scant information exists about the role of NGF in postischemic brains. Forebrain ischemia leads to cell death in the hippocampus, the target area of basal forebrain neurons. The present works addressed the question, if ischemic loss of hippocampal cells indirectly affects the survival of basal forebrain neurons. Forebrain ischemia was induced by reversible occlusion of both common carotid arteries and simultaneous lowering of blood pressure to 40 mmHg for 10 min. Blood pressure was restored to normal levels by reinfusion of the shed blood. After survival of 3, days, 3 weeks or 3 months (N=4) rats were perfusion-fixed and cryoprotected with sucrose solution. Hippocampal damage was assessed by staining sections with celestine blue/acid fuchsin. Sections of the septum were stained immunocytochemically with a monoclonal antibody against NFG-receptors (IgG clone 192, Boehringer Mannheim). Loss of pyramidal cells was almost complete in the CA1 sector at all time points investigated. However, cells showing NGFr immunoreactivity in the medial septum and in the diagonal band did not differ in number or in morphology between controls and the various postischemic rats.

The results suggest that loss of pyramidal cells does not lead to loss of NGFr positive cells in the basal forebrain.

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COMPARISON OF VASOCONTRACTILE AGONISTS UPON THE TONE OF HUMAN TEMPORAL-, OCCIPITAL- AND PIAL ARTERIES

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This investigation was initiated to study the efficacy of various vasoconstrictors and to compare their effects upon different vessel specimens: human temporal-, occipital and pial arteries.
The vessels were obtained during neurosurgical operations. After removal of connective tissue vessels of 600-800 μ were cut into cylinders of 4 mm length. These vessel cylinders were mounted into an organ bath containing oxygenated (20% O_2 in 75% N_2 and 5% CO_2) Krebs-Henseleit buffer (pH 7.4; 37°C). Isometric measurements were attained using tensions of 0.5 g for pial and 1.0 g for temporal and occipital arteries.
Cumulative addition of 5-Hydroxytryptamine (5-HT) (0.001-10 μmol/l), noradrenaline (NA) (0.01-10 μmol/l), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (0.01-30 μmol/l), 9,11-Dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U 46.619) (0.001-3 μmol/l), KCl (4-400 mmol/l) and human endothelin 2 (END) (0.00001-0.01 μmol/l) was followed by dose dependent vasoconstrictions. Histamine (HIS) (0.001-100 μmol/l) did not cause any changes in vessel tone. The mentioned contractile agents did not influence meningeal arteries.
Concerning the concentration and the absolute contraction of the vessel cylinders the assessment of the contractile responses are as follows: END > U 46619 > $PGF_{2\alpha}$ > 5-HT/NA > KCl.
Comparison of the vessel specimens led to the conclusion, that temporal and pial arteries are ideal objects for the study of vasoconstriction whereas occipital arteries should be neglected because of their weak and inconstant reaction.

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EFFECTS OF VINCAMINE AND VINPOCETINE ON INFARCT SIZE IN FOCAL CEREBRAL ISCHEMIA

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Vincamine, a major alcaloid from vinca minor, has a long history in the treatment of cerebrovascular disorders. Only recently, in several models of cerebral ischemia neuroprotectant drug efficacy could be demonstrated for the structurally-related analogue, Vinpocetine. The present study was set up to evaluate the effects of both compounds on infarct size after permanent occlusion of the middle cerebral artery (MCA-O) in the mouse.
MCA-O was induced in male NMRI mice (30-35g body weight) by electrocoagulation of the vessel under tribromethanol (500 mg/kg i.p.) anesthesia. Forty-eight hours after surgery the animals were reanesthetized and injected with neutral red (1%; 0,5 ml i.p.). After sacrifice, brains were dissected out and the infarcted brain surface was quantified planimetrically. Vincamine was given 30 min prior to MCA-O by the oral route at doses of 1, 2, 3, 5, 10 and 30 mg/kg. Vinpocetine was investigated at doses of 1, 10 and 30 mg/kg. Drug treatment was repeated 4 and 24 hours after the vessel occlusion.
Vincamine significantly ($p<0.05$) reduced cortical infarct size at doses of 3 and 5 mg/kg by 26 and 30%, respectively. Vinpocetine also significantly ($p<0.05$) reduced the infarct size at the 10 mg/kg dose. In the other doses employed both compounds failed to improve brain morphology.
The data presented here demonstrate neuroprotectant efficacy for Vincamine and Vinpocetine in a model of permanent focal cerebral ischemia.

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Adenosine-A1-Agonist R-PIA protects cortical tissue in focal cerebral ischemia in the rat

G.Wagener

Adenosine is known to be an inhibitory neuromodulator in central nervous system. Several studies investigated the role of adenosine in cerebral ischemia; administration of antagonists of adenosine caused aggravation of neuronal damage, whereas application of adenosine agonists caused attentuation of ischemic neuronal damage. The purpose of the present study was to investigate the effects of adenosine-A1-agonist R-Phenyl- Isopropyl-Adenosine, R-PIA, on the infarct volume after permanent occlusion of middle cerebral artery (MCA-O).
MCA-O was performed by microbipolar coagulation of left middle cerebral artery under light halothane/nitrose oxide anaesthesia. After 48 hours brains were perfusionfixed. Coronal sections were taken every 0.5 mm and stained with cresylviolett. Infarcted areas were determined planimetrically.
Intraperitoneal administration of R-PIA caused transient reduction in arterial blood pressure from 110 ± 12 mm Hg to 75 ± 17 mmHg (0.1mg/kg) and to 60 ± 9 mm Hg (0.3 mg/kg). A slight decrease in arterial oxygen tension and a slight increase in carbon dioxide tension were observed at both doses.
Administration of R-PIA at a dose of 0.1 mg/kg exhibited a strong tendency towards lower total infarct volumes. Administration of 0.3 mg/kg R-PIA led to significant reduction of cortical and total infarct volumes. Neither 0.1 mg/kg nor 0.3 mg/kg R-PIA influenced striatal damage.

	Dose (mg/-kg)	Infarct Volume Cortex	Striatum	Total
Control	-	49.9±18.5	17.8±4.6	67.8±21.1
R-PIA	0.1	28.5±25.6	14.4±8.2	42.9±31(*)
Control	-	88.7±34.5	17.9±8.1	106.6±40.9
R-PIA	0.3	60.8±29.3*	14.5±11	75.3±35.5*

Values are given as means ± SD from 6 - 10 experiments. Different from controls (U-test): (*) $p<0.1$; * $p<0.05$.
The results of the present study demonstrate that adenosine has protective action on cortical tissue via its A1 receptor in focal cerebral ischemia.
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FLUNARIZINE IMPROVES CEREBRAL RECOVERY FROM CORTICAL SPREADING DEPRESSION AS INDICATED BY MEASUREMENTS OF EXTRACELLULAR LACTATE.

D. Scheller, J. Kolb, and F. Tegtmeier

During cortical spreading depression (CSD) anaerobic glycolysis is maximally stimulated and lactic acid accumulates (1). Thus, lactate might leak into the extracellular space (ECS) where it can be measured by means of microdialysis. Changes of extracellular lactate (lac_e) might indicate the degree of damage and of recovery from an insult. To test that, flunarizine, which has been shown to accelerate postischemic tissue lactate elimination (2), was applied. A microdialysis (MD) probe (length 2 mm, tip diameter 500 μm; Carnegie Medicin, Stockholm, perfusate: artificial CSF; flow rate: 2 μl/min; fraction volume: 8 μl) was inserted into the cerebral cortex of anaesthetized rats together with a remote microelectrode (ME) to measure DC (distance to the MD probe 200 to 300 μm). Lactate was quantified by a combined HPLC/enzymatic assay. Switching the perfusate from CSF to K^+-CSF (K^+=128 mmol/l) for 2 min, a single CSD was induced. Between the 2nd and the 3rd K^+-application (repetitive applications were done in intervals of 60 to 90 min), the animals received i.v. either cyclodextrine (10%, 2.0 ml/ kg, n=5) or 5 mg/kg Flunarizine (in cyclodextrine, n=5; data are presented in mean ± SD).
CSD was identified as a fast, transient negative DC shift of 15 to 18 mV lasting for about 30 s (1). With a slight delay, extracellular lactate increased and returned to basal levels within 45 min. Lactate removal occurred in two steps: a fast recovery phase I was followed by a slower phase II. Areas under the curve of the two phases were separately quantified: areas during phase I (1st, large peak) for the 2nd and the 3rd CSD were 3.14 ± 0.50 and 3.01 ± 1.38 respectively for the control group and 2.80 ± 1.14 and 2.30 ± 0.68 respectively for the treated group; areas during phase II (2nd, small peak) for the 2nd and the 3rd CSD were 2.02 ± 1.33 and 1.57 ± 1.29 respectively for the control group and 1.59 ± 1.34 and 0.31 ± 0.36 respectively for the treated group. Treatment affected only phase II significantly (α = 0.03 for intraindividual MWU test and α = 0.02 for interindividual MWU test).
These data show, that lactate appears in the ECS during CSD. Afterwards, extracellular lactate is removed from the ECS within two steps with flunarizine accelerating phase II of lactate disappearance. This illustrates that flunarizine improves substantially the recovery from CSD possibly by amelioration of oxidative phosphorylation.

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(2) Scheller, D. et al. (1989), Biomed. Biochim. Acta 48(2/3): 161-165

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R56865 AND TTX ANTAGONIZE VERATRIDINE INDUCED INTRASYNAPTOSOMAL (Ca^{2+})i INCREASE. B. A. Osikowska-Evers, N. Müller, R. Korolkiewicz and T. Peters

Prevention of calcium overload in brain occuring during ischemia/hypoxia, increases chances of neuronal cell survival.

R56865 (N-{1-{4-(4-fluorophenoxy)butyl}-4-piperidinyl}-N-methyl-2-benzothiazolamine) is protective in cerebral ischemia (1) and attenuates intracellular (Ca^{2+})i increase in veratridine treated synaptosomes (2). Therefore, further studies to investigate molecular mode of action of R56865 were carried out, and compared with the mode of action of TTX (tetrodotoxin).

Both substances (conc. range $1x10^{-8}$M-$1x10^{-5}$M) were investigated in synaptosomal preparations from the rat cortex before and after (3 min.) application of veratridine (conc.$1x10^{-5}$M). Intrasynaptosomal changes in (Ca^{2+})i level were measured using fluorimetric technique with fura-2. All results are $\bar{x} \pm$ sd.

Veratridine increased (Ca^{2+})i in control synaptosomes from 204±41 nM (n=30) to 511±138 nM (n=15) within 3min. (pretreatment controls) and to 767±147 nM (n=15) within 8min. (post-veratridine controls).

R56865 (conc. range as above, n=8) given before veratridine inhibited the veratridine induced intrasynaptosomal calcium increase by 7±10% to 95±3% in a dose dependent manner with an IC_{50} of $0.26x10^{-6}$M. R56865 applied 3 min. after veratridine also inhibited (Ca^{2+})i increase (7±7%-67±9%; respectively) with an IC_{50} of $0.30x10^{-6}$M. TTX (conc. range as above; n=7) treated synaptosomes showed inhibition between 8±9% and 95±2%, with an IC_{50} of $0.11x10^{-6}$M. TTX given 3 min. after veratridine inhibited (Ca^{2+})i increase by 12±12% to 80±3%; respectively; with an IC_{50} of $0.12x10^{-6}$M. It is shown that R56865 and TTX inhibit in a dose dependent manner veratridine induced (Ca^{2+})i increase and show the same IC_{50} values independent of the application time. However, application 3 min. after veratridine failed to return (Ca^{2+})i to baseline levels, leaving 20-30% increase of baseline (Ca^{2+})i (TTX and R56865, $1x10^{-5}$M, respectively).

TTX and R56865 in pretreated synaptosomes abolish membrane depolarization caused by prolonged opening of Na^+ channel and influx of Na^+. This, in turn, prevents opening of the voltage operated Ca^{2+} channels and Na^+/Ca^{2+} exchanger operating in a reverse mode. Obviously this is not the case when these compounds follow veratridine application. Possibly, Ca^{2+} still enters synaptosomes via voltage operated Ca^{2+} channels and/or Na^+/Ca^{2+} exchanger.

1.Scheller et al., Naunyn-Schmied.Arch.Pharmacol., Suppl 339 R107 (1989)
2.Osikowska-Evers et al., Naunyn-Schmied.Arch.Pharmacol., Suppl 343 R49 (1990).

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NEUROPROTECTIVE EFFECT OF THE 5-HT_{1A} AGONIST ROXINDOLE AFTER FOCAL CEREBRAL ISCHEMIA IN MICE AND IN RATS
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Because of their inhibitory effect on neurons 5-hydroxytryptamine-1A (5-HT_{1A}) receptor agonists are suggested to reduce neuronal damage after focal or global cerebral ischemia. In the present study, the effect of the new 5-HT_{1A} receptor agonist roxindole against brain damage caused by permanent occlusion of the left middle cerebral artery (MCA-O) was tested in rodent models of focal ischemia.

Male Fischer-344 rats, SHR rats or NMRI mice were subjected to MCA-O (Tamura et al. 1981). Anaesthesia was induced with halothane/nitrous oxide in rats and tribromoethanol (500mg/kg) in mice. Fortyeight hours after surgery, the infarct volume was quantified by planimetry and subsequent integration on cresyl violet stained serial sections in rats and by planimetric analysis of the damaged cortical surface after staining with carbon black in mice. Roxindole was dissolved in a mixture of 1,2-propanediol/physiological saline solution (35/65) and was applied intraperitoneally 30 min before MCA-O in doses of 0.3, 1, and 3 mg/kg.

The 5-HT_{1A} agonist roxindole significantly reduced infarct area of mice brains in doses of 1 mg/kg (17.4 ± 4.2 mm^2) and 3 mg/kg (15.7 ± 3.8 mm^2, controls: 22.2 ± 3.2 mm^2). Infarct volume of the cortex was diminished significantly in treated (1 mg/kg) Fischer-344 rats (54.7 ± 30.1 mm^3) in comparison with the accompanying controls (88.9 ± 28.0 mm^3). Administration of roxindole in SHR rats had no effect on the volume of the infarct.

The results demonstrate a clear ameliorative effect of roxindole on brain infarct area in mice and on cortex infarct volume in Fischer-344 rats. Administration of roxindole in SHR rats had no neuroprotective effect on brain tissue under the experimental conditions used.

Tamura A, Graham DI, McCulloch J, Teasdale GM (1981) J Cereb Blood Flow Metab 1:53-60

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LACK OF DOPAMINE D2-RECEPTOR SENSITIVITY CHANGES AFTER LONG-TERM TREATMENT WITH ROXINDOLE
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Roxindole (ROX, EMD 49980) is a structurally novel dopaminergic drug which apart from actions on 5HT-systems, displays high selectivity for dopamine D_2-autoreceptors, thereby reducing dopaminergic transmission. Such synaptically selective D_2-agonists might represent an alternative approach to the treatment of schizophrenia. However, a prerequisite for clinical application would be that neither sub- nor supersensitization of pre- or postsynaptic D_2-receptors, respectively, occur after prolonged treatment. Especially the latter property has been related to the induction of serious neurological side effects. Therefore, subchronic studies (21 days) in rats were carried out with ROX. For presynaptic actions, inhibition of GBL-induced DOPA accumulation was compared after acute or daily treatment with 0.3 or 3 mg/kg i.p. in striatum (ST) and t.olfactorium (TO). Differences were not significant in any case (% of contr., acute/subchronic at 0.3 and 3 mg/kg, respectively: 78/72, 42/45 in ST; 68/74, 48/53 in TO). For postsynaptic actions, potentiation of apomorphine-induced stereotypies was determined after a withdrawal period of 2 days. No significant effects were observed (110-121 % of contr. after 1-10 mg/kg i.p. ROX), whereas haloperidol (HAL) induced massive potentiation (161-190 % of contr., 1 mg/kg i.p. or p.o.). Clozapine (CLZ) was only marginally active (30 mg/kg i.p. or p.o.). Interestingly, ROX (1 mg/kg/d i.p., 10 days) failed to increase striatal neurotensin (NT) levels significantly in contrast to HAL (1 mg/kg i.p.) and similar to CLZ (20 mg/kg i.p.), whereas in the n.accumbens, all three drugs induced significant increases (striatum: 131, 236, 113; n.accumbens: 194, 260, 194 % of contr. for ROX, HAL and CLZ, respectively). The results show that ROX induces no changes in D_2-receptor sensitivity even at high doses. In accordance, no case of EPS side effects was observed in early clinical studies (Klimke and Klieser, Pharmacopsychiat. 24 (1991), 107). With respect to NT, ROX displays similarities to CLZ and other atypical D_2-blockers which are known to increase selectively limbic NT levels without effects in the striatum.

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COMPARISON OF THE EFFECTS OF NOOTROPIC DRUGS AND BENZO_ DIAZEPINES ON THE ELEVATED - X- MAZE. L . de Angelis.

A large body of experimental and clinical evidence has accumulated in recent years indicating that nootropic drugs, whose prototype is piracetam, improve the efficiency of the higher telencephalic functions of the brain involved in cognitive processes, such as learning and memory. Furthermore, available clinical evidence has indicated that piracetam may have anxiolytic properties. In light of foregoing information, the present study was designed to assess further the acute effects of piracetam and oxiracetam in an animal model predictive of anxiolytic activity. The test of anxiety selected was the 5 min. elevated - X - maze. An increase in the percentage of time spent on or in the percentage of number entries into the open-arms indicated a decrease in anxiety. In mice, the effects of the above mentioned drugs were compared with those of established references drugs, i. e. diazepam and lorazepam. DL-propanolol, a β-blocker with 5HT1A and 5HT1B antagonistic properties, was also studied.In the elevated - X-maze , benzodiazepines and d,l-propanolol displayed a marked anxiolytic effect. Piracetam and oxiracetam failed to alter significantly the index of anxiety.

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BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF ESUPRONE, A NEW SELECTIVE AND REVERSIBLE INHIBITOR OF MONOAMINE OXIDASE A

M. Traut, H. Weifenbach, H.-J. Teschendorf, H.-P. Hofmann

In vitro inhibition of rat brain monoamine oxidases A and B by esuprone (7-hydroxy-3,4-dimethylcoumarin ethanesulfonate) has been studied using [^{14}C] tryptamine (0.4 µM) and [^{14}C] phenylethylamine (0.4 µM) as substrates. Esuprone turned out to be a potent inhibitor of MAO A (IC_{50} = 8.4 nM), whereas MAO B is affected only at much higher concentrations (IC_{50} = 5 000 nM). Inhibitory potency and selectivity are comparable to those of harmaline (IC_{50} for MAO A 7.3 nM, for MAO B ≥1 000 nM), and brofaromine (5.3 nM and ≥ 1 000 nM), respectively. IC_{50} values of irreversible inhibitors clorgyline (5.7 nM; ≥ 1 000 nM) and pargyline (1 300 nM; 32 nM) have been determined under the same assay conditions but may vary depending on preincubation and incubation times.

Reversibility of MAO A inhibition was assessed by 1-h incubation of the enzyme with the inhibitor and subsequent dilution. Whereas enzyme activity is restored by dilution in the case of esuprone, harmaline, and brofaromine, it remains inhibited in the case of clorgyline.

No substantial affinity of esuprone to any neurotransmitter receptor has been detected.

Ex vivo MAO inhibiting activity of rat brain extracts was tested after oral administration of esuprone. About 60 % MAO A inhibition was found 2 h after 0.316 mg/kg; even at 10 mg/kg MAO B was inhibited by no more than 10 %. From the time course a half-life of MAO A inhibiting activity in brain of 12-14 h is estimated. In rats after a single oral dose of esuprone (1 - 31.6 mg/kg) brain neurotransmitter concentrations are increased reaching maximum levels of 145 ± 8 % of control (noradrenaline), 134 ± 8 % (dopamine), and 157 ± 19 % (serotonin), respectively; MAO-dependent metabolites are decreased, DOPAC (ED_{50} ~ 1 mg/kg), and HVA (ED_{50} ~ 3 mg/kg) responding much more sensitively than MOPEG-sulfate (ED_{50} ~ 8.5 mg/kg), and 5-HIAA (ED_{50} ~ 20 mg/kg). Overall esuprone is about 3 times more active than moclobemide, which exerts essentially the same effects. In vivo esuprone antagonizes reserpine-induced hypothermia (ED_{50}: 1.6 mg/kg p.o. in mice; 1.3 mg/kg p.o. in rats) and potentiates the effects of threshold doses of L-DOPA in mice or L-5-HTP in rats (ED_{50}: 0.7 mg/kg p.o. and 1.2 mg/kg p.o., resp.). Furthermore, esuprone decreases dose-dependently (2 - 21 mg/kg p.o.) the duration of paradoxical sleep in rats and cats with a concomitant slight increase in the duration of the waking stage.

As a potent and highly selective, reversible inhibitor of MAO A esuprone is a promising candidate for further development as antidepressant.

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IS THERE A CORRELATION OF THE ANTIAMNESTIC EFFECT IN A STRESSFUL AVOIDANCE TASK WITH THE ANXIOLYTIC EFFECT IN A CONFLICT TEST A.Rostock, Ch.Siegemund

The benzodiazepine-$GABA_A$ mechanism are involved in processes of memory storage. A rat exposed to an active aviodance test combined with foot shock stimulation becomes anxious or stressed. A degree of anxiety or stress may be necessary for learning. But too much of either will hinder learning. Recent studies indicate that there are several GABA systems, mediated by $GABA_A$ receptor complexes, that downregulate memory.

Avoidance is widely used in memory experiments. The anticonvulsants carbamazepine (Finlepsin$^{(R)}$), Ca-valproate (Convulsovin$^{(R)}$) and clonazepam (Antelepsin$^{(R)}$) where evaluated. Clonazepam and Ca-valproate interfere with GABAergic processes, the anticonvulsive mechanism of carbamazepine is still unknown.

Carbamazepine 5 mg/kg/d has antiamnestic effects in an active avoidance (pole jumping) test which is combined with foot shock stimulation. Ca-valproate 30 mg/kg/d is weakly active, clonazepam 0,3 mg/kg/d downregulates learning and memory in this test.

The anxiolytic properties were tested in Vogel's conflict test. Carbamezepine 5-20 mg/kg is uneffective. Ca-valproate 10-100 mg/kg acts anxiolytic in a dose dependend manner. Clonazepam 0,25-2,5 mg/kg was significant effective.

These results indicate an inverse correlation of the antiamnestic and the anxiolytic efficacy of substances.The antiamnestic activities measured in this stressful aviodance test are not mediated by anxiolytic activities.

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CNS-EFFECTS OF RACEMATE AND ENANTIOMERS OF AN 1.5-BENZODIAZEPINE IN THE RAT USING DIFFERENT ROUTES OF ADMINISTRATION

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7-Chloro-3-ethyl-1,3-dimethyl-5-phenyl-1H-1,5-benzodiazepine-2,4 (3H,5H)-dione (rac. 1) was synthesized as well as S(+)- and R(-)-1 (using optically active precursors). The water insoluble substances were injected **i.p.** in Wistar rats (weight about 200 g) as tragacanth stabilized suspension in 0.9 % NaCl and prolongation of hexobarbital sleeping time (i.v. 25 mg/kg given 30 min after the pretreatment) was used to evaluate their CNS-activity. Clobazam, an 1,5-benzodiazepine closely related to 1, was used as reference substance. Hexobarbital sleeping time (control: 4.0±1.5 min; n=47) was prolonged by application of rac., S(+)- and R(-)-1 (tested up to 100 mg/kg) only about 2-fold, whereas clobazam was much more active (25 mg/kg:>4-fold and 50 mg/kg: 8-fold). 30 min after i.p. application of 50 mg/kg, concentration of rac. 1 and clobazam in brain tissue of rats UV-photometrically measured after extraction with benzene and TLC was for rac. 1 below the limit of detection (n=8) whereas it amounted for clobazam 13.9±1.3 µg/g tissue wet weight (n=6) explaining that large difference in the depressing CNS-effect between both substances. **I.v.** injection of rac., S(+)- and R(-)-1 (dissolved in 30% Cremophor EL^R/0.9 % NaCl) revealed a quite other pattern of CNS-activity for the substances: S(+)- (5 mg/kg) and rac. 1 (10 mg/kg) caused immediately after administration pentetrazol-like seizures (with loss of consciousness) whereby a tonic phase was followed by clonic convulsions (n=8-15); most rats died during seizures if higher doses of both substances were given. In contrast, rats which received R(-)-1 (15 mg/kg) did not show any signs of CNS-excitation (n=13); hexobarbital which was injected 5 min after pretreatment produced loss of righting reflexes during 9.6 ±1.5 min (n=5); after administration of clobazam (i.v. 5 mg /kg) hexobarbital sleeping time lasted 18.4±1.4 min (n=5).

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INTRAVENOUS VALPROATE: COMPARISON WITH DIAZEPAM AND PHENYTOIN IN A RODENT MODEL OF STATUS EPILEPTICUS.

D. Hönack

Status epilepticus is defined as a condition characterized by an epileptic seizure that is so prolonged or so frequently repeated as to create a fixed or lasting epileptic condition.

Conventionally intravenous diazepam is the drug of choice against status epilepticus (S.E.). However frequently seizures are refractory to this treatment, so that phenytoin or barbiturates have to be used, which have a slower onset of action or severe side-effects.

Valproate (VPA) may be an alternative drug for treatment, but is not available in a form for intravenous administration. Few reports showed successful treatment of S.E. after rectal administration. Only one clinical study used intravenously applicated VPA.

Therefore we studied the effect of i.v. VPA in comparison to the clinically effective anticonvulsants diazepam and phenytoin in a model of S.E. in mice. We used 5 repeated maximal electroshock (MES) stimulations (13 mA, 0.2 sec, ear electrodes) within 30 min to test the efficacy and time course of action of different doses of these 3 drugs versus control groups. In controls, all 5 stimulations induced generalized tonic-clonic seizures. Diazepam depressed the MES-scores of the treated mice significantly in doses from 2.5-10 mg/kg which exhibited marked side-effects. The effect was present 30 sec p. appl.

Ten to 30 mg/kg Phenytoin were effective, but had a slower onset of action except at the highest dose. VPA decreased the MES-scores significantly in doses from 100-400 mg/kg with a dose-dependent onset of action. The peak effect of 200 mg/kg occurred 3 min p. appl. correlating with brain concentrations.

The present results indicate that the model described here may be useful to test the efficacy of drugs against S.E. Intravenous VPA seems to be a promising drug for the control of refractory S.E.

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VALPROATE, BUT NOT TRANS 2-EN VALPROATE DEPRESSES PETIT-MAL LIKE SEIZURES IN RATS DURING CHRONIC TREATMENT R. Scherkl and M. Voits

In a rat model, petit-mal like spike wave activity in the electrocorticogramm was depressed after single doses of valproate and the active metabolite trans 2-en valproic acid (Löscher et al., Eur. J. Pharmacol. 99, 211-218, 1984). We studied the efficacy of these drugs during chronic treatment. Groups of 6 rats were treated and ECoG recordings were carried out 15 min after the morning dose (at 07.45 h) for 30 minutes on every day of treatment, as well as for 1-2 weeks after cessation of treatment. For habituation the animals were put into the recording cage immediately after administration.
Treatment with valproate (75 mg/kg i.p., t.i.d. for 3 weeks) had only a weak but constant effect on number and duration of spike wave discharges in the ECoG, in spite of plasma concentrations known as therapeutically active from man (140-150 µg/ml during registration time, i.e. 30 min after the morning dose). In previous studies 170 mg/kg valproate t.i.d. were necessary to suppress the number of discharges (Wahle and Frey, Eur. J. Pharmacol. 181, 1-8, 1990). Subchronic treatment with trans 2-en valproic acid (100 mg/kg i.p., t.i.d., 1st week and 150 mg/kg, 2nd week) leading to plasma concentrations of about 170 µg/ml and 240 µg/ml, respectively, during the time of ECoG recording had no depressing effect on spike-wave activity, it even seemed to be increased in the second week of treatment (DFG).

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DIFFERENT INFLUENCE OF ANTICONVULSIVE DRUGS ON HIPPOCAMPAL LONG-TERM POTENTIATION IN FREELY MOVING RATS.
M.Krug, R.Matthies, A.Pfeiffer, and M.Wagner

Relationships between long-term potentiation (LTP) and mechanisms of epileptogenesis have frequently been discussed. If such a relationship exists,that means, if there are common mechanisms underlying LTP and the development of epileptic processes, classical anticonvulsive drugs should also be able to suppress LTP. In order to test this assumption, phenytoine (50 mg/kg), pentobarbitone (40 mg/kg, 20 mg/kg), diazepam (0.5 mg/kg), and dextrometorphan (40 mg/kg) were given 30 min prior to tetanization of the perforant pathway. LTP in the dentate gyrus, as measured by an increase of the population spike of the monosynaptic evoked field potential, was influenced in different ways. While all the substances have clear anticonvulsive properties and suppressed for instance kindling development in the dosages used in our experiments, dextromethorphan and pentobarbitone suppressed LTP induction and diazepam was without any effect. Interestingly, phenytoine, which has been reported to do not influence LTP in short-lasting slice experiments, did also not suppress the induction of LTP but prevented its maintenance.These results point to the participation of LTP mechanisms in epileptogenesis but also to the fact that LTP-like processes might be only one component in epilepsy

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POST-CONVULSIVE AND POST-HYPOXIC CHANGES OF ADENOSINE METABOLISM IN THE BRAIN OF RODENTS
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By reason of its hyperpolarizing action and its inhibitory effect on the release of the excitatory transmitter glutamate, adenosine was thought to be an endogeneous neuroprotective agent. In rats, adenosine does exhibit anticonvulsive properties, which are more pronounced after stable analogues, like 2-chloroadenosine or 6-N-cyclohexyladenosine. In the present study, the metabolism of adenosine was investigated after development of the kindling phenomenon by repeated administration of subconvulsive doses of pentylenetetrazol (PTZ), after implantation of cobalt into the cortex of rat brain, and after hypoxic exposure of mice.
Brains of mice and rats were quickly removed from animals and homogenized in phosphate buffer. After centrifugation, the supernatants containing adenosine and its metabolites inosine, hypoxanthine, and xanthine were analyzed by use of an HPLC gradient elution procedure.
The repeated administration of PTZ causing a lowered threshold for convulsive stimuli led to an enhanced conversion of adenosine to xanthine. Similar, but more moderate changes of adenosine metabolism were observed after implantation of cobalt into rat brain cortex and after hypoxic exposure of mice.
The results support the assumption of an involvement of adenosine in endogeneous neuroprotective processes, independently of the kind of noxious stimuli.

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KINDLING INDUCED POTENTIATION EFFECTS ARE ABSENT IN POORLY KINDLED ANIMALS

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The hippocampal formation has a particular propensity to seizure activity. From studies in rats it is known that the stimulation of the perforant pathway results in some forms of synaptic plasticity, such as long-term potentiation or the kindling phenomenon. Kindling is an experimental model of seizure research and epilepsy. We induced a chemical kindling by repeated administration of subconvulsive doses of pentylenetetrazol (PTZ). Previously, we have demonstrated, that PTZ application produces different changes on evoked field potentials in the dentate gyrus in fully kindled rats, for instance a long-lasting kindling induced potentiation effect, which refers to an increase in amplitude of the evoked response. The slope function of the field EPSP is depressed until 90 to 120 min after injection, but is only potentiated in the following time in kindled animals which have shown seizure stage 4 or 5.
In some of the animals it seems not possible to induce the kindling phenomenon by the same procedure. The results of the present studies demonstrates that in these "poorly PTZ kindled animals" the kindling induced potentiation effect in the granule cells of the dentate gyrus is absent.
It can be suggested that the poteniation effect is connected with the enhanced seizure susceptibility induced by the kindling procedure.

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MODULATION OF THE DOPAMINERGIC SYSTEM AS A CONSEQUENCE OF CHEMICAL KINDLING

Gisela Grecksch

Kindling is considered to be an useful experimental model for human epilepsy. This phenomenon can be induced by repeated administrations of initial subconvulsive doses of the convulsant agent pentylenetetrazol (PTZ), resulting in the development of generalized motoric seizures.
We intended to investigate the involvement of the dopaminergic system by behavioural pharmacological methods. The locomotor activity of kindled rats is not changed in comparison to control animals. But the administration of subconvulsive PTZ doses induces in kindled animals a dose-dependent activation of the locomotor behaviour. This activation is antagonized by a low dose of the dopamine receptor blocker haloperidol. On the locomotor activity of controls treated with PTZ is haloperidol without any effect. On the other side the apomorphine-induced hyperlocomotion is reduced in kindled rats compared to controls. These results suggest an involvement of dopaminergic mechanisms in the induction of the kindling phenomenon by PTZ. Wether there is a direct influence on processes of dopaminergic transmission or the effectiveness of the dopaminergic system is modulated by other systems should be investigated in further experiments.

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THE INFLUENCE OF ß-CASOMORPHIN DERIVATIVES ON ARECOLINE CATALEPSY

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The influence of ß-Casomorphin (ß-CM) derivatives on arecoline (AREC) catalepsy was studied in comparison to standard drugs after intracerebroventricular (ICV) administration in rats. Atropine (ATR) or amphetamine (AMPH) were able to diminish the cataleptic state induced by 20 mg/kg intraperitoneally applied AREC. Only a lower clozapine (CLO) dose decreased the cataleptic behaviour, whereas higher doses were without effect. Morphine (MOR) or 5-hydroxytryptamine (5-HTP) increased AREC catalepsy. The AREC cataleptic state was significantly enhanced by tyrosine (TYR)-containing ß-CM derivatives with opiate activity. TYR-containing derivatives without opiate activity did not influence catalepsy. The des-TYR-ß-CM analogs des-TYR^1-D-PHE^3-, des-TYR^1-D-PRO^4-ß-CM or PHE-D-PRO-GLY diminished the cataleptic state, des-TYR^1-D-pipecolinic acid (des-TYR^1-D-PIP^4-ß-CM) had no influence on the AREC catalepsy. The work reported here suggests an involvement of opiate mechanism in the induction of catalepsy by cholinergic drugs. The mode of the inhibiting action of des-TYR-ß-CM derivatives on this model is discussed.

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INFLUENCE OF ß-CASOMORPHINS ON AMPHETAMINE-INDUCED HYPERLOCOMOTION IN RATS

Heide-Linde Rüthrich

There are some reasons to assume that the casomorphin sequences of milk ß-casein exhibit effects on the central dopaminergic systems in addition to their well-known opiate-like activity. To test this assumption structural analogues of the ß-casomorphin 5 (Tyr_1-Pro-Phe-Pro-Gly) and their corresponding des-Tyr^1-derivatives were investigated on the model of amphetamine-hyperlocomotion.
Experiments were performed using 8-week-old male Wistar rats. For intracerebroventricular (icv) application of casomorphins a hole (lat. 1.6 mm, AP 0.25 mm) was drilled under deep anaesthesia through the skull 3 days before the experiment. The locomotor activity was registered for 40 min. The casomorphin solutions were applied in a volume of 5 ul into lateral ventricle 5 min before systemic application of 5 mg/kg amphetamine.
All ß-casomorphin-5-derivatives suppressed amphetamine-induced hyperlocomotion.
The most prominent inhibitory effects were observed after administration of 2 nmol D-Pip^4-CM 5 and 5 n mol D-Pro^4-CM 5. The corresponding des-Tyr^1-CM analogs were without effect.
These as well as other results cannot explain the mechanism of action of casomorphin peptides concerning hyperlocomotion. we may speculate that opioide as well as non-opioide casomorphin derivatives interfere with different neurotransmitter systems. Furthermore, these substances may modulate neurotransmitter circuits.

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SYNERGISTIC EFFECTS OF THE NMDA-ANTAGONIST CPP AND THE AMPA-ANTAGONIST NBQX WITH L-DOPA IN ANIMAL MODELS OF PARKINSON'S DISEASE

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Based on recent evidence suggesting an abnormal activity of the subthalamic nucleus and its basal ganglia output projections in the MPTP-treated primate, beneficial effects of NMDA (N-methyl-D-aspartate) antagonists for the treatment of Parkinson's disease have been suggested.

Quisqualate receptors (AMPA/Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) are present in the basal ganglia in higher densities than NMDA-subtypes. The effects of NBQX (6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3(1H,4H)-dione), a selective AMPA-antagonist and those of the NMDA-antagonist CPP (3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) were therefore tested in substantia nigra lesioned (6-OHDA) rats and MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated primates alone and in combination with L-dopa. Both compounds were effective when administered in combination with a threshold dose of L-Dopa plus benserazide. Contraversive rotations were significantly stimulated by NBQX and CPP. Similarily, in MPTP-treated common marmosets both, NBQX and CPP combined with L-Dopa stimulated locomotor activity, indicating the potential usefulness of NMDA and AMPA-antagonists for treatment of Parkinson's disease when combined with L-Dopa.

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NEUROPHARMACOLOGICAL CHARACTERISATION OF ROPINIROLE, A NOVEL DOPAMINE AGONIST H.Wachtel, V. Schulze, M. Kunow, T. Akai, P. Böttcher, K.-J. Rettig and M. Lehmann*)

The agonistic activity of the indolone derivative ropinirole (ROP) on various central dopamine (DA) systems was investigated after systemic administration in mice, rats and dogs. In addition, studies comparing the influence of the route of administration (i.p. vs. p.o.) and of hepatic drug metabolism (p.o. vs. p.o. plus proadifen) on the locomotor effect of a fixed ROP dose served as functional measure to estimate bioavailability and biotransformation.

Studies in mice: ROP, 0.025 - 6.25 mg/kg i.p., caused a short-lasting (<1 h) lowering of rectal temperature in normal mice [minimal effective dose (MED) 6.25 mg/kg] and reversed the reserpine (RES)-induced hypothermia (MED 0.39 mg/kg).

Studies in rats: Pretreatment with ROP (0.025 - 6.25 mg/kg s.c.; 2 h) decreased serum prolactin levels in normal rats (MED 0.39 mg/kg); prolactin determinations 2 h and 8 h, respectively, after the injection of ROP (0.01 - 10 mg/kg i.p.) to RES-pretreated rats revealed that only the highest dose suppressed the RES-induced hyperprolactinaemia (MED 10 mg/kg). ROP i.p. evoked short-lasting ($\leq$1 h) stereotyped behaviour in (a) normal, (b) RES-treated and (c) monoamine-depleted rats (ED_{50} 0.5 h: 2.21, 5.06 and 4.43 mg/kg, respectively). ROP, 0.1 - 25 mg/kg i.p., stimulated locomotor activity for about 1.5 h (MED 6.25 mg/kg). In contrast to i.p. administration, ROP 6.25 mg/kg p.o. was ineffective in producing hyperactivity, but even slightly inhibited locomotor activity. In rats pretreated with proadifen (50 mg/kg i.p., 0.5 h), however, ROP 6.25 mg/kg p.o. caused marked and long-lasting ($\gg$ 2 h) locomotor stimulation.

Studies in dogs: ROP s.c. had a rather strong emetic action (ED_{50}: 0.044 mg/kg) occurring within 20 - 30 min and rarely exceeding 1.5 h post injection.

Our investigations indicate that ROP is a directly acting central DA agonist with rather short duration of action and moderate efficacy in vivo resulting from poor bioavailability, at least in rats, due to marked metabolic inactivation; these preclinical findings contrast with published preliminary data on the favourable pharmacokinetics and the powerful therapeutic action of ROP in parkinsonian patients (Boothman BR and Spokes EGS, Lancet 336: 314, 1990; Vidailhet MJ et al., Lancet 336: 316 - 317, 1990).

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INFLUENCE OF CIPROFLOXACIN ON BRAIN METABOLISM OF BIOGENIC AMINES IN THE RAT

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Ciprofloxacin is one of the fluorinated quinolone carboxylic acids with high activity against gram-positive and gram-negative bacteria. The decision to apply a drug depends not only on the efficacy but also on the frequency and the strength of side effects. In this respect a high safety is characteristic for quinolones. The incidence of side effects on CNS is 1.5% but severe side effects as convulsions, hallucinations, depersonalisations and depressions were described. It has been shown that quinolones inhibit receptor binding of GABA (Hori et al. Abstract No: 396, Symposium of the ICAAC, 1985), but the required concentrations are very high and not attained under therapeutical conditions (Stahlmann and Lode in "The Quinolones" (Ed. Andriole V.T), Academic press, London, 201, 1988). The present study was performed to investigate the interactions between ciprofloxacin and the metabolism of biogenic amines in brain of rats. We treated rats (male Sprague-Dawley-rats, about 200 g) for 7 days with 5, 15 and 45 mg/kg/d ciprofloxacin by osmotic minipumps; those doses are simular to such used in human treatment (1 g/d). We observed dose dependent stady-state-concentrations in plasma (19.5, 47.7, 336 ng/ml) and in different organs. In cortex, as an example for pentetration into central nervous tissue, the concentrations reached 10% of the plasmaconcentrations. The noradrenaline concentrations of the pons as well as adrenaline, dopamine, some metabolites (MOPEG, DOPAC, normetanephrine), 5-Hydroxytryptamine and tryptophane were significantly increased versus control. The activity of the monoamine oxidase in the pons was significantly decreased by about 20% compared to the group receiving the highest dose of ciprofloxacin. Our results demonstrate an increase of excitatory stimuli of the central neuronal transmission and could therefore contribute to an explanation of the convulsive side effects during high dose quinolone therapy.

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CENTRAL NERVOUS SYSTEM (CNS) EFFECTS OF FLUOROQUINOLONES IN ANIMALS

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The quinolones can cause severe CNS effects in man even after short-term use. Convulsive seizures are rare events. In animals convulsions associated with quinolones are uncommon and may be symptoms of intoxication, rather than specific pharmacodynamic effects. Flumequine can produce convulsions in mice after very high doses (1300-2500 mg/kg).

Methods and Results: We investigated the epileptogenic potential of different quinolones after oral and intraventricular administration and the stimulating effects on the CNS using the Ungerstedt turning model. **Oral administration:** Among the newer fluoroquinolones, only fleroxacin can produce convulsions in mice after high doses (800 mg/kg). Equivalent doses of ofloxacin, enoxacin, ciprofloxacin, pefloxacin or norfloxacin had no effect. **For intraventricular administration**, a steel cannula was implanted stereotactically into the right lateral ventricle of rats. Enoxacin, ciprofloxacin and pefloxacin were administered intraventricularly in doses of 100 ug each. Pronounced and long-lasting (for 40 min) clonic-tonic convulsions were produced by enoxacin and ciprofloxacin. Pefloxacin in doses of 100 ug and 200 ug had no effect. Only in a dose of 400 ug was pefloxacin shown to produce convulsions.

Stimulating effects: As an approach to measure any stimulating effects on the CNS, the circling model of Ungerstedt was used as a quantifiable behavioural index. Female rats (Lewis strain, weighing 145-155 g) were lesioned unilaterally in the nucleus caudatus. The lesion was verified by following the response to cocaine (20 mg/kg i.p.). The following quinolones were investigated: ciprofloxacin; pefloxacin; norfloxacin; lomefloxacin; ofloxacin; and enoxacin (dose range: 100-400 mg/kg). These quinolones did not produce any effect in this model. When the quinolones were administered 15 min. before cocaine, the circling behaviour caused by cocaine was decreased dramatically.

Conclusion: Up to date it is impossible to present a convincing hypothesis concerning the sub-cellular mechanisms involved in the CNS reactions of the quinolones. The preliminary conclusion of our findings is that quinolones are not themselves stimulants of CNS overactivity, mediated by the dopaminergic system.

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IS THE PAROXYSMAL DYSTONIA IN MUTANT dt^{SZ}HAMSTERS INFLUENCED BY ALTERATIONS IN SEX HORMONE LEVELS ?

T. Blanke

It is known, that sex hormones influence GABAergic, dopaminergic and cholinergic neurotransmitter systems. These neurotransmitters are involved in dystonic syndromes. Thus the possibility was investigated, whether sex hormone levels may influence the time course or the severity of dystonic attacks in a mutant hamster model, which was described recently (Löscher et al., Mov. Dis. 4, 219-232,1989).

Mutant dt^{SZ}-hamster were weaned at the 21. day of life and tested every 2 or 3 days. The dystonic attacks were graded by a score system. There were no significant differences between males and females. In one group, plasma was taken each time of testing and measured for progesterone, estradiol and testosterone by RIA. Estradiol in the females and testosterone in the males increased at the time of onset of puberty. Males and females had nearly equal progesterone levels (about 1.5 ng/ml). The hormone levels did not correlate with the severity of the dystonic attacks. In a second group, hamsters of both sexes were either castrated or sham-operated at the day of weaning. Plasma was taken every 7 days and measured for sex hormones. In females, castration reduced progesterone levels significantly, showing that in female hamsters the main source of progesterone are the ovaries. With castrated females and both castrated and sham-operated males progesterone levels were equal, showing that in the male hamster progesterone is mainly produced by the adrenals. Castration abolished the increase of testosterone levels in the males and of estradiol levels in the females at the onset of puberty. Neither the severitiy nor the time course of the dystonic attacks were changed by castration. In the female hamsters vaginal smears were taken to determine the onset of puberty and the estrous cycle of the animals. In these animals no changes in the severity of the dystonic attacks during the estrous cycle were visible.

Thus, under our testing conditions sex hormone levels do not seem to influence the dystonic attacks in the dt^{SZ}-hamster.

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BEHAVIOUR PHYSIOLOGICAL DIFFERENCES BETWEEN TWO SUBLINES OF WISTAR RATS

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Two sublines of 8-week-old Wistar rats referred to as Shoe: Wist/2 and Crl: (WI)BR were compared for their usefulness in behavioural experiments. Animals from both sublines are lively, good-natured, and easy to handle. The (WI)BR-males are on an average 50g heavier.
Learning performance of the rats was compared in three different tests: the pole jumping-box (one-way active avoidance), the shuttle-box (two-way active avoidance), and the Y-chamber (brightness discrimination).
Concerning one-way active avoidance WIST/2 acquired the learning task, the instrumental reaction as well as the conditioned reaction rapidly. In contrast, the learning performance of (WI)BR was significantly lower.
The same results were obtained in two-way active avoidance. In brightness discrimination the savings percentage of (WI)BR is significantly higher than that of WIST/2.
In the open field test, rats of the (WI)BR line were more active in comparison to WIST/2 measured in terms of higher ambulation scores.
Finally, we tried to induce long-term potentiation in hippocampal slices. Interestingly, it was not possible to induce LTP in slices from (WI)BR animals. The data obtained show clearly that the experimenter has to put more attention to the question about the biological parameters of the test animals that should be used.

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INVESTIGATION OF DOSE-RELATED ANALGESIC EFFECTS OF IBUPROFEN USING TONIC AND PHASIC PAINFUL STIMULI

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The aim of the study was to investigate the dose-related effects of ibuprofen (0, 400 and 800 mg, p.o.) using a new experimental technique which is characterized by the application of tonic and phasic painful stimuli. 18 volunteers (9 female, 9 male, mean age 25.8 years) participated in the randomized, double-blind, 3-fold cross-over study. Measurements were obtained before and 90 min after the drug's administration to the fasted subjects. Painful pulses of CO_2 (200 ms, interval approx. 40 s; 65 and 70 % v/v) were applied to the left nostril. In addition, the contralateral nasal mucosa was stimulated with a constant air-stream of dry air, which produced a tonic painful sensation often described as dull and burning. Subjects rated the intensity of the painful stimuli via visual analogue scales. EEG was recorded from 3 recording sites (Fz, Cz, Pz, versus A1/A2; bandpass 0.2 to 30 Hz, sampling frequency 250 Hz). Thus, in response to phasic stimuli, pain-related chemosomatosensory evoked potentials (CSSEP) were obtained. Additionally, both, acoustically evoked potentials (AEP) and frequency analyses of the spontaneous EEG were obtained in order to control for unspecific CNS effects of ibuprofen. Intensity estimates for both, tonic and phasic painful stimuli decreased in a dose-related manner. However, comparably to previous studies, a dose-related significant decrease ($p<0.05$) could be observed for CSSEP amplitudes, whereas spontaneous EEG and AEP were not affected by the drug. Thus, CSSEP appear to be a more sensitive measure to investigate analgesic drug effects when compared to psychophysically obtained responses. Additionally, since in previous experiments interactions between tonic and phasic painful stimuli had been established when applied homotopically, it was demonstrated that there was no such interaction in the perception of tonic and phasic pain after bilateral, heterotopical stimulation.

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An animal model of the development of ethanol addiction

Jochen Wolffgramm

High levels of drug self-administration by an experimental animal have been often - and rashly - interpreted as indications of an addiction. However, the development towards an addiction is a long-lasting process including several distinct stages. Measures taken at an early time may reflect "controlled" drug intake rather than "psychic" (better: behavioral) dependence. Recently we have established an animal model of ethanol taking which comprises all stages of the development (Wolffgramm J, Neurosci. Biobehav. Rev., in press). In addition to tap water three differently concentrated drug solutions are continuously offered to male rats for 9 months. After several months of forced abstinence a retest is performed which uses the same free choice paradigm as before. Four subsequent stages of ethanol taking can be discriminated: (1) During the first one or two weeks the rat becomes acquainted with the drug. Initial levels of consumption are high and variable. After some days they reach lower, but more stable values. Individual stability contrasts with interindividual variability. (2) A period of "controlled" drug intake follows lasting for 5-8 months. The rat adjusts its ethanol intake according to external situation (social conditions, distress) and internal state. Short term and long term isolation reversibly increase the preference for ethanol, dominant rats consume less than subordinates. (3) During the subsequent 3-4 months drug consumption reveals a positive temporal trend. This increase is not accompanied by a detectable loss of ethanol's effects (drug tolerance). (4) After this period nearly all the rats have become behaviorally dependent on ethanol and remain so for their lifetime. After an abstinence of 9 months they reveal a preference for ethanol (4 g/kg/day) which is more than twice as high compared to controls. The preference can no longer be suppressed by adulteration with quinine. Modifying factors like social situation and individual disposition lose their influence. Ethanol reveals an altered pattern of action (motor depression by very low doses, excitation with higher ones). The described animal model enables to investigate exogeneous and endogeneous variables facilitating or inhibiting the development of drug addiction, to test possible therapeutics, and to look for the neurobiological base "controlled" and "dependent" drug consumption.

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Influence of the Dopamine Agonist Lisuride on Ethanol-Effected Reward in the Rat

Ulrike Wolf

In the mesolimbic "reward system" dopaminergic transmission is mediating both attraction and aversion produced by psychoactive drugs. It has been postulated that administration of a dopamine agonist might be helpful in the treatment of drug addiction. To test this hypothesis by means of an animal model of alcoholism (Wolffgramm J & Heyne A, Pharmacol. Biochem. Behav. 38: 389, 1991) three experimental series were performed with male Wistar rats ($N=40$) which either were behaviorally dependent on ethanol (D) or not dependent (ND). Series 1 tested effects of lisuride (L), ethanol (ETOH) and ETOH + L in a home cage situation by means of a circadian registration. Similar to the experiments of series 2 administration of L was forced, whereas ETOH was offered in addition to water (free choice). In ND rats locomotor activity was significantly stimulated by L (83 ug/kg/day) and depressed by ETOH (2.2 g/kg/day) whereas D rats were not affected. Vertical exploration declined in both D and ND after ingestion of ETOH, L, and ETOH + L. In the second series D and ND rats had the choice between water and 5, 10, and 20 Vol.% ETOH for six weeks. Half of the animals received L (1.5 mg/l) in all drinking fluids. These rats were subchronically pretreated with L for one week. D rats took significantly more ETOH (3.7 g/kg/day) than ND rats (1.8 g/kg/day). L increased ETOH consumption in both D and ND animals (4.7 and 3 g/kg/day, resp.; $p<0.05$) although the total fluid intake was lower in L-treated rats. The third series comprised an operantly conditioned place preference. The experimental setup consisted of two compartments one of which enabled access to 5 Vol.% ETOH, the other one to water. After 19 h of fluid deprivation the test sessions took place. Subsequently the rats received in their home cages either water or 1.5 mg/l L for two hours. The conditioning period lasted one week. In the following test trials, only L-treated rats preferred the ETOH compartment ($p<0.01$). There were no significant differences between ND and D. The results demonstrate that chronic treatment with L increases the preference for ETOH by enhancing its reinforcing properties. This effect is not specifically linked to ETOH dependence.

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Voluntary but not forced intake of an opiate causes "psychic" dependence in an animal model of drug addiction A. Heyne

In an animal model of drug addiction the development of physical and behavioral ("psychic") dependence on the opiate etonitazene (ETZ) was studied. Male rats (n=46) either had the choice between water and 2,4, or 8 mg/l ETZ-solutions (voluntary intake: V) or received only ETZ (2 mg/l) as drinking fluid (forced intake: F). Similar to ethanol intake different stages of ETZ taking appeared. In the free choice situation "controlled" intake was characterized by individual stability and by marked influences of both external (social housing conditions) and individual (dominance rank) factors on ETZ taking. Group housed rats took only 4 ug/kg/day whereas the intake of socially deprived rats was three times as high (12 ug/kg/day). High-ranking rats took significantly less ETZ (9 ug/kg/day) than subordinate ones (14 ug/kg/day). Daily doses were in general constant until week 25, but then socially deprived rats increased ETZ consumption. After 30 weeks of voluntary or forced intake ETZ-solutions were withdrawn. Both socially deprived V-rats and F-rats revealed a significant hyperreactivity to electric foot shock and a loss of body weight compared to controls. All symptoms of withdrawal disappeared after 4 days. In contrast, behavioral dependence developed only in socially deprived V-rats but not in rats having been forced to consume ETZ. When water and ETZ-solutions were re-offered after 20 weeks of abstinence, V-rats revealed a high and even increased preference for ETZ (109 ug/kg/day). Even after adulteration with quinine, their ETZ intake remained high (95 ug/kg/day). F-rats which had previously been forced to take 200 ug/kg/day consumed in the re-test significantly less ETZ than V-rats and even did not significantly differ from drug-naive controls (F: 40 ug/kg/day, controls: 21 ug/kg/day). When quinine was added, both groups strongly reduced ETZ intake (16 ug/kg/day, 13 ug/kg/day). Thus both forced and voluntary intake of the opiate led to physical dependence but only socially deprived rats with long term voluntary intake became behaviorally dependent, i.e. they revealed an irreversible high demand for the drug.

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ADAPTIVITY DURING ONTOGENESIS MODIFIED BY PRENATAL TREATMENT WITH DIFFERENT NOXAE B. JÄNICKE, AND H. COPER

According to the hypothesis of adaptivity ageing is characterized by the differentially decreasing ability to cope with endogenous and exogenous stimuli. This hypothesis is strongly supported by cross-section analyses of different species (Wallace et al. J. Gerontol. 3, 364-370, 1980; Ingram et al. Exp. Gerontol 17, 295-303, 1982; Cheal et al., Physiol. & Behav. 36, 1047-1051, 1986). To sufficiently consider the individual biographical data as a factor influencing ontogenesis, results obtained so far will be substantiated in life-span studies. In these the proven method to determine critical values of various system qualities such as capacity, stability or reserve was used (Coper et al.: Biopsychological Research on Adaptivity across the Life-span of Animals. In: Life-span Development and Behavior. P.Baltes et al. (eds.) 207-232, LEA, 1986).

Three life-span studies were conducted with rats to test to what extent moderate prenatal damage would affect the ageing process. In the first study the F1 generation of rats, treated with alcohol (6 g/kg/d) from day 7 - 17 of the pregnancy did not show any physiological differences in their development vs. controls. Until the age of 20 months motoric and cognitive performances were hardly diminished. Beyond this age one could recognize, an earlier, more pronounced impairment of motoric-coordinative, cognitive and physiologic-adaptive abilities in prenatally alcohol-exposed rats depending on task difficulty. In the second study the F1 generation, whose mothers had been kept under 12% normobaric hypoxia during pregnancy, showed quantitatively and qualitatively a slightly different performance profile. The motoric abilities were already permanently reduced in the adult phase. In learning tasks only a partial approximation vs. controls occurred. Even the capacity of physiological functions (e.g. adaptation to 10% hypoxia) was likewise clearly restricted. In the senile period performance deteriorated further. In the third study in which rats were prenatally treated with diazepam (20 mg/kg/d) only slight consequences were observed. The results reveal the scope of adaptive processes during ontogenesis and its restriction by prenatal damage.

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POSTNATAL DEVELOPMENT AND BEHAVIOUR OF WISTAR RATS BORN TO STREPTOZOTOCIN-INDUCED DIABETIC DAMS
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Infants of diabetic mothers are suspected to be at risk for signs of an impaired psychomotor development. It remains unclear whether diabetes during pregnancy influences the development of the central nervous system of the offspring. A delayed myelination has been observed in children of diabetic mothers, which could be the reason for a developmental retardation. To study this more closely, diabetes mellitus was induced in Wistar rats by a single i.v.-injection of 40 mg/kg streptozotocin (STZ) on day 8 of pregnancy. Blood glucose levels were determined on days 10 and 20 of gestation in blood samples taken from a tail vein. The STZ treatment (N = 42) resulted in a marked increase of the blood glucose concentration [mmol/l] of 21.7 $\pm$ 4.5 on day 10 and of 23.8 $\pm$ 4.5 on day 20 [control (N = 26): 5.98 $\pm$ 0.65 day 10 and 5.45 $\pm$ 0.68 day 20]. During the first 21 days postnatally (pn) there were no significant differences in litter size and mean body weight of the pups, the body weight of the mothers was significantly reduced on day 1 pn. Although the newborns were cross-fostered, only about 50% surviving litters could be obtained. A retarded myelination was demonstrable in the STZ-offspring. After weaning, a rota rod test (day 20 - 29 pn) and the measurement of the locomotor activity (day 30 - 35 pn) was performed (individual animals simultaneously in 5-min-intervals with four infra-red light photocells per cage over a 120-hr-period). The capability to stay on a rotating rod was delayed in the STZ-offspring. On day 25 postnatally only 54% were successful compared to 92% of the controls. The locomotor activity was comparable to that of the controls in all three parameters investigated (frequency, duration and intensity). It is very likely that diabetes during pregnancy is able to influence postnatal brain development in rats.

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