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Transistor recordings with Nav 1.2 IFM-QQQ expressed in oocyte

M. dal Maschio, M. Scorzeto, S. Vassanelli
dept. Anatomy and Physiology Padua University

We investigated the gating of non-inactivating mutated sodium channels at the Xenopus Leavis oocyte-semiconductor interface. The interface was described by an equivalent electrical mode of a cylindrical_bidimensional core-coat conductor. A bidimensional cable equation describes the potential Vj (x,y,t) in the junction. Due to the additional voltage Vj and the peculiar geometry in the cleft, the behaviour of sodium channels differed in the attached membrane with respect to the free membrane. The open probability of channels was bistable: smooth gating was trasformed into an all-or-non process with switching, hysterisis and memory. These results show the peculiar behaviour of sodium channels at the oocyte-semiconductor interface. The sodium conductance is characterized by bistability: channel gating with switching and hysterisis was observed experimentally and simulated using an equivalent electrical model.On these basis we supposed that sodium channel gating may be similarly effected by adhesion in mammalian cells, but only in conditions of a very high channel density and a large area of adhesion.

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Modulation of Gj of Connexin36 (Cx36) Channels expressed in N2A cells: the role of CaM Kinase II

C. del Corsso[1], M. Srinivas[1], R. Dermietzel[2], G. Zoidl[2], C. Alev[2], D. C. Spray[1]
[1]Albert Einstein College of Medicine, [2]Ruhr-University-Bochum

The gap junction protein Cx36 interconnects GABAergic interneurons and other neuronal populations and also is found between pancreatic islet cells. In dual whole cell recordings from transfected neuroblastoma (N2A) cells, we have found that junctional conductance (g_j) of both C-terminally GFP-tagged and untagged Cx36 channels display the remarkable property of "run-up", in which g_j increases by ten-fold or more within minutes following cell break in with patch pipettes. In order to determine mechanisms underlying the run-up phenomenon, we have applied a variety of pharmacological tools and methods. KN-93, a selective inhibitor of CaM Kinase II (CaM KII) blocked the run-up and a similar effect was also observed when the cells were dialyzed with a peptide whose sequence overlaps the binding site for CaMKII of Cx36. Experiments with synthetic peptides overlapping the potential phosphorylation sites of Cx36 showed that phosphorylation of the S315 residue in the C-terminal (CT2), but not the S111 residue in the cytoplasmic loop (CL1) contribute to the "run up". Other pharmacological inhibitors including colchicine and AMP-PNP also attenuated the "run up" implying that the run-up in the junctional currents observed in Cx36 transfectants may be due multiple pathways. Experiments are underway to determine whether these different pathways are parallel and synergistic or converge within a simple common signaling mechanism.

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Incorporation of the Voltage Dependent Anion Channel in tethered lipid bilayer

A. Deniaud[1], C. Rossi[2], A. Berquand[3], J. Homand[3], J. Chopineau*[2], C. Brenner*[1]
[1]UVSQ, CNRS FRE2445, Versailles, France, [2]CBS, Centre Universitaire de Nîmes, CNRS UMR5048-UM1-INSERM UMR554,30000 Nîmes, France, [3]UTC, CNRS UMR6022, Compiègne, France

The outer mitochondrial membrane protein VDAC (voltage-dependent anion channel) mediates the passage of metabolites into and from the mitochondria. Recently, a new role of VDAC in apoptosis emerged. Indeed, VDAC could constitute or contribute to a mitochondrial membrane permeabilization event. We have developed a VDAC-incorporated tethered lipid bilayer. Polymer-tethered membranes on solid support were obtained from vesicles containing N-hydroxysuccinimide (NHS)-terminated poly(ethyleneglycol) (PEG)-phospholipids as anchoring molecules. VDAC/POPC/DSPE-PEG-NHS mixture liposomes were injected on the top of an amine grafted surface (cysteamine-coated gold or silanized glass); vesicles linked to the surface and disrupted, leading to the formation of a bilayer. The membrane construction was followed by surface plasmon spectroscopy while membrane fluidity and continuity were vizualized by fluorescence microscopy. Each step of the self-assembling process was imaged by atomic force microscopy. Different approaches such as protein binding and enzymatic activity assay, are developed to prove that the incorporated VDAC is correctly folded and active in such supported biomimetic membranes.

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Conformational analysis, lytic and biological activity of bioactive peptides

M. P. dos Santos Cabrera[1], K. Konno[2], L. C. Salay[3], J. Procopio[3], J. Ruggiero Neto[1]
[1]Physics Dept.,UNESP, S.J. Rio Preto, SP, [2]CAT/CEPID/FAPESP, S.Paulo, SP, [3]Inst. of Chemistry and Inst. Biosciences USP, S.Paulo, SP

The mechanisms of action of four mastoparan-like peptides, extracted from wasp *Eumenes micado*, have been studied through conformational analysis and lytic activity and correlated with biological activities. These are supposedly centered in the membrane lipid phase. Besides a mild citotoxic activity, two of these peptides are also broad-spectrum antimicrobials. We accessed peptide secondary structure by circular dichroism and lytic activity in zwitterionic and anionic vesicles through dye leakage experiments using fluorescence spectroscopy. We also investigated the interaction of EM1 in net negatively charged planar lipid bilayers (BLM) of azolectin.

Peptides showed α-helical structure dependent on the amino acid sequence but mainly on the environment. The lytic activity on anionic vesicles indicates that EM1 (LKLMGIVKKVLGAL-NH2) and analog EM2 (M4L;L14I) are slightly more efficient than EM3 (FDIGIIIKKVVSGL-NH2) and analog EM4 (L3I). In zwitterionic vesicles the peptides showed intensive lytic activity, around 90% leakage for peptide concentrations 10-25 μM. EM1's displayed ion channel activity in BLMs. It formed large conductance channels with long residence times ($\sim$ seconds) and there are indications of more than one open pore.

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Structure function relationship of the ssDNA channel VirE2 of Agrobacterium tumefaciens

M. Duckely, H.-W. Rémigy, I. Callebaut, J.-P. Mornon, F. Axthelm, V. Olivieri, P. Ringler, S. A. Müller, A. Engel
M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, CH-4056, Switzerland

The pathogen *Agrobacterium tumefaciens* is unique in its ability to pass ssDNA and protein through five lipid bilayers to integrate the ssDNA molecule into the host genome. Central to this process, the multifunctional VirE2 protein protects the ssDNA from nuclease degradation, allows interaction with the nuclear import machinery. Recently, the VirE2 protein was further found to integrate in lipid bilayers and form channels of defined conductance. These channels are voltage-gated and anion selective and allow passage of ssDNA into unilamellar vesicles. Hence we proposed that the VirE2 protein is additionally a translocator and mediates the passage of ssDNA through the host plasma membrane. Here we report our analysis of the sequence of the VirE2 protein to identify the transmembrane domains, our biochemical work to determine the oligomeric state of the protein and a low resolution electron microscopy study of the oligomer formed by VirE2 in solution. VirE2 mutants impaired *in vitro* in channel formation or properties were further analyzed *in vivo* for transfer ability. Their unability to form channels correlated with reduced DNA transfer ability.

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Specificity in the dimerization of the transmembrane domains of ErbB receptor proteins

J.-P. Duneau[1], A. P. Végh[2], J. N. Sturgis[1]
[1]LISM, CNRS/Université Aix-Marseille 2, Marseilles, France, [2]IBRB, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Hydrophobic transmembrane domains are not solely passive anchors of membrane proteins to cellular membranes. In the case of Receptor tyrosine kinases (RTKs) evidences were shown that the transmembrane domain plays an active role in the dimerization process that leads to the receptor activation. Among RTKs, the four members of the ErbB/HER subclass can homo or heterodimerize to trigger specific role in biological processes like growth control and tissular differentiation.
We have synthesized the transmembrane domains of the different HER proteins (HERtm) and investigated their homo- and heterodimerization abilities in a micellar environment. We make use of a FRET test to determine the affinity and specificity of the different transmembrane helices for each other. Our results show that measured dissociation spans 3 decades in Kd, ranging from submicromolar to virtually no association. A hierarchy in association preferences mimics that found for the whole receptors (1250 residues) in a cellular context. We propose a homology based model to explain how the single transmembrane domains might contribute to the receptor association energetics and to the selection of the partner for signaling purpose.
Keywords : Membrane proteins, α-helix association, FRET, tyrosine kinase receptors

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Chemical cross-linking photosensitized by a ruthenium chelate as a new tool for signaling studies

I. Duroux-Richard[1], J.-F. Guichou[1], G. Subra[1], B. Mouillac[2], P. Vassault[1], J. Marie[1], A. Chavanieu[1], J.-C. Bonnafous[1]
[1]Centre de Biochimie Structurale, 29 rue de Navacelles, 34090 Montpellier, France, [2]Institut de Génomique Fonctionnelle, 141 rue de la Cardonille, 34094 Montpellier-Cedex, France

A new strategy involving oxidative cross-linking reactions photosensitized by the ruthenium chelate $Ru(bipy)_3{}^{2+}$ in the presence of ammonium persulfate has been successfully applied to the covalent labeling of several G-protein coupled receptors. The B_2 and B_1 bradykinin, AT_1 angiotensin, V_{1a} vasopressin and oxytocin receptors were labeled in intact cells by synthetic agonist or antagonist ligands possessing a radio-iodinatable phenol moiety, including biotin- or metal chelate-functionalized ligands : high labeling yields were obtained for very short irradiation times (3 seconds) by visible light. The receptor amino-acids candidates to phenolated ligand cross-linking are tyrosine, tryptophan and cysteine residues. Ongoing refinements leading to improved specificity and yield (up to 40%) concern «auto-photosensitized» cross-linking, using ligands possessing, in addition to a tyrosine, a covalently attached ruthenium chelate. The potential applications of these strategies include stabilization of ligand-receptor complexes or supramolecular edifices for structural studies and the luminescence detection of $Ru(bipy)_3{}^{2+}$-derivatized ligands for signaling mechanism studies in living cells.

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Store-operated Ca^{2+} entry is regulated by mitochondria in acinar cells of rat submandibular salivary

N. V. Fedirko[1], O. V. Kopach[2], P. G. Kostyuk[3], N. V. Voitenko[4]
[1]Lviv National University, 4 Grushevsky St, Lviv, Ukraine, [2]Lviv National University, 4 Grushevsky St, Lviv, Ukraine, [3]Bogomoletz Institute of Physiology, 4 Bogomoletz St, Kiev, Ukraine, [4]Bogomoletz Institute of Physiology, 4 Bogomoletz St, Kiev, Ukraine.

In non-excitable cells the major route for Ca^{2+} influx is through store-operated Ca^{2+} channels in the plasma membrane that is activated by emptying of intracellular Ca^{2+} stores following the stimulation. However, little is known about the downstream consequences of depletion of internal Ca^{2+} stores in submandibular cells. We have studied the extent of the cytosolic Ca^{2+} increase ($Ca^{2+}{}_i$) to 3 μM thapsigargin (Tg) in cells preloaded with fura-2/AM. In the absence of external Ca^{2+}, Tg triggered small transient Ca^{2+} release from the internal stores with the amplitude 51 $\pm$ 14 nM; in the presence of external Ca^{2+} the $Ca^{2+}{}_i$ signal was more sustained with the amplitude 112$\pm$11nM. We found that mitochondria (Mit) are required to support Ca^{2+} entry in the acinar cells. In these experiments, the stores were depleted by exposing cells to Tg for 20 min in Ca^{2+}-free solution and then to CCCP (10 μM) for further 15 min. The rate of Ca^{2+} entry and amplitude of $[Ca^{2+}]_i$ rise, measured following readmission of external Ca^{2+}, were significantly lower when Mit had been depolarized. Thus, the emptying of intracellular Ca^{2+} stores in acinar cells activates the sustained store-operated Ca^{2+} entry and Mit may have a crucial physiological role in regulating store-operated influx.

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Correlation between residue ionization and ionic selectivity in OmpF porin

J. J. García-Celma, M. Aguilella-Arzo, J. Cervera, A. Alcaraz, V. M. Aguilella
University Jaume I, Department of Experimental Sciences. Biophysics Unit., P.O. Box 8029. E-12080 Castellón, Spain

The bacterial porin OmpF found in the outer membrane of E. coli. is a wide channel, characterized by its poor selectivity and almost no ion specificity. Its asymmetric structure, with relatively large entrances and a narrow constriction is similar to that of general diffusion porins. By applying continuum electrostatic methods we determine the ionization states of titratable amino acid residues in the protein and calculate self-consistently the electric potential 3D distribution within the channel. The average electrostatic properties are then represented by an effective fixed charge distribution along the pore, which is the input for a Poisson-Nernst-Planck procedure. Measurements of reversal potential performed under different salt gradients and pH are satisfactorily compared to the model predictions. The sensitivity of reversal potential and conductance to the direction of the salt gradient and the solution pH is captured by the model allowing a discussion on the unidirectional insertion of the channel. The correlation found between atomic structure and ionic selectivity shows that the transport characteristics of wide channels like OmpF are mainly regulated by the collective action of a large number of residues, rather than by the specific interactions of atoms at particular locations.

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A quantitative structure-activity relationship study on a novel class of calcium-entry blockers

S. P. Gupta, A. N. Mathur, A. N. Nagappa, D. Kumar, S. Kumaran
Birla Institute of Technology and Science, Pilani, India

A quantitative structure-activity relationship (QSAR) study has been made on two different series of 1-[{4-(aminoalkoxy)phenyl}sulphonyl]indolizines acting as calcium entry blockers, using some physicochemical and structural parameters. Two different assays were reported for both the series: $(IC_{50})_A$, referring to the molar concentration of the compound required to reduce [^{3}H] nitrendipine binding by 50%, and $(IC_{50})_B$, referring to that required to block Ca^{2+} induced concentration of K^+ depolarised rat aorta by 50%. For series 1, where the 2-position substituents of indolizine ring were varied along with the aminoalkoxy moieties of the phenyl ring, the QSAR analysis shows that the 2-position substituents can equally affect both the activities through their hydrophobic and electronic properties and the aminoalkoxy moiety through some steric effects. For series 2, where the indolizine ring has been replaced by varying heterocyclic rings, along with the changes in aminoalkoxy moiety of the phenyl ring, the QSAR exhibits that these different heterocyclic rings affect both the activities through some steric roles, altering the conformations of the receptors from system A to system B.

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An atomistic simulation study of the nicotinic acetylcholine receptor transmembrane domain

A. Hung, M. S. P. Sansom
Department of Biochemistry, Laboratory of Molecular Biophysics, University of Oxford, U.K.

The adult muscle-type nicotinic acetylcholine receptor (nAChR) is currently the most well-characterised member of the superfamily of ligand-gated ion channels. Recent electron microscopic studies (Unwin, J. Mol. Biol. 346(4):967-989) have elucidated the structure of the transmembrane, extracellular and part of the intracellular domains. Additionally, numerous experimental studies have demonstrated the influence of the lipid environment on its channel activity. We have performed molecular dynamics (MD) simulations on the transmembrane domain of the nAChR within a lipid bilayer in order to study its dynamics in a membrane environment, and to elucidate the nature of lipid-protein interactions at the atomic level. The evolution of the TM domain structure during the MD trajectory is described, with particular focus on the motions of the M2 helices and their influence on the dimensions of the pore. Correlations between M2 hinge-bending motions and fluctuations of the M2-M3 extracellular loops were observed, suggesting one possible mechanism by which conformational changes at the ligand binding domain may influence the dynamics of the pore-lining helices. Ion desolvation energies along the pore were estimated using a continuum solvent model and discussed with respect to the physical dimensions and polarity of specific regions within the channel. Addtionally, we characterise the structural and dynamical properties of lipids in proximity to the protein.

P-256

Cholesterol recognition by the peripheral-type benzodiazepine receptor

N. Jamin[1], J.-M. Neumann[1], M. Ostuni[2], T. K. Ngoc[1], Z.-X. Yao[3], S. Murail[1], J.-C. Robert[2], C. Giatzakis[3], V. Papadopoulos[3], J.-J. Lacapère[2]
[1]CEA-Saclay, DBJC/SBFM and URA CNRS 2096, 91191 Gif sur Yvette Cedex, France, [2]Unité INSERM U410, Faculté de Médecine Xavier Bichat, 75870 Paris Cedex 18, France, [3]Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20057, USA

The peripheral-type benzodiazepine receptor (PBR, 18kDa), a transmembrane protein containing five putative transmembrane domains, plays a key role in cholesterol transport across the outer mitochondrial membrane in various steroid and bile acid producing tissues[1]. Site-directed mutagenesis and in vitro expression studies identified a region of the cytosolic carboxyl-terminus of the PBR that contains a cholesterol recognition amino acid consensus sequence (CRAC)[2].
Structural NMR data obtained on the C-terminal domain of this protein containing the CRAC, combined with functional data obtained on wild type and mutated recombinant PBR reconstituted in proteoliposomes are reported. These results reveal a cholesterol binding concavity and indicates essential residues involved in cholesterol binding[3].

1. Lacapere J-J & Papadopoulos V 2003, Steroids 67, 569
2. Li et al. 2001, PNAS. 98, 1267
3. Jamin et al. 2005, Mol. Endocrinol. 19, 588

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Roles of transmembrane regions in determining activation kinetics of heag potassium channels

M. Ju, D. Wray
School of Biomedical Sciences, University of Leeds, Leeds, UK, LS2 9JT

Heag1 and heag2 potassium channels are highly homologous, but activation kinetics is different between the two channels. We showed previously that the intracellular N terminus and the S1-S6 transmembrane regions are involved in determining these differences; here we focus on which parts of the transmembrane regions are involved. For this, chimeras were constructed by transferring corresponding regions of heag1 to heag2. These chimeras were expressed in Xenopus oocytes and currents were recorded two days later by using two-electrode voltage clamp. Oocytes were depolarised from -80 mV to 0 mV and activation times were measured as the time for 20-80% of maximal current. Chimera I with the S1 transmembrane region of heag2 replaced by that of heag1 showed an activation time (32.7 ± 1.4 ms, n=8) faster than heag2 (54.5 ± 7.2 ms, n=5), indicating an involvement of the S1 region. Chimera II with S2-S3 regions of heag2 replaced by that of heag1 showed an activation time remaining slow (51.2 ± 3.0 ms, n=8) like heag2, suggesting that S2-S3 regions are not involved. Chimera III with S5-S6 regions of heag2 replaced by that of heag1 showed an activation time (28.3 ± 4.7 ms, n=10) faster than heag2, indicating that S5-S6 regions are also involved. Taken together, these data suggest that S1 and S5-S6 regions are involved in determining differences in activation kinetics between heag1 and heag2 channels.

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Calorimetric study of SAP97 PDZ domain-peptide interactions

A. Kangasniemi, L. Von Ossowski, K. Keinänen, A. Annila
Department of Biological and Environmental Scienses, University of Helsinki, Finland

PDZ domains are widely spread in different organisms and known to interact with specific sequence motifs at the C-termini of target proteins. Synapse associated protein 97 (SAP97/Dlg1) contains three PDZ domains which associate with a number of synaptic signaling proteins. In this study, isothermal scanning calorimetric measurements will be used to characterize the binding of different SAP97 target peptides to the separate PDZ domains. Binding affinities, interaction specificity and possible binding motifs in the synthetic 13-mer target peptides will be presented. Preliminary analyses indicate that the C-terminal peptide of the NMDA receptor subunit NR2A binds to PDZ2 of SAP97 with a μM level dissociating constant, in good agreement with values obtained from other types of assays.

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Conformational studies of Vpu_{1-32} and its interaction with drug in membrane bilayers using SS-NMR

Y. H. Lam, D. Fischer, W. Fischer, A. Watts
Biomembrane Structure Unit, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom

Vpu is an 81-residue accessory protein of HIV-1. This protein enhances the release of new virus particles from cells infected with HIV-1 and induces the intracellular degradation of the CD4 receptor protein. The Vpu-mediated enhancement of the virus release from HIV-1 infected cells is correlated with ion channel activity associated with the transmembrane hydrophobic helical domain. Structural information on Vpu has been derived from NMR, CD and FTIR spectroscopy (reviewed in Fischer, FEBS letter, 2003). Recently, a derivative of amiloride, 5-(N,N-hexamethylene)amiloride has been found to block ion channel activity and thereby hampering the release of particles from the infected cell (P. W. Gage et al, Eur. Biophys. J (2001). With this study we aim to obtain experimental evidence of the site of drug-protein interaction.
Vpu consists of one transmembrane hydrophobic helix and two amphipathic helices in its cytoplasmic domain. We focus on the transmembrane domain part of the Vpu_{1-32}, especially using ^{13}C and ^{15}N labeling in proximity to the proposed drug-binding region (Arg30, Tyr29, Ser23, Ile27, Val25). Solid-state NMR was used to study conformational changes of Vpu_{1-32} upon drug binding by comparison of its membrane-inserted structures in both its drug-free and drug-bound forms.

P-260

Electrogenic Na+ transport in access channels of Na,K-ATPase studied by the admittance measurements

A. A. Lenz[1], V. S. Sokolov[1], K. Grishanin[1], A. A. Scherbakov[1], K. V. Pavlov[1], H.-J. Apell[2]
[1]Frumkin Institute of Physical Chemistry and Electrochemistry RAS, Leninsky prosp.,31, b.5, 119071, Moscow, Russia, [2]Department of Biology, University of Konstanz, Fach M635, 78457 Konstanz, Germany

Non steady-state electrogenic transport of sodium ions in the extracellular and cytoplasmic access channels of the Na,K-ATPase was studied by measuring small increments of capacitance and conductance of bilayer lipid membranes with adsorbed protein-containing membrane fragments triggered by photorelease of ATP. A fit of the frequency dependences of these increments by a sum of Lorentzians allowed the resolution of two steps of the sodium transport in the extracellular access channel and one on the cytoplasmic side. The amplitudes and corner frequencies of the Lorentzians characterize the relative dielectric distances and rate constants of charge movements in the channels. They were dependent on the experimental conditions, such as sodium concentration and pH. A decrease of sodium concentration leads to a disappearance of the fast transport steps in the extracellular channel and to an increased contribution of the step corresponding to ion movements in the cytoplasmic channel. The results are explained by a model of sodium transport by the Na,K-ATPase consisting of a sequence of sodium binding through cytoplasmic channel and subsequent release of these ions through the extracellular channel.

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From simple to complex: state-dependent interaction between neighboring RyRs makes the regular array a powerful and flexible signalling machine

X. Liang[1], X. Hu[1], K. Chen[1], H. Xie[2], P. Zhu[2], J. Hu[1]

[1]Bio-X Research Center, SJTU, Shanghai 200030, China, [2]Key Laboratory of Neuroscience, SIBS, CAS, Shanghai 200031, China, [3]SIAP, CAS, P. O. Box 800-204, Shanghai 201800, China

How biomolecules achieves the highly flexible modulation of their signalling is quite a fundamental question in biology. Recently, data from experiments and modeling both strongly indicate the regular lattices formed by membrane receptors might be an exciting way. Ryanodine receptors (RyRs), one of the SR Ca^{2+} release channels, conservatively form paracrystalline arrays in SR membrane. Some evidence has suggested, in a certain extent, the functional interaction between RyRs is essential for the maintenance of a quiescent Ca^{2+} release machine in the resting states, as well as for the coordinated activation and termination of SR Ca^{2+} release in muscle cells.

In the present work, the modulation of RyR-RyR interaction by their functional states was investigated by using AFM and PCS. Consequently, we found that the interaction strength between RyRs is tightly correlated with channels' functional states. For the physiological sense of this interesting phenomenon, a mathematical model was operated and the results definitely show that such state-dependent interaction between neighboring RyRs is essential for the stabilization of RyRs under the resting condition and also for the efficient closure of individual RyRs to achieve the rapid termination of the SR Ca^{2+} release events by dynamic modulating the Ca^{2+} sensitivity and opening duration of arrayed RyRs.

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Rat brain Nav1.2 channel sorting: auxiliary subunits/ ECM proteins interaction in cultured neurons

M. Maschietto, S. Girardi, S. Vassanelli

Dept. of Human Anatomy and Physiology

Rat brain voltage-dependent $Na_v1.2$ channels are heteromultimeric complexes that include a pore-forming α subunit and several accessory β subunits. Experiments *in vitro* suggest interactions between β subunits (in particular β1 and β2) and some ExtraCellular Matrix proteins (i.e. contactin and tenascins). On these bases the aim of our work is studying the channel sorting in cultured rat hippocampal neurons in order to polarize the channels localization. To reach this purpose we create chimerae between the auxiliary β subunits (β1 or β2) and a fluorescent protein and express them in neurons. The interactions between the chimerae and the ECM proteins can be monitored analyzing the different fluorescence patterns given by neurons cultured over an extracellular matrix protein coating, compared to neurons growing without a specific substrate. Moreover, electrophysiology experiments could evidence differences in the channel expression levels or changes in the voltage dependence.

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Viral channel investigations: The kinetics of Vpu channels from HIV-1

T. Mehnert[1], A. Routh[1], P. Judge[1], T. Lam[1], D. Fischer[1], A. Sauerwein[1], A. Watts[1], W. B. Fischer[2]

[1]Biomembrane Structure Unit, Department of Biochemistry, South Parks Road, OXFORD, OX1 3QU, UK, [2]Bionanotechnology IRC, Clarendon Laboratory, Department of Physics, Parks Road, OXFORD, OX1 3PU, UK

A short integral membrane protein called Vpu (virus protein unknown) with 81 residues is encoded in the genome of the HIV-1 virus. Vpu is proposed to be an accessory protein with two distinct functions: (1) the degradation of the CD4 receptor and (2) the enhancement of the release of new virus particles from the infected cell due to ion channel activity of self-assembled Vpu bundles. A broad series of non-voltage-dependent conductance states is found when channel data are recorded from bilayers with incorporated peptides, corresponding to the transmembrane part of Vpu. Even if small or no potential is applied those channels can open and remain open. The mean open time in each conductance state is found to be voltage independent whereas the frequency of gating, means the transition from the shut to any of the conductance states, increases with the external electric field. The data support a model, already established for larger cell ion channels, in which an internal twist of the individual transmembrane helices causes a rotation of the channel sealing kinks, controlling the pore radius and gating the channel.

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Probing the pore-forming region of FhaC of Bordetella pertussis: ion channel measurements in lipid bilayers

A. Méli[1], G. Molle[1], F. Jacob-Dubuisson[2], N. Saint[1]

[1]UMR 5048 CNRS, U554 INSERM, UM1, 29 rue de Navacelles, 34090 Montpellier, France, [2]U629 INSERM, Institut Pasteur de Lille, 1 rue Calmette, 59019 Lille Cedex, France

FhaC is an outer membrane transporter of the Omp85/TpsB family involved in secretion of the filamentous hemagglutinin of *B. pertussis.* This 63-kDa protein, shown to form ion channels in artificial membranes (Jacob-Dubuisson *et al*, *J.Biol.Chem.*, 1999, 274, 37731-37735) is most likely a two-domain protein: an N-terminus domain of ill-defined structure and function and a C-terminal domain, most likely presenting a β−barrel type structure and involved in pore formation (Guédin *et al*, *J.Biol.Chem.*, 2000, 275, 30202-30210). In order to confirm that the C-terminal part is the pore-forming region of the transporter, we first looked at the ion channel properties of FhaC mutants with mutations located in the putative β-barrel domain. Thus, we have shown that short insertions or deletions in transmembrane segments of the β-barrel or in loops connecting the β strands alter or abolish both the FHA secretion activity *in vivo* and the channel properties *in vitro.* Secondly, we looked at the pore-forming activity of additional FhaC mutants, with mutations located in the N-terminal region. The results showed that ions channels formed by these latter mutants are hardly affected, supporting the hypothesis that the N-terminal part of FhaC is not directly involved in pore formation.

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Monitoring activity of olfactory receptors expressed in yeast cells, and in their membrane fraction

J. Minic[1], J. Grosclaude[2], M.-A. Persuy[1], J. Aioun[1], I. Connerton[3], R. Salesse[1], E. Pajot-Augy[1]
[1]INRA, NOPA-RCC, Jouy-en-Josas, France, [2]INRA, VIM, Jouy-en-Josas, France, [3]U. Nottingham, Loughborough, UK

The functional expression of olfactory receptors is a primary requirement to examine the molecular mechanisms of odorant perception and coding and to screen receptor-odorant couples. Membrane expression of such receptors was optimized in engineered *S. cerevisiae* yeast.
Their functional activity was first evaluated in living cells, where receptor stimulation by its odorant ligand induces activation of the transduction pathway to the synthesis of a luciferase reporter, evaluated by its bioluminescence. Secondly in membrane fragments, the receptor undergoes conformational change upon activation and interacts with trimeric G protein still present in the membrane fraction, resulting in $G_{\alpha olf}$ dissociation monitored by surface plasmon resonance.
Both approaches yield a same bell-shaped concentration-dependence response, for receptors stimulated by their ligands, in terms of threshold concentration and concentration at the maximum. This result underlines that the receptor functional response in the living cell indeed arises from its own behavior upon odorant stimulation, and is not due to subsequent signal transduction.
We demonstrate that olfactory receptors maintain their activity in membrane fragments, which can thus be used for specific odorant detection and recognition in olfactory biosensors.

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β3- and β2-adrenoreceptor agonists: In vitro relaxation effects on human pregnant uterus contractions

B. Modzelewska[1], A. Pedzinska[1], R. Urban[2], A. Kostrzewska[1]
[1]Department of Biopysics, Medical University of Bialystok, Mickiewicza 2A, 15-089 Bialystok, Poland, [2]Department of Perinatology, Medical University of Bialystok, University Hospital, Sklodowska-Curie 24a, 15-276, Bialystok, Poland

Preterm birth is the major cause of prenatal mortality and morbidity. A leading cause of all preterm births is due to spontaneous idiopathic preterm labour for which the main therapeutic intervention is tocolytic therapy. Beta2-adrenoreceptor agonists are the most commonly used tocolytic drugs (ritodrine). However, its use is associated with serious maternal cardiovascular side effects. There is substantial in vivo evidence that the beta3-adrenergic agonists have potent non-vascular smooth muscle relaxant effects while having significantly less potent vascular smooth muscle inhibitory effects. The purpose of our study was to investigate the functional selectivity of the beta3 agonists: BRL 37344, Cl 316243 and the beta2-adrenoceptor agonist ritodrine for their putative receptors in human pregnant myometrium in vitro. To investigate this effect - biopsy specimens of human myometrial tissue during pregnancy were obtained at elective caesarean delivery. Uterine activity was recorded under isometric conditions by means of force transducers with digital output. Concentration-responses curves to BRL 37344 and Cl 316 243 and ritodrine were constructed in the absence and presence of beta-adrenoreceptor antagonists. Quantification of the responses was done by calculation of the area under the curve, the amplitude and the frequency of contractions. The effects were evaluated by comparing experimental responses with the controls.

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β3- and β2-adrenoreceptor agonists:In vitro relaxation effects on human pregnant uterus contractions

B. Modzelewska[1], A. Pedzinska[1], R. Urban[2], A. Kostrzewska[1]
[1]Biopysics Dpt., Medical Univ., Bialystok, Poland, [2]Perinatology Dpt., Medical Univ., Bialystok, Poland

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To investigate this effect - biopsy specimens of human myometrial tissue during pregnancy were obtained at elective caesarean delivery that was performed at term. Uterine activity was recorded under isometric conditions by means of force transducers with digital output. Concentration-responses curves to BRL 37344 and Cl 316 243 and ritodrine were constructed in the absence and presence of beta-adrenoreceptor antagonists. The spontaneous contractile activity was treated as a control. Quantification of the responses was done by calculation of the area under the curve, the amplitude and the frequency of contractions. The effects were evaluated by comparing experimental responses with the controls.

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Electron-conformational model of cooperative cardiac ryanodine receptors gating

A. S. Moskvin[1], M. P. Philipiev[2], O. E. Solovyova[2], P. Kohl[3], V. S. Markhasin[2]
[1]Ural State University, Ekaterinburg, 620083, Russia, [2]Institute of Immunology and Physiology, Ekaterinburg, 620219, Russia, [3]Laboratory of Physiology, University of Oxford, UK

We propose an electron-conformational model to describe the cooperative dynamics of the lattice of ryanodine receptors (RyR) which regulate the release of the intracellular activator calcium from calcium stores in the sarcoplasmic reticulum (SR) by a process of Ca^{2+}-induced Ca^{2+} release (CICR). We characterize a single RyR channel by a fast electronic and slow conformational degrees of freedom, respectively. The former is assumed to be dihotomic variable with only two ("open" and "closed") bare electronic RyR states, while a conformational degree of freedom Q is described by a classical continuous variable which specifies the RyR channel conductance with regard to Ca^{2+}. The RyR lattice cooperative high-gain response to L-type channel triggering evolves due to a nucleation process with a step-by-step domino-like opening of RyR channels. Typical mode of RyR lattice functioning in a CICR process implies the fractional release with a robust termination due to the depletion of SR with a respective change in effective conformational strain. The SR overload leads to an unconventional auto-oscillation regime with a spontaneous calcium release. The model is shown to consistently describe the main features of CICR, that is its graddedness, coupled gating, irreversibility, inactivation/adaptation, and spark termination.

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– Channels and Receptors –

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Dissecting the mode of action of the nuclear receptor car through biophysical and functional studies

V. Nahoum[1], C. Benod[1], J.-M. Pascussi[2], A. Chavanieu[1], W. Bourguet[1]

[1]Centre de Biochimie Structurale, CNRS-UMR5048 NSERM UMR554 UM1, Faculté de Pharmacie, 15 avenue Charles Flahault, 34093 Montpellier, France, [2]Physiopathologie Hépatique, INSERM U632, 34293 Montpellier, France

The constitutive androstane receptor (CAR), that belongs to the nuclear receptor superfamily, has been shown to play a central role in detoxification. Upon binding of a variety of endo- or xenobiotics, CAR regulates the expression of various metabolic enzymes that in turn facilitate the deactivation and clearance of these toxins. Unlike classical nuclear receptors that require a ligand for transcriptional activation, CAR displays a high level of constitutive activity. Fluorescence anisotropy assays were performed to characterize the recruitment of transcriptional co-regulators by CAR and the influence of ligand binding on these interactions. Our data reveal unexpected CAR/co-regulators interactions either in the presence or absence of ligands. Studies of CAR functions are difficult due to the weak affinity and specificity of CAR ligands. A structure-based approach was used to identify new CAR modulators. The efficiency and the biological effect of these new putative ligands were checked on primary human hepatocytes by RTQ-PCR, and transient transfection assays on HepG2 cells. Together, these studies will provide important insights into the unusual regulation of this receptor and will help for the development of improved therapeutic agents.

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Prediction of Interfaces for oligomerization of GPCRs

W. Nemoto[1], H. Toh[2]

[1]Graduate School of Information Science, Nara Institute of Science and Technology (NAIST), Japan, [2]Bioinformatics Center, Institute for Chemical Research, Kyoto University, Japan

Because over 45% of clinically marketed drugs are active at this family, GPCRs are one of the most important target classes of proteins for drug. The formation of GPCR homo/hetero oligomer has been suggested by biochemical and pharmacological evidence. To clarify the mechanism of signal transduction, it is important to elucidate the oligomeric patterns of GPCRs. Accordingly, prediction of interface for the oligomerization would be the first step to understand the mechanism of the oligomerization. We focused on the distribution of conserved residues on molecular surface and developed a novel method to predict the interface for the GPCR oligomer. Predicted interfaces of mouse rhodopsin, D2 dopamine receptor (D2R) and β2 adrenergic receptor agreed with the experimentally confirmed interfaces. This indicates that our method is useful for predicting the interfaces. Moreover, a highly conserved residue detected from the D2R corresponded to a residue involved in a missense change found in the large family of myoclonus dystonia. Our observation suggests the possibility that the disease is caused by the disorder of the oligomerization, although the mechanism of the disease has not been revealed yet. The benefits and the pitfalls of the new method will be discussed, based on the results of the applications. (Nemoto W, Toh H. Proteins. 2005;58:644-60)

P-271

Contribution of BK channels to guinea-pig ileum contractile response evoked by hydrogen peroxide

V. L. A. Nouailhetas, E. F. Rosa, A. Simonato, J. Aboulafia

Department of Biophysics - UNIFESP

Hydrogen peroxide (H_2O_2) stimulates a slow transient smooth muscle contraction. We evaluated the participation of K+-channels on isometric contraction of the guinea-pig ileum muscular longitudinal layer (IMLL) evoked by addition of H_2O_2 in the presence of K+-channel blockers, and on unitary currents in freshly dispersed myocytes, bathed in high K+ solution, recorded by the cell-attached configuration. H_2O_2 (1 mM) induced a transient slow isometric contraction, corresponding to 100% of the KCl tonic response. The H_2O_2 contraction became faster after 20-min tissue pre-treatment with 89 nM IbTx and was abolished by 1μM verapamil. In the case of 1 mM TEA, this response was also fast but had amplitude similar to the phasic component evoked by 40 mM KCl. No effect was verified by previous incubation with 20 mM TEA or 1 mM 4-AP. At single channel level, the H_2O_2 transiently activated Ca^{2+}-dependent K^+-channel (BK) (NPo increased from 0.002 up to 0.088), reaching its maximum in 2-3 min, and returning to basal level in 9-10 min. Altogether these results clearly demonstrate that the H_2O_2 contraction in guinea pig ileum is due to simultaneous activation of Ca^{2+}- and BK population.

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Redox Interactions Mediate the Effects of Non-thiol Reagents on the SR Ryanodine Receptor

R. O. Olojo, B. S. Marinov, J. J. Abramson

Department of Physics, Portland State University, Portland, OR 97201

The Ca^{2+} release channel (CRC) from sarcoplasmic reticulum (SR) is rich in thiol groups and is strongly regulated by thiol reagents. Oxidation of thiols results in activation of the Ca^{2+} release mechanism, while reduction of the disulfides formed closes down the CRC. Although most channel regulators are not thiol reagents and do not appear to directly interact with thiols, it is demonstrated that all channel activators tested act as electron acceptors, while all inhibitors of the CRC act as electron donors. Channel activators such as ryanodine, doxorubicin, and caffeine slow down dye photobleaching, and the reduction of NBD-Cl by acting as electron acceptors. In contrast, the CRC antagonists tetracaine, procaine, and the poly unsaturated fatty acid docosahexaenoic acid (DHA) facilitate photo-bleaching and reduction of electron acceptors, revealing electron donor activity. In spite of the apparent structural diversity among numerous Ca^{2+} release channel regulators, it appears as if many allosteric channel activators are electron acceptors, and many channel inhibitors act as electron donors. These observations support a model in which activation of the Ca^{2+} release mechanism leads to the oxidation of critical thiols, while closing down of the release channel involves the donation of electrons and the reduction of protein disulfides.

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– Channels and Receptors –

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Single channel conductance of recombinant CFTR of mitochondria-rich cells

C. F. Pavel, S. E. Hansen, J. Amstrup, E. H. Larsen
Institute of Molecular Biology and Physiology, August Krogh Building, University of Copenhagen, Denmark

Transcellular voltage clamp of single isolated mitochondria-rich cells [1] showed that isoproterenol activates a glibenclamide inhibitable chloride conductance of the apical membrane. In studying toad skin CFTR in HEK293 cells, *bbcftr* [1] was cloned into a construct with EGFP C-terminally to *bb*CFTR. Expression of CFTR was investigated by ion channel patch clamp from 24-72 hrs after transfection. In cell attached patches, the addition of 10 μM forskolin to bath resulted in activation of several slightly outwardly rectifying chloride channels (γ_{Cl}=5.18$\pm$0.07 pS, -V_P=60mV), NPo $\approx$ 0 and 0.6 prior to and after forskolin, respectively. 200 μM glibenclamide in bath induced channel flickering. As an alternative construct, we expressed CFTR and EGFP on a new vector (pEGFP-C2) resulting in CFTR chloride channels of similar conductance as the fusion protein (γ_{Cl}=5.6$\pm$0.12 pS, -V_P=60mV). We conclude that the apical membrane of mitochondria-rich cells expresses a CFTR homologue with a conductance that is smaller than CFTR in amphibian acinar cells (γ_{Cl}=10.3$\pm$0.3 pS) [2].

[1] Larsen EH, Amstrup J, Willumsen NJ. Biochim Biophys Acta 1618: 140-152, 2003.
[2] Sørensen JB, Larsen EH. J.Gen.Physiol 112:19-31, 1998. Supported by DNSRC and The Carlsberg Foundation.

P-275

Inhibition of nonpregnant human uterine contractions: new in vitro pharmacological approaches

A. Pedzinska, B. Modzelewska, A. Kostrzewska
Department of Biopysics, Medical University of Bialystok, Mickiewicza 2A, 15-089 Bialystok, Poland.

The numerous physiological mechanism that control myometrial contractility have led to the elaboration of various therpeutic methods. Of the various pharmacotherapeutic alternatives, beta2-adrenoceptor agonists are applied most frequently, even though their efficacy is not fully satisfactory. The more recently described beta3-adrenoreceptor has many functions in different human tissues but intimately linked to smooth muscle relaxation in gastrointestinal and respiratory tract or vascular systems. To our knowledge there are only two papers reporting beta3 -adrenergic receptors in myometrial pregnant tissue. This recently published data reported expression of beta3 -adrenergic receptor mRNA in human myometrium from pregnant uterus and assessed the effects of beta3 -adrenergic agonists on isolated human myometrial contractions. Human nonpregnant myometrium was obtained from non-pregnant women undergoing hysterectomy for benign gynaecological disorders. To investigate the effect of a selective beta3-adrenergic agonists the contractility of uterine strips was recorded under isometric conditions. The main parameter to measure drug effects was area under curve. We also used basic force, amplitude of contraction, frequency of contractions and duration of contraction. The purpose of this study was to investigate the effects of a known selective beta3- adrenergic agonist on the contractility of human nonpregnant uterine smooth muscle in vitro, and to compare the effects with those of the known beta2-adrenergic agonist ritodrine.

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Electrical properties and voltage-gated ion-channels of the snail, Helix pomatia salivary gland cell

Z. Pirger, K. Elekes, T. Kiss
Dep. Exp. Zool., Balaton Limnological Research Institute, Hungarian Academy of Sciences, Tihany, Hungary

Applying current-clamp technique a homogeneously distributed membrane potential (-56.6 mV) of the *Helix pomatia* salivary gland cells of the acini have been shown, suggesting a strong electrical coupling between the cells. Low resistance electrical coupling was demonstrated using two microelectrodes inserted in cells separated from each other by a distance of 0.7 mm. The coupling ratio was found to be of 0.1. Ultrastructural studies revealed the occurrence of different types of intercellular contacts, such as desmosomes and gap junctions between the cells in the acini. Using an immunohistochemical approach the expression of the innexin2-like protein was identified along the gland cell membranes. The resting membrane potential was mainly determined by the potassium electrochemical gradient and, in addition, by the contribution of an electrogenic Na-pump. Electrical stimulation of the salivary nerve elicited depolarisation, which could be mimicked by extracellular application of Ach, DA, 5HT. It is suggested that the depolarisation evoked by nerve stimulation or putative transmitters is accounted for the secretory potential. Using one microelectrode voltage-clamp technique four major conductances were identified and characterised as follows: I_K, I_A, $I_{K(Ca)}$ and I_{Ca}. One or more of these conductances may give rise to a stimulus activated secretory potential leading to the excitation-secretion coupling. Others may play a role in the mechanisms of the release of secretory materials, including the apoptotic way of release of mucus.

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Phosphatidic acid inhibits GTPγS binding activity of Gαo by its direct interaction with

L. Qu, Y. Cao, Y. Huang
National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

G proteins consisting of G alpha and G beta-gamma play a pivotal role in G protein-coupled signal transmembrane transduction. At present interaction between phosphatidic acid (PA) and Go alpha has not yet been reported. For this purpose, the assays of ^{35}S-GTPrS binding activity and the binding of purified Go alpha to liposomes made of phospholipids were performed. The results indicate that PA inhibits the activity of the Go alpha specially and dose-dependently with an IC_{50} of 50uM and the maximum inhibition is over 90%, since the other phospholipids (PC, PS, PE, PG) did not. To further elucidate the interaction, the effects of PA analogs (DPPA, DOPA and LPA) on the Go alpha were analyzed. While DPPA and DOPA differed little in inhibition, lysophosphatidic acid (LPA), with only one fatty acid chain, shows potent inhibition with an IC_{50} of 15uM. The binding results of Go alpha to liposomes show that Go alpha specially and directly interact with PA/LPA, while not significantly bound to other lipids. The preliminary results indicate that conformation of Go alpha with LPA/PA is different from that caused by other lipids as detected by CD spectroscopy and endogenous fluorescence change. We proposed that the specific inhibition of PA/LPA on Go alpha might be related to the conformational change caused by the direct interaction between PA/LPA and the Go alpha. The mechanism on the inhibitory effect of PA/LPA is still further undertaken by the mutagenesis of the Go alpha.

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– Channels and Receptors –

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Phosphatidic acid inhibits GTPγS binding activity of G o alpha by its direct interaction with

L. Qu, Y. Cao, Y. Huang

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

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P-279

Cortex neurotoxicity following exposure to agonist and antagonists of ionotropic glutamate receptors

L. L. Radenovic[1], V. M. Selakovic[2]

[1]Department of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, [2]Institut for Medical Research, Military Medical Academy, Belgrade, Serbia and Montenegro

Increasing evidence suggests that glutamate neurotoxicity is partly mediated by reactive oxygen species, formed as a consequence of several processes, which in turn modulates glutamate transmission. We investigated superoxide production and the activity of mitochondrial manganese-superoxide dismutase (MnSOD) after kainate, non-NMDA receptor agonist (0.5 mg/ml, pH 7.2), kainate with CNQX, non-NMDA receptor antagonist (0.5 mg/ml, pH 7.2) and kainate with APV, NMDA antagonist (0.5 mg/ml, pH 7.2), injection unilaterally into CA3 region of the rat hippocampus. The measurement took place in the ipsi- and contralateral forebrain cortex, at different times (5 min, 15 min, 2 h, 48 h, 7 days) following intracerebral drug injection. Used glutamate antagonists APV and CNQX both expressed sufficient neuroprotection in sense of decreasing superoxide production and increasing MnSOD levels, but with differential effect in mechanisms and time dynamics. Our findings suggest that NMDA and AMPA/kainate receptors are differentially involved in superoxide production. Following intrahippocampal antagonists injection they, also, interpose different neuroprotection effect on the induction of MnSOD activity in forebrain cortex.

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Contractile proteins participate in chemosignal transfer of plant microspores

V. V. Roshchina

Institute of Cell Biophysics RAS, Pushchino, Moscow Region, 142290, Russia, E.mail: roshchina@icb.psn.ru

Participation of actin and tubulin linked with Na^+, K^+ and Ca^{2+} ion channels in a transduction of chemosignal (neurotransmitters acetylcholine, dopamine and serotonin or hydrogen peroxide and *tert*-butyl peroxide) from cellular surface inside to organelles has been shown on plant vegetative microspores of *Equisetum arvense* and pollen of *Hippeastrum hybridum*..

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The study of calcium-dependent protein/membrane association using supported biomimetic membranes

C. Rossi[1], D. Ladant[2], J. Chopineau[3]

[1]UMR 6022 CNRS, U T C, BP 20529, 60205 Compiègne, [2]URA 2185 CNRS , Institut Pasteur, 75724 Paris, [3]CBS, CNRS, Centre Universitaire de Nîmes, 30000 Nîmes

Two different types of supported biomimetic membranes were designed to study the membrane binding properties of two different proteins that both interact with cellular membranes in a calcium-dependent manner. The first one, neurocalcin is a member of a subfamilly of EF-hand calcium-binding proteins that exhibit a calcium-myristoyl switch. The second protein is a bacterial toxin, the adenylate cyclase produced by *Bordetella pertussis*, the causative agent of whooping cough. The biomimetic membranes were either hybrid bilayer membranes or polymer tethered membranes. The biomimetic membrane constructions were followed by surface plasmon spectroscopy while membrane fluidity and continuity were observed by fluorescence microscopy. Protein/membrane binding properties were determined by resonance surface plasmon measurements. The tethered bilayer, designed here, is very versatile as it can be adapted easily to different types of support. The results demonstrate the potentialities of such polymer-tethered artificial membrane for the study of proteins that insert into biological membranes such as toxins and/or integral membrane proteins.

claire.rossi@utc.fr ; joel.chopineau@unimes.fr

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– Channels and Receptors –

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Ion channel properties of OmpF in micro- and nano-BLMs

E. K. Schmitt, C. Steinem
Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Germany

The protein OmpF, a general porin of the outer membrane of *Escherichia coli*, forms channels permeable for ions and small polar molecules. In order to investigate the ion channel activities of this integral membrane protein, artificial membranes, which are exposed to an aqueous phase on both sides are desirable. Classical black lipid membranes (BLMs) serve this purpose but the lack of stability limits their use. Thus our research focuses on micro- and nano-BLMs, lipid bilayers, that suspend the pores of a porous material, which combine the stability of solid supported bilayers and freestanding lipid membranes. As porous material, we use porous silicon and aluminum oxide with pore sizes of 5 μm and 200 nm, respectively. To obtain micro- and nano-BLMs one side of the porous material is coated with a thin gold layer followed by chemisorption of 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (DPPTE). The hydrophobic monolayer on top of the upper surface is a prerequisite for the formation of suspending membranes. The pore suspending membranes are analysed in terms of their capacitive and resistive behaviour by means of impedance spectroscopy. OmpF is reconstituted into these novel artificial membranes and its activity is investigated depending on the pore size of the porous material. The ion channel properties of the protein are measured by voltage clamp and conductivities are expected to be in the range of 1.4 nS.

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Single-channel analysis: Two pairs of rate constants determine current reduction in the K+/Tl+-AMFE

I. Schröder, U.-P. Hansen
Center of Biochemistry and Molecular Biology, University of Kiel, 24098 Kiel, Germany

The effect of Tl^+ on single channel behaviour was investigated in native K^+ channels in *Chara* and in human MaxiK channels expressed in HEK-cells. In *Chara*, an AMFE (anomalous mole fraction effect) was found. Farokhi et al. (2000) could show that the apparent current reduction is caused by averaging over gating effects normally hidden by temporal resolution. Such a current reduction was not found in human MaxiK. Nevertheless, gating analysis based (Schröder et al. 2005) on a Markov model (AOGCZ) with two open (A, O) and three closed (G, C, Z) states showed that the basic mechanism of Thallium action was the same, i. e., a shift in the occupation probability of A and O. In either case, the ratio of the rate constants of the transitions between these states was changed by a factor of 50-200. The absence of the AMFE in human MaxiK is due to the rate constant k_{OG} which is 5- to 30-fold slower than in *Chara*, whereas k_{GO} is of the same order of magnitude. Increasing k_{OG} to the value in *Chara* induces apparent current reduction in simulated time series also of this human MaxiK model. The gating related to the AMFE is compared with the gating causing a negative slope in the MaxiK IV-curve at positive membrane potentials.

Farokhi et al. 2000, Biophys. J. 79 : 3072-3082
Schröder et al. (2005) J. Membrane Biol. 202: (in press)

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Single-channel analysis: New approaches for jump detection and temporal resolution

I. Schröder, U.-P. Hansen
Center of Biochemistry and Molecular Biology, University of Kiel, 24098 Kiel, Germany

Kinetic analysis of single-channel recordings has to cope with a serious problem, i.e., detection of jumps between different current levels. Noise and filtering limit temporal resolution. We present two extensions of classical analysis: 1. The generation of distributions per level. 2. Improvements of the direct fit of the time series. The benefits of splitting the overall amplitude histogram into histograms per level are: visualization of the errors of jump detectors, revealing of small deviations from gaussian distributions (indicating fast gating), distinction between different putative Markov models. In the second approach, the direct fit of the time series (HMM-fit) is followed by a fit of the amplitude histogram (Beta fit), and simulations are employed to generate higher-order beta distributions. Simulation studies with two and five Markov states show that this approach enables the detection of very slow and very fast dwell times existing in one record. Both approaches can reveal rate constants above 1 μs^{-1} at a filter frequency of 50 kHz. The high resolution is shown to be necessary to reveal fast gating as the origin of sublevels, negative resistance or current reduction in the AMFE (see second poster of Schröder and Hansen).

Schröder et al. (2004) J. Membrane Biol. 197: 49-58
Schröder et al. (2005) J. Membrane Biol. 202: (in press)

P-285

Ca-activated potassium channels drive agonist-stimulated NO synthesis in human endothelial cells

J.-Z. Sheng, A. P. Braun
Dept of Pharmacology and Therapeutics, University of Calgary, Calgary, AB, Canada

The production of nitric oxide (NO) by the vascular endothelium affects hormone-induced vasodilation, platelet adhesion and angiogenesis. Pharmacologic studies have demonstrated that agonist-stimulated vasodilation can be largely prevented by apamin and charybdotoxin (ChTx), selective inhibitors of small and intermediate conductance, Ca-activated K^+ channels (SK_{Ca} and IK_{Ca} channels), respectively. Both types of channels have been recently identified in vascular tissue, however, the cellular mechanism(s) by which they contribute to vasorelaxation remains controversial. We have hypothesized that activation of endothelial SK_{Ca} and IK_{Ca} channels by Ca-mobilizing stimuli serves as a critical step in the cellular pathway leading to NO production. Experimentally, agonist-induced changes in membrane potential or SK_{Ca} and IK_{Ca} channel currents were performed in single patch clamped HUVECs loaded with either DAF-FM or Fluo-3 to monitor NO synthesis or cytosolic $[Ca^{2+}]$, respectively. Selectively inhibition of SK_{Ca} and IK_{Ca} channels by apamin and ChTx prevented NO synthesis, membrane hyperpolarization, and increased cytosolic $[Ca^{2+}]$, in response to either histamine or ATP. Similar effects were produced by 80 mM external K^+ or removing external Ca^{2+}. However, agonist-stimulated NO synthesis was unaffected by 50 μM Ba^{2+} and 100 μM ouabain. These data suggest a mechanistic model in which endothelial SK_{Ca} and IK_{Ca} channels 'drive' NO production in response to Ca^{2+} mobilizing agonists via membrane hyperpolarization.

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– Channels and Receptors –

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Kinetics of gramicidin channel-formation in muscle fibre membrane

N. Shvinka
Institute of Medical Education (LUMPI), Skolas Str. 1a, Riga, Latvia, LV-1010

The aim of our experiments was to study the changes of cation conductance and efflux induced by gramicidin A and synthetic covalently linked gramicidin dimer ($HOCH_2CH_2NH–CO(CH_2)_2CO–NHCH_2CH_2OH$) in frog muscle membrane to characterize the kinetics of channel-formation. Efflux was studied on whole sartorius muscles by means of flame emission photometry. Conductance was measured on single frog skeletal muscle fibres under current-clamp conditions using a double sucrose-gap technique. Gramicidin A ($2.5\times10–^7–1\times10–^6$ mol/l) increased the rate constants of potassium and rubidium effluxes but does not influence the effluxes of sodium and lithium. Gramicidin A and its dimer increased the potassium conductance due to newly formed gramicidin channels. At room temperature the channel-formation by gramicidin was much faster than that of the head-to-head covalently linked gramicidin dimer. The increase of temperature by 8–10 °C resulted in a considerable rise of both gramicidin- and gramicidin dimer-induced conductances. The temperature dependence of adsorption was more pronounced than that of desorption. Free energy of channel formation ranged from 0.140 to 0.163 kJ/mol for monomer and from 0.126 to 0.221 kJ/mol for dimer. The effect of temperature on adsorption kinetics was much greater than in the case of bilayers, indicating a remarkable entropy change in muscle fibre membrane.

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Cardiac calcium handling proteins studied with a novel high resolution colocalization algorithm

C. Soeller, X. Song, R. Gilbert, M. Cannell
Department of Physiology, University of Auckland, New Zealand

The close apposition of surface and SR membranes in the region where cardiac excitation-contraction (E-C) coupling takes place profoundly alters free Ca^{2+} levels so that 'local control' of E-C coupling is now a central theme. The subcellular gradients characteristic of local control could result from the close proximity between proteins involved in moving Ca^{2+} and (and other ions). We have therefore investigated the colocalization of several key E-C coupling proteins using high-resolution antibody mapping. Isolated rat ventricular myocytes labeled with antibodies against ryanodine receptors, L-type Ca^{2+} channels and SERCA2a were imaged by confocal microscopy. The data was enhanced by digital deconvolution to improve signal-to-noise ratio and resolution. The resulting data was analyzed using novel colocalization algorithms. While previous reports have generally determined global whole cell colocalization indices we developed techniques to calculate colocalization at nearly the full resolution of the input data. Our results suggest that the degree of protein colocalization varies throughout the cell from regions of almost complete signal overlap to negligible proximity. This data implies the existence of spatial heterogeneities in E-C coupling and should also provide input data for spatial models of cardiac Ca^{2+} handling. Our novel techniques for high-resolution proximity analysis should also be widely applicable to other biological tissues.

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Membrane diffusion of oxygen: bare lipid bilayers versus membranes reconstituted with aquaporin-1

V. S. Sokolov[1], A. G. Ayuyan[2], A. Lenz[1], E. Sokolenko[1], B. Wiesner[2], P. Pohl[3]
[1]Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Science, Moscow, Russia, [2]Forschungsinstitut für Molekulare Pharmakologie, Campus Berlin-Buch, Robert-Rössle-Str. 10, D-13125 Berlin, Germany, [3]Institut für Biophysik, Altenberger Str. 69, A-4040 Linz, Austria

Oxygen permeation across the cell plasma membrane is believed to occur so fast that it is not rate-limiting for cellular respiration. Because of their high oxygen content, membranes have been hypothesised to be involved in lateral transport of oxygen. As we show here by confocal fluorescence spectroscopy, fluorescence correlation spectroscopy, and measurements of intrinsic membrane potentials, lateral diffusion may be the prevailing mode of transport for bare planar lipid bilayers. Only a tiny amount of $^1O_2$ reacted with reporter molecules at the inner membrane leaflet after being released by a photosensitiser adsorbed to the outer membrane leaflet. Monitoring lipid peroxidation revealed the same result. If located exclusively in the inner leaflet of an asymmetric planar bilayer, unsaturated lipids were protected from peroxidation. $^1O_2$ was not able to cross even the outer leaflet. However, $^1O_2$ transmembrane diffusion was facilitated by reconstitution of the water channel protein aquaporin-1.

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Extended point-contact model of the cell-transistor coupling

F. Sommerhage[1], G. Wrobel[1], S. Ingebrandt[1], U. B. Kaupp[2], C. Fahlke[3], A. Offenhäusser[1]
[1]Institute of Thin Films and Interfaces (ISG2), Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany, [2]Institute of Biological Information Processing (IBI1), Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany, [3]Institute of Physiology, University Hospital Aachen, D-52074 Aachen, Germany

The coupling of electrogenic cells to non-metallized field-effect transistors (FET) has been subject of intense studies during the past years. Recently, the interest of this non-invasive technique has grown, because it holds great promise for biomedical studies. Understanding the recorded FET signal shapes is important for the application of cell-transistor hybrids.

To advance the knowledge of the recorded FET signals, we coupled HEK293 cells that expressed either voltage-gated K^+- or Cl^- channel to FETs. Membrane currents of cells, which adhered directly on a FET-gate, were simultaneously recorded with transistors and patch-clamp pipettes.

The FET signals clearly showed an electrical and an electrodiffusive signal component. Here we demonstrate, that the FET signal shape could be quite accurately simulated with an extended Point-Contact Model (PCM): the standard PCM, which describes the FET signals in terms of an equivalent electrical-circuit, and an additional electrochemical calculation, which describes the accumulation or depletion of ions in the small cleft between the FET surface and the attached cell.

Posters

– Channels and Receptors –

P-290

Epithelial sodium channel stoichiometry

A. Staruschenko[1], E. Adams[1], R. E. Booth[2], J. D. Stockand[1]
[1]Univ Texas Health Sci Centr San Antonio, [2]Texas State Univ

Ion channels, including the epithelial Na^+ channel (ENaC), are intrinsic membrane proteins comprised of component subunits. ENaC is comprised of three subunits, alpha, beta and gamma. For maximal ENaC activity, each subunit is required. The subunit stoichiometry of functional ENaC within the membrane remains uncertain and controversial. We combined fluorescence intensity ratio (FIR) analysis and fluorescence resonance energy transfer (FRET), used to assess relative subunit stoichiometry and whether the channel contains more than one copy of each type of subunit, respectively, with total internal reflection fluorescence (TIRF) microscopy, which enables isolation of plasma membrane fluorescence signals, to determine the limiting subunit stoichiometry of ENaC within the plasma membrane. Our results demonstrate that membrane ENaC contains equal numbers of each type of subunit and at least two copies of each type of subunit. Moreover, we find that at steady-state, subunit stoichiometry is fixed. Electrophysiological results testing effects of ENaC subunit dose on channel activity, co-immunoprecipitation experiments and resolution of ENaC mass on native gels were consistent with TIRF-FIR and FRET findings. We conclude that membrane ENaC contains equal numbers of each channel subunit possibly at a 2:2:2 or 3:3:3 stoichiometry.

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Ion-induced conformational changes in channels. Order and Chaos

M. E. Starzak
State University of New York at Binghamton, Binghamton, NY, USA

Conformational changes at sites in a membrane channel are induced by permeant ions passing the site so that the probability of the transition is proportional to the ion current in the channel. Since the rate parameters for the conformers can be different, each conformer at a site can produce a different net current through the channel. The channel can then produce a series of discrete current levels directly proportional to the ion injection current. First order conformational transitions, however, produce an output current-injection current ratio independent of the injection current. Non-linear conformation change kinetics are generally unstable; the channel produces irregular current oscillations produced by rapid kinetic transitions between the site conformers. The kinetic restrictions necessary to produce stable multi-level transitions with increasing injection current are discussed. The nature of the irregular currents observed for unstable systems are analyzed as well.

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Interplay between huntingtin and syntaxin 1A regulates N-type calcium channels

L. A. Swayne, L. Chen, M. A. Colicos, G. W. Zamponi, J. E. Braun
Hotchkiss Brain Institute, Cellular and Molecular Neurobiology Research Group, University of Calgary, Calgary, Alberta, Canada

We have discovered an association between the N-type calcium channel, a calcium channel central in neurotransmitter release, and huntingtin. The synprint region located in the domain II-III linker of the N-type channel $Ca_v2.2$ subunit interacts with huntingtin^{exon1} containing both wildtype and mutant numbers of glutamine repeats. The native proteins coimmunoprecipitate from transfected HEK cell lysates and rat and mouse brain homogenates. The association of huntingtin^{exon1} with the synprint region perturbs the well-characterized syntaxin 1A-mediated effects on both steady-state inactivation and G protein inhibtion. Analysis of the huntingtin^{exon1}- and syntaxin 1A-binding properties of synprint reveals that, *in vitro*, these proteins bind to overlapping sites on, and compete with one another for association with $Ca_v2.2$ synprint. Immunofluorescence in hippocampal neurons confirms the presynaptic localization of the $Ca_v2.2$ and full length huntingtin. Further examination of huntingtin N-terminal fragments suggests enhanced localization of these fragments in axonal varicosities. Our results suggest a novel pathway for modulation of calcium fluxes through N-type calcium channels.

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Single molecule analysis of the binding of cardiac ryanodine receptor and ryanodine

Y. Takeuchi, T. Ichikawa, T. Aoki, T. Ide
JST, Laboratory for Nanobiology , Graduate School of Frontier Bioscience, Osaka University, 1-3 Yamada-oka, Suita, Japan.

Ion selectivity and the gating are two essential properties of ion channels. The study of ion selectivity has been in much progress by the development of X-ray structure analysis. However, the conformational changes of channel proteins accompanied by the change of activity have not been directly detected yet. Channel gating is regulated by the voltage or binding of specific ligands or blockers. For analysis of the gating mechanism, the biding constants for the average of a large number of ligands or blockers have been used until now. However, in such cases, the dynamic, kinetic and fluctuating properties of biomolecules remain hidden.
In this study we have used total internal reflection fluorescence (TIRF) microscopy to visualize single channel proteins in lipid vesicles on the glass surface to observe the interaction between channels and ligands.
We have also developed a novel method for simultaneous measurement of the optical and electrical properties of single-channel protein. In this study, we have succeeded for the first time the simultaneous observation of the Ryanodine receptor(RyR) isolated from excitable membranes.
These innovative technology will greatly advance the study of channel proteins as well as signal transduction processes that involve ion permeation processes.

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– Channels and Receptors –

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Biophysical characterization of the human mu opioid receptor purified from Pichia pastoris

F. Talmont, I. Muller, A. Milon
Institute of Pharmacology and structural biology, 205 route de Narbonne 31077 Toulouse, France

The first aspect of our work consists in the development of methods for the overexpression and purification of G-protein coupled receptor (GPCR) in order to produce milligrams of pure receptor which are required for structural biology. In this context we are mainly interested with GPCRs involved in pain perception (opioid receptors). The second part of our work is the establishment of physical and chemical conditions allowing the renaturation of unfolded membrane proteins initially produced in inclusion bodies. In collaboration with J. Kleinschmidt (University of Konstanz, Germany) and J.L. Popot (IBCP, Paris) we are studying receptor refolding in detergents micelles, amphipols and in lipid bilayers. The characterization of refolding is performed using biophysical techniques such as circular dichroism, mass spectrometry, dynamic light scattering, and fluorescence spectroscopy as well as pharmacological binding experiments. The availability of non limiting quantities of receptors is essential to perform such biochemical, molecular and spectroscopic studies. We plan to use NMR methods (transferred NOE, solid state MAS NMR, TROSY) to determine the conformation of ligands in their binding site. Spatial organization of the secondary structures of the receptors will also be studied by NMR after isotopic labeling of the proteins.

P-295

BmK I, an Alpha-Like Scorpion Toxin Enhances Persistent Current of Rat Brain Sodium Channel Type II expressed in Xenopus oocytes

M. Tan, M. M. Zhu, Y. H. Ji
YonghuaJi lab, Chinese Academy of Sciences, P.R. China

In the present study, the selective role of BmK I, an alpha-like scorpion toxin on the rat brain Na^+ channel type II functionally expressed in Xenopus oocytes has been investigated using the two electrodes voltage clamp. The results showed that the transient current I_{Na} was not changed any more, however, an interesting sodium current, called as the persistent current I_{NaP} activated near -60 mV, was recorded and persistent at negative potentials.
The new finding fruited in the study indicates that á-like scorpion toxins such as BmK I may modulate the rat brain Na^+ channel type II with a distinct way. Thus BmK I may provide as a valuable tool for the investigation of the persistent currents.

P-296

The spider peptide GsMTx-4 inhibits stretch-activated BK channel as a negative gating modifier

Q. Y. Tang[1], K. Naruse[2], M. Sokabe[2]
[1]ICORP/SORST Cell Mechanosensing, JST. Japan, [2]Dept.Physiol. Nagoya Univ. Grad. Sch. Med. Nagoya, Japan

GsMTx-4, a spider peptide, was reported to be a candidate for a specific inhibitor of mechanosensitive ion channels (MSCs), yet little is known on its inhibition mechanism. To address this problem, we examined the effects of GsMTx-4 on the Stretch-Activated and Ca-activated big K channel (SAKcaC) in chick heart (Tang, et al., J Membr Biol, 2003) by use of the back-fill method with excised inside-out patches. The open probability (Po) of SAKcaC was significantly reduced by nM range of GsMTx-4, irrespectively of suction in the pipette. This inhibition was caused by a rightward shift of Po-V curves of SAKcaC without changing the single channel conductance, while suction in the pipette shifted leftward the curve to antagonize the peptide effect. These results indicated that membrane stretch positively modulates, while GsMTx-4 negatively modulates the gating of SAKcaC. Analyses of gating kinetics suggested that GsMTx-4 may interact with a certain site other than the voltage sensor and Ca^{2+} bowl in the channel. As we found that the sensitivities of SAKcaC to stretch and GsMTx-4 required 59 amino acids sequence called STREX in the c-terminus and an associated membrane protein (AMP), they may be the target of GsMTx-4. However, the positively charged peptide GsMTx-4 would not pass through the bilayer to directly interact with STREX, we speculate that GsMTx-4 may bind AMP and impair the interaction between AMP and STREX resulting in a negative modulation of SAKcaC gating.

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Additive modulatory effects of extracellular pH and zinc ions on potassium channels Kv1.3

A. Teisseyre, J. W. Mozrzymas
Medical University, Department of Biophysics, ul. Chałubińskiego 10, 50-368 Wrocław, Poland

We applied the whole-cell patch-clamp technique to study the influence of extracellular pH (pH_o) on the modulatory effect exerted by zinc ions (Zn) on voltage-gated potassium channels Kv1.3 expressed in human lymphocytes. Lowering of pH_o from 7.35 to 6.4 shifted the activation midpoint by 16 mV towards positive membrane potentials, reduced the current amplitude by 50% and slowed the current activation rate. Application of 300 μM Zn at pH_o=6.4 shifted additionally the activation midpoint by 33 mV, reduced the current amplitude by 50% and slowed the activation rate. Both the magnitude of the shift and the degree of current inhibition by 100, 300 μM and 1 mM Zn was comparable at pH_o=6.4 and 7.35. Raising of pH_o from 7.35 to 8.4 did not affect the current significantly. It also had no effect on the shift of the activation midpoint and reduction of the current amplitude upon application of 100 μM and 1 mM Zn. Changing the holding potential from –90 mV to –60 mV abolished the inhibition of current amplitudes at Zn concentrations below 300 μM. The inhibitory effects caused by Zn and protons at pH_o=6.4 were also diminished when changing the holding potential, however to a lesser extent. In contrast, changing the holding potential did not afftect the Zn- and proton-induced shift of the voltage dependence of the activation midpoint. In conclusion, lowering of pH_o augments the modulatory effects exerted by Zn on Kv1.3 channels.

Posters

– Channels and Receptors –

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Molecular dynamics of migration of ions and complexes through the glycine receptor TM2 domain

K. B. Tereshkina, K. V. Shaitan
Moscow State University, Faculty of Biology, Dept. of Biophysics, 119992 Moscow, Russia

In the present work the structures of natural and modified homomeric glycine receptors were studied. Extended TM2 domains were studied as functionally critical for ion migration. The structure of natural receptor TM2 domain was considered to have two positively charged rings (Asp) and one negatively charged ring (Arg). These rings are important at channel conductance [1].
Steered MD was used that including standard parameters [2] and a constant force 5kcal/(mole.A) applied to the ion. Three types of migration were found: free migration, stop due narrow channel and attraction to charged atoms of the channel. Only negatively charged ions passed through the channel. The Arg residues play an important role during migration of negatively charged ions, except for I^- that are attracted to Gln. For positive charged ions it is not possibly to detect residues that influence ion trajectory predominantly. Diffusion coefficient (depend on a part of the channel) for Cl^- are 1.38 10^{-5}-1.45 10^{-6}, Br^- 1.25 10^{-5}-3.25 10^{-6}, F^- 1.66 10^{-6}-1.47 10^{-6}, I^- 2.04 10^{-6}-3.63 10^{-6} cm^2/ps. Thus, the most rapid diffusion was detected for Cl^- and Br^-. Cl^- was found to be bound to residues of receptor less time in comparison with other ions. [1] A Keramidas, et al. // Biophys. J. (2000), 78, 247-259. [2] KB Egorova, et al. // IJQC (2004), 94, 219-225. This work was supported by MES of Russia (prs. No 0431, 01.106.11.0001, 01.165.11.0001); RFBR (pr. No 04-04-49645).

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Analogs of lysophosphatidic acid as novel PPAR-gamma ligands

J. Tigyi[1], G. G. Durgam[2], V. Gududuru[2], T. Tsukahara[3], S. Yasuda[3], T. Virag[3], D. M. Walker[3], N. Makarova[3], D. D. Miller[2], P. Allison[4], H. Yuan[4], A. Parrill[4], G. Tigyi[3]
[1]Inst. of Biophysics, University of Pecs, Hungary, [2]Dept. of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN USA, [3]Dept. of Physiology, University of Tennessee Health Science Center, Memphis, TN USA, [4]Dept. of Chemistry, University of Memphis, Memphis, TN USA

Lysophosphatidic acid (LPA), a pleiotropic phospholipid growth factor, exerts its biological effects through the activation of three-EDG family G protein-coupled receptors (GPCR) and the transcription factor PPARγ. Computational docking of LPA to the crystal structure of PPARγ identified distinct differences in its interaction with the ligand binding pocket as compared to rosiglitazone. Site-directed mutagenesis confirmed that R288 is uniquely important for LPA binding but has no effect on that of rosiglitazone. H323 and H449 were essential for rosiglitazone binding but not that of LPA. Alkyl ether and unsaturated fatty acyl analogs of LPA are agonists of PPARγ and LPA GPCR, whereas LPA with saturated fatty acids and cyclic phosphatidic acid do not activate PPARγ. To achieve a better understanding of the physiological and pathological role of these receptors, we describe the pharmacological characterization of analogs based on three LPA-like scaffolds, fatty alcohol phosphates, short chain phosphatidates and acetalphosphates. Modifications of these scaffolds have led to analogs that distinguish between PPARγ and the different LPA GPCR subtypes. Supported by NIH-CA 92160

P-300

Trafficking Defective SCN5A Brugada Syndrome Mutations Rescue by different Class I Antiarrhythmic drugs

C. R. Valdivia, K. M. Ruwaldt, J. C. Makielski
University of Wisconsin-Madison

Mutations in the cardiac Na channel gene, *SCN5A,* that cause decreased Na current underlie arrhythmia syndromes such as the Brugada syndrome (BS). We reported that a BS mutation G1743R could be "rescued" by mexiletine (500 uM). Are other BS mutations rescued by this or other antiarrhythmic drugs? Five previously reported trafficking defective BS mutations (R367H, R1232W, R1232W/T1620M, R1432G and G1743R) were engineered into the SCN5A, transiently transfected into HEK-293 cells and incubated for 24 hours with or without the antiahythmic drugs: Quinidine (QUIN), Mexiletine (MEXI) and Flecainide (FLEC), as representatives of the Class Ia, Ib, and Ic (respectively). Voltage clamp was performed in the absence of drug. The BS mutations had negligible Na current compared to wild type (WT). Except for R367H that was not rescued by any drug, the BS mutations significantly increased the Na^+ current density after incubation with MEXI compared to WT in MEXI. However, only three out of the five mutants responded to drug rescue with QUIN or FLEC. Also, the increase in Na current was still less than WT. Location of these BS mutations may be important for drug binding interaction as a mechanisms of drug rescue, because only the mutations located in extracellular loops of SCN5A were rescue by the antiarrhythmic drug, and R367H which hides deep inside the P loop of domain I was not rescued. More practically, these findings may have implications for possible novel therapy to correct the Na channel trafficking defect in some BS patients.

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Physical properties of lipid monolayers at presence of gramicidin A

P. Vitovič[1], M. Weis[2], P. Tomčík[2], J. Cirák[2], T. Hianik[1]
[1]Laboratory of Biophysics, FMFI UK, Mlynská dolina F1, 842 48 Bratislava, Slovakia, [2]Department of Physics, FEI STU, Ilkovičova 3, 812 19 Bratislava, Slovakia

We studied the electrical and mechanical properties of monolayers composed of pure model peptide gramicidin A (GRA) as well as lipid monolayers containing various mole ratio of GRA/Phospholipid. The area-pressure isotherms were measured both in gel and liquid–crystalline state. The measurements of area-pressure isotherm of pure GRA monolayers confirmed results previously obtained by the other authors. In addition to these studies we for the first time measured the changes of Maxwell currents flowing between the surface of monolayer and ground, caused by reorientation of dipole moments of the monolayer. We have shown, that after initial growth of the current during monolayer compression at relatively large area per molecule, the growth was changed by decrease of the current at area per molecule around 4.7 nm^2. This effect is the evidence of the GRA dipole moments orientational changes and changes of the secondary structure of the molecule, that switches to double helix conformation. GRA affect the mechanical and thermodynamical properties dimyristoylphosphatidylcholine (DMPC) monolayers. Based on the L-B isotherms of mixed DMPC-GRA monolayers below and above phase transitions of phospholipids, we confirmed that aggregation of GRA exists both in liquid-crystalline and in a gel state of the monolayer and take place at DMPC:GRA mole ratio 3:1. This is in good agreement with the results obtained by Diociaiuti et al. (Biophys. J. 82 (2002) 3198).

Posters

– Channels and Receptors –

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Inverse regulation in axonal and somatic plasticity

J. H. Wang
Institute of biophysics, Chinese Acdemy of Sciences, Beijing, China

N-terminal fragments of staphylococcal nuclease (SNase) with different chain lengths were used as a model system in the folding study. The detailed characterization of conformational states of 1-79 and 1-110 residues SNase fragments (SNase79 and SNase110) and their V66W and G88W mutants can provide valuable information on the development of conformations in the folding of SNase fragments of increasing chain lengths in *vitro*. In this study, the presence of retained capacity for folding and residual structures in SNase79 and SNase110 is detected by CD, fluorescence, FTIR, and NMR spectroscopy. SNase79 is represented as an ensemble of interconverting conformations. The fluctuating nascent helix- and â-sheet-like structures, localized in regions of A58-A69 and T13-V39, respectively are transiently populated in SNase79. The native-like tertiary conformations are obtained for G88W110 and V66W110 and for SNase110 in the presence of 2.0 M TMAO. Analysis of the results of such studies indicate that folding of SNase fragments is dominated by developing the local and non-local nucleation sites from native-like secondary structures and by intensifying the long-range interactions of residues at nucleation sites with residues further removed in sequence.

P-303

A new mechanistic model for Interleukin-4 receptor signaling across the lipid bilayer

T. Weidemann, S. Höfinger, M. Auer
Novartis Institutes for BioMedical Research Vienna, Brunnerstr. 59, A-1235 Vienna

The Interleukin-4 receptor (IL-4R) is a single-pass transmembrane receptor of the hematopoietin receptor superfamily. This receptor class is defined by the cytokine binding module which contains the highly conserved WSXWS motif in the membrane proximal fold. By including the membrane itself as a structure inducing determinant, we propose the WSEWS motif of the IL-4R (amino acids 187-191) to function as a rotational toggle switch. Essential elements of the model are (1) a striking 3-fold symmetry of the W, S, E residues along the polypeptide backbone, (2) their physicochemical propensity to reside at the membrane interface, and (3) the presence of amphiphilic arginines at the adjacent backbone. Free energy calculations were carried out to estimate the energy necessary for both, a liberation of tryptophan W190 from the arginine stack followed by exposure to a membrane environment. Preliminary results indicate a surprisingly small energy penalty of about +1.0 kcal/mol to tilt W190 away from the interior core of the receptor. A significantly higher free energy contribution of about 5 to 10 kcal/mol was determined for chemical change from water to n-octanol (membrane mimic). In a molecular mechanistic model, IL-4 mediated signaling is resolved into a sequence of molecular rearrangements of the receptor for which implications are thoroughly aligned with published results.

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Insights into the binding of acetylcholine to the acetylcholine receptor by solid-state NMR

P. T. Williamson[1], K. W. Miller[2], A. Watts[3], B. H. Meier[4]
[1]Institut ISIS (CNRS FRE2446), Université Louis Pasteur, 8 rue Gaspard Monge, 67000 Strasbourg, France, [2]Harvard Medical School, Department of Anaesthesia and Critical Care, Massachusetts General Hospital, Boston MA 02114 USA, [3]Biomembrane Structure Unit, University of Oxford, Oxford, OX1 3QU, England, [4]Department of Physical Chemistry, ETH-Zurich, CH-8093 Zurich, Switzerland.

Magic-angle spinning (MAS) and oriented sample solid-state NMR have been employed to characterize the conformation of the high-affinity agonist, acetylcholine whilst resident in its binding site on the nicotinic acetylcholine receptor. Employing MAS, high-resolution spectra have been obtained of the carbon-13 labelled acetylcholine within the agonist binding site. Exploiting recent advances in the determination of dipolar couplings within uniformly labelled systems we have been able to obtain multiple high resolution structural constraints suitable for the structure refinement of the agonist within the binding site. Deuterium NMR of oriented nicotinic acetylcholine receptor membranes labelled with the deuterated agonist analogue bromoacetylcholine indicate the quaternary ammonium group is oriented at $\sim 42^{\circ}$ with respect to the bilayer normal and is relatively restricted within the binding site. These restraints have enabled us to create a model for the acetylcholine within its binding site enabling us to identify putative residues important in the binding of acetylcholine to its receptor.

Posters
– Redox Enzyme Mechanisms –

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Profile of expression of peroxiredoxins in human endothelial cells treated with glucocorticoids

A. Balcerczyk[1], L. Pulaski[2], M. Soszynski[1], C. Kieda[3], G. Bartosz[1]
[1]Department of Molecular Biophysics, University of Lodz, Poland, [2]Center of Medical Biology PAS, Lodz, Poland, [3]Centre de Biophysique Moleculaire, CNRS UPR, Orleans, Cedex, France

Redox balance of endothelial cells is very important, because its disturbance can lead to impaired endothelial function, changes in bioavailability of nitric oxide and consequently to pathological states. We have examined the effect of glucocorticoids (dexamethasone, prednisone and hydrocortisone - anti-inflammatory drugs known to stimulate endothelial cells to produce reactive oxygen species), in the presence and absence of externally added H_2O_2, on the expression of peroxiredoxins (Prx), a family of small antioxidant proteins which can catalyze the reduction of hydrogen peroxide. We have also compared the profile of expression of Prx in primary endothelial cells isolated from umbilical cord, HUVECs and in HUVEC-ST, an endothelial cell line immortalized by telomerase gene transfection.
We found that 48 h of incubation of cells with 100 nM glucocorticoids increases the expression of all peroxiredoxin (Prx1-Prx6) mRNAs in HUVECs, while only a subset of peroxiredoxin genes is upregulated by H_2O_2 treatment in the concentration range of 30-70 μM. The response pattern of peroxiredoxin expression at the protein level was confirmed by Western blotting. In spite of alterations in the expression pattern of peroxiredoxins in endothelial cells, we did not observe any statistically significant changes in total antioxidant capacity and in the level of thiol groups measured in intact cells.

P-306

Pulsed ELDOR study on tyrosyl radical in the dimer R2 of mouse Ribonucleotide reductase

D. Biglino, P. P. Schmidt, E. J. Reijerse, W. Lubitz
Max-Planck-Institute for Bioinorganic Chemistry, D - 45470, Mülheim an der Ruhr, Germany

Ribonucleotide reductase (RNR) is an enzyme devoted to the catalytic reduction of the nucleoside diphosphates into the correspondent deoxynucleoside diphosphates; this reaction is the rate determining step for the duplication of DNA. Mouse RNR belongs to the class Ia and is formed by two homodimeric subunits: R1 and R2. In R2 a tyrosyl radical (Y) and an antiferromagnetically coupled diiron(III) centre is stored. Electron paramagnetic resonance (EPR) has been broadly employed to study the tyrosyl radical in RNR. From quantitative analysis an average of about 1.2 Y per dimer has been found, but in some case 1.7 Y per dimer have been measured. This means that in a substantial amount of protein both radical sites are populated. Pulsed ELDOR (electron electron double resonance) has recently been successfully employed to determine the distance between the two Ys in *E. coli* R2 ([1]). In the case of R2 of mouse RNR, no crystal structure is available under physiological conditions. Therefore information such as the distance between the two radicals are of great importance. 3- and 4-pulse ELDOR spectra have been recorded of R2 mouse samples in both X-band and Q-band and the analysis of spectra provides distance between the two tyrosine radicals of 3.2 ± 0.1 nm.
([1]) Bennati M. et al., *J. Am. Chem. Soc.* **2003** (125), 14988-14989

P-307

Radicals in the *paracoccus denitrificans* cytochrome *c* oxidase

K. Budiman[2], F. MacMillan[1], H. Michel[2]
[1]Institut für Physikalische und Theoretische Chemie, Goethe Universität, Frankfurt, Germany, [2]Max-Planck-Institut für Biophysik, Frankfurt, Germany

Cytochrome *c* oxidase couples the reduction of O_2 to water with the translocation of protons across the mitochondrial or bacterial membrane. The mechanism of electron coupled proton transfer is however still poorly understood. The P_M-intermediate of the catalytic cycle is an oxoferryl-state whose generation requires one additional electron, which cannot be provided by the two metal centres. The missing electron is suggested to be donated to this binuclear site by a tyrosine residue that forms a radical species, and which can be detected in the P_M and F• intermediates. One possibility to produce these intermediates artificially is the addition of H_2O_2 and a tyrosine radical species has been observed using EPR [1]. From activity measurements as well as optical and EPR experiments on several variants the origin of the radical species was found to be Y167 [2]. This result is surprising because Y167 is not part of the active site, where H_2O_2 reacts with the enzyme. Upon inspection of the active site it becomes evident that W272 could be the actual donor of the missing electron, which can then be replenished from Y167 (in the case of the reaction with H_2O_2) or from the Y280-H276 cross link in the natural cycle. The difference between the reaction with H_2O_2 and the natural cycle might be that H_2O_2 carries two electrons plus two protons into the active site whereas in the natural cycle only one proton enters the active site.
[1] MacMillan, F. *et al.*, (1999) Biochemistry **38**, 9179
[2] Budiman, K. *et al.*, (2004) Biochemistry **43**, 11709

P-308

Prooxidant activity of 3-(dimethylamino-)phenol to human erythrocytes

B. Bukowska, W. Duda
Institute of Biophysics, University of Lodz, Poland

The molecular mechanism of the toxic properties of phenolic derivatives in humans and animals has been insufficiently studied. In this study, levels of reduced glutathione (GSH); the activity of glutathione peroxidase, catalase and superoxide dismutase; lipid peroxidation, hemolysis, cell morphology and the level of free radicals were measured in human erythrocytes exposed *in vitro* to 3-(dimethylamino-)phenol (3-DMAP). 3-DMAP is a main product of aniline transformation 3-DMAP exist in the environmental as a metabolite of ureic herbicides, and may be also disused as a derivative of phenoxyherbicides. Human erythrocytes were incubated with DMAP at the concentrations from 10 to 500 ìg of 1 ml erythrocytes of 5% hematocrite, for 1hours. The results showed, that the 3-(dimethylamino-)phenol increase the level of methemoglobin, the level of free radicals and concentrations of products of lipid peroxide (TBARS) and decrease GSH and glutathion peroxidase, catalase, SOD, changed cell morphology and hemolysis. All these results convince thesis that DMAP causes creation of oxidative stress in cell.

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– Redox Enzyme Mechanisms –

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Free radicals induced by 2,4-D and phenolic compounds in human erythrocytes

B. Bukowska, W. Duda, M. Koter
Institute of Biophysics, University of Lodz, Poland

In the work 2,4-dichlorophenoxyacetic acid, 2,4-dichlorophenol, 2,4-dimethylphenol and catechol in doses of 5 ìg to 500 ìg per 1 ml of erythrocytes (hematocrit 5%) were used to estimate formation of free radicals *in vitro*. The rate of 6-carboxy-2',7'-dichlorofluoresceine diacetate oxidation was measured by flow cytometry. We observed an increase in DCFH-PA fluorescence for all of investigated compounds. 2,4-D caused a significant increase in free radicals at doses above 100 ppm. This increase confirmed the pro-oxidant action of 2,4-D. The larges increase was observed in erythrocytes incubated with catechol. Oxidation of catechols leads to formation of semiquinone radicals, which immediately react with oxygen generating superoxide radicals. The obtained results revealed that toxic activity of 2,4-DCP and 2,4-DMP was similar, however mechanism of action of these compounds was different. Strong oxidation of hemoglobin by 2,4-DMP was induced. This conversion is associated with superoxide anion production and thereby with products such as hydrogen peroxide or hydroxyl radicals. Chlorophenols provoked a lesser oxidation of hemoglobin but damaged the structure of antioxidant enzymes such as catalase and superoxide dismutase.

P-310

Understanding enzymes by what they do not do: The paradox of reactivity in cyt bc complexes

J. L. Cape[1], A. Kanazawa[1], I. Forquer[1], M. K. Bowman[2], D. M. Kramer[1]
[1]Institute of Biological Chemistry, 335 Clark Hall, Washington State University, Pullman, Washington, 99163, [2]Structural Biology and Microimaging, Battelle Northwest Laboratory, PO Box 999 K8-98, Richland, Washington 99352-0999

Current models for the proton motive Q-cycle in the cyt *bc* complexes rationalize how the highly reactive intermediates of quinol (QH_2) oxidation avoid a variety of harmful, but thermodynamically favored, side reactions. The cyt *bc* complexes steer the fate of QH_2 oxidation intermediates away from "bypass" reactions through a strict control mechanism, though the exact details are still controversial. Four general models address the avoidance mechanism of Q-cycle bypass reactions in the *bc* complexes: 1) Concerted QH_2 oxidation, 2) Gated QH_2 oxidation, 3) Stabilized semiquinone intermediates and 4) Kinetic steering of the semiquinone. Each model makes specific predictions about the relationship between the thermodynamic properties of the QH_2 substrate and the yield of the Q-cycle relative to its bypass reactions. Here we provide critical tests of these models by tuning the substrate and cofactor redox properties and characterizing reaction intermediates using both physical and computational methodologies. Contrary to the mechanisms of many other enzymes, our findings suggest that the *bc* complexes actually destabilize QH_2 oxidation intermediates in order to achieve a high yield of productive energy conservation. This seemingly paradoxical control mechanism might represent a new paradigm for the control of reactive intermediates in other enzymatic systems.

P-311

Best guess catalytic cycle for Marinobacter hydrocarbonoclasticus 617 membrane nitrate reductase

C. Correia[1], S. Besson[2], C. D. Brondino[1], G. Fauque[3], J. Lampreia[1], I. Moura[1], J. J. G. Moura[1]
[1]REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, 2829-516 Caparica, Portugal, [2]Departamento de Ciências Naturais, Ambientais e Biotecnológicas, Universidade Lusófona, Campo Grande 376, 1749-024 Lisboa, Portugal, [3]Laboratoire Microbiologie, IRD UR 101, IFR-BAIM, Universités Provence et Méditerranée, ESIL Case 925, 163 Av Luminy, 13288 Marseille Cedex 9, France

A membrane nitrate reductase was purified from *Marinobacter hydrocarbonoclasticus* 617 (formerly *Pseudomonas nautica* 617) and its molybdenum-containing catalytic centre was probed through Electron Paramagnetic Resonance spectroscopy. This nitrate reductase is one of four enzymes involved in the denitrification pathway, the enzymatic reduction of nitrate to dinitrogen. As Mo(IV) and Mo(VI) species are both EPR silent, only Mo(V) species could be investigated. These Mo(V) species are usually in equilibrium with the corresponding Mo(VI) species and thus provide an insight into the coordination of the latter. EPR spectra of Mo(V) were recorded with the as-purified enzyme (at various pH values), as well as in the presence of nitrite or nitrate, and under turnover conditions. All EPR signals that had been previously described for other membrane nitrate reductases were also observed for this enzyme. However, in the turnover experiment, and upon electron donor shortage, a new Mo(V) EPR signal was described. Based on these results, a catalytic cycle is proposed in which a coordination pattern is tentatively assigned to each individual state of the cycle.

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Proton transfer in A. vinelandii Ferredoxin I: simulations using a dissociable force field

J. Danielsson, S. Lammers, M. Meuwly
Department of Chemistry, University of Basel, Basel, Switzerland

A. vinelandii ferredoxin I is a redox protein involving two iron-sulphur clusters, one of [4Fe-4S] type and one of [3Fe-4S] type. In this protein, electron transfer is coupled to proton transfer from the solvent to the buried [3Fe-4S] cluster. Structural and kinetic data of all redox and protonation states are experimentally available for wildtype as well as for several mutants [1]. The proton transfer reaction is here investigated by the use of a recently developed technique for describing potential energy surfaces allowing bond dissociation [2] in combination with classical molecular dynamics. We study the influence on this reaction of the redox state of the protein and solvent accessibility to the active site, since mechanistic proposals have been made both with [3] and without [1] involvement of water molecules adjacent to the cluster. Emphasis is put on accounting for dynamics effects and proper sampling of the protein and solvent degrees of freedom to calculate the free energy profiles. In addition, some experimentally characterized mutants have been studied to rationalize the observed influence on kinetic parameters and structural perturbations.

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P-313

Electron transfer studies in the flavohaemoprotein from ralstonia eutropha

P. de Oliveira, A. Ranjbari, B. Keita, T. Bizouarn, P. Sebban, L. Nadjo, L. Baciou
Laboratoire de Chimie Physique, UMR8000, Université Paris-Sud

Bacterial haemoglobins constitute a large protein family, but the biological role of each family member remains to be unambiguously established in several cases.This applies to a protein from the bacterium *Ralstonia eutropha*, known as flavohaemoprotein (FHP). It consists of a 43 kDa protein containing both a FAD and a five-coordinated, *b*-type haem moieties non-covalently bound to the protein. Its biological role could be that of a NO dioxygenase, *ie* it catalyses the detoxification of NO.
Using external light-activated flavins, we have investigated the electron transfer kinetics in the FHP by flash-induced absorption change spectroscopy. We have estimated the potential difference between FAD and Fe^{2+}-heme at about 30 mV and found a rather different external flavin accessibility for FAD and heme, a result which is not obvious judging from the three-dimensional structure.
The FHP has also been studied by cyclic voltametry, potentiometry and double potential step chronocoulometry. The electrochemical data obtained has been used in an attempt to resolve the redox potential values of its redox sites (*ie*, the FAD and the heme), as well as to calculate the electron transfer rates between the protein and the electrode. The need for mediators turned out to be unavoidable in many circumstances in order to obtain reliable results. The study has been complemented with experiments carried out with a semi-apo-FHP form deprived of the FAD moiety.

P-314

Fragile design of the electron transfer chain in NiFe hydrogenase

S. Dementin[1], V. Belle[1], P. Bertrand[1], A. Delacey[2], B. Guigliarelli[1], M. Rousset[1], C. Léger[1]
[1]Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Chemin J. Aiguier, 13402 Marseille Cedex 20, France., [2]Instituto de Catalisis, CSIC, Campus de Cantoblanco, 28049 Madrid, Spain

In NiFe hydrogenases, electrons are transfered from the active site to the redox partner via a chain of three Iron-Sulfur clusters. The surface-exposed [4Fe4S] cluster has an unusual $His(Cys)_3$ ligation. When this Histidine (H184 in D. fructosovorans) is changed into a Cysteine or a Glycine, a distal cubane is still assembled but the oxidative activity of the mutants is only 1.5 and 3% of that of the WT, respectively.
We compared the activities of the WT and engineered enzymes for H_2 oxidation, H^+ reduction and H/D exchange, under various conditions: (i) either with the enzyme directly adsorbed onto an electrode or using soluble redox partners (MV, MB, cytochrome), and (ii) in the presence of a number of exogenous ligands whose binding to the exposed Fe of H184G affects the turnover rate: for example pyridine substitutes reversibly for the imidazole ring that is absent in H184G and rescues this mutant, whereas thiols deal it the final blow and this mirrors the very low activity of H184C.
We conclude that the conserved Histidine is involved neither in partner recognition nor in proton transfer and that both intermolecular and intramolecular electron transfers are impaired in the mutants. This contrasts with the idea that a small distance between redox centers in multicentered enzymes is enough to make intramolecular electron transfer fast and resistant to mutational changes.

P-315

Mechanism of periplasmic nitrate reductase as viewed by EPR, electrochemistry and mutagenesis

B. Frangioni[1], C. Leger[1], P. Bertrand[1], D. Pignol[2], P. Arnoux[2], B. Guigliarelli[1]
[1]Bioénergétique et Ingénierie des Protéines, UPR9036, CNRS & Université de Provence, Marseille, France, [2]Laboratoire de Bioénergétique Cellulaire, DSV-CEA, St Paul lez Durance, France

The molybdoenzymes from the DMSO-reductase family show a large diversity of subunit compositions, metal center contents, Mo ion coordinations, and enzymatic reactions. The periplasmic nitrate reductase from *Rhodobacter sphaeroides* (NapAB), which belongs to this family, contains the Mo-cofactor and a Fe-S cluster in NapA, and two *c*-type hemes in NapB. Its three-dimensional structure was recently determined and this enzyme is a good model for studying the electron and proton transfers associated to the catalytic mechanism at the Mo-Cofactor. By using EPR spectroscopy in combination with protein film voltammetry, we have shown that the formation of the NapAB complex lead to a strong variation of the Fe-S redox potential which favours the electron transfer from the hemes. Moreover, the catalytic voltammograms show a peculiar shape with an optimal potential window for catalysis, which reveals a strong increase of the Mo-Cofactor redox potential upon substrate binding. In addition, the effects of amino-acid replacements performed by site-directed mutagenesis in the vicinity of the Mo-Cofactor are presented and discussed in light of the possible role of these residues in the catalytic mechanism and substrate specificity.

P-316

Investigation into the kinetics of the direct electron transfer to a multi-centered membrane protein

M. G. Friedrich, J. Robertson, R. Naumann, W. Knoll
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz

By immobilising a bacterial Cytochrome c Oxidase-mutant from *Rhodobacter sphaeroides* in a lipid architecture [1], it is possible to orient this protein with its natural electron pathway entrance towards the electrode surface. Potential-dependent SERRS in comparison with resonance raman spectra of CcO in solution shows that the redox site structures of the surface confined enzyme are preserved. During the immobilisation [2].
Attempts are made to use the potential dependent behavior of specific marker bands in the finger-print region, to observe the kinetics of the electron transfer inside the protein including the catalytic centre. Complementary to raman spectroscopy, electrochemical measurements such as scan-dependent cyclic voltammetry (CV), differential pulse voltammetry (DPV) and voltammetry at the rotating disc electrode (RDE) are performed and analyzed in detail. Trumpet plots are used to calculate the kinetic rate constants of electron transfer to the first redox site of the multi-centred protein [3]. Differential pulse voltammetry shows catalytic O_2 reduction [*A*] and discharge of H^+ transported actively across the protein [B]. Binding of CN^- to the O_2-reduction site of CcO results in the complete inhibition of the H^+ pumping.
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P-317

Nitric oxide synthase inhibitor reduces noise-induced cochlear damage in guinea pigs

W. Gao[1], M. Diao[1], C. Da[2]
[1]Physiology Dpt., Second Military Medical Univ., Shanghai, [2]Otorhinolaryngology Dpt., EENT Hospital, Shanghai

N^G-nitro-L-arginine methyl ester (L-NAME) could reduce cochlear damage in acoustic trauma. Sixty guinea pigs (300∼350g) were divided randomly into four groups [non-exposed group, noise + saline group , noise +L-NAME group and noise +L-NAME+L-arginine (L-Arg, a precursor of nitric oxide) group]. Auditory brainstem responses (ABR) were measured Cochlear tissue was assayed for the nitric oxide (NO) level and immunoreactivity to inducible nitric oxide synthase (iNOS) was observed with StrepAvidin-Biotin Complex (SABC) method. The hearing thresholds in the noise + saline group was significantly higher than those in the noise + L-NAME group (P<0.05). There was no significant difference in the thresholds between the noise + L-NAME + L-Arg group and the noise + saline group (P>0.05). The NO concentration in the noise + saline group was significantly higher than that of the non-exposed group (P<0.05). The cochlear NO concentration in the noise +L-NAME group was significantly lower than that of the noise + saline group (P<0.05), while there was no significant difference in NO level between the noise + saline group and the noise + L-NAME + L-Arg group (P>0.05). The immunoreactivity was more intense in the noise + saline group and noise +L-NAME+L-Arg group than that in the noise + L-NAME group. Conclusion: iNOS plays an important role in the formation of noise-induced hearing loss (NIHL) and L-NAME reduces cochlear damage through inhibiting the activity of this isoform of enzyme.

P-318

EPR and kinetic studies on NapA from Desulfovibrio desulfuricans ATCC 27774

P. J. González, M. G. Rivas, C. D. Brondino, I. Moura, J. J. Moura
REQUIMTE, Chemistry Department, CQFB, FCT, UNL, Portugal

Periplasmic Nitrate Reductase from the sulphate-reducing bacteria *Desulfovibrio desulfuricans* ATCC 27774 (*Dd* NapA) is an 80 KDa monomeric periplasmic enzyme having the bis-molybdopterin guanine dinucleotide cofactor (bis-MGD) and a [4Fe-4S] cluster [1,2]. EPR and enzymatic studies were performed to identify catalytic and non-catalytic relevant species. The sample in the *as-prepared* form presents a Mo(V) EPR signal that was assigned as non-catalytic relevant. The Dithionite reduced sample lacks Mo(V) ion EPR signal and presents a rhombic signal that is associated with the [4Fe-4S] cluster. The addition of nitrate to this sample yielded a Mo(V) rhombic signal with resonance lines split by a weakly interacting proton which is not solvent exchangeable. Addition of cyanide to the fully reduced sample yielded a new Mo(V) signal and decrease the g_3 component of the Fe-S EPR signal. Kinetic studies show that *Dd* Nap has a complex enzymatic mechanism with more than one binding site for nitrate. Inhibition studies demonstrated that cyanide and azide inhibits nitrate depletion.

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P-319

Cloning and overexpression of Bacterioferritin from Desulfovibrio vulgaris Hildenborough

M. Guilherme, P. Tavares, A. S. Pereira
REQUIMTE, CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal

Iron is an important nutrient, required in almost every aspects of cellular function. However, at physiological pH and under oxidizing conditions, the predominant form is Fe^{3+}, which is highly insoluble. Ferritin and bacterioferritin (Bfr) perform dual functions of iron detoxification, by oxidizing the Fe^{2+} ions in solution and iron sequestration and by storing the Fe^{3+} ions in its inner cavity in the form of ferrihydrate mineral (1,2). Ferritins are a broad superfamily of iron storage proteins present in all type of organisms. Bfrs are heme proteins with a b-type heme per dimer. Recently a Bfr was isolated from *Desulfovibrio (D.) desulfuricans* ATCC 27774. Besides the heme group this protein was purified with a carboxylate-bridged diiron center at the ferroxidase site (3). A Bfr encoding gene was isolated from *Desulfovibrio vulgaris* genome. The gene was inserted into an expression vector and induction conditions in *E. coli* were studied. The recombinant *D. vulgaris* Bfr was purified and preliminary biochemical and spectroscopic characterization will be presented.
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M.G. (SFRH/BD/18825/04) and A.S.P. (POCTI/QUI/47273/02) wishes to acknowledge to FCT for financial support.

P-320

High resolution crystal structures of two of the intermediates in the peroxide reaction of myoglobin

H.-P. Hersleth[1], T. Uchida[2], Å. K. Røhr[1], T. Teschner[3], V. Schünemann[3], K. Nilsson[4], Y.-W. Hsiao[4], T. H. Rod[4], A. X. Trautwein[3], U. Ryde[4], T. Kitagawa[2], C.-H. Görbitz[1], K. K. Andersson[1]
[1]University of Oslo, Norway, [2]Okazaki National Institutes of Natural Sciences, Japan, [3]Medical University of Lübeck, Germany, [4]Lund University, Sweden

We have previously reported high resolution (1.35 Å) structures of the myoglobin compound II intermediate at pH 5.2 [1], now confirmed by microspectrophotometry in the pH range 5.2 to 8.7. These structures show a relatively long Fe···O distance of 1.9 Å compared to the 1.6 Å distance of the commonly proposed oxoferryl [Fe^{IV}=O] species. This long Fe···O bond is supported by the newly observed rRaman Fe-^{18}O mode below 700 cm^{-1}. Quantum refinement best fit either a $Fe^{III}OH^{-\cdot}$ or a $Fe^{IV}OH$ state [2], while the Mössbauer spectroscopy indicates a Fe^{IV}-state. From compound II we were able to generate compound III (an oxy-complex). This intermediate was reduced by the synchrotron radiation giving an equivalent of compound 0 (Fe^{III}-peroxide) for which we have solved the 1.25 Å resolution structure. The different states were confirmed by microspectrophotometry, which also indicated that the normal high spin ferric state can be reduced by radiation.
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Crystal structures and catalytic mechanism of undecaprenyl pyrophosphate synthase

T.-P. Ko[1], R.-T. Guo[2], A. P.-C. Chen[1], C.-J. Kuo[1], A. H.-J. Wang[2], P.-H. Liang[1]

[1]Institute of Biological Chemistry, and, [2]Taiwan International Graduate Program, Academia Sinica, Taipei 11529, Taiwan

Undecaprenyl pyrophosphate synthase (UPPs) catalyzes the consecutive condensation reactions of a farnesyl pyrophosphate (FPP) with eight isopentenyl pyrophosphates (IPP), in which new *cis*-double bonds are formed, to generate undecaprenyl pyrophosphate that serves as a lipid carrier for peptidoglycan synthesis of bacterial cell wall. The structures of *Escherichia coli* UPPs have been determined in an orthorhombic crystal as an apo-enzyme, in complex with Mg^{2+}/sulfate/Triton, and with FPP. In further search of its catalytic mechanism, the wild-type UPPs and the Asp26Ala mutant are crystallized in a new trigonal unit cell with Mg^{2+}/IPP/FsPP (farnesyl thiopyrophosphate) bound to the active site. In the wild-type enzyme, Mg^{2+} is coordinated by the pyrophosphate of FsPP, the carboxylate of Asp26, and three waters. In the mutant it is bound to IPP. [Mg^{2+}] dependence experiments show that the activity of UPPs is maximal at 1 mM, but decreases when it is in excess. Without Mg^{2+}, IPP binds to UPPs only at high concentration. Mutation of Asp26 to other charged amino acids results in significant drop of the UPPs activity. The role of Asp26 is likely to assist the migration of Mg^{2+} from IPP to FPP, and thus initiating the reaction by ionization of the pyrophosphate from FPP. Other conserved residues including His43, Ser71, Asn74 and Arg77 may serve as general acid/base and pyrophosphate carrier. Our results here improve the understanding of the UPPs enzyme reaction significantly.

P-322

Identification of quinone binding site in the respiratory nitrate reductase complex from E.coli

P. Lanciano[1], S. Grimaldi[1], A. Magalon[2], F. Blasco[2], P. Bertrand[1], B. Guigliarelli[1]

[1]Bioénergétique et ingénierie des protéines, UPR9036, CNRS & Université de Provence, Marseille, France, [2]Laboratoire de Chimie bactérienne, UPR9043, CNRS, Marseille, France

Bacterial nitrate reductases are molybdoenzymes which show a large variety of structural organization and metal center content. The NarGHI complex from *E. coli* is a respiratory enzyme which catalyzes nitrate reduction in the cytoplasm coupled to the oxidation of membrane–bound quinol. This heterotrimeric complex is composed of a catalytic subunit (NarG) containing a Mo-bisMGD cofactor and a Fe-S cluster, an electron transfer subunit (NarH) carrying four Fe-S clusters, and a membrane anchor subunit (NarI) with two b-type hemes. The three-dimensional structure of the enzyme was recently solved but a number of questions concerning the catalytic mechanism at the Mo-cofactor and the intramolecular electron transfer remain, especially regarding the quinone binding site. To address this point, detailed redox titrations of NarGHI from *E. coli* were monitored by EPR spectroscopy. We evidenced a radical species which could be assigned to a semiquinone intermediate on the basis of its properties analyzed by multifrequency EPR and ENDOR spectroscopies. This species is strongly stabilized both in the whole enzyme and in the NarI subunit alone. By removing each of the b-type hemes by site-directed mutagenesis, we showed clearly that the quinol binding site of the enzyme is located in the vicinity of the distal heme in the enzyme.

P-323

Electron transfer process between cytochrome c552 and models of its redox partner

N. Leygue[1], S. Bernad[1], T. Soulimane[2], S. Lecomte[1]

[1]LADIR, CNRS/UPMC UMR7075, 2 rue Henry Dunant F-94320 Thiais, France, [2]Paul Sherrer Institut, Life Sciences, OSRA/008, CH-5232 Villigen, Switzerland

Cytochrome c_{552} (Cyt-c_{552}) from *Thermus thermophilus* does not exhibit a lysine-rich domain on the protein surface. This structural peculiarity suggests that the molecular interactions with the terminal ba_3 oxidase and, hence, the mechanism of the interprotein electron transfer are quite different compared to the mitochondrial redox couple cytochromes c-cytochrome c oxidase. This work is dedicated to analyse the heterogeneous electron transfer reactions of Cyt-c_{552} adsorbed on silver electrodes coated with functionalised alkanethiols which mimics the redox partner environment. SERR spectroscopy was used to probe the conformation of the heme group and to study the potential-dependent equilibria of adsorbed Cyt-c_{552}. Cyclic voltammetry experiments have been performed to determine the redox potential of the adsorbed Cyt-c_{552}, the reversibility of the electrochemical process, and the rate constant of the electron transfer. The electron transfer is not efficient for Cyt-c_{552} adsorbed onto the electrostatic model. A surface formed with hydrophobic and uncharged polar alkanethiolates seems to be a good model for ba_3-oxidase. The structure of the heme is not modified and the electron transfer is effective and reversible. The rate constant for electron transfer is 210 s^{-1}.

P-324

NO-Synthases and oxidative stress: a novel nitration activity

A. Marechal[1], T. A. Mattioli[1], D. J. Stuehr[2], J. Santolini[2]

[1]Laboratoire de Biophysique du Stress Oxydant, DBJC CEA-Saclay, 91191 Gif-sur-Yvette cédex, France, [2]Department of Immunology, Cleveland Clinic foundation, 9500 Euclid Avenue, 44195 Cleveland, U.S.A.

Nitric oxide (NO) is a major biological mediator that is involved in numerous physiological processes. It is exclusively generated in mammals by proteins from the NO-Synthase family (NOS). It has been recently suggested that NOSs, after NO biosynthesis, can partition in two distinct catalytic cycles via two different complexes: i) $Fe^{III}NO$ complex whose dissociation leads to NO ii) $Fe^{II}NO$ complex, whose oxidation produces other reactive nitrogen species. We previously suggested that $Fe^{II}NO$ oxidation lead to the transient formation of peroxynitrite (PN). We checked this hypothesis by investigating the model reaction: Fe^{III} + PN. Rapid-scanning Stopped-flow experiments show that iNOS rapidly reacts with PN with the build-up of a 445 nm intermediate. This PN activation, that is pH- and iNOS concentration-dependent, is abolished by the binding of substrate or product. iNOS modifies PN reactivity and tunnels it towards its sole nitration activity. Our results showed that, via PN-activation, iNOS promotes self-catalytic nitration and inhibition. We investigated the interaction between PN and other NOS isoforms. Each isoform displays a distinct PN-activation mechanism which could relate to their specific biological role.

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Nitric oxide reductase from pseudomonas nautica

C. E. Martins, C. M. Cordas, C. G. Timoteo, P. Tavares, A. S. Pereira, J. J. G. Moura, I. Moura
REQUIMTE, Centro de Química Fina e Biotecnologia, Dept. de Química, Fac. de Ciências e Tecnologia, Univ. Nova de Lisboa, 2829-516 Caparica, Portugal

A Nitric Oxide Reductase (NOR) was purified from the denitrifying *Pseudomonas nautica* 617. It is a membrane bound enzyme, so the detergent dodecyl maltoside was used for solubilization. The purified enzyme consists of two subunits: NOR B which has a molecular weight of about 54 kDa and contains two *b* type heme and one non-heme iron; and NOR C which has a molecular weight of about 17 kDa and contains one *c* type heme.
The EPR spectrum of the as-isolated form exhibits a typical sets of ressonaces that can be attributed to low spin heme *b* and *c* and to a high spin heme *b*. Incubation of the reduced enzyme with NO produces a triplet EPR signal around g=2 characteristic of a heme-nitrosyl complex and a S= 3/2 NO signal attributed to a high-spin non-heme iron-nitrosyl complex.
Preliminary results reveal that cytochrome c_{552} might be the physiologic electron donor. NaN_3 seems to have an inhibitory effect on the NOR activity.
A first evaluation of the NOR electrochemical behaviour was performed in presence and absence of NO.
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Arg265 as the proton source in the radical transfer pathway of mouse ribonucleotide reductase R2

A. J. Narváez[1], N. Voevodskaya[1], L. Thelander[2], A. Gräslund[1]
[1]Department of Biochemistry and Biophysics, Stockholm University, Sweden, [2]Department of Medical Biochemistry and Biophysics, Umeå University, Sweden

Ribonucleotide reductases class I consist of two non-identical components, R1 and R2, the latter containing an iron-tyrosyl free radical center (Fe/$Tyr^{\bullet}$) essential for activity. Catalysis includes the long-range transport of an electron and a between the $Tyr^{\bullet}$ in R2 and the active site in R1. These sites are connected by a hydrogen bonded chain of conserved amino acid residues. Site directed mutagenesis, enzymatic activity and electron paramagnetic resonance spectroscopy (EPR) were used to study the radical transfer pathway (RTP). Mutants of the highly conserved R2 Arg^{265} were generally catalytically inactive. However, upon reconstitution with Fe(II) and O_2, R265E showed about half of the enzymatic activity compared to native R2 at standard pH. Furthermore, a distinct pH dependence on the enzymatic activity was observed for native and the R265E mutant with a 2.5-fold increase in activity in native R2 protein and a 4-fold increase in R265E when the pH is shifted from 7.5 to 8.5. Activity was almost non-existent at pH 6.5. All mutants when reconstituted formed normal $Tyr^{\bullet}$. Kinetic differences in $Tyr^{\bullet}$ formation were also observed at different pH values between native and R265E. The overall results suggest Arg^{265} is involved in the RTP and is most likely the proton source.

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Intriguing conformation changes associated with the trans/cis isomerization of a prolyl residue in the active site of the DsbA C33A mutant

E. Ondo-Mbele, C. Vivès, A. Koné, L. Serre
IBS-LPM CEA/CNRS/UJF, 41 rue Jules Horowitz, 38027 Grenoble cedex 01 France

E.coli DsbA belongs to the thioredoxin family and catalyzes the formation of disulfide bonds during the folding of proteins in the bacterial periplasm. It active site (C30-P31-H32-C33) consists of a disulfide bridge that is transferred to newly translocated proteins. The work reported here refers to the DsbA mutant termed C33A that retains, towards reduced unfolded thrombin inhibitor, an activity comparable with the wild type enzyme. Besides, C33A is also able to form a stable covalent complex with DsbB, the membrane protein responsible for maintaining DsbA in its active form.
We have determined the crystal structure of C33A at 2.0Å resolution. Although the general architecture of *wt* DsbA is conserved, we observe the *trans/cis* isomerization of P31 in the active site and further conformational changes in the so-called "peptide binding groove" region. Interestingly, these modifications involve residues that are specific to DsbA but not to the thioredoxin family fold. The C33A crystal structure exhibits as well a hydrophobic ligand bound close to the active site of the enzyme. The structural analysis of C33A may actually explain the peculiar behavior of this mutant in regards with its interaction with DsbB and thus provides new insights for understanding the catalytic cycle of DsbA.

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The role of Y167-W272 in the cleavage of the O-O bond by cytochrome c oxidase

E. Pinakoulaki[1], T. Ohta[2], K. Budiman[3], O.-M. H. Richter[4], T. Kitagawa[2], B. Ludwig[4], H. Michel[3], C. Varotsis[1]
[1]Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece, [2]Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787 Japan, [3]Max-Planck Institüt fur Biophysik, D-60439 Frankfurt am Main, Germany, [4]Institute of Biochemistry, Biozentrum, Johann Wolfgang Goethe-Universität, Marie-Curie-Str. 9, D-60439 Frankfurt/M., Germany

We report the first resonance Raman evidence for the formation of the two oxoferryl species with characteristic Fe(IV)=O stretching modes at 790 and 804 cm^{-1} at the same oxidation level (P_M) in the cytochrome *c* oxidase (CcO) reaction cycle. The present data and those previously reported in the peroxide reaction demonstrate that the proximal H-/non-H bonded His-411 equilibrium determines the strength of the distal Fe(IV)=O bond. With the identification of the 790 and 804 cm^{-1} Fe(IV)=O modes at the P_M intermediate level (three electron reduced) we propose a mechanistic pathway for the cleavage of the O-O bond by CcO. These results indicate that the mechanism of oxygen reduction must be reexamined.

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Detection of the primary His-heme Fe(2+)-NO intermediate in the reduction of NO by ba3-oxidase

E. Pinakoulaki[1], T. Ohta[2], T. Soulimane[3], T. Kitagawa[2], C. Varotsis[1]
[1]Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece, [2]Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787 Japan, [3]Deparment of Biological sciences, University of Limerick, Limerick, Ireland

Reaction pathways in the enzymatic formation and cleavage of the N-N and N-O bonds, respectively, are difficult to verify without the structure of the intermediates, but we now have such information on the primary intermediate in the reaction of ba_3-oxidase with NO from resonance Raman spectroscopy. We have identified the primary His-heme $a_3{}^{2+}$-NO/$Cu_B{}^{1+}$ species by its characteristic Fe-NO and N-O stretching frequencies at 539 and 1620 cm^{-1}, respectively. From these results and earlier Raman and FTIR measurements, we demonstrate that the protein environment of the proximal His384 that is part of the Q-proton pathway, controls the strength of the Fe-His384 bond upon ligand (CO vs NO) binding. We also show by time-resolved FTIR spectroscopy that $Cu_B{}^{1+}$ has a much lower affinity for NO than for CO. We suggest that the reduction of NO to N_2O by ba_3-oxidase proceeds by the fast binding of the first NO molecule to heme a_3 with high-affinity, and the second NO molecule binds to Cu_B with low-affinity, producing the temporal co-presence of two NO molecules in the heme-copper center. The low-affinity of Cu_B for NO binding also explains the NO reductase activity of the ba_3-oxidase as opposed to other heme-copper oxidases.

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ATR-FTIR analysis of the PM intermediate of cytochrome c oxidase using isotope-labelling

A. Puustinen[1], M. Iwaki[2], M. Wikström[1], P. R. Rich[2]
[1]Helsinki Bioenergetics Group, Institute of Biotechnology, University of Helsinki, FINLAND, [2]Glynn Laboratory of Bioenergetics, Department of Biology, University College London, U.K.

The structure of P_M intermediate of *Paracoccus denitrificans* cytochrome *c* oxidase is investigated by perfusion-induced attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. In prior work (Biochemistry 2004 (43) 14370), unlabelled, universally ^{15}N-labelled and tyrosine ring-d_4-labelled proteins were used to assign vibrational frequencies to the unusual covalently-linked histidine-tyrosine ligand of Cu_B that has been found in the catalytic site and probably functions as a proton and electron donor during the reaction. Comparison with model material data suggests that the tyrosine hydroxyl group is protonated when the binuclear center is oxidized but deprotonated in the P_M intermediate. The observed band shifts with tyrosine ring-d_4-labelling were characteristic for ν_{7a}(C-O) and ν(C-C) bands of neutral phenolic radicals and most likely reflect the formation of the neutral radical state of histidine-tyrosine in P_M. In the present study, redox difference spectra and P_M *minus* oxidized difference spectra of tyrosine ^{15}N, $^{13}C_9$-labelled and tyrosine ring-$^{13}C_6$-labelled proteins are compared with the previous data to further test the earlier assignments. The results are discussed in relation to the oxidase catalytic cycle in terms of chemical changes of the his-tyr and structural alterations in the HPEVY region.

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Catalytic properties and EPR studies of Desulfovibrio desulfuricans formate dehydrogenase

M. G. Rivas, P. J. González, C. D. Brondino, J. J. Moura, I. M. Moura
REQUIMTE, Chemistry Department, CQFB, FCT, UNL, Portugal

Formate dehydrogenases (FDH) comprise a heterogeneous group of enzymes found in both prokaryotes and eukaryotes that catalyse the oxidation of formate to carbon dioxide and hydrogen (1,2). FDH from *D. desulfuricans* (*Dd* FDH) present an heterotrimeric structure (α= 88kDa, β= 29kDa, and γ = 16kDa) and contains three types of redox-active centers: four *c*-type hemes, four $[4Fe\text{-}4S]^{2+/1+}$ clusters and a Mo-*bis*MGD site (3). Kinetic parameters of *Dd* FDH have been determined using both formate (HCOOH) as well as deutero-formate (DCOOH). Inhibition studies with azide and cyanide showed that they are strong mixed inhibitors. Nitrate was shown to be a competitive inhibitor. The role of the solvent in the reaction mechanism is unveiled from EPR studies in slow turnover conditions.

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Acknowledgement: We thank The Fundação para a Ciência e a Tecnologia for financial support.

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Orientation Selective Pulse 1H / 2H ENDOR at 34 GHz on the Tyrosine Radical in Mouse RNR R2-Protein

P. P. Schmidt, E. Reijerse, S. Sinnecker, W. Lubitz
Max-Planck Institute for Bioinorganic Chemistry, D - 45470 Mülheim an der Ruhr, Germany

Ribonucleotide Reductase (RNR) is the sole source for deoxyribonucleotides. Class I RNRs are $\alpha_2\beta_2$ enzymes, in which α_2 carries regulatory sites and the active site. Active β_2 (R2) harbors an essential tyrosyl radical (Y*) adjacent to a μ-oxo-bridged, antiferromagnetically coupled diiron(III) center involved in generation and stabilization of Y*. Mouse R2 is one of the few RNRs in which this Y* is H-bonded. Davies pulse ^1H ENDOR spectra at 34 GHz revealed the hyperfine couplings (hfcs) of all seven protons (two β-, four ring-, and the H-bonded proton). As supported by DFT calculations the symmetry related 3,5-protons are slightly different caused by the single asymmetric hydrogen bond. Orientation selective ^2H-cw ENDOR at 34 GHz on the exchanged ^2H in the H-bond displayed well-resolved spectra regarding hfc and nuclear quadrupole coupling (nqc). The hfc and nqc values are in good agreement with existing data and were, supported by DFT calculations, used to build a geometrical model of this part of the mouse R2 protein. A consistent data set from simulations of all measured Q- and X-band spectra is presented.

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IR spectroscopic characterization of the caa3 oxygen reductase from Rhodothermus marinus

F. L. Sousa[1], M. M. Pereira[1], R. M. Nyquist[2], J. Heberle[2], M. Teixeira[1]
[1]Instituto de tecnologia química e biológica, UNL, Portugal, [2]Forschungszentrum Jülich, IBI-2, Germany

The *caa$_3$* HiPIP:oxygen oxidoreductase is one of the oxygen reductases present in the respiratory chain of the thermohalophilic bacterium *Rhodothermus marinus.*This oxygen reductase, a member of the A_2 haem copper oxygen reductases family [1], is characterised by having four subunits and lacks the putative essential glutamate residue (E278 in *Paraccocus denitrificans*) in the D-channel. Homology modelling and sequence analysis showed that this glutamate carboxyl could be substituted by the phenolic group of a tyrosine residue. This residue, together with a serine residue located between the reaction centre and the tyrosine, forms the so-called YS motif [2]. In order to try to elucidate this different intraprotein proton pathway and to further characterise *R.marinus caa$_3$* oxidase in terms of its catalytic cycle and proton translocation coupling [3], redox and kinetic proprieties were studied. By visible spectroscopy it could be observed the establishment of the canonical P_M and F intermediates. Based on the ATR-FTIR difference spectra of those intermediates the involvement of at least two different tyrosine residues in the protonation/deprotonation mechanism is proposed, one being possibly the tyrosine of the YS motif and the other the tyrosine residue covalently linked to a histidyl ligand of Cu_B.
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P-334

A novel cluster and spectroscopic studies of the radical-iron site in mouse ribonucleotide reductase

K. R. Strand[1], Å. K. Røhr[1], B. Dalhus[1], M. Kolberg[1], C. H. Gørbitz[1], S. Karlsen[1], A.-L. Barra[2], K. K. Andersson[1]
[1]Depts Mol Biosciences & Chemistry, University of Oslo, NO-0316 Oslo, NO, [2]GHMFL, CNRS, BP 166, FR 38042 Grenoble, FR

Ribonucleotide reductase (RNR) is the enzyme that converts ribonucleotides to their corresponding deoxyribonucleotides. The R2 protein reacts with ferrous iron and dioxygen to generate a tyrosyl radical that is essential for enzymatic activity [1]. We have studied the mouse R2 protein with several spectroscopic methods e.g., Raman, HF EPR at 285 GHz [2], ENDOR and MCD [1]. We find cooperative binding of Fe(II) (or Co(II)) and hydrogen binding to tyrosyl radical [1, 2]. Here we present structures of mouse R2 soaked in iron [3]. We now observe a biologically unusual new type of tri-nuclear metal ion cluster, clearly different from a tri-nuclear metal ion cluster recently observed in an R2 protein from *Corynebacterium ammoniagenes*. In methanol soaked mouse R2, the tri-nuclear cluster is located ∼10 Å from the di-nuclear iron cluster and is attached to the protein by a surprising and novel type of coordination. The cluster is ∼15 Å away from the electron/radical transport chain in mouse R2, and therefore probably not involved in radical transfer. It is not yet clear whether the observed tri-nuclear cluster is an artefact from the soaking conditions, or if it has some biological relevance. [1] Kolberg, Strand, Graff, Andersson *Biochem. Biophys. Acta* **2004**, 1699, 1-34 [2] Andersson *et al. J. Biol. Inorg. Chem.*, **2003**, 8, 235-247: [3] Strand *et al. J. Biol. Chem.* **2004**, 279, 46794-46801. Norwegian Cancer Soc.

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How molecular hydrogen approaches the active site of [NiFe]-hydrogenases: A theoretical analysis

V. H. Teixeira, A. M. Baptista, C. M. Soares
Instituto de Tecnologia Quimica e Biologica - Universidade Nova Lisboa

[NiFe]-hydrogenases are a group of enzymes, present in sulphate reducing bacteria, that can reversibly catalyse the oxidation of H_2 to electrons and protons. The NiFe active site is deeply buried inside the protein and H_2 has to permeate the protein in order to reach it. The H_2 approach to the active site is not clear, and different hypotheses were put forward by experimental and quantum chemical works. Our objective here is to characterise possible permeation pathways of H_2 towards the active site, using molecular dynamics simulations.
The starting point was the X-ray structure of [NiFe] hydrogenase from *Desulfovibrio gigas*. Quantum chemical calculations were performed on the metallic centres to determine their partial atomic charges using the Gaussian98 program for generating electrostatic potentials, and the RESP program for fitting these potentials. Continuum Electrostatic and Monte Carlo simulations were used to predict the protonation states of all residues in the protein oxidation state that binds H_2. Using these states we did molecular dynamics simulations in a box containing explicit water and H_2 molecules to search for pathways inside the protein.
We were able to observe H_2 permeation into the interior of the protein, reaching the active centre through various possible pathways. This allows the characterization of important residues in this process and gives us clues on the reaction mechanism.

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Complex electron transfer kinetics between the photoactive label TUPS and the heme of cytochrome c

K. Tenger[1], P. Khoroshyy[1], B. Leitgeb[1], G. Rákhely[1], N. Borovok[2], A. Kotlyar[2], L. Zimányi[1]
[1]Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary, [2]Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

Electron transfer between the photoinduced covalent redox label thiouredopyrene-trisulfonate (TUPS) and the heme of cytochrome c deviates from the expected monoexponential kinetic behavior. TUPS has been attached to two lysine residues (K8 and K39) at opposite sides of the protein, as well as to cysteines at the same positions introduced by site directed mutagenesis. Neither the overall rate, nor the individual exponential components of electron transfer, as followed by kinetic absorption spectroscopy, correlate with the length of the covalent link connecting the dye with the protein. Molecular dynamics calculations show that TUPS can approach the protein surface and occupy several equilibrium positions stabilized by ionic interactions. This heterogeneity may explain the multiexponential electron transfer kinetics. The optimal electron transfer pathways calculated using the program HARLEM (www.kurnikov.org) do not follow the covalent link but involve through space jumps from the dye to the protein moiety. The correlation of the experimental rates with the calculated pathways and protein packing density which follow from the molecular dynamics simulations will be evaluated.

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– Redox Enzyme Mechanisms –

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Characterisation of the redox behaviour of the cbb3 oxygen reductase from Bradyrhizobium japonicum

A. F. Veríssimo, M. M. Pereira, S. Todorovic, M. Teixeira
Instituto de Tecnologia Química e Biologica, Universidade Nova de Lisboa, Av. República (EAN) Apt127, 2781-901 Oeiras, Portugal

Bradyrhizobium japonicum, a diazotrophic Gram-negative soil bacterium has a c*bb*$_3$ oxygen reductase encoded by the f*ix*NOQP operon, expressed in symbiosis conditions. This oxygen reductase belongs to the C group of haem-copper oxygen reductases superfamily (Pereira, M.M. *et al* (2001) *BBA* 1505, 185-208). In this study, we purified and further characterized this oxygen reductase carrying a histidine-tagged FixN (Arslan, E. *et al* (2000) *FEBS Letters* 470, 7-10) from *B. japonicum* cells grown in microaerophilic conditions. Different spectroscopic techniques, namely UV-Vis, EPR and Resonance Raman, as well as anaerobic potentiometric titrations at different pH values, followed by visible spectroscopy, were used to study the 5 redox centres present in this complex.

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Mitochondria dysfunction based on the disorder of mitochondrial radical metabolism

J.-X. Xu
National Laboratory of Biomacromolecule, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China.

A concept of mitochondrial radical metabolism is suggested based on the two cytochrome c-mediated electron-leak pathways of respiratory chain are the reactive pathways for $O_2^{-\cdot}$. Four reactive pathways initiated by $O_2^{-\cdot}$, which is linked to the electron leakage of respiratory chain, are defined as mitochondrial radical metabolism. The model of mitochondria having a radical metabolism to share electrons with ATP synthesis makes it possible to describe mitochondria dysfunction by abnormal higher level of electron leakage in the respiratory chain or abnormal ratio of the four $O_2^{-\cdot}$ reactive pathways of radical metabolism. In proving this idea, a number of animal models were randomly selected and the mitochondria were prepared from those models. KCN-insensitive respiration was tested based on the oxygen consumed by the leaked electrons not being inhibited by KCN. Results showed that the level of electron leakage in the respiratory chain is always higher when the animal in pathological and disease states.

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A thioredoxin reductase inhibitor induces growth inhibition/apoptosis in human carcinoma cell lines

F. Zhao, J. Yan, J. S. Deng, X. L. Lan, F. He, B. Kuang, H. H. Zeng
School of Pharmaceutical Sciences, Peking University, Beijing, 100083, China

Human Thioredoxin reductase (TrxR) system is associated with cancer cell growth and anti-apoptosis process. Effects of 1, 2-[bis (1,2-Benzisoselenazolone-3 (2H) -ketone)]ethane (BBSKE), a novel TrxR inhibitor, were investigated on A549, HeLa, Bel-7402, BGC823 and KB cell lines. After treated with BBSKE, a good linear correlation coefficient ($r \geqslant 0.989$) between TrxR activity and cell viability exists in each cell line together with cell growth/proliferation inhibition and apoptosis through Bcl-2/Bax and Caspase-3 pathways. These results suggest that there exists some relationship between TrxR inactivation and growth/proliferation inhibition or apoptosis in the investigated cell lines.

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– Single Molecule Biophysics –

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The effect of peripheral substituent on interaction of meso-tetra-(3N-pyridyl)porphyrins with DNA

L. R. Aloyan, V. I. Vardanyan, Y. B. Dalyan
Chair of Molecular Physics, Department of Physics of the Yerevan State University,1 Al. Manoogian St., Yerevan 375025, Armenia

By spectrophotometry and circular dichroism methods the influence of peripheral substituent of porphyrins on their interaction with calf thymus DNA was investigated. The new *meso*-tetra-(3N-pyridyl) porphyrins [TOEPyP(3), MetAlPyP(3), TAlPyP(3)] was used. The induced CD spectra of DNA/porphyrin complexes in visible region are shown, that this porphyrins have intercalation binding mode. And the CD spectral changes in UV region of complexes are similar of the B-C transition of DNA. But it can be result of porphyrin's induced CD in this region, because the porphyrins have absorption spectra in UV region. The shapes of changes of the Soret absorption band are similar for all porphyrins: upon adding DNA the maximum of Soret band showed hypochromic effect and batochromic shift. Analysis of differential absorption spectra has demonstrated that there are different binding site's numbers on DNA for porphyrins with different peripheral substituents. For DNA/MetAlPyP(3) complexes the binding isotherm has two-stage. It can be result of presence two biding modes of this porphyrin.

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Two-hybrid fluorescence cross-correlation spectroscopy detects protein-protein interactions *in vivo*

N. Baudendistel[1], G. Müller[1], W. Waldeck[1], P. Angel[2], J. Langowski[1]
[1]Division Biophysics of Macromolecules, DKFZ Heidelberg, [2]Division Signal Transduction and Growth Control, DKFZ Heidelberg

Fluorescence cross-correlation spectroscopy (FCCS) uses the correlated motion of two distinct fluorophores to detect their interaction. While FCCS has been used with chemically or genetically labeled interaction partners *in vitro*, FCCS has never been demonstrated *in vivo* between two autofluorescent proteins. At least one reaction partner was always chemically labeled. Fos and Jun, two components of the AP-1 transcription factor, are known to exert their function as a dimer and can therefore serve as a reference for dimer formation. Expressing fusion proteins between Fos and the enhanced green fluorescent protein (EGFP), as well as Jun and the monomeric red fluorescent protein 1 (mRFP1) in HeLa cells, we show here for the first time *in vivo* detection of protein-protein interaction by FCCS. The mobility of the dimerized species is slow, indicating that DNA-binding might stabilize dimerization. The technique has rich potential applications for the rapid screening of protein-protein interactions *in vivo*, which are able to clarify events during the whole life of cells.

P-342

Dynamics of single GABAAR in nerve growth cone : from molecular interactions to axonal guidance

C. I. Bouzigues[1], A. Triller[2], M. Dahan[1]
[1]Laboratoire Kastler Brossel, Ecole Normale Supérieure, Paris, France, [2]Laboratoire BCS, Ecole Normale Supérieure, Paris, France

During axonal growth, the distal part of the axon – the growth cone (GC)- is able to detect spatial gradients of guidance cues, such as GABA, netrins or BDNF, leading to accurate axonal pathfinding and formation of a functional network. Although the signal transduction pathways leading to GC steering have been widely investigated, the remarkable sensitivity of GCs in gradient sensing remains mostly unexplained. We address this issue by studying membrane dynamics of single quantum dot-tagged GABA receptors ($GABA_AR$) in GCs of spinal neurons.

In addition to Brownian motion, we measured directed movements for 80% of the receptors. A statistical analysis of trajectories combined with pharmacological treatments by nocodazol and taxol revealed that these motions were due to transient interactions with microtubules.

To investigate the functional role of these interactions, we analyzed the dynamics of $GABA_ARs$ in the presence of an external GABA gradient. We observed a redistribution of receptors in the direction of the source, which is specific of $GABA_AR$ activation and is microtubule-and calcium-dependent.

These observations lead us to propose a model for guidance signal amplification based on the autocatalytic spatial redistribution of receptors through interactions with microtubules.

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Probing molecular free energy landscapes by non-equilibrium measurements and periodic loading

O. Braun[2], A. Hanke[1], U. Seifert[2]
[1]University of Texas at Brownsville, USA, [2]University of Stuttgart, Germany

Single-molecule pulling experiments provide information about interactions in biomolecules that cannot be obtained by any other method. However, the reconstruction of the molecule's free energy profile from the experimental data is still a problem, in particular for the unstable barrier regions. The challenge is to deduce equilibrium information from non-equilibrium data. This problem has been resolved by recent advances in statistical mechanical theory based on a non-equilibrium equality for free energy differences obtained by Jarzynski in 1997. We propose a new method for obtaining the full molecular free energy profile by introducing a periodic ramp and using Jarzynski's relation. Our simulated experiments show that this method delivers significant more accurate data than previous methods, under the constraint of equal experimental effort.

Posters

– Single Molecule Biophysics –

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Functionalized luminescent oxide nanoparticles for Na-channel imaging at the single molecule level

D. Casanova[1], D. Giaume[2], E. Beaurepaire[1], M.-P. Sauviat[1], E. Auksorius[1], V. Buissette[2], K. Lahlil[2], J.-L. Martin[1], T. Gacoin[2], J.-P. Boilot[2], A. Alexandrou[1]
[1]Laboratory for Optics and Biosciences, Ecole Polytechnique - CNRS UMR 7645 - INSERM U696, Palaiseau, France, [2]Laboratory of Condensed Matter Physics, Ecole Polytechnique - CNRS UMR 7643, Palaiseau, France

We here demonstrate functionalized lanthanide-ion doped oxide nanoparticles as a promising new class of biological fluorescent probes. This system possesses distinct advantages: (i) colloids are synthesized directly in water, which greatly facilitates their functionalization, (ii) no blinking behaviour is observed due to the large number of dopant ions in each nanoparticle, (iii) good photostability and (iv) very narrow emission linewidths (<15 nm).
We functionalized 20-30 nm diameter YVO_4 :Eu nanoparticles with guanidium groups in order to mimick the blocking effet of saxitoxin on Na^+ channels. Electrophysiological measurements in frog cardiomyocytes confirmed that functionalized nanoparticles specifically target Na^+ channels and mimick the effect of saxitoxin. We achieved imaging of individual functionalized nanoparticles on the membrane of live cardiomyocytes, revealing the distribution of Na^+ channels. Moreover, we showed that a simple time-gated detection takes advantage of the long excited state lifetime of lanthanide ions ($\sim$1 ms) and filters out the short-lived (ns) cellular fluorescence.
Functionalized oxide nanoparticles thus appear as a versatile tool particularly attractive for long-term single-molecule tracking.
E. Beaurepaire et al, Nano Lett. **4**, 2079 (2004).

P-345

Pre-unfolding Resonant Oscillations of Single Green Fluorescent Protein

G. Chirico, F. Cannone, G. Baldini
University of Milano Bicocca, Physics Department, Istituto Nazionale per la Fisica della Materia, INFM, Italy

Fast fluorescence spectroscopy investigations of a Green Fluorescent Protein mutant at single molecule resolution have led to the discovery of a remarkable oscillatory behaviour that can also be driven by applied fields. We show that, immediately before unfolding, several periodic oscillations among the protonation states of the protein chromophore occur. The presence a few oscillation frequency values suggests their connection with relevant conformational substates of the protein. We show also that applied alternate electric or acoustic fields give rise to conspicuous resonance effects when tuned to the protein characteristic frequencies. A model involving chemical and conformational changes of the protein substates near unfolding gives a preliminary rationale of the results and new applications of the molecule as a nanodevice could be foreseen

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Dynamics of single cytoplasmic proteins labeled with quantum dots

S. Courty[1], C. Luccardini[1], G. Cappello[2], Y. Bellaiche[3], M. Dahan[1]
[1]Laboratoire Kastler Brossel, Ecole Normale Supérieure, Paris, France, [2]Institut Curie, CNRS UMR 168, Paris, France, [3]Institut Curie, CNRS, UMR 144, Paris, France

The observation of biomolecules at the single molecule level opens new prospects for cellular imaging since it provides informations which are often inaccessible by conventional optical detection (e.g. GFP fusion proteins). By combining recent imaging methods with semiconductor quantum dots (QDs) and well established tools of molecular and cellular biology, we have studied *in vivo*:
- the intracellular dynamics of kinesins labelled with QDs-streptavidin. We have measured the motion of individual kinesins along microtubules and found an average velocity of <v>=0.56 μm.s^{-1} and an average residence time <t>=1.72s. These velocity and processivity are directly compared to the results of *in vitro* motility assays.
- the dynamic localisation of a cell fate determinant (Pon) during asymmetric cell division[2] in Drosophilae neuroblasts. The Pon protein is labelled specifically with quantum dots and injected in neuroblast cells. Tracking and image analysis of spatio-temporal motions are performed during the division.
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Bellaiche Y, Radovic A, Woods DF, Hough CD, Parmentier ML, O'Kane CJ, Bryant PJ, Schweisguth F. *Cell* (2001) 106(3): p. 355-66.

P-347

Observation of EcoRV sliding along DNA at single-molecule level

A. Crut[1], I. Bonnet[1], W. Wende[2], P. Desbiolles[1]
[1]LKB, Département de Physique de l´ENS, 24 rue Lhomond, 75005 Paris, France, [2]Justus-Liebig-Universitaet Giessen, Institut für Biochemie, FB08, Heinrich-Buff-Ring 58, 35392 Gießen, Germany

The time necessary for the localization of specific sequences on DNA by proteins can be much shorter than the upper limit estimated for a 3-D diffusion-controlled process involving macromolecules. Several mechanisms have been proposed to account for this sequence localization, especially one-dimensional diffusion along DNA molecules. The related processes (sliding and/or hopping) are supported by kinetic studies, many of them conducted on the type II restriction enzyme EcoRV.
We have studied the interaction of individual EcoRV enzymes with single T7 DNA molecules using fluorescence microscopy. In our experiments, DNA molecules were stretched and attached to a surface specifically by their extremities. Proteins were labeled with quantum dots. We observed and analyzed individual association/dissociation events of enzymes on DNA. Our experiments evidence a sliding of the enzymes along non-specific parts of DNA and provide quantitative information about the process. In addition, our results support the conclusions of previous kinetic studies regarding the large contribution of sliding to target localization by enzymes.

Posters

– Single Molecule Biophysics –

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Protein immobilization at polyelectrolyte brushes

C. Czeslik[1], G. Jackler[1], M. Ballauff[2], E. Gratton[3], T. Hazlett[3]
[1]Universität Dortmund, Physikalische Chemie I, D-44221 Dortmund, Germany, [2]Universität Bayreuth, Physikalische Chemie I, D-95440 Bayreuth, Germany, [3]University of Illinois, Laboratory for Fluorescence Dynamics, Urbana, IL 61801, USA

There is a series of examples where immobilized proteins play an important role, such as solid-phase immunoassays for medical diagnostics or protein biochips that are used in protein analysis. However, proteins may undergo severe conformational changes at a solid surface which can lead to a loss of biological activity. To optimize protein adsorption, the solid surface can be modified by a poly(acrylic acid) (PAA) brush. We used two-photon excitation fluorescence fluctuation spectroscopy, neutron reflectometry and CD spectroscopy to study the binding of proteins to spherical PAA brush particles and planar PAA brushes. It has been found that a PAA brush binds huge amounts of protein at low ionic strength, whereas a salt concentration of a few 100 mM in the protein solution renders the PAA brush protein resistant. Remarkably, this effect of salt appears to be independent of protein net charge. Without added salt, protein molecules penetrate deeply into the PAA brush even under electrostatic repulsion. Applying a simple mean field model these results can be explained by a counterion release as driving force for protein adsorption to a PAA brush. Furthermore, a PAA brush provides a mild environment for immobilized protein molecules, since it does not induce major protein conformational changes. All experiments indicate that a PAA brush represents a novel and unique surface coating for the controlled immobilization of proteins.

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RuvAB mediated Holliday Junction migration at the single molecule level

A. Dawid[1], V. Croquette[2], M. Grigoriev[3], F. Heslot[1]
[1]LPA, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris (France), [2]LPS, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris (France), [3]LBME, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse (France)

The RuvAB complex is a molecular motor of the homologous recombination in E. coli. An essential intermediate of this process is the Holliday Junction (HJ) : a cruciform DNA structure formed by two homologous double-strands DNA that exchange their single strands. The RuvAB complex drives the strand exchange between homologous DNA molecules by acting directly on the branch point of the HJ.
We use a magnetic tweezers setup to study the RuvAB mediated strand exchange activity between two 7kb-long homologous DNA double-strands. These experiments allow to observe the RuvAB complex activity in real time and reveal some details of its functioning such as its processivity or its speed.
Ref. : Dawid A. *et al.* (2004) PNAS (101) 11611

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Micro-mechanics of cruciform DNA at the single molecule level and DNA helical pitch measurement

A. Dawid[1], F. Guillemot[1], C. Breme[1], V. Croquette[2], F. Heslot[1]
[1]LPA, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris (France), [2]LPS, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris (France)

Using a magnetic tweezers setup, we directly demonstrate that a cruciform DNA structure (a Holliday Junction) can be extruded from the centre of a palindromic DNA molecule by applying a torsional constraint. Moreover, the strand exchange and the branch point migration can be reversibly driven on several kilo base-pairs by controlling the degree of supercoiling of the system.
With this experimental configuration, we can measure the total number of DNA helical turns contained into the tethered molecule. Surprisingly, from the literature, the pitch of DNA in solution has not been precisely measured. With the above experiment, using the Worm-Like-Chain model of DNA elasticity, we obtain a very precise measurement of the DNA helical pitch in solution.

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Self-organized structures of polyA molecules on the stearic acid LB monolayer

E. V. Dubrovin, S. N. Staritsyn, S. A. Yakovenko, I. V. Yaminsky
Physics Department, Moscow State University, Leninskie Gory, Moscow, 119992, Russia

It is well-known that nucleic acids doesn't tend stretching during its adsorbtion onto freshly cleaved mica or highly oriented pyrolytic graphite (HOPG). Therefore a number of modification procedures of these substrates has been elaborated.
In our experiments Langmuir-Blodgett (LB) films of stearic acid were transferred from NaCl aqueous subphase containing synthetic polyA onto the freshly cleaved mica.
We used atomic force microscopy (AFM) to investigate the surface topography of the samples. The surface consists of domains of stretched parallel polyA molecules, and each domain mostly has its own direction of alignment. Salt solution allows negatively charged polyA molecules to reach stearic acid and to bind with it. NaCl salt also diminishes electrostatic interaction between polyA molecules allowing them to be close to each other and to pack compactly. The surface density of the network from polyA molecules depended on the concentration of polyA in aqueous subphase: while the concentration increases polyA lays more compact onto the substrate.
We have developed a new promising method of creation of self-organized nanostructures of genetic molecules which can be used not only for alignment of these molecules onto the substrate but also in other applications.

Posters

– Single Molecule Biophysics –

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Single molecule study of glycine receptor dynamics and interactions with scaffolding proteins

M.-V. Ehrensperger[1], C. Hanus[2], C. Charrier[2], S. Lévi[2], A. Triller[2], M. Dahan[1]
[1]Laboratoire Kastler Brossel, ENS Paris, [2]Laboratoire de Biologie Cellulaire de la Synapse, ENS Paris

The use of quantum dots as probes for receptors for neurotransmitters allows us to track the motion of single receptors over durations inaccessible with conventional fluorophores.
Previous studies for both inhibitory and excitatory neurotransmitters have revealed a high diversity of lateral dynamics, related to the localisation of the receptors [1], the neuronal activity [2] and the cytoskeleton.
To better analyze the diffusion-capture processes that govern the membrane dynamics, we focus here on the role of the scaffolding protein, gephyrin. Using Hela cells and young neurons (before synapse formation), transfected with both gephyrin-GFP and myc-tagged receptors able (R1) or not (R2) to interact with gephyrin, we aim to quantify in vivo the gephyrin-receptor interactions and their influence on the receptor diffusion. First results in Hela cells shows that R1 are slowed down (compared to R2) even if they are not colocalized with gephyrin cluster. Therefore, we will discuss the parameters that govern the membrane viscosity and how to analyze them.
[1] Dahan et al., Science 302, 442 (2003)
[2] Groc et al., Nature neuroscience 7, 7 (2004)

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Characterization of lipid supported bilayers using parallel AFM-FCS

C. Espenel, E. Margeat, P. Dosset, C. A. Royer, P.-E. Milhiet
Centre de Biochimie Structurale, CNRS UMR 5048-Univ. Montpellier I, INSERM UMR554, 29 rue de Navacelles, 34090 Montpellier Cedex, France.

Lateral heterogeneity in eukaryotic plasma membranes is now well established and involved in several cellular functions. It is mainly explained by the coexistence of phase separated lipid microdomains within the membrane and by lipid-protein interactions. However, static and dynamic organization of the membrane needs to be further investigated and new technical approaches are necessary to understand molecular mechanisms underlying membrane heterogeneity.
Using model supported bilayers on glass or mica we report the study in parallel of the dynamic and the structural organization of membranes by Two-Photon Fluorescence Correlation Spectroscopy and Atomic Force Microscopy.

P-354

Quantitative DNA separation in self-assembled magnetic matrices and microfluidic flow control

C. Fütterer, N. Minc, K. D. Dorfman, J.-L. Viovy
Institut Curie, Laboratoire Physicochimie-Curie, Paris, France

Abstract
We present an experimental/theoretical study of the microfluidic electrophoresis of long DNA in self-assembling matrices of magnetic bead columns. Microfluidic technology proffers great potential for performing rapid, reproducible, inexpensive separations of DNA. In contrary to other separation methods, single molecule observations are possible during the separation. These observations allow to determine the collision parameters for models. Experimental results are presented for the rapid separation of ?-phage DNA and its dimer, where separation resolutions greater than unity are achieved in times as short as 87 s. The use of a new flow control principle allows to eliminate hydrodynamic and electroosmotic flows resulting in high reproducibility between separations. We compare our experimental results with theoretically predictions from an exactly solvable lattice Monte Carlo model. The theory predicts band broadening and resolution, and furnishes reasonable quantitative agreement.

P-355

Single molecule FRET spectroscopy on individual nucleosomes in solution

A. Gansen, F. Hauger, K. Toth, J. Langowski
Biohysics of Macromolecules, German Cancer Research Center (DKFZ), Heidelberg, Germany

Fluorescence resonance energy transfer (FRET) is an important tool to investigate conformational and structural dynamics *in vivo* and *in vitro*.
We describe the confocal single molecule detection system which has been set up in our group. Using a sub-femtoliter observation volume we create single molecule conditions at picomolar concentrations. Single photon counting is applied to analyse particles diffusing freely in solution. Test experiments on double stranded DNA prove the ability to resolve different subpopulations and point out the limitations of one-color FRET experiments. Recent experiments focus on the analysis of the structure and dynamics of reconstituted mononucleosomes under ambient conditions. First experiments verified the bulk measured FRET efficiency of 25-30 % for mononucleosomes consisting of a 160 bp fragment of DNA endlabelled with Alexa488 and RhodamineX, which is wrapped around a HeLa histone octamer. Internal labelling of the DNA fragment greatly enhances energy transfer up to values of 80 %.
The effect of various chemical and physical factors on the nucleosomal structure is investigated, which locally influences the compaction of the chromatin inside the nucleus. Special attention is paid to monitor dynamical fluctuations in FRET efficiencies, while the molecule transits the observation volume. These fluctuations may arise from intrinsic dynamics of the DNA on the nucleosome, where spontaneous un- and rewrapping is supposed to occur.

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– Single Molecule Biophysics –

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Diffusion of spheres in a dynamic network of rods – A simple model of cellular environment

J. Gapiński[1], M. Ratajczyk[1], A. Patkowski[1], K. Kang[2], J. K. G. Dhont[2], M. P. Lettinga[2], J. Buitenhuis[2], G. Meier[2]
[1]Physics Department, A. Mickiewicz University, Poznań, Poland, [2]Forschungszentrum Juelich, IFF, Weiche Materie, D-52425 Juelich, Germany

To a large extent the cellular environment is defined by the dynamic networks of microtubules and actin filaments. All the diffusion controlled biochemical reactions depend on the mobility of many particles acting as substrates, ligands or transporters. For static networks it was found that the particle mobility scales with the ratio a/ξ, where a is particle diameter and ξ is the mesh size. In this work we studied the diffusion of spherical tracers in a dynamic network of rigid rod-like particles (*fd*-virus) whose dynamics was fast enough to couple to the translational motion of the tracers. The long-time self-diffusion coefficient of fluorescently labeled spherical particles (sizes from 3 nm to 500 nm) were measured in a broad range of *fd*-virus concentration both in isotropic and in nematic phase by means of fluorescence correlation spectroscopy (FCS). In the isotropic phase it was found that the factor a/ξ does not scale tracers' diffusion properly. A new theory was developed and successfully applied to the description of sphere diffusion in dynamic networks. In the nematic phase we found splitting of the diffusion coefficient concentration dependence showing the distinction between the diffusion along the director and perpendicular to it. Application of the FCS technique allowed reducing the tracer molecule concentration to nano-molar level, not available to other techniques.

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Fractons in proteins: can they lead to anomalously decaying time-autocorrelations?

R. Granek[1], J. Klafter[2]
[1]Dept. of Biotechnology Engineering and NIBN, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel, [2]School of Chemistry, Tel-Aviv University, Tel-Aviv 69978, Israel

Motivated by recent experimental and theoretical studies focusing on the fractal nature of folded proteins, we analyze the time-dependent autocorrelation function of the distance between two points on a thermally vibrating fractal. Using known results for fractons, the vibrational excitations on a fractal, and neglecting damping, we show that this correlation function is anomalously decaying, and shows a crossover from a nearly stretched exponential decay at short times to a slow algebraic decay at long times. Similar relaxation is found for strongly overdamped fractons. Implications for single molecule experiments detecting protein dynamics are discussed.

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Carbocyanine dye as efficient reversible single-molecule optical switch

M. Heilemann, E. Margeat, R. Kasper, M. Sauer, P. Tinnefeld
Applied Laserphysics and Laserspectroscopy, University of Bielefeld, Germany.

We demonstrate that commercially available carbocyanine dyes such as Cy5 (excited at 633 nm) can be used as efficient reversible single-molecule optical switch at room temperature, whose fluorescent state after apparent photobleaching can be restored upon irradiation at shorter wavelengths. Ensemble photobleaching and recovery experiments of Cy5 in aqueous solution irradiating first at 633 nm, then at 337, 488, or 532 nm, demonstrate that restoration of absorption and fluorescence strongly depends on efficient oxygen removal and the addition of the triplet quencher β-mercaptoethylamine. Single-molecule fluorescence experiments show that individual immobilized Cy5 molecules can be switched optically in milliseconds by applying alternating excitation at 633 and 488 nm between a fluorescent and nonfluorescent state up to 100 times with a reliability of >90% at room temperature. Because of their intriguing performance, carbocyanine dyes volunteer as a simple alternative for ultrahigh-density optical data storage. Measurements on single donor/acceptor (TMR/Cy5) labeled dsDNA demonstrate first insights into the switching mechanism.
We further present first results of a more general view of fluorescent chromophores at the single molecule level. The influence of triplet and radical ion states onto the photophysics of fluorescent dyes, and hence their stability, has been investigated by using triplet quenching molecules and changing the redox potential of the microscopic environment.

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Bioconjugation and applications of fluorescent quantum dots for single-molecule biophysics

C. D. Heyes[1], V. V. Breus[1], A. Y. Kobitski[1], G. U. Nienhaus[1]
[1]Department of Biophysics, University of Ulm, 89069 Ulm, Germany, [2]Department of Physics, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA

Quantum Dots (QDs) are promising new fluorescent probes for biophysics. Their brightness, multiple colors and photostability can be taken advantage of in long-timescale single-molecule studies of biomolecules. However, the bioconjugation strategy of QDs becomes very important for their use in such ultrasensitive studies. We have been systematically studying the bioconjugation strategies with respect to the following criteria: total QD size, quantum yield, surface composition, bioavailability, chemical stability and photochemical properties. Here, we present details of their bioconjugation together with applications in single-molecule FRET of labeled proteins and single particle tracking (SPT) of labeled lipids. The advantages and potential pitfalls of using quantum dots for such ultrasensitive experiments are also discussed.

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– Single Molecule Biophysics –

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Single hydrogen bonds of DNA base pairs detected by intermolecular force microscopy

M. Hiroshima[1], M. Tokunaga[2]
[1]Natl. Inst. of Genetics, RIKEN, [2]Natl. Inst. of Genetics, RIKEN, Grad. Univ. for Advanced Studies

Single hydrogen bonds of DNA base pairs have been resolved by unzipping double-stranded DNA molecules with intermolecular force microscope (IFM). To detect such ultrafine forces, high force resolution as well as high accuracy in probe positioning is required. Subpiconewton resolution was achieved using ultra-sensitive cantilevers. The probe position is controlled with nanometer accuracy using laser radiation pressure with feedback.
Force-extension curves showed repeated force peaks of 10-15pN. The previous studies showed that the force for separating poly(G-C) DNA was 1.5 to 2 times stronger than that for poly(A-T). However, we found no such difference in the force between individual G-C and A-T base pairs.
The force-extension curves are averaged over sequence repeats using correlation functions. The force curve of individual G-C and A-T pairs is composed of three and two peaks, respectively, which are assigned to single hydrogen bonds.
The force is variable depending on the disruption length but the work is constant. The work to disrupt single hydrogen bonds is 5.4 $\pm$ 1.7 pN·nm, which is approximately 1.3-fold of the thermal energy. This is the first report to detect directly the force of single hydrogen bonds in biological macromolecules.

P-361

Simultaneous optical and electrical recording of single ion-channels

T. Ide, Y. Takeuchi
Japan Science and Technology Agency

Purpose: The purpose of this study was to develop an apparatus for simultaneous measurement of electrical and spectroscopic signals from single ion-channel proteins.
Methods: The microscope designed for single-fluorophore detection was combined with the artificial bilayer single-channel recording system. The artificial membranes were formed horizontally in an aqueous environment or on a thin polymer (agarose or polyethyleneglycol) layer. Single molecules in the bilayer were observed under an epi-fluorescence or an objective-type total internal reflection fluorescence micoscope. Single-channel currents across the bilayers were measured by a patch clamp amplifier under voltage clamped conditions.
Results: The apparatus developed in this study was sensitive enough to detect the optical signals from single-fluorophores in the bilayer simultaneously with the single-channel current recording. Using this apparatus, the following results were obtained: (i) The diffusion constant of a lipid molecule was calculated from the trajectories of single molecules as D=8.5x10^{-8}cm^2/s, indicating that the lipids in the membrane moved freely. (ii) The Cy5-conjugated K(Ca)-channel was incorporated into the bilayer and the fluorescence image was recorded simultaneously with the channel current recording at the single-channel level. (iii) Various types of channels could be incorporated into the bilayers on a polymer layer. The properties of the channels were identical to those determined in the bilayer formed in an aqueous environment.

P-362

Protein-induced interactions with the plasma membrane studied by atomic force microscopy

M. Janke, A. Janshoff
Universität Mainz; Institut für Physikalische Chemie

The attachment of cytoskeletal elements to the plasma membrane is a very sophisticated process that is not fully understood in detail so far. In order to identify and quantify the involved forces it is important to develop a model system which separates the individual interactions and enables their direct examination. The AFM is an appropriate tool to serve this purpose because it allows the visualization and characterization of the mimicking system and subsequently the performance of force spectroscopy experiments. The intention of this study was the establishment of a proper model system to examine the resulting forces of the interaction between actin filaments and the plasma membrane, mediated by particular cross-linking proteins. Consistently the adsorption of Ezrin, a member of the ERM-family, on solid supported membranes has been visualized by AFM. By functionalization of the cantilever with Actin, pulling experiments could be performed.

P-363

Single-molecule investigation of calmodulin: conformations, dynamics, and target activation

C. K. Johnson, B. D. Slaughter, A. Mandal, J. R. Unruh, E. S. Price
University of Kansas, Lawrence, KS, USA

The calcium signaling protein calmodulin (CaM) binds and activates a diverse array of target enzymes. The conformations and dynamics of CaM in solution are essential to the activation of target enzymes but remain poorly understood. We used single-pair Förster resonance energy transfer (spFRET) to probe the dynamics and conformations of CaM. Burst-integrated spFRET measurements demonstrate the presence of three conformational substates, of which the dominant population does not match any structure predicted by x-ray crystallography. These populations interchange on the millisecond time scale. Cross correlation of spFRET signals reveals dynamics on the 100s of microsecond time scale. The populations of the conformational substates of CaM in solution are shown to depend on Ca^{2+}, pH, ionic strength, and oxidative modification, suggesting that electrostatic interactions between negatively charged residues play an important role in determining the conformation of CaM in solution. The existence of conformational substates of CaM also raises questions about its unfolding pathway. spFRET demonstrates the existence of at least one unfolding intermediate, possibly corresponding to unfolding of one of the two terminal lobes of CaM. Activation of a target enzyme, the plasma-membrane calcium ATPase, was investigated by single-molecule polarization modulation spectroscopy, which revealed states of CaM having different orientational mobilities when bound to the enzyme in a manner that correlates with the activity of the enzyme.

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Imaging of S-DNA - New DNA form with 2 Å rise per base pair

O. Y. Limanskaya, A. P. Limanskii
Institute of Microbiology and Immunology of UAMS

Supercoiled pGEMEX DNA with length of 3993 nucleotides was immobilized on the different substrates (freshly cleaved mica, standard aminomica, modified aminomica with increased and decreased aminogroups surface density in comparing with standard aminomica) and it was visualized by atomic force microscopy.
DNA molecules with extremely high level of supercoiling were visualized on modified aminomica characterized by increased surface charge density (i.e. density of protonated aminogropes). Contour length of oversupercoiled DNA molecules was measured from AFM images and distance between base pairs along duplex axis was determined. Rise value per base pair was varied from H = 1.94 Å up to H = 2.19 Å for different molecules. These compressed supercoiled DNA molecules like a spring with decreased rise/base pair in comparison with well known DNA forms were called new DNA form – S-DNA. Determined base slope for S-DNA molecules was in the range $27^{\circ} < \gamma < 30.5^{\circ}$ for different molecules.
Molecular models of S-DNA and A-DNA were assembled. S-DNA looks like a compressed spring and S-DNA molecules are characterized by considerably decreased base pair rise in comparison with well known A- and B-DNA families. Possible mechanisms of DNA oversupercoiling are discussed. Crucial influence of mice surface charge density on the DNA conformation was shown.

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Compactization of single supercoiled DNA molecules on the modified aminomica

O. Y. Limanskaya, A. P. Limanskii
Institute of Microbiology and Immunology of UAMS

Supercoiled DNA pGEMEX with length of 3993 nt was immobilized on the different substrastrates (freshly cleaved mica, standard aminomica and modified aminomica) and visualized by atomic force microscopy. Plectonomically supercoiled DNA molecules as well as molecules with extremely high level of compactization (i.e. molecules with considerably higher superhelix density values in comparing with previously experimentally measured and theoretically investigated ones) were visualized on modified aminomica which was characterized by increased charge surface density. At the further increasing of the compactization level an axis length of supercoiled molecule is decreased. Length of molecules superhelix axis of the first order is decreased from $\sim$ 580 nm to $\sim$ 370 nm and following formation of superhelix axis of second and third orders with length of $\sim$ 260 nm and $\sim$ 140 nm ($\sim$ 10 % of contour length of relaxed molecules) respectively at the increasing of the compactization level. Formation of minitoroids with $\sim$ 50 nm diameter and molecules in spheroid conformation were final stages of the single molecules compactization.
Our data indicate that DNA compactization up to level of spheroids and minitoroids are caused by influence of increased surface charge density and changing substrate hydrophobic properties. Model of possible conformational transitions of supercoiled DNAs in vitro without proteins is proposed.

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Effect of porphyrin sensitizers on grow rate of G361 human melanoma cell line

J. Macecek[1], H. Kolarova[1], R. Bajgar[1], P. Nevrelova[1], M. Tomecka[1], K. Tomankova[1], J. Mosinger[2], M. Strnad[3]
[1]Department of Medical Chemistry, Faculty of Medicine, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Rebublic, [2]Department of Inorganic Chemistry, Faculty of Science, Charles University in Prague, Czech Republic, [3]Laboratory of Growth Regulators, Palacky University, Slechtitelu 11, 78371 Olomouc, Czech Republic

Photodynamic therapy (PDT) is relatively new treatment modality based on cytotoxic effect of sensitizers excited by appropriate light source with a suitable wavelength in the oxygen-rich environment. Sensitizers used in clinical practice should have low cytotoxicity without light excitation and high phototoxicity in presence of light. In this study we were tested cytotoxic effect of new generation of sensitizers *meso*-tetrakis (4-sulfonatophenyl) porphyrin ($TPPS_4$) and its zinc and palladium metallocomplexes $ZnTPPS_4$ and $PdTPPS_4$. The result quantifies grow of malign cells according to different concentration of sensitizers and sensitizers with 2-hydroxypropyl-cyclodextrins (hpCD's). Cyclodextrins are able to form noncovalent host-guest complexes to prevent aggregation of guest and facilitate transfer into cells. All three types of porphyrin sensitizers and also host-guest complexes have no cytotoxic effect on grow rate of melanoma cells. Due to lipophilic character of sensitizers, preferential penetration to tumours, low cytotoxicity and high phototoxicity are suitable for photodynamic therapy of human melanoma G361 *in vitro*. This work was supported by the grant project of Ministry of Education MSM No.6198959216.

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Real time imaging of DNA ejection from single phage particles

S. Mangenot[1], M. Hochrein[2], J. Radler[2], L. Letellier[1]
[1]CNRS, Université ParisSud, Orsay, France, [2]Ludwig Maximilan Universität, Münich, Germany

Infection by tailed dsDNA phages is initiated by release of the viral DNA from the capsid and its polarized injection into the host. The driving force for the genome transport remains poorly defined. Among many hypotheses it has been proposed that the internal pressure built up during packaging of the DNA in the capsid is responsible for its injection. Whether the energy stored during packaging is sufficient to cause full DNA ejection or only to initiate the process was tested on phage T5 whose DNA (121 kbp) can be released *in vitro* by mere interaction of the phage with its *E.coli* membrane receptor FhuA. Fluorescence microscopy was used to investigating in real time the dynamics of DNA ejection from single T5 phages adsorbed onto a microfluidic cell. The ejected DNA was fluorescently stained and its length was measured at different stages of the ejection after being stretched in a hydrodynamic flow. We conclude that DNA release is not an all-or-none process but occurs in a stepwise fashion and at a rate reaching 75000 bp/sec. The relevance of this stepwise ejection to the *in vivo* DNA transfer is discussed.
Mangenot S., Hochrein M., Rädler J. and Letellier L. (2005), Real-time imaging of DNA ejection from single phage particles. Current Biology, 15, 430-435
Letellier, L., Boulanger, P., Plancon, L., Jacquot, P., and Santamaria, M. (2004). Main features on tailed phage, host recognition and DNA uptake. Front. Biosci. *1*, 1228-1239.

Posters

– Single Molecule Biophysics –

P-368

Observation of abortive initiation on single transcription complexes, using TIR microscopy with ALEX

E. Margeat[1], A. N. Kapanidis[1], P. Tinnefeld[1], Y. Wang[1], J. Mukhopadhyay[2], R. H. Ebright[2], S. Weiss[1]
[1]Single Molecule Biophysics Group - UCLA - USA, [2]HHMI - Rutgers University - USA

Using total-internal-reflection fluorescence microscopy with alternating-laser excitation, we are able to detect abortive initiation and promoter escape within single immobilized transcription complexes. Our approach involves monitoring of Förster resonance energy transfer (FRET), and thus distance, between a fluorescent donor incorporated on RNA polymerase (RNAP) and a fluorescent acceptor incorporated on DNA. We observe small, but reproducible and abortive-product-length-dependent, decreases in distance between the RNAP leading edge and DNA downstream of RNAP upon abortive initiation, and we observe large decreases in distance upon promoter escape. Inspection of population distributions and single-molecule time traces for abortive initiation indicates that, at a consensus promoter, at saturating NTP concentrations, abortiveproduct release is the ratelimiting step.

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A new view of homologous recombination provided by single molecule nanomanipulation

J. Mine[1], R. Fulconis[1], A. Bancaud[1], M. Dutreix[2], J. L. Viovy[1]
[1]Laboratoire Physico-Chimie Curie, UMR CNRS 168, Institut Curie, Paris, France, [2]Laboratoire Génotoxicologie et Cycle Cellulaire, UMR CNRS 2027, Institut Curie, Orsay, France

RecA protein is the central enzyme involved in homologous recombination in *Escherichia Coli* bacterium. Homologous recombination consists of exchanging DNA strands between homologous molecules. This process has two functions: (i) it is a major source of genetic diversity, (ii) it plays a fundamental role in DNA repair and genome maintenance. We use magnetic tweezers to study homologous recombination at the single molecule level. It enables us to exert both a stretching force in the pN range and a torsional constraint on a single DNA, and to measure its extension in real time with a precision of 10 nm. We investigate the mechanism of recombination by RecA protein, observing the process in real time on a single DNA held in magnetic tweezers. These experiments confirm the buildup of torsional stress and the formation of a three-strand synapsis during exchange. Moreover, they reveal some features at odds with the current view of recombination, in particular a fast depolymerization of RecA upon strand-exchange, and a limited size of the synapsis.

Fulconis R., Bancaud A., Allemand J-F., Croquette V., Dutreix M., Viovy J-L.
Twisting and Untwisting a Single DNA Molecule Covered by RecA Protein.
Biophys J. 2004 Oct

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Herpes simplex virus heterogeneity studied by two-color fluorescence analysis of single virions

N. Monnier[1], H. Li[1], D. Zhou[1], H. Browne[2], D. Klenerman[1]
[1]Dept of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK, [2]Division of Virology, Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

Many structural features of enveloped viruses have been difficult to study because both X-ray crystallography and cryo-electron microscopy require regular geometries. The herpes simplex virus icosahedral capsid is surrounded by an irregular protein layer (tegument) and lipid envelope. To study the protein composition of these viral structures, we have analyzed virion-to-virion variability in levels of a tegument protein (VP16) and an envelope glycoprotein (gD) of herpes simplex virus, relative to levels of a capsid protein (VP26), using two-color coincidence fluorescence spectroscopy. Measurements were made on single virions containing either YFP-VP26 or GFP-VP16 fusions and coated with an Alexa-647-labeled monoclonal antibody to gD. Coincident fluorescent bursts in both color channels were selected and their intensity ratios calculated to obtain histograms showing the variation in relative levels of these proteins between individual virions. Our results show clear differences between the variation of gD, VP16, and VP26 in herpes virions, providing new insights into enveloped virus composition and packaging and demonstrating a new and general method for studying virus heterogeneity.

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Fluorometric assessment of ROS production after photodynamic reaction

P. Nevrelova, H. Kolarova, R. Bajgar, J. Macecek, M. Tomecka, K. Tomankova
Department of Medical Biophysics, Medical Faculty, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic

Photodynamic therapy (PDT) offers an alternative, less invasive treatment for several types of cancers. It involves the use of sensitizer, light, molecular oxygen and biological substrate. After light activation, sensitizer is excited to triplet state and can react with biomolecules via type I or II mechanism, resulting in cell death and tumor necrosis. Type I mechanism involves electron transfer reactions to form free radicals and radical ionts, that react rapidly with oxygen, leading to the production of reactive oxygen species (ROS). Type II reactions (energy transfer) produce a highly reactive singlet oxygen. In this study, we engaged in photodynamic effects of free sensitizer $ZnTPPS_4$ or sensitizer bound to cyclodextrine carrier hpâCD on G361 human melanoma and MCF7 mamma carcinoma cells. The production of ROS after PDT was detected using nonfluorescent probe CM-H_2DCFDA. After probe oxidation the fluorescence was recorded by luminescence spectrometer. For timedrive measurements the suspension of cells was irradiated by LEDs (420 nm). The production of ROS in photosensitized cells was the highest for $ZnTPPS_4$ without hpâCD carrier. This work was supported by the grant project of Ministry of Education FRVS No. 562/2005 and MSM 6198959216.

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– Single Molecule Biophysics –

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Measuring fast conformational dynamics of freely diffusing single DNA Hairpin

E. Nir, S. Weiss
Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, California, 90095

We report advances towards measuring fast conformational dynamics of freely diffusing macromolecule in solution using single pair FRET (spFRET). The methodology, based on combining nanosecond or microsecond alternated laser excitation (ALEX) with cross-correlation and spFRET histogram analysis of ALEX resolved subpopulations in a complex mixture, extends the dynamic range for temporal fluctuations measurements down to a few microseconds. Such measurements could quantify the amplitude and the time scale of rapid, small fluctuations in proteins and nucleic acids.
Several DNA hairpins, with different opening and closing rates were synthesized, site-specifically labeled and used as model systems for measuring fast conformational dynamics. These measurements were backed-up by detailed monte-carlo simulations that account for the key factors in the experiment, including: diffusion, photophysics, detection volumes, background and detection efficiency. Comparing simulations with experiments assist in understanding and in elimination of artifacts.
By comparing the spFRET histograms of a few different two-state-DNA-hairpins to simulation we are able to resolve the opening and closing rates of those hairpins.
The new methodology provides information about energy landscapes and kinetics of fluctuating macromolecules such as proteins and nucleic acids.

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A dual DNA molecule experiment reveals details of H-NS mediated bridging of DNA

M. C. Noom, R. T. Dame, G. J. Wuite
FEW, Complex Systems, Vrije Universiteit Amsterdam, noom@nat.vu.nl

Bacterial chromatin is organized and compacted at least in part by interaction with nucleoid-associated proteins. We have shown (with Scanning Force Microscopy) that one of these proteins, H-NS, compacts DNA by the formation of bridges (links) between adjacent DNA duplexes. This property is exploited not only for providing a means of compaction, but is also essential to the regulatory role of H-NS in transcription. DNA bridging directly explains the recognition of curved DNA and transciptional repression by H-NS.
We have developed an experimental setup that features quadruple optical tweezers which allows the simultaneous and independent manipulation of two DNA molecules. Using this setup, we demonstrate the formation of H-NS mediated physical links between two DNA molecules. Disassembly can be induced by applying an external force to unzip or shear the bridged complexes. Applying a shear force results in the gradual disruption of bridged tracts, whereas the application of an unzipping force leads to the disruption of such an area in a stepwise manner. Qualitative and quantitative analysis of the steps and forces involved leads us to propose a refined model for the compaction and the organisation of DNA by H-NS.

P-374

Single Molecule Monitoring of Transmembrane Receptor Proteins

A. Noskov, R. Steinmeyer, G. S. Harms
Rudolf-Virchow-Zentrum für Experimentelle Biomedizin der Universität Würzburg, Versbacher Str. 9, 97078 Würzburg, Germany

The change of lateral mobility between the active and inactive state of membrane receptors plays an important role in the regulation and in signaling of cells. The approach of single-molecule tracking allows the study of mobility of specific proteins separately in the active or inactive state. Fluorescence labeled ligands could also reveal differences in diffusion patterns of single receptors upon activation. Our experimental setup consists of a laser used for wide field fluorescence excitation on an inverted microscope equipped with an ultra sensitive CCD-camera. This allows us to obtain images of individual fluorophores in membranes of living cells with a rate of up to 30Hz.
By the method, receptor systems for Bone Morphogenetic Proteins were examined for: a) inhomogeneities and differences in diffusion b) stoichiometry of effector-receptor interaction d) random diffusion processes as well as directed and confined motion of receptors in free and activated state. These single-molecule observations were correlated with such events like initiation of distinct signaling cascades by single receptor complex upon activation, and involvement of specific lipid microdomains and cytoskeleton elements in regulation of signaling processes for this family of cytokines.

P-375

Determining the oligomeric state of a membrane protein using Atomic Force Microscopy

M. Odorico, D. Marcellin, J.-M. Teulon, L. Bellanger, O. Averseng, P. Guérin, P. Parot, E. Darrouzet
CEA VALRHO, DIEP/SBTN, Bagnols sur Ceze, France

As membrane protein studies increase, more and more of these hydrophobic polypeptides are found to function in oligomeric state. However, due to the detergent shell that surrounds the protein during purification, it is often tedious to determine with confidence their oligomeric state in the different fractions of a purification process. In addition, we are interested to know if high molecular weight populations correspond to randomly aggregated (and denatured?) proteins or to organized assembly. In this work, we will report on the usefulness and relative simplicity of AFM to answer these questions taking the human sodium-iodide symporter (NIS) as example. We deposited purification fractions of NIS obtained from exclusion chromatography on freshly cleaved mica and present molecular images of various oligomeric states of NIS using the intermittent contact mode of AFM.

Posters

– Single Molecule Biophysics –

P-376

How to grow a large single crystal for neutron macromolecular crystallography

Y. Onishi[2], T. Ito[2], T. Ishikawa[1], Y. Kobayashi[1], D. Yagi[1], T. Ichige[1], I. Tanaka[1], K. Kurihara[3], N. Niimura[1]
[1]Ibaraki University, [2]Kaken Co. Ltd, [3]Japan Atomic Energy Research Institute

Neutron diffraction provides an experimental method of directly locating hydrogen atoms in proteins and hydration structure of proteins. The disadvantage, however, of neutron macromolecular crystallography is that large crystals are needed: currently, the volume of samples to be studied should be larger than 1 mm^3. We have found that one rational way to find the proper conditions to grow large single crystals is to establish a detailed crystallization phase diagram. Generally speaking, a large single crystal can be grown under supersaturated conditions close to the solubility boundary. As a matter of fact, large single crystals of three macromolecular samples, which were successfully used in neutron protein data collection.
For drawing the phase diagram and subsequently growing a large crystal, we have designed a novel crystallization device by modified dialysis method.
Neutron diffraction experiments for basic proteins such as pH dependent of cubic insulin, 2Zn-insulin, β-lactoglobulin, and α-amylase are scheduled to obtain the hydration structure and the crystallization of these proteins based on phase diagram strategy are currently under way.

P-377

Light generated and light driven micomachines for single particle manipulation

P. Ormos, P. Galajda, L. Kelemen, H. Kirei, L. Oroszi, S. Valkai
Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, Szeged

Photopolymerisation offers a relatively simple method to produce microscopic particles of arbitrary shape that are practical to expand the possibilities of optical manipuation. There are different ways to influence the orientation of non spherical objects in the optical traps. For example, propeller shaped micrometer sized rotors are rotated in optical tweezers, while flat objects are oriented in traps formed by linearly polarized light. The force and toque exerted by these devices falls in the range of those occuring in biology. Consequently, such elements and the possibilities opened up by their use would find numerous applications biology, both in fundamental science and biotechnology (lab-on-a-chip devices) by realising new types of manipulation.
Photopolymerisation is also a practical method to create microchannels, microvessels, as well as optical waveguides. By the combination of these elements complex, fully integrated systems are built where the manipulation and observation of single molecules, cells or other biological objects of up to micometer size can be realised in a practical manner.
We demonstrate the function of the different elements and show how they can be applied in single particle experiments.

P-378

Single PrOTein NanObioSEnsor griD array IST-2001-38899-SPOT-NOSED European project

E. Pajot[1], .. European Consortium[2]
[1]INRA Jouy-en-Josas France, [2]see website

The objective is to develop nanobiosensors based on single olfactory receptors (ORs) anchored between nanoelectrodes, by detecting impedancemetric changes arising from ORs conformational change upon odorant binding. They should benefit from ORs individual properties ; specificity, low detection thresholds, high reproducibility, and could constitute new electronic noses for rapid detection/caracterization of products (dangerous compounds, medical diagnosis, food safety ...), for de-orphanizing receptors, or as a substitute for human sensorial analysis. Integration of individual nanosensors into multisensors arrays could increase sensitivity or widen the detection spectrum.
An OR is modelled by an equivalent impedance network, by associating an elemental RC impedance to each link between neighbour aminoacids, predicting a detectable impedance change. Nanoelectrodes with a 40 nm inter-electrode distance are obtained by focused ion beam. ORs are specifically immobilized onto conducting substrates via a mixed self-assembled monolayer, neutravidin, specific biotinylated antibody, and grafting of 40-60 nm nanosomes prepared from membrane fraction of yeast expressing the receptor. A transimpedance preamplifier specially suited for low-noise wide-bandwidth measurements was designed and directly connected to the nanoelectrodes.
www.nanobiolab.pcb.ub.es/projectes/Spotnosed/

P-379

Analysis of flow profiles in microchannels by scanning fluorescence correlation spectroscopy

X. Pan[1], H. Yu[3], T. Wohland[2]
[1]Graduate Programme in Bioengineering, National University of Singapore, [2]Department of Chemistry, National University of Singapore, [3]Department of Physiology, National University of Singapore

There is a growing need for simultaneous imaging and characterization of samples by confocal microscopy and spectroscopy. In this work we introduce a setup based on a combination of laser scanning confocal microscopy (FV300, Olympus) and fluorescence correlation spectroscopy (FCS). This setup allows imaging and FCS flow profile measurements by simple laser beam scanning without the need to move the sample stage. To measure flow directions we introduce a novel method based on scanning FCS (sFCS). We demonstrate that by scanning the laser beam with a defined speed and direction we can measure flow directions in microchannels with a resolution of 3μm. The system is assessed by measuring flow profiles in a standard microchannel and its accuracy is determined. To further verify the technique, a microchannel incorporating micropillars with an unknown flow pattern is used. A 2D map of the flow distribution across the microchannel and around micropillars is measured and plotted, and different flow directions between micropillars both at the inlet and the central part of the microchannel are obtained by sFCS. This method is a good tool to determine flow conditions in tissues which are an important factor for tissue function and viability.

Posters

– Single Molecule Biophysics –

P-380

Importance of the surface for studying DNA/ligand complexes by Atomic Force Microscopy

D. Pastré[1], O. Piétrement[2], F. Landousy[2], L. Hamon[1], J. Jeusset[2], M.-O. David[1], A. Zozime[1], E. Le Cam[2]
[1]Laboratoire Milieux Nanométriques, Université d´Evry - Rue du Père Jarlan, 91025 Évry Cedex, France., [2]Laboratoire de Microscopie Moléculaire et Cellulaire, UMR 8126 CNRS-IGR-UPS, Institut Gustave-Roussy - 39 rue Camille Desmoulins, 94805 Villejuif

The Atomic Force Microscope (AFM) is a useful tool for imaging DNA/ligand complexes at a nanometric scale in liquid. Weak DNA adsorption is obtained by the sharing of divalent counterions between DNA and mica which are both highly negative bodies. In order to analyse the information provided by AFM, it is necessary to get further insights about the influence of the surface on the formation of DNA/ligand complexes. Here is presented the study of two drugs: ethidium bromide which is a DNA intercalator and, bleomycin which induces double and single-strand breaks. We show that the surface friction between DNA and mica induced by multivalent counterions prevents intercalation by ethidium depending on the mica surface charge. Besides, DNA cleavage by bleomycin is inhibited by a positively charged surface. This study shows that the information provided by AFM about DNA/ligand complexes could be biased by the surface.

P-381

Single hairpin ribozymes provide insights into structural roles of conserved nucleotides

G. Pljevaljcic, D. P. Millar
The Scripps Research Institute, Department of Molecular Biology (MB-19), 10550 North Torrey Pines Road, La Jolla, CA 92037

The hairpin ribozyme is a popular paradigm for RNA tertiary structure formation. Its complex, catalytically-active docked conformation is stabilized by a network of 14 hydrogen bonds connecting the two loop-bearing arms of the ribozyme. The G+1 pocket consists of 5 H bonds which are placed around the conserved G+1 nucleotide, 5 additional H bonds are located around the conserved U42 nucleotide and the remaining 4 H bonds involve a ribose zipper motif. Initial studies to evaluate the energetic contributions and couplings of individual H bonds to tertiary structure formation are complicated by the presence of a quasi-docked conformation that is indistinguishable from the specifically docked tertiary structure in standard bulk experiments. However, these two species are readily distinguishable in single-pair FRET measurements of freely-diffusing ribozymes labeled with donor and acceptor dyes. We examined a series of different ribozyme variants lacking one or more H bonds at the docking interface. Surprisingly, the hydrogen bonds within the G+1 pocket contribute little to the stabilization of the docked state, whereas specific H bonds within the U42-pocket contribute immensely to tertiary structure stabilization. Our results provide new insights into the structural roles of the conserved nucleotides G+1 and U42.

P-382

End synapsis in transposition of IS911 studied by a single molecule approach

N. Pouget[1], C. Turlan[2], N. Destainville[3], L. Salome[1], M. Chandler[3]
[1]IPBS UMR CNRS 5089, [2]LMGM UMR CNRS 5100, [3]Laboratoire Physique Théorique UMR CNRS 5152

The transposition process, involved in genome plasticity, consists of the mobilisation of genetic elements from one site to another. We study IS*911* a member of the widespread IS*3* family of bacterial insertion sequences which follows a two-step transposition mechanism involving the formation of a circular transposon intermediate. Like other insertion sequences, IS*911* is bordered by short imperfectly repeated sequences, IRL and IRR, in an inverted orientation. These are essential for productive transposition, since they provide the specificity for both the binding of the transposase, OrfAB, and for the cleavage and strand transfer reactions required for the displacement of the element.
Plenty of informations, unaccessible by classical approaches but necessary for the full understanding of the transposition mechanism, can be obtained from experiments performed on single DNA molecules. For this purpose, we developed the « Tethered Particle Motion » technique, based on the videomicroscopy observation of the movement of a bead tethered by the DNA molecule to a glass surface. We present here the results of the analysis of the protein binding to a sequence end and the paired end complex (giving rise to a loop) formation and stability in the presence of a derivative transposase OrfAB149, truncated for its C-terminal catalytic domain.

P-383

Metal-bound protein – antibody interactions in vitro SPR and AFM studies

L. Reiser, L. Bellanger, M. Odorico, J.-M. Teulon, J.-L. Pellequer, P. Parot, C. Vidaud
CEA-Valhrô, DSV/DIEP/SBTN Bagnols sur Cèze France

Heavy metals such as actinides pose important human health hazards and the investigation of their uptake by specific biomolecules is a major field in human toxicology. Immediate health risks associated with uranium exposure include kidney disorders and respiratory problems. To characterize the mechanisms of intermolecular interaction between proteins and uranyl ions, we explore a model system using antibodies as protein receptors. Immunology offers a sensitive approach to obtain highly specific proteins for uranyl ion recognition. We selected two monoclonal antibodies for their capacity to bind to uranyl $(UO_2)^{2+}$ cation bound to a phenanthroline hapten. We characterized the binding properties of these two antibodies using Surface Plasmon Resonnance (BIAcore technology) and using atomic force microscopy high-resolution imaging of single antibody-antigen molecules.

Posters

– Single Molecule Biophysics –

P-384

Probing membrane dynamics in neuronal synapses

M. Renner[1], L. Cognet[2], B. Lounis[2], D. Choquet[1]
[1]CNRS UMR 5091 Université de Bordeaux-2, Bordeaux, France, [2]CNRS UMR 5798 Université de Bordeaux-1, Talence, France

Recent studies have shown that neurotransmitter receptors exchange between synaptic and extrasynaptic spaces by lateral diffusion, being mobile also inside synapses. Their diffusion might play an important role in synaptic transmission as the modification of synaptic strength depends on changing the amount of synaptic glutamate receptors. We use single molecule and single particle techniques to study the properties of the diffusing space inside synapses, analyzing the diffusion of two lipids and AMPA receptor subunit GluR2. The lipids chosen were Gm1 (visualized by cholera toxin, ChTx) and dioleoyl-phosphatidylethanolamine (DOPE). Outside synapses, DOPE showed anomalous or free diffusion while GluR2 and ChTx showed confined or anomalous diffusion. Inside synapses all the molecules are confined. This behavior could be changed by the disruption of the cytoskeleton, as after F-actin depolymerization by latrunculin A, ChTx and DOPE showed free diffusion. In case of GluR2, latrunculin treatment causes a major loss of receptors in synapses and the remaining receptors have reduced mobility, suggesting that cytoskeleton disruption allows the mobile fraction to escape. In conclusion, synapses are a structured space that allows rapid although confined diffusion and cytoskeleton integrity is important for this confinement.

P-385

Characterization of the interaction between the lipopolysaccharide-binding protein and membranes

S. Roes, M. Müller, U. Seydel, T. Gutsmann
Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Division of Biophysics, Parkallee 10, 23845 Borstel, Germany

During an infection with Gram-negative bacteria, lipopolysaccharides (LPS) are eventually released from bacterial outer membranes and trigger a cascade of events starting with the activation of mononuclear cells (MNCs) of the host, leading to the release of proinflammatory cytokines, and finally causing sepsis and septic shock. A number of proteins are involved in the transmembrane signaling of MNC, and in this study we investigated the function of the LPS-binding protein (LBP) using atomic force microscopy and other biophysical methods including reconstituted planar bilayers. Reconstituted phospholipid membrane systems were used to characterize the interaction of LBP. At low LBP concentrations, the protein was embedded in a transmembrane configuration and could only be localized in the membrane using a cantilever tip functionalized with an anti-LBP antibody. At higher protein concentrations, LBP tends to form clusters of several molecules and leads to cross-linking of lipid bilayers. We could show that LBP functions as a fusion protein between lipid membranes and aggregates of lipid A, the endotoxic principle of LPS.

P-386

Single molecule DNA stretching study of T4 gene 32 protein binding to single-and double-stranded DNA

I. Rouzina[1], K. Pant[2], R. Karpel[3], M. C. Williams[2]
[1]University of Minnesota, [2]Northeastern University, [3]University of Maryland Baltimore County

Bacteriophage T4 gene 32 protein (gp32) is a single-stranded DNA (ssDNA) binding protein, which is essential for DNA replication, recombination and repair. The primary function of gp32 is to polymerize on exposed ssDNA regions behind the moving helicase in the T4 replication fork at the rate of up to 1000 base pairs per second. While being a very strong and fast binder of ssDNA, gp32 is, however, unable to melt dsDNA. In this work we resolve this paradox based on the results of our single DNA molecule studies of the kinetics and thermodynamics of gp32 interaction with both ss- and ds-DNA. By measuring both equilibrium, and pulling rate dependent DNA melting force in the presence of gp32 we were able to obtain its binding constants to ss- and ds- DNA, as well as to determine the kinetic mechanism of gp32-suppotred ds-DNA melting. We show that the slow step of this melting process is the thermal melting of 7 base pairs at the duplex end, followed by the one-dimensional search for melted site by gp32 molecule pre-bound to ds-DNA. In addition, by studying gp32 mutants we found that gp32 binding to both ss- and ds-DNA is regulated by the salt dependent opening off its C-terminal domain. *In vivo* interaction of this domain with other proteins of the replication fork provides the mechanism of regulation of the gp32 ds-DNA melting ability.

P-387

Why is the counterion-induced DNA compaction preferred at higher temperature?

T. Saito, T. Iwaki, K. Yoshikawa
Department of physics, Graduate school of science, Kyoto university, Japan

Upon the addition of multivalent cations, a giant DNA chain exhibits a large discrete transition from an elongated coil into a folded compact state. To clarify the mechanism of the DNA compaction, we performed single-chain observation of long DNAs in the presence of tetravalent cations (spermine), by changing the temperature and monovalent salt concentration. It was found that the compact state is more stable at higher temperature and at lower salt concentrations. Our results suggest that ionic exchange plays an important role in the manner of transition, i.e., the effects of both temperature and the monovalent salt concentration on compaction originate from the increase in net translational entropy due to ionic exchange between higher and lower valence ions.
arXiv:cond/mat/0411028v1 1 Nov 2004

Posters

– Single Molecule Biophysics –

P-388

Single molecule measurements of the motor protein FtsK

O. A. Saleh[1], S. Bigot[2], F.-X. Barre[3], J.-F. Allemand[1]
[1]Laboratoire de Physique Statistique, Ecole Normale Superieure, Paris, France, [2]Laboratoire de Microbiologie et de Génétique Moléculaire, Toulouse, France, [3]Centre de Génétique Moléculaire, Gif-sur-Yvette, France

FtsK is a bacterial motor protein that translocates DNA in order to move newly-replicated chromosomes to their proper positions just before cell division. In order to understand this process, we have performed a series of experiments in which we detect the activity of a single FtsK motor as it interacts with a single stretched DNA molecule. We have measured both FtsK's 'linear' motion[1], such as its velocity and the distances it travels, and its 'rotational' motion[2]- due to DNA's helical structure, most DNA-based motor proteins rotate as they move linearly. We have found that FtsK is quite an exceptional motor: it translocates both quickly (up to 7 kbp/s) and for long distances (several microns per binding event), but rotates relatively slowly while doing so. This slow rotation indicates FtsK does not track along DNA's groove, but it is consistent with our previous estimate of FtsK's step size. We hypothesize that FtsK's rotational behavior is linked to the supercoil density of the *E. coli* chromosome; this idea indicates an evolutionary origin for FtsK's unique translocation characteristics.

1. Saleh et al., The EMBO Journal (2004)
2. Saleh et al., Nature Structural and Molecular Biology (2005)

P-389

Dynamical high-accuracy observations of individual membrane protein molecules using X-rays

Y. C. Sasaki
JST/CREST SASAKI-team, SPring-8/JASRI, 679-5198 Sayougun, Hyougo-ken, Japan

Recently, we succeeded *in vitro* time-resolved (-ms) x-ray observations of picometer-scale slow Brownian motions of individual membrane protein molecules in aqueous solutions. Diffracted X-ray Tracking can monitor dynamics of the individual molecules or specific sites in individual single protein molecules. Now, we observed Brownian motions and momentarily structural change of individual single Bacteriorhodopsin (BR) molecules, which is a light-driven proton pumping found in the purple membrane, in the light irradiation (560nm). We have consequently confirmed Brownian motions, and that the average size of the momentarily structural changes by light irradiation in 35^{th} residue of BR was $76\pm$ 48.2pm.

P-390

Mechanically activated proteins - new aspects in cell adhesion using flat microfluidics -

M. F. Schneider[1], S. W. Schneider[2], A. Wixforth[1]
[1]University of Augsburg, Experimantalphysics I, Biophysics Group, Germany, [2]University of Muenster, Universitatsklinikum, Dept. of Dermatology, Germany

Von Willebrand Factor (VWF) is the essential adhesive protein in mediating platelet adhesion and aggregation during vessel injury. In the underlying adhesion mechanism a transition from a compact (inactive form) to an elongated (active form) conformation of the protein is claimed. However, biophysical investigations are completely missing.

Here we present the shear force induced transition of VWF from the compact to its elongated state on a novel designed micro flow chamber. Using acoustically driven microfluidics (surface acoustic wave "nanopumps") we were able to extract mechanical properties (elasticity) as well as the dynamics (relaxation time between the two states) of this protein.

Based on these physical properties we are able to explain the relation between platelet adhesion and blood flow velocity. Using our microfluidic system we further proofed our interpretations by culturing endothelial cells on a chip (mimicking the vessel wall) and monitored the flow driven blood platelets at different velocities. The results clearly confirmed all our interpretations.

P-391

Two-color single molecule imaging of photoconverting protein molecule Kaede

S. P. Schäfer[1], P. S. Dittrich[2], P. Schwille[1]
[1]Institute of Biophysics, TU Dresden, Tatzberg 47, 01307 Dresden, Germany, email: stephan.schaefer@biotec.tu-dresden.de, [2]ISAS, Dep. Miniaturization, Bunsen-Kirchhoff-Str. 11, D-44139 Dortmund, Germany

Fluorescent proteins are now widely used in fluorescence microscopy as genetic tags to any protein of interest. Recently, a new fluorescent protein *Kaede*, a tetrameric GFP analog, was introduced [1] that exhibits an irreversible color-shift after photoactivation with λ=350-410nm and thus, allows for a very specific cellular tracking of proteins before and after exposure to the illumination light. The conversion of *Kaede*'s green into the red form proceeds probably via a cleavage reaction of the protonated non-fluorescent state [2]. The protein gains attractivity for intracellular applications since both green and red forms can be excited by blue light, e.g. 488nm.

In this work we present a detailed study of the dynamics of *Kaede*'s conversion process by single molecule 2-color imaging and quantitative image analysis. Either green or red fluorescence for single molecules was observed before and after illumination with 405nm light. No indications of fluorescent intermediates during the reaction were found. The complex interplay between the partially converted monomers within the molecules tetramer could be observed over several hundred milliseconds even without previous activation by violet light.

[1] Ando et al., 2002. Proc. Natl. Acad. Sci. U.S.A. **99**, 12651-12656
[2] Mizuno et al., 2003, Mol. Cell **12**, 1051-1058

Posters

– Single Molecule Biophysics –

P-392

Observing the conformational dynamics of single DnaK chaperones

J. D. Seelig[1], M. P. Mayer[2], M. Prummer[3], A. Renn[1], V. Sandoghdar[1]
[1]Nano-Optics Group, Laboratory of Physical Chemistry, Swiss Federal Institute of Technology (ETH), [2]Zentrum für Molekulare Biologie Heidelberg, Universität Heidelberg, [3]Laboratory of Physical Chemistry of Polymers and Membranes, Institute of Biomolecular Sciences, Swiss Federal Institute of Technology

The 70 kDa heat shock proteins (Hsp70) are central components of the cellular chaperone system. They control protein folding processes such as the folding of de novo synthesized polypeptides and the repair of misfolded proteins [1, 2]. To perform their chaperone function Hsp70s enclose short stretches of target polypeptides. The rate of substrate binding depends on the interaction with ATP, which induces large conformational changes in the binding domain. This process is regulated by cochaperones which stimulate ATP hydrolysis and nucleotide exchange.
We have investigated the structural dynamics of the E. coli Hsp70 homologue DnaK at the single molecule level. We labeled DnaK with dye pairs appropriate for fluorescence resonance energy transfer (FRET). We monitored the dependence of the FRET signal on peptide substrate, ATP and cochaperones. Both freely diffusing proteins as well as surface immobilized proteins were studied.
[1] Bernd Bukau and Arthur L. Horwich, The Hsp70 and Hsp60 protein machines. Cell 92, 351-366 (1998).
[2] Stefan Walter and Johannes Buchner, Molecular Chaperones – Cellular machines for protein folding. Angew. Chem. Int. Ed. 41, 1098-1113 (2002)

P-392-B

Scanning Ion Conductance Microscopy imaging of single protein dynamics in membranes of living cells

A. Shevchuk[1], D. Sánchez[1], J. Gorelik[1], R. Jones[2], P. James[2], G. Frolenkov[3], M. Lab[1], D. Klenerman[4], Y. Korchev[1]
[1]Division of Medicine, Imperial College London, London W12 0NN, U.K., [2]The Babraham Institute, Cambridge CB2 4AT, U.K., [3]National Institute on Deafness and Other Communication Disorders, NIH, Rockville MD, 20850, U.S.A., [4]Department of Chemistry, Cambridge University, Cambridge CB2 1EW, U.K.

Until now, any ultrastructural studies of membranes and membrane proteins in native cell environment have been restricted to either fixed or frozen samples, hiding the details of the formation and maintenance of functionally important membrane structures. Here we report a non-contact scanning probe imaging technique that can visualize dynamic changes in surface of the living cells in the physiological conditions with a resolution sufficient to image directly the individual proteins. In a relatively well-studied model cell, a spermatozoon, we have found that the majority of proteins that appear on the specialized equatorial region after acrosome reaction have very low mobility suggesting that they may be linked to the cytoskeleton at specific locations. Another, smaller fraction of these proteins exhibits fast dynamic re-arrangement. This is the first direct *in vivo* observation of ultrastructural changes of plasma membrane proteins.

P-393

Tertiary and secondary structure elasticity of repeat proteins

M. Sotomayor, K. Schulten
Department of Physics and Beckman Institute, University of Illinois at Urbana-Champaign, USA

Repeat proteins are thought to mediate protein-protein interactions and have been successfully utilized as models for design of synthetic proteins and in protein-folding studies. Repeat proteins like ankyrins or leucine-rich repeats (LRRs), contain typically 4-30 repeats of 20-40 amino acids each forming a distinctive structural motif. By using molecular dynamics simulations we have investigated ankyrins and characterized, for the first time, tertiary and secondary structure elasticity of a synthetic four-ankyrin-repeat structure, 12 and 24 repeats of human ankyrin-R, and a model of 17 ankyrin repeats of TRPA1. The published [1] predictions of a low force elastic spring behavior reflecting tertiary structure elasticity and high force secondary structure elasticity involving sequential unraveling of repeats are presently investigated by atomic force microscopy experiments. The results explain mechanotransduction in vertebrate hair cells that involves a biophysically defined elastic element (the "gating spring") pulling on the transduction channels, members of the TRP family including ankyrin repeats in their N-termini. We also studied by means of molecular dynamics simulations Internalin, a major invasion, leucine-rich repeat protein of Listeria monocytogenes alone and in complex with E-cadherin; first results will be reported.
[1] M. Sotomayor, D. P. Corey, and K. Schulten, Structure 13: 669-682 (2005)

P-394

Single molecule imaging of signal input of nerve growth factor into the growth cone

T. Tani, Y. Harada
The Tokyo Metropolitan Institute of Medical Science

Growth cone is a motile structure located at the distal tip of nerve fiber. Nerve growth factor (NGF) locally controls the axonal growth and the survival of sensory and sympathetic nerve cells. Local interactions of single growth cones with NGF were observed by the use of a novel fluorescent analogue of NGF, Cy3-NGF, and a technique of fluorescent single-molecule imaging. Single-molecule imaging of the fluorescent NGF enabled us to count the number of bound molecules to evoke the motile response of the growth cones. In addition, *in situ* kinetic analyses of the interactions between NGF and the receptors on a growth cone allowed us to estimate the number of binding sites with different affinities to NGF. 40 molecules of Cy3-NGF, which occupied approximately 4% of the total binding sites on a growth cone, could evoke the expansion of lamellipodia. Tracking of single molecules of NGF provided new findings to account for the molecular mechanisms of NGF uptake at the growth cones. After binding to the receptors, Cy3-NGF showed lateral diffusionÅ@followed by actin-driven rearward movement toward the central region of growth cone, which resulted in the accumulation of Cy3-NGF at central region. During rearward movement, Cy3-NGF was thought to be endocytosed at the vicinity of central region. Behaviors of single molecules of Cy3-NGF on the growth cones were discussed in relation to the local and /or global responses of nerve cells upon application of NGF.

Posters

– Single Molecule Biophysics –

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Binding and unbinding forces between a single antibody molecule and a metal-bound ligand

J.-M. Teulon, M. Odorico, J.-L. Pellequer, P. Parot
CEA-Valhrô, DSV/DIEP/SBTN - BP 17171, 30207 Bagnols Sur Cèze. France

Atomic force microscope in force spectroscopy mode is a powerful tool to study the interaction energy between biological complexes. The adhesion mechanism between uranyl-phenantroline functionalized tip and antibody coated surface was determined by recording a large collection of force-distance curves. Specific binding, atomic interactions involved in the recognition between the ligand uranyl-phenantroline and the antibody, is distinguished from non-specific binding by recording forces in the presence and absence of uranyl ions. Energies involved during the retraction phase is estimated in the range 100-400 pN using a loading rate of about 3000 pN/s. Analysis of force curves allow us to localize the breaking points and to estimate the binding energies of the complex around 50 kJ/mol.

P-396

Unravelling the mechanism of RNA-polymerase forward motion by using mechanical force

P. Thomen[1], P. J. Lopez[2], F. Heslot[1]
[1]LPA-ENS 24 Rue Lhomond 75005 Paris, France, [2]LSMD-ENS 46 Rue d´Ulm 75005 Paris, France

Polymerases form a class of enzymes acting as molecular motors as they move along nucleic acid during catalysis, incorporating nucleotides triphosphate at the end of the growing chain and consuming chemical energy. A debated issue is how the enzyme converts the chemical energy into motion. In a single molecule force assay, we studied how a mechanical force opposed to forward motion affects the rate of the T7 RNA-polymerase. Our measurements show that force acts as a competitive inhibitor of nucleotide binding. This dynamic feature is interpreted in view of possible models, and also with respect to published crystal structures of the T7 RNA polymerase. The transcribing complex appears to utilize only a small fraction of the energy of to perform mechanical work, with the remainder being converted to heat.

P-397

Single molecule multistep FRET: revealing complex molecular interactions

P. Tinnefeld, M. Heilemann, J. Ross, P. Buschkamp, A. Donnermeyer, M. Sauer
Laserphysics & Laserspectroscopy, University of Bielefeld, Bielefeld, Germany

During recent years, fluorescence resonance energy transfer (FRET) has become a key method for the analysis of molecular interactions with exquisite sensitivity down to the single molecular level. As nearly every major process in a cell is carried out by assemblies of multiple dynamically interacting molecules it is of great interest to extend single molecule FRET-technologies to the study of three and more chromophores. We show the applicability of single molecule multistep FRET for the design of DNA based molecular photonic wires including FRET over five chromophores. To make more complicated energy transfer in complex 3D biomolecular interactions analysable, a general approach based on alternate 3 color laser excitation making use of new acousto-optical devices to rapidly switch laser lines and optimize detection efficiency is presented. Besides enabling absolute distance determination of three chromophores and colocalization of chromophores beyond the FRET-range, correlative movements of different segments within a molecule can be unravelled.

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Stepping kinetics of myosin-V under high loads

M. Vilfan[1], A. E. Clemen[2], J. Jaud[2], M. Rief[2]
[1]J. Stefan Institute, Ljubljana, Slovenia, [2]Technical University Munich, Germany

We used optical tweezers with a long-range feedback for studying myosin-V kinetics under controlled external loads. Both forward and backward forces were applied and the step-size and dwell-time distributions were analyzed. Our results show that the mean step size corresponded to the helicity of actin filament and did not change with applied force over a wide range of loads: from 5 pN forward to 1.5 pN backward load. Two force dependent transitions in the chemo-mechanical cycle were observed. The rate limiting ADP release showed only a weak force-dependence whereas the faster rate was strongly dependent on the applied load. At super-stall forces of 5 pN, the motor performed continuous backward steps with the average step size of 36 nm.

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Single wavelength fluorescence cross-correlation spectroscopy: principles, limits and applications

T. Wohland[1], L. C. Hwang[1], M. Goesch[2], T. Lasser[2]
[1]National University of Singapore, Department of Chemistry, Biophysical Fluorescence Laboratory, 3 Science Drive 3, Singapore 117543, [2]Ecole Polytechnique Fédérale de Lausanne (EPFL), Laboratoire d´Optique Biomédicale, CH-1015 Lausanne, Switzerland

Fluorescence Cross-correlation Spectroscopy (FCCS) is a tool that allows the measurement of protein dynamics and interactions. Unlike classical fluorescence correlation spectroscopy (FCS) it is not limited by mass differences of the interaction partners, but only by the possibility to label two interaction partners with different fluorescent labels. However, FCCS has some drawbacks. Either it needs the difficult alignment of two lasers which results in an incomplete overlap of excitation volumes due to the difference in excitation wavelength, or it requires the use of expensive pulsed near-infrared lasers for multi-photon excitation, which result usually in comparatively small count rates per particle, limiting the signal to noise ratio. Recently we have shown that FCCS can be performed even when using only one single wavelength for one-photon excitation (SW-FCCS). In this work we discuss the principle and limits of the method as derived from FCCS theory and present data showing the applicability of the method to different fluorophores. The method is not only simpler to align but does not suffer from incomplete overlap of excitation volumes and results in high count rates per particle. Finally, we expand this approach to include up to three channels with pair wise cross-correlations to elucidate interactions of complexes.

P-400

DNA compaction: Relationships between higher-order structure and transcriptional properties

A. Yamada, F. Luckel, K. Yoshikawa
Department of Physics, Graduate School of Science, Kyoto University

Recently, it has been confirmed that long, linear ds-DNA molecules larger than several tens of kilo-base pairs, exhibit an on/off conformational transition between an elongated coil and a compact globule states. By single-molecule observations, we studied the relationship between the higher-order conformation of a 40 kbp DNA and its transcriptional activity (RNA production) at the single chain level. In the case of linear DNA, it is shown that the discrete folding transition induces an all-or-none switching of transcriptional activity at the level of single DNA molecules. In contrast, in the case of circular DNA, we found that the change in transcriptional activity is rather mild. Significant effect of torsional stress on circular DNA molecules will be reported.
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Nanopipette: a novel tool for manipulating biomolecules and biosensing

L. Ying, D. Klenerman
Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

Glass nanopipettes have been used in a special form of scanning probe microscopy called scanning ion conductance microscopy (SICM) for reliable non-contact imaging over the surface of a live cell. We have found recently that the nanopipette can be employed for local voltage driven application of reagents or biomolecules and this can be used for controlled deposition and the local delivery of probes for mapping of specific species. This will be demonstrated as nanowriting of DNA and protein molecules on functionalized surface and as a nanosensor probing pH and Calcium concentration. We have also observed dielectrophoretic trapping of biomolecules in the tip of the nanopipette due to the strong non-uniform electric field created near the tip region when a moderate voltage ($\sim$ 1 V) is applied between the bath and the glass pipette. We have extended the use of nanopiptte further as a femtolitre volume nanoreactor for the enzymatic reaction and a nanomixer for probing folding and unfolding of single biomolecules. The strong local electric field within the pipette tip has been exploited for changing the optical properties of a donor/acceptor FRET pair attached in the same end of a double-stranded DNA, making a reversible rapid electric-field driven single molecule optical switch.
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Development of a novel microscope for single molecule measurement of DNA/protein interaction

H. Yokota[1], J.-F. Allemand[2], X. Xi[3], V. Croquette[2], D. Bensimon[2]
[1]Department of Molecular Physiology, The Tokyo Metropolitan Institute of Medical Science, Japan, [2]ECOLE NORMALE SUPERIEURE, LPS, [3]ECOLE NORMALE SUPERIEURE de Cachan, LBPA, France

Recently developed single molecule methodology has revealed mechanical properties and functions of biomolecules. However, most of the research employed either single molecule fluorescence imaging or single molecule manipulation so that obtained information was limited.
To understand dynamic features of DNA/protein interaction in more detail, we have been developing a novel microscope that simultaneously allows the manipulation (stretching or twisting) of a single DNA molecule and the visualization of enzymes that are interacting with it. With this microscope, DNA/protein interactions could be investigated both temporally and spatially at the single molecule level by monitoring the change in extension of the DNA and the fluorescence emitted by appropriately labeled DNA, enzymes or nucleotides.
We present preliminary results on the use of this microscope.

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Characterization of an artificial antimicrobial peptide by fluorescence correlation spectroscopy

L. Yu[1], J. L. Ding[2], B. Ho[3], T. Wohland[1]

[1]Department of Chemistry, National university of singapore, [2]Department of biological science, National university of singapore, [3]Department of microbiology, National university of singapore

Antimicrobial peptides are ancient defense weapons of many organisms, which rapidly lead to antibacterial activity. Because of the difficulty for bacteria to develop a new membrane system within a short time, antimicrobial peptides have been considered a promising drug candidate. An artificial antimicrobial peptide named V4 has been designed based on a known amphipathic cationic pattern BHPHB (B: basic; H: hydrophobic; P: polar residue, respectively) and showed a good combination of high antimicrobial activity, low cytotoxic activity and low hemolytic activity. Fluorescence Correlation Spectroscopy was used to investigate the interaction of V4 with liposomes composed of artificial lipids which are used to mimic mammalian and bacterial membranes. The study demonstrated that at low peptide/lipid ratio, V4 peptide specifically binds negatively charged lipids, POPG, compared to zwitterionic lipids POPC, while at a high peptide/lipid ratio, V4 not only induced leakage of negatively charged liposomes and showed a strong permeabilising ability compared to magainin, but also caused vesicle fusion. The strong interaction between V4 and the negatively charged lipids showed that electrostatic force is a prerequisite for its selective action on bacterial membranes, in contrast to mammalian membranes.

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– Sensing with Ion Channels –

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Functional tethered membranes: a sensing platform for ion channels

P. Atanasova, V. Atanasov, C. Breffa, N. Knorr, I. Vockenroth, W. Knoll, I. Koeper
Max Planck Institute for Polymer Research, Mainz, Germany

Tethered lipid bilayer membranes have been proven to be a powerful architecture for the study of membrane protein in an artificial, but quasi-natural environment. Based on a lipid bilayer membrane, which is coupled via a spacer group to a solid substrate, they provide not only excellent stability but possess also the necessary conditions for functional incorporation of membrane proteins, especially fluidity and high electrical resistance.
We could synthesize several lipids, which allow the construction of membrane architecture both on gold as well on silicon surfaces and provide good electrical sealing properties. We could functionally incorporate a variety of membrane proteins, e.g. ion channels and shuttle proteins.
By variation of the anchor group, we have a generic model for all kinds of substrates. Modification of the spacer architecture allows the adaptation of the system to different membrane proteins.
We will give an overview about the systems obtained so far. Experimental characterization is made by divers surface analytical tools such as SPR, QCM, FRAP and impedance spectroscopy.
Our construction kit offers now the possibility to combine directly microelectronic read-out systems with a biological compound. We will present a device concept, where ion channels, embedded in a tethered membrane are used as sensing elements of a new type of bio-electronic sensor.

P-405

Investigations of tethered bilayer lipid membranes

P. Atanasova[1], V. Atanasov[1], C. Breffa[1], I. Vockenroth[1], W. Knoll[1], T. Jenkins[2], I. Koeper[1]
[1]Max Planck Institute for Polymer Research, Mainz, Germany, [2]University of Bath, Bath, UK

The tethered bilayer lipid membranes (tBLMs) investigated in this study consist of a planar support (that is given by a gold electrode) and a monolayer of thiolipids that is completed to a bilayer by vesicle fusion. The monolayer is covalently attached via a spacer unit to the gold substrate. Insertion of a spacer between substrate and lipid bilayer serves as an ionic reservoir underneath the membrane, that mimics the cellular interior.
Thiolipids differing in spacer length and anchor group are investigated for their self assembly properties and their ability to form a good proximal layer for the following vesicle fusion. The membrane architectures can be investigated using a variety of surface analytical tools, such as electrical impedance spectroscopy (EIS), surface plasmon resonance spectroscopy, atomic force microscopy or ellipsometry.
The first test on functionality of the membrane assembly was the addition of valinomycin, a small ion carrier peptide, which is known to intercalate into biological membranes, transporting ions from one side to the other. The high selectivity for potassium ions compared to other ions like sodium could be shown by EIS. Furthermore the well known antibiotic pore gramicidin could be incorporated, as shown by a decrease of the membrane resistance upon incorporation. The transmembrane fragment M2 of the nicotinic acetylcholine receptor could be incorporated and its cation selectivity and blocking could be shown.

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New synthetic strategies towards tethered bilayer lipid membranes

C. Breffa, I. Vockenroth, P. Atanasova, V. Atanasov, N. Knorr, I. Koeper, W. Knoll
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz Germany

Tethered lipid bilayers on solid supports are of great practical and scientific interest for use as model systems of the biological membrane. As such they provide a natural environment for the immobilization of proteins under non-denaturing conditions. In respect to functional applications they allow the preparation of ultrathin, high-resistance lipid layers on metals or semiconductors. Proteins can be incorporated into these insulating layers in order to design biosensors, bioelectronic or other biomimetic devices.
The aim of the project is to design a new molecule for the construction of a tethered membrane system. The molecule developed in former work [1] will be improved, to enable a better introduction of common transmembrane proteins and ions channels.
The tether molecule can be divided into 3 parts : a tether to bind the molecule on the surface, a spacer to provide an ionic reservoir between the gold surface and the membrane, and to decouple the membrane from the surface, and a lipid headgroup (DiPhytanyl-Glycerol) to help build the membrane.
The use of heterofunctionalized defined oligoethyleneglycols [2] seems to be a promising route to achieve longer spacers and, therefore, a better incorporation of the proteins.
[1] Schiller S. M. et al, Angew. Chem. Int. Ed., 42 (2), 208 (2003)
[2] Burns C. J. et al, Synth. Commun., 29(13), 2337-2347 (1999)

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Modulation of Acid-Sensing Ion Channels (ASICs) by nitric oxide

H. Cadiou[1], M. Studer[1], N. G. Jones[2], E. S. J. Smith[1], S. B. Mcmahon[2], P. A. Mcnaughton[1]
[1]Department of Pharmacology, Tennis Court Road, Cambridge CB2 1PD, UK, [2]Neurorestoration Group, Wolfson CARD, King´s College London, London SE1 1UL, UK.

Acid Sensing-Ion Channels (ASICs) are a class of ion channels gated by extracellular protons and are believed to mediate acid-induced pain caused by tissue acidosis. Although ASICs have been widely studied, little is known about their regulation. Here, we provide evidence that nitric oxide (NO) potentiates the activity of ASICs. First, patch clamp whole cell recordings were carried out on neonatal rat cultured dorsal root ganglia neurones and on different ASIC isoforms expressed in CHO cells. Application of the NO donor S-nitroso-N-acetylpenicillamine (SNAP), increased acid-gated ion currents in both systems. The use of cGMP/PKG pathway modulators and patch clamp studies at the single channel level showed a direct external action of NO, possibly involving oxidization of cysteine residues. Complementary psychophysical studies were carried out using iontophoresis of acidic solutions through skin of human volunteers. Topical application of glyceryl trinitrate significantly increased acid-evoked pain but did not affect heat or mechanical pain thresholds. Our work suggests that ASICs may play an important role in pathologies where tissue acidosis and high level of NO are present.
We thank Prof M. Lazdunski for the gift of ASIC clones.

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Modulation of the channel activity of Vpu from HIV-1

P. J. Judge[1], T. Mehnert[2], G. Patargias[2], A. Sauerwein[2], Y. Lam[2], D. Fischer[2], A. Watts[2], W. B. Fischer[1]

[1]Bionanotechnology IRC, Clarendon Laboratory, Department of Physics, Parks Road, OXFORD, OX1 3PU, UK., [2]Biomembrane Structure Unit, Department of Biochemistry, South Parks Road, OXFORD, OX1 3QU, UK

Vpu is a viral ion channel protein encoded by HIV-1 to enhance the rate of virus particle release from an infected host cell by altering the electrochemical potentials across host lipid membranes. The 81 amino acid monomers have a single transmembrane á helix, which oligomerises within the host's sub cellular membranes forming bundles which enable ion flux. The transmembrane (TM) domain has been synthesised by continuous-flow solid phase peptide synthesis and the full-length wild type protein has been expressed in recombinant *E. coli*. Conductance measurements have been obtained by bilayer recording of Vpu reconstituted into membranes of a variety of different compositions and the inhibition of ion permeation by the amphipathic ligand HMA has been studied. The binding of HMA and a putative peptide inhibitor resembling the TM domain of the TASK channel has been simulated using a computer docking approach.

P-409

Ligand-operated peptide ion-channels as biosensors

C. S. Pilz, C. Steinem

Institute for Analytical Chemistry, Chemo- and Biosensors; University of Regensburg, Germany

In recent years a variety of ion-channel sensors mimicing biological sensory functions have been reported. The ability of ligand-operated ion-channels to amplify an incoming signal by several orders of magnitude and the possibility to operate as single molecule detection systems make them attractive candidates for biosensoric applications. In the case of natural ion-channels binding of a single ligand causes an ion flux of 10^4 – 10^5 ions/ms.

In nature, conformational changes within properly folded proteins due to the ligand-binding give rise to selective channel-opening events. Since the development of tailored sensor systems by protein engineering is very complex, we utilize amphipatic channel forming peptides which are covalently linked to water exposed receptors. To perform single channel recordings our receptor-functionalized amphipatic peptides are introduced into artificial model membranes, black lipid membranes (BLMs). An applied transmembrane voltage induces the insertion of the peptides into the membrane where they form helix bundle structures. Upon addition of an appropriate analyte, which is capable of binding two receptors, the formation of ligand-receptor couples will connect helices in the membrane leading to defined helix-bundle structures. Due to an altered bundle formation, we expect different channel characteristics such as conductance, mean open lifetime or frequence of channel openings.

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Voltage-gated currents of crypt cells in the fish olfactory epithelium

O. Schmachtenberg

Centro de Neurociencias de Valparaiso CNV, Universidad de Valparaiso, Chile

Crypt cells from the olfactory epithelium of the Pacific jack mackerel *Trachurus symmetricus* were characterized by light and electron microscopy and analyzed in dissociation with the patch clamp technique in its perforated patch and normal whole cell mode. Isolated crypt cells remained united with their supporting cells and both were electrically coupled through gap junctions. Immunocytochemistry suggests that connexin Cx 45 is expressed in the contact area of crypt and supporting cell. Crypt cells had an average cell capacitance of 4.2 pA and an apparent resting membrane potential of -41 mV. Depolarizing voltage steps triggered a transient sodium current, a sustained calcium current and two types of potassium currents with fast and slow inactivation kinetics. No calcium-dependent potassium current could be observed. The sodium current was blocked by saxitoxin, the calcium current by cobalt and furnidipine and the potassium currents by TEA. These first recordings of individual crypt cells show that they are amenable to patch clamp analysis and provide the basis for future studies of their odorant response properties and general function in the fish olfactory epithelium.

P-411

A novel μ-conotoxin potently blocks TTX-R sodium channels in rat dorsal root ganglion neurons

H. Zhang[1], C. Z. Wang[2], C. W. Chi[2], Z. Q. Zhao[1]

[1]Institute of Neurobiology, Fudan University, Shanghai, 200433, China, [2]Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China

Mu-conotoxin SIIIA, a novel blocker of tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels (VGSCs) has been identified from the fish-hunting cone snail, *Conus striatus*, via cDNA cloning. By means of the whole-cell patch clamp technique, the present study is to examine the effects of mu-conotoxin SIIIA on neuronal sodium currents. The experiments are performed in 127 acutely isolated dorsal root ganglions (DRG) neurons (<25um) from the Sprague-Dawlay male rats, which express both subtypes of sodium currents. After aether anaethesia, The L_{4-6} DRGs were picked out and dissociated into cells by enzyme. mu-conotoxin SIIIA has no significant effect on TTX-Sensitive sodium current, but produces an irreversible, concentration and time-dependent inhibition of TTX-R sodium current amplitude, but doesn't affect the voltage range of channel activation, suggesting a frequency-dependent blocking effect.

In the behavioral test, intraplantar injection of ì-conotoxin SIIIA (6 ug) significantly slows the thermal hyperalgesia development and increases the paw withdrawal latency to noxious heating in the carrageenan-induced inflammatory rat, which suggests that TTX-R sodium channels expressing in the peripheral afferent fibers innervating the receptive field are blocked by ì-conotoxin SIIIA.

The present results indicate that mu-conotoxin SIIIA is a novel potent inhibitor of TTX-R sodium channel.

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P-412

Probing DHPR-RYR1 interaction with fluorescent proteins

R. A. Bannister, S. Papadopoulos, K. G. Beam
Department of Biomedical Sciences, Colorado State University, Ft. Collins, CO, USA

Excitation-contraction (EC) coupling in skeletal muscle relies on conformational coupling between the dihydropyridine receptor (DHPR) and the sarcolemmal Ca^{2+} release channel (RyR1). Skeletal-type EC coupling is bidirectional; orthograde coupling is observed as depolarization-induced myoplasmic Ca^{2+} release and retrograde coupling is manifested by increased L-type Ca^{2+} current. The DHPR α_{1S} II-III loop contains the "critical domain" (aa 720-765) that is essential for bidirectional coupling with RyR1. In this study, we examined the functional consequences of fluorescent protein insertion within the critical domain. Introduction of a ($\sim$56 kDa) CFP-YFP tandem between α_{1S} residues 726 and 727 resulted in minimal L-type current and barely detectable Ca^{2+} transients, whereas insertion of a single YFP ($\sim$27 kDa) between α_{1S} residues 726 and 727 allowed partial bidirectional coupling. Immuno-labelling with a monoclonal antibody directed against α_{1S} aa 737-744 demonstrated the conformational integrity of the critical domain for both constructs. Taken together, these results suggest that the large CFP-YFP tandem sterically denies the α_{1S} II-III loop access to junctional interaction partners, while the single YFP only partially interferes with EC coupling. We are currently examining the effect of introduction of a single YFP at α_{1S} residue 760. Supported by NIH grants NS24444 and AR44750 to KGB.

P-413

Linear dichroism, applied to structural analysis of protein fibres, membrane proteins and DNA

T. R. Dafforn[1], M. Hicks[1], R. Marrington[2], R. Mortimer[3], S. G. Addinall[2], D. J. Halsall[4], A. Rodger[2]
[1]Biosciences, University of Birmingham, UK, [2]Department of Chemistry, University of Warwick, UK, [3]CPO, Rugby, UK, [4]Addenbrookes NHS, University of Cambridge, UK

Understanding the organisation of molecules in naturally occurring ordered arrays (eg membranes, protein fibres and DNA strands) is of great importance in understanding their biological function. Unfortunately few biophysical techniques provide detailed structural information in these non-crystalline systems. Ultra-violet, and visible linear dichroism (LD) has the ability to provide such information and may hold the key to understanding molecular mechanisms of such fundamental biological processes as amyloid fibre formation, and membrane protein folding. We have developed LD apparatus that has allowed us to study the structures of number of systems including protein fibres (Alzheimer's fibres, actin, tubulin and the bacterial cell division protein FtsZ). Data from these studies has provided novel information of the orientation of these proteins within the fibre as well as showing the details of conformational changes that occur during fibre function. We have also expanded the technique to encompass studies of the conformation of membrane proteins in lipid vesicles as well as proteins binding to and drugs binding to DNA. Taken together this work provides an exciting glimpse of the importance of LD in the future biophysical studies.

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Theoretical study of force development in smooth muscle cells

A. Dobovišek[1], A. Fajmut[1], M. Brumen[2]
[1]University of Maribor, Faculty of Education and Faculty of Medicine, Slomškov Trg 15, SI - 2000 Maribor, Slovenia, [2]Josef Stefan Institute, Jamova 39, SI - 1000 Ljubljana, Slovenia

Phosphorylation and dephosphorylation of myosin are essential steps in the cross bridge cycling between myosin and actin filaments and in the concomitant force development. An increase in cytosolic Ca^{2+} concentration is generally considered to activate this process. In our study the 4-state kinetic scheme of cross bridge cycling proposed by Rembold & Murphy (Am. J. Physiol, 259 (1990) 251) is taken into account as a basic model scheme. Instead of considering the experimentally determined Ca^{2+} dependent phosphorylation we introduced a simple kinetic scheme of Ca^{2+}-calmodulin activation of myosin light-chain kinase proposed by Kato et al. (Biophys. J. 46 (1984) 35), and combined the both models in an unique mathematical model which enables to predict the force development as a function of phosphorylation as well as cytosolic Ca^{2+} concentration. Furthermore, a model biphasic Ca^{2+} transient signal is applied in order to study the appearance of sustained force with respect to characteristic parameters of the model signal. A concentration plateau attained after an initial peak determines a sustained force. The model predictions are analysed with respect to the previous models and relevant experimental data. They indicate an improvement in mathematical modelling of Ca^{2+} mediated smooth muscle contraction.

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Effect of remifentanil on ach-induced hyperpolarization and relaxation in human umbilical arteries: physiological roles and properties of K^+ -channels

M. Emre, H. Ünlügenç, A. Demirkazık, S. Kavak, A. Pelit, M. Şan
Çukurova University department of Biophysics, Adana - Turkey

Influence of remifentanil on endothelium-dependent hyperpolarization evoked by acetylcholine (ACh) in smooth muscle of the human umbilical (HUA) artery were investigated using conventional microelectrode techniques. We have studied the effects of K^+-channel blockers on the hyperpolarization responses to ACh in resting and phenylephrine-contracted HUA. Glinbenclamide had no significant effect on the hyperpolarization or relaxation. Tetraethylammonium (TEA, 5 mM) inhibited the hyperpolarization to ACh significantly to a similar extent in both the resting and phenylephrine-stimulated arteries. Charybdotoxin (CTX) (150 nM) caused a significant inhibition. Wherase apamine (300 nM) caused only a small in inhibiting the hyperpolarization in resting of HUA. A combination CTX and Apamin caused significant depolarization of the resting membrane potential (RMP). In resting arteries treated with CTX+Apamin, the hyperpolarizations were nearly abolished and a depolarization of 5.9 ± 0.6 mV (n=8) was evoked by 3 μM ACh. We have shown that endothelium-dependent hyperpolarization to ACh is significantly increased by remifentanil (10^{-6} mM/L).

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Calcium signals induced by insulin and electrical stimuli in myotubes. Possible role of PI3K and ROS

A. Espinosa, J. M. Eltit, M. Pena, C. Hidalgo, E. Jaimovich
Centro de Estudios Moleculares de la Célula, ICBM, Fac.de Medicina, Universidad de Chile

We have described that both insulin and electrical stimulation produce slow transient calcium increases in myotubes. Insulin induced signals are a rapid (2-10 s), transient signal and in some cells, a prolonged calcium signal with oscillations. The fast signal is dependent on ryanodine receptors. When 400 1 ms pulses at 45 Hz were applied on myotubes, a slow calcium signal starting 20-30 seconds after the end of the stimulation protocol and lasting several minutes is evident. Calcium signals induced by insulin were inhibited by DPI and apocynin. None effect of both inhibitor was shown with electrical stimulation. When we measured ROS production using 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFA), we observed delayed (50 to 100s) fluorescence increases in both conditions. To test the possible role of phosphatidyl inositol 3 kinase (PI3K) in these signal, we used inhibitors as 40 μM LY 294002 that blocked the calcium increase induced by insulin. With 100 nM wortmanin the calcium signal induced by electric stimuli was completely abolished. We concluded that the PI3K pathway is necessary for the onset of the calcium signal induced by insulin and that electrical stimuli induces both ROS production and slow calcium signal mediated by PI3K.

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Complex model of interactions between Ca2+, calmodulin and myosin light chain kinase

A. Fajmut[1], M. Brumen[2]
[1]University of Maribor, Medical Faculty and Faculty of Education, Slomškov trg 15, SI-2000 Maribor, Slovenia, [2]Institute Jozef Stefan, Jamova 39, SI-1000 Ljubljana, Slovenia

Active Ca^{2+}/calmodulin dependent myosin light chain kinase (MLCK) determines the rate and extent of myosin light chain (MLC) phosphorylation which is necessary for cross-bridge cycling between myosin and actin filaments in smooth muscles and consecutive muscle contraction. We propose a novel complex binding-scheme of interactions between Ca^{2+}, calmodulin (CaM) and MLCK taking into account eight different aggregates. The corresponding dynamic system is formulated, concentrations of intermediates with respect to time-dependences as well as their stationary values are predicted. The active form of MLCK is defined as being proportional to concentration of Ca_4CaM•MLCK aggregate, and the main model results refer to predictions of its dependence on $[Ca^{2+}]$ at different CaM and MLCK concentrations. The model results are in good agreement with the recently published experimental results (Geguchadze et al. (2004) FEBS Lett. 557,121) and may argue in favour of notable presence of Ca_2CaM•MLCK aggregate at low $[Ca^{2+}]$ in the system. Moreover, the sensitivity of active MLCK to small perturbations in parameter values of single reaction steps is studied and discussed regarding the influence of MLCK phosphorylation on its activity.

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Mechanism of myosin-V processivity

E. Forgacs[1], M. Webb[2], S. Cartwright[1], B. Belknap[1], H. White[1]
[1]Eastern Virginia Medical SchooL,Norfolk, VA, USA., [2]National Institute of Medical Research, London, UK.

To investigate the kinetic basis of myosin-V processivity, we have compared the kinetics of product dissociation of myosin-V-HMM and S1 using the substrate 7-diethylaminocoumarin-3-carboxylic (deac)ATP. This novel fluorescent ATP derivative provides a much better signal/noise ratio than the conventionally used 2'deoxy3'mantATP (mdATP). Double-mixing stopped-flow fluorescence was used to measure deacADP dissociation from actomyosinV-S1-deacADP-Pi and from actoHMM-ADP-Pi, using ATP or ADP chase. With the ADP chase, the decrease in fluorescence was fit to a single exponential for myosin-V-S1 and a double exponential for the HMM construct with the second component $\sim$1000 fold slower. With an ATP chase, the fluorescence signal is single exponential for both S1 and HMM. ADP (unlabeled) dissociation occurs in a single step from actoS1-ADP-Pi and in two steps from actoHMM-ADP-Pi. The later has two observed rate constants, one similar to actoS1-ADP-Pi and a second slower process. Our solution kinetic data provide a very accurate comparison of the kinetics of product dissociation from actomyosinV-S1-ADP-Pi and actomyosinV-HMM-ADP-Pi. The data support a model for the processive movement of the myosin-V molecule in which slow dissociation of ADP from the lead head produces directionality of movement and provides a mechanism to reduce the possibility of simultaneous dissociation of both heads from actin. Although similar mechanisms had been proposed by others these data provides the first compelling evidence to support the model.

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Influence of unloading on sarcolemmal dystrophin and permeability of sarcolemma to macromolecules

N. M. Gasnikova, B. S. Shenkman, I. M. Larina
RF SRC Institute for Biomedical Problems of RAS

Two experiments performed on rats were purposed to study influence of unloading on the number of sarcolemmal dystrophin disruptions; to compare damage induced by downhill running in normal and unloaded muscle; to study the dystrophin disruptions during recovery from hindlimb suspension (HS); to compare sensitivity of different parts of dystrophin molecule to damage; to estimate the number of muscle fibers (MF) containing Evans blue dye (EBD) and serum creatine kinase (CK) levels; to investigate whether the increased level of intracellular calcium plays role in dystrophin destruction during HS. Unloading was modulated by HS for 14 days, animals were injected with EBD and blood samples were taken. In order to decrease the accumulation of calcium during HS in the second experiment animals were injected with EGTA. It was shown that HS leads to destruction of dystrophin which became more during recovery; different parts of dystrophin molecule have the same sensitivity to the damage induced by downhill running in normal conditions and the different sensitivity to the damage induced by HS, running after HS and reloading; there were no differences in CK levels though some influences on the number of MF containing EBD were marked; EGTA decreases destruction of dystrophin during HS.

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Dielectric Spectroscopy and Delayed Luminescence measurements in dehydrated bovine Achilles´ tendon

M. Gulino[1], P. Bellia[1], F. Falciglia[1], F. Musumeci[1], A. Pappalardo[1], A. Scordino[1], A. Triglia[1], M. W. Ho[2], L. Brizhik[3]
[1]Dip. Metodologie Fisiche e Chimiche per l´Ingegneria, Catania University (Italy), [2]King´s College, London (UK), [3]Bogolyubov Institute for Theoretical Physics, Kiev (Ukraine)

By using Dielectric Spectroscopy measurements in the frequency range 500 Hz-10 MHz, corresponding to the β dispersion region of biological materials, it has been studied how the dielectric permittivity of the tendon tissue change as a function of its water content, in order to investigate the role of water network in the structure of the collagen molecules constituting the tendons. In parallel measures of Delayed Luminescence (DL), a phenomenon consisting of the prolonged ultra-weak emission of optical photons as a consequence of an excitation of the system by illumination, have been performed on dehydrated tendon samples. The results show that both the dielectric permittivity and DL total number of emitted photons suddenly change at the same threshold humidity value, which corresponds, according to other authors' measurements, to the removal of the most external hydration shell. Results also accord the prediction of a theoretical model, developed in order to explain DL from biological systems, based on the idea of a possible existence of auto-localized states in the triple helical conformation of collagen with lateral inter-chain hydrogen bonds and in the presence of hydrogen bonds to water molecules.

P-421

Depolarization-induced slow calcium transients stimulate transcription of genes in skeletal muscle cells

N. Juretić, J. M. Eltit, P. García-Huidobro, J. A. Iturrieta, N. Riveros, E. Jaimovich
Centro de Estudios Moleculares de la Célula, ICBM, Fac. Medicina, Univ. de Chile

Inositol 1,4,5-trisphosphate (IP_3) mediated slow calcium signals evoked by hormones and electrical stimulation of skeletal myotubes have been shown to be involved in regulation of gene expression. Using semi-quantitative RT-PCR, we have shown that K^+ depolarization of rat myotubes and C_2C_{12} cell line induces a transient increase in IL-6 mRNA level, which peaks at 3-4 h, and is independent of extracellular calcium. Inhibitors that suppress slow calcium signals, as U73122 (phospholipase C inhibitor), 2-APB (inhibitor of IP_3 induced signals) and xestospongin-C (IP_3 receptor blocker), decreased activation of IL-6 gene expression. Ryanodine, which inhibits the fast calcium transient had no effect on IL-6 induction. Depolarization of myotubes transfected with a luciferase reporter gene containing nucleotides – 651 to +1 of the human IL-6 promoter, induced a two fold increase on IL-6 promoter activity. Increased luciferase expression was abolished in the presence of either 2-APB or U73122 whereas remained unaffected after ryanodine treatment. Site directed mutagenesis of the parental construct allowed us to identify AP-1 and NF-κB sequences as cis regulatory elements responsible for the upregulation of IL-6 expression in transfected myotubes. Our results provide evidence for the involvement of IP_3 mediated calcium signals on IL-6 transcription in skeletal muscle cells and further support its regulatory role in gene expression.
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Solution Structure of Chicken Skeletal Muscle Troponin via Small-Angle Neutron and X-ray scattering

W. A. King[1], D. B. Stone[2], P. A. Timmins[3], N. Theyencheri[4], A. A. Von Brasch[1], R. A. Mendelson[2], P. M. Curmi[1]
[1]School of Physics, University of New South Wales, Sydney NSW 2052, Australia, [2]Department of Biochemistry and Biophysics and Cardiovascular Research Institute, University of California, San Francisco, CA 94143-2240, USA, [3]Large-Scale Structures Group, Institut Laue-Langevin, 6, rue Jules Horowitz, BP 156 38042 Grenoble Cedex 9, France, [4]European Synchrotron Radiation Facility, 6, rue Jules Horowitz, BP 156 38042 Grenoble Cedex 9, France

Troponin is a Ca^{2+} -sensitive switch that regulates the contraction of vertebrate striated muscle by participating in a series of conformational events within the actin-based thin filament. It consists of a Ca^{2+} -binding subunit (TnC), an inhibitory subunit (TnI), and a tropomyosin-binding subunit (TnT). Small angle neutron scattering data has been collected from TnC-TnI-TnT2 ternary complexes, in which all combinations of the subunits have been deuterated, in both the + Ca^{2+} and - Ca^{2+} states. Small angle x-ray scattering data was also collected from the same complex. Starting with a model based on the human cardiac troponin crystal structure (Takeda, Yamashita et al. 2003), a rigid-body Monte Carlo optimization procedure, using the programs CRYSOL and CRYSON (Svergun et al.), was used to yield models of chicken skeletal muscle troponin, in the presence and absence of regulatory calcium. The optimized models show significant differences when compared to the cardiac troponin crystal structure in the + Ca^{2+} state and provide a structural model for the switch between + Ca^{2+} and – Ca^{2+} states.

P-423

Contribution of calcium-dependent mechanisms to the unloading-induced cellular alterations in soleus fibers

K. Litvinova
SRC Institute for Biomedical Problems RAS, Laboratory of Myology; Moscow, Russia

Effects of Ca^{2+}-binding agent on soleus fiber contractile characteristics and Sarco-Endoplasmatic Reticulum Ca-ATPase isoform distruction in hindlimb-suspended rats. In this study, the tail-suspended rats injected with physiological saline did not differ from those injected with the Ca^{2+} chelator EGTA in the degree of muscle atrophy. Values of absolute tension in rats treated with physiological saline were less than in the control group by 45%, and in EGTA-treated rats-by 28%. The decrease of maximal specific tension of muscle fibers after exposure to unloading gives evidence that the decrease of the fiber contractile properties should be in part explained by the mechanisms other than fiber atrophy. The Ca/tension curve in hindlimb-suspended saline-treated rats shifted to the right and the pCa thresholds from 7.02±0.05 in cage controls to 6.69±0.02 that means the less Ca sensitivity of myofibrils of unloaded soleus muscle. At the same time pCa threshold in EGTA-treated hindlimb-suspended rats was 7.10±0.05. Analysis of sections stained with monoclonal antibodies against SERCA showed quite another evolution of fiber distribution. The hindlimb suspension induced the increased number of fibers stained positively for both isoforms (15% increase for so-called "fast" SERCA1 and 10% increase for so-called "slow" SERCA2) with the huge amount of fibers expressing both isoforms. In EGTA treated unloaded animals the increase of SERCA1 positive fibers was completely inhibited.

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Spin-spin relaxation of ^{1}H NMR signals from myofibril suspension of rabbit skeletal muscle

T. Ohno, M. Chiba, M. Kimura, M. Yamaguchi, S. Takemori
Department of Physiology, The Jikei University School of Medicine

Spin-spin relaxation of ^{1}H NMR signals from myofibril suspension of rabbit skeletal muscle.
Tetsuo Ohno , Masahumi Chiba, Masako Kimura, Maki Yamaguchi and Shigeru Takemori
Department of Physiology, The Jikei University School of Medicine
We observed spin-spin relaxation process of ^{1}H-NMR signals from suspension of myofibrils prepared from rabbit psoas muscle. As was the case in tissue skeletal muscle, decomposition analysis of the relaxation process could be well represented by the summation of several exponentials indicating that water molecules in the suspension could be conveniently grouped into several components based on the relaxation time constant (T_2). The slowest two components dominated over faster relaxation components at the myofibril concentration ranges studied. With increase in the concentration of myofibrils, water component that relaxed with T_2 around 0.15 s progressively replaced the slowest component of T_2 > 0.4 s. An equivolumic point for these two components was found at 12 mg/ml myofibril concentration at 20 Åé in the absence of MgATP. Water components that relaxed more rapidly existed at small fractions. Since the average separation between the myofibrils is estimated to be 1.72 fÊm at the myofibril concentration of 10 mg/ml, myofibril affects water molecules within a significant distance from its surface differently from water molecules in the bulk solution.

P-425

Concerted functioning of cardiac myosin and titin isoforms in adaptive and pathological processes

Z. Podlubnaya
Institute of Theoretical and Experimental Biophysics RAS

At the first stages of dilated cardiomyopathy (DCM) we have revealed the appearance of atrial light chains 1 (ALC-1) up to 30% in human ventricular myosin, which disappear at the terminal stage of the disease. In DCM ventricle, the amount of long cardiac titin isoforms increases by ∼12% (Makarenko et al., 2004). In sarcomere, titin is bound to myosin and functions with it in concerted manner. To test the functional sense of the above isoforms changes in DCM, the hibernation as a nature model of temporal suppression of contractile capacity of ground squirrels (GS) was used. In ventricles of awaking GS, the appearance of ALC-1 up to 30% has been revealed. Their appearance increases ATPase activity of ventricular myosin and thereby accelerates the restoration of heart function inhibited upon hibernation. Changes in titin isoform composition also contribute to the recovery of normal function of GS heart. In ventricles of hibernating GS, the content of long titin isoforms increases by 12-15%. It leads to low heart rate necessary upon hibernation. In ventricles of active animals they replaced by short titin isoforms necessary for normal work of GS heart. These data attest an adaptive nature of similar changes of myosin and titin isoform composition in DCM. They can be used for estimating the stages of DCM (compensation, decompensation) and for choice of optimal terms of heart transplantation.
Work is supported by RFBR grants 03-04-48487, 04-04-48599 and 04-04-97305.

P-426

Ileum contractile responsiveness is not affected either by aging or prolonged aerobic exercise

E. F. Rosa[1], J. Aboulafia[1], A. C. Silva[2], V. L. A. Nouailhetas[1]
[1]Department of Biophysics - UNIFESP, [2]Department of Physiology - UNIFESP

Aging (AG) and exercise alter organism oxidant status, thus likely to affect some tissue functions. We investigated the effects of AG and prolonged aerobic exercise (PAE) on the contractile response (CR), lipid peroxidation (LP) and morphology of murine ileum. C57BL/6 male mice (n=30) were divided in, 3-, 18-month old sedentary, and exercised groups. PAE consisted of a 60 min treadmill running session, at 13-21m/min, 5day/week for 15 months. Tissue responsiveness was evaluated by E_{max} and EC_{50} obtained from carbachol and KCl concentration-isometric contraction curves; LP by [MDA] reaction with TBARs; and morphology by optical and electronic micrographs. E_{max} (KCl: 1,00±0,12; 1,12±0,14; 0,83±0,10 g; and CCh 1,30±0,21; 1,47±0,20; 1,26±0,20 g) and $logEC_{50}$ (KCl: -1,88±0,04; -1,80±0,03; -1,82±0,02; and CCh: -6,23±0,08; -6,21±0,05; -6,17±0,04) values were similar for the three groups. AG caused hypertrophy of muscular cell, mitochondria deterioration, and enhanced LP level (48%), while exercise associated with AG caused muscular layer thinness (24%), maintenance of cell structure and decreased LP level (57%). Despite the LP and morphological alterations promoted by aging and avoided with aerobic exercice, the non-sensitivity of CR is likely related to the large reserve capacity of this tissue.
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P-427

Effect of osmotic compression on the state of water in demembranated fibers of skeletal muscle

M. Yamaguchi, S. Takemori
Jikei university school of medicine

Transverse relaxation process of ^{1}H-NMR from demenbranated frog muscle fibers could be decomposed into four exponential components with distinct time constants; extremely slow (T_2>0.4 sec), slow (T_2∼0.12 sec), intermediate (0.03<T_2<0.05 sec), and rapid (T_2<0.03 sec) exponentials (Takemori et al., 2001). Among these, the extremely slowly and slowly relaxing components were considered to represent mainly extracellular water group, and the other two were considered to be intracellular.
When the fiber was osmotically compressed by various concentration of dextran (mw. ∼500,000), the intermediately relaxing component, which is considered to represent water molecules restricted moderately by macromolecules in the myofilament lattice, decreased its amplitude linearly with the lattice volume estimated by X-ray diffraction. The other intracellular component did not change its amplitude significantly. On the other hand, none of the time constants, which would represent the averaged strength of the restriction on the water by macromolecules, was significantly affected by the osmotic compression. This shows that the strength of the restriction on the water is not a simple function of distance from the macromolecule surface. A possible mechanism for the restriction will be discussed.

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– Membrane Microdomains –

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Influence of resveratrol on properties of lipid bilayers

B. Łania-Pietrzak[1], D. Mosiądz[1], M. Komorowska[2], A. B. Hendrich[1], K. Michalak[1]
[1]Department of Biophysics, Wrocław Medical University, Chałubinskiego 10, 50368 Wrocław, Poland, [2]Institute of Physics, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50370 Wrocław, Poland

Influence of trans-3,5,4'-trihydroxystilbene (trans-resveratrol), biologically active compound abundant in some fruit and vegetables, on properties of model phospholipid membranes was investigated. Perturbation of lipid bilayer formed of various phospholipids was studied using spectrofluorimetry, EPR and microcalorimetry. Alterations in fluorescence emission spectra of resveratrol observed after its incorporation into lipid bilayers indicate the differences in interaction of this stilbene with various phospholipids (DPPC, DMPC, DMPG or EYPC). Decrease in enthalpy, cooperativity and temperature of main phase transition in the presence of resveratrol were observed by microcalorimetry. EPR measurements performed with EYPC revealed increase in environment polarity and order parameter at level of fifth carbon of alkyl chains, shortening of correlation time of spin probe located in region of polar head groups and increase in correlation time of spin probe located at level of sixteenth carbon of alkyl chains. Obtained results indicated that resveratrol strongly affected all regions of bilayer.

P-429

Plasma membrane microdomains of heterotrimeric G proteins characterized by FRET

D. Abankwa, H. Vogel
Ecole Polytechnique Fédérale de Lausanne (EPFL), Laboratoire de Chimie Physique des Polymères et Membranes, CH-1015 Lausanne, Switzerland

Biochemical and biophysical experiments have shown that G proteins of the Ras-family have different activity dependent affinities for membrane microdomains. Similarly, transducin was shown to redistribute to detergent resistant membrane fractions after light-activation. However, it is not known, whether other heterotrimeric G proteins respond in this way and how biochemical fractionation behavior correlates with plasma membrane microdomain localization in living cells.
We expressed various pairs of cyan and yellow variants of the green fluorescent protein fused to membrane targeting sequences of heterotrimeric G proteins or G proteins of the Ras-family, in order to determine acceptor density dependence of the fluorescence resonance energy transfer (FRET) in living cells as a measure for clustering in microdoamins. We correlate fluorescence data with data derived from biochemical extraction protocols. This approach may help to integrate the heterotrimeric G protein activation cycle into the raft-model and might provide some new ideas about the molecular mechanisms that contribute to the specificity of GPCR to G protein coupling.

P-430

Relationship between bilayer binding and catalytic activity of PI-specific phospholipase C

H. Ahyayauch, A. V. Villar, F. M. Goñi, A. Alonso
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, P.O. Box 644, 48080 Bilbao, Spain

Abstract
Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* appears to be very sensitive to bilayer composition. Certain non-substrate lipids , e.g. galactosylceramide or cholesterol, inhibit PI-PLC in a dose-dependent way. Simultaneous measurements of enzyme activity, enzyme binding and fluorescence of different probes, on a variety of bilayer compositions, reveal that enzyme activity decreases with increasing lipid order, as measured by the fluorescence polarisation of the hydrophobic probe diphenylhexatriene. In contrast, no correlation is found for enzyme activity with fluorescence changes of probes, e.g. laurdan, that report on phenomena occurring mainly at the lipid-water interface. Sphingomyelin has a dual effect, up to 40 mole % it increases PI-PLC activity, with little effect on bilayer fluidity. At higher proportions, the increased lipid chain order causes a decrease in enzyme activity. These results support the "two-stage model" for PI-PLC binding to lipid bilayers, and underline the significance of the enzyme partial penetration into the membrane hydrophobic matrix for its catalytic activity.

P-431

Lipid domains in prokaryotic inner membrane models

S. Alexandre[1], A. Delaune[1], C. Lafontaine[1], V. Norris[2], J.-M. Valleton[1], Z. Zerrouk[1]
[1]UMR 6522 CNRS-Université de Rouen 76821 Mont-Saint-Aignan Cedex, [2]FRE 2829 CNRS-Université de Rouen 76821 Mont-Saint-Aignan Cedex

One of the questions about lipid organization in bacterial membranes is the existence of domains. We elaborated different models of the inner membrane of *E. coli* using Langmuir technique. These models are based on natural lipid mixtures (commercial membrane extracts of *E. coli,* inner membrane extracts of *E. coli* obtained in our group) and mixtures of two or three pure lipids (phosphatidylethanolamine, phosphatidylglycerol and cardiolipins) with proportions mimicking the composition of the inner membrane of *E. coli*.
The Langmuir technique allowed us to elaborate monolayers at the water/air interface. The monolayers were imaged directly at the water/air interface using Brewster angle microscopy and after transfer on a solid substrate (muscovite) with atomic force microscopy.
For all these systems, we observed lipid domains in a range of interfacial pressures coherent with pressures found within biological membranes. In the case of natural extracts, domains could not be identified in terms of the nature of lipids; in the case of artificial lipid mixtures, the nature of domains could be identified taking into account the proportions of the lipid involved in the mixed system.
These results are discussed on the basis of a comparison with the rafts observed within the membranes of eukaryotes.

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P-432

Fission of a multi-domains membrane induced by molecules absorption: an analytical treatment

J.-M. Allain, M. Ben Amar
Laboratoire de Physique Statistique, Ecole Normale Superieure, Paris

The study of giant vesicles made of a lipid mixture have shown a possible phase separation, leading to domains with specific composition. In particular, domains made of cholesterol and sphingomyelin have motivated recently a large number of experiments. These domains, which are more structured than the classical lipid bilayer, are used to model cellular structures called 'raft', which are possibly implicated in various cellular processes such as signaling or intracellular transport. Recent experiments on tense giant vesicles have shown a spectacular deformation, with the ejection of domains, induced by either osmotic shocks or detergent absorption. We present here a theoretical treatment of these experiments. We use an approach based on the energy of the system, including the chemical absorption, to predict the possible shapes for the vesicle. This model leads to a classical bifurcation diagram with, as control parameter, an effective line tension function of the elastic moduli, of the osmotic pressure and of the molecules concentration. In the vicinity of the bifurcation point, the inhomogeneous vesicle becomes instable. At the bifurcation, the fission occurs and one phase is transformed into a daughter independent vesicle.

P-433

The role of lipidic environment on the activity of the human delta opioid receptor

A. Andre, C. Lebrun, G. Gaibelet, M. Welby, A. Lopez
Institut de Pharmacologie et de Biologie Structurale UMR 5089 205 Route de Narbonne, 31077 TOULOUSE Cedex 4

The human delta opioid receptor (hDOP) belongs to the family of G-Protein Coupled Receptors and mediate the effects of opioid alkaloids and peptides on many physiological functions like modulation of pain perception and euphoria. Activation of hDOP regulates different classes of G-proteins causing the activation of multiple G-protein signal pathways.
It has been shown that lipid environment can modulate activities of hMOP (Lagane *et al.*, 2000). Furthermore it has been shown that modulation by lipid environment can correspond to a conformational change of the receptor. Indeed a 10% increase of the hydrophobic thickness of hDOP and rhodopsin receptors has been observed after agonist stimulation (Salamon *et al.*, 2000). Based on these observations we plan to study the role of the lipidic composition and the organization of the lipids (microdomains, ...) in the fonctionality of hDOP.
For this purpose, a fusion protein T7-hDOP-GFP containing a signal peptide to adress the chimeric receptor to the plasma membrane was constructed and stably overexpressed in HEK 293T cells. The cell line containing the fusion protein localised at the plasma membrane and its pharmacological properties are conserved.
So this tool will allow us to investigate the importance of cholesterol and thickness of the membrane on the functionality of the hDOP.

P-434

Interaction of sphingomyelin bilayers with Triton X-100: a calorimetric study

C. Arnulphi, J. Sot, A. Alonso, F. M. Goñi
Unidad de Biofísica, Departamento de Bioquímica Universidad del País Vasco, Spain

The aim of this study is to explore the nature of the interaction of sphingomyelin membranes with the non-ionic detergent Triton X-100 at concentrations below its CMC, i.e. under non-solubilizing conditions, in a range of temperatures that comprises the gel and fluid state of the membrane. Isothermal titration calorimetry was used to characterize and quantify the binding parameters of the reaction. We found that a simple partition model was unable to describe the interaction, which was interpreted in terms of sequential binding isotherms. The thermodynamic parameters reflected the dual behaviour of the interaction depending on the lipid phase of the bilayer: below the Tc the reaction was endothermic, becoming exothermic at higher temperatures. These results are relevant for the interpretation of the mechanisms of detergent solubilization and detergent-resistant membranes.

P-435

Formation of cubic phases from vesicles of DOPG/MO membranes induced by low concentrations of Ca^{2+}

T. S. Awad, S. M. Masum, M. Yamazaki
Dept. Physics, Fac. Science, Shizuoka University, 836 Oya, Shizuoka 422-8529, Japan

Elucidation of stability of cubic phases of biomembrane is essential for understanding of biomembrane dynamics. Recently we found that electrostatic interactions due to the surface charges in the membrane interface induce transitions between cubic phases and L_α phase, and also between different IPMS cubic phases.[1−3] In the present study, we have investigated ion-induced transformation of large unilamellar vesicles (LUVs) into cubic phase membranes. Phase stability of membranes of monoolein (MO) and negatively-charged dioleoylphosphatidylglycerol (DOPG) mixtures (DOPG/MO membrane) depended greatly on the surface charge density of the membrane interface; with an increase in DOPG concentration, the most stable phase changed as follows, $Q^{224} \Rightarrow Q^{229} \Rightarrow L_\alpha$. Low concentrations of Ca^{2+} induced L_α to cubic phase transitions in the DOPG/MO-MLV; e.g., for 30%DOPG/70%MO-MLV in excess water, 15-30 mM Ca^{2+} induced the Q^{229} phase, and ≥ 30 mM Ca^{2+} induced Q^{224} phase. DOPG/MO membranes containing $\geq 25\%$ DOPG also formed LUVs. Low concentrations of Ca^{2+} transformed these LUVs into cubic phases (Q^{224} or Q^{229} phase depending on Ca^{2+} concentration); e.g., for 30%DOPG/70%MO-LUV, 50 mM Ca^{2+} induced the formation of the Q^{224} phase.
[1] Aota-Nakano et al., *BBA*, **1461**, 96, 1999 [2] Li et al., *Biophys. J.* **81**, 983, 2001
[3] Masum et al., *Langmuir*, **19**, 4745, 2003

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– Membrane Microdomains –

P-436

Electroporation and electropermeabilization of lipid bilayer membranes from tissues of vertebrates

N. M. Ayvazian, A. E. Zaqaryan
Dpt. Biophysics, Yerevan State University, Armenia

All living cells characterized by availability of stationary difference of electrical potential caused by ionic asymmetry in both sides of membrane and its transmembrane diffusion. In course of excitement of nervous and muscle cells it is taking place the quick depolarization and polarization of membranes by influence of temper. Own or external electrical potential can be a reason of instability or even distraction of lipid bilayer. We isolated lipid fractions from the brain, heart, liver and muscle of vertebrates: crucian carp (*Carassius carassius*), marsh frog (*Rana ridibunda*), caucasian agama (*Stellio caucasicus*), rats by the method of Keits. Model membranes were formed from the total lipid fraction on a teflon aperture by the method of Muller. The electrical parameters of the BLMs were determined on an electrometric devise equipped with a Keithley 301 differential feedback amplifier (USA) in a voltage-fixation mode. The potential of membrane rupture was taken as the threshold value of the voltage applied. There are supposed that the process of electrical rapture of BLM underlies the process of electroporation, in which course appear huge defects like inverted pores. As we waited, mostly stabile are BLMs from brain lipids, which breaking-potential have most value in brain membranes of reptiles, what surely depend of cholesterol content. BLMs from lipids of fishes and amphibian are more stabile in media of K^+, than Na^+, Li^+ and Ca^{2+}.

P-437

Analytical expressions for chain order parameter and lateral pressure profiles in lipid membrane

S. V. Baoukina, S. I. Mukhin
Theoretical Physics department, Physics and Chemistry faculty, Moscow State Institute for Steel and Alloys

Flexible string model of hydrocarbon chain is used to analytically derive various thermodynamic characteristics of lipid membrane. We obtained analytical expressions for lateral pressure distribution and chain orientational order parameter across hydrophobic part of lipid bilayer. Pressure profile influences the functioning of integral membrane proteins and is difficult to measure experimentally. Chain order parameter is experimentally observable quantity and provides good verification for our theory. Temperature and area-per-lipid dependences of lateral pressure and order parameter are found theoretically. Chain contribution to the area compressibility modulus and the temperature coefficient of area expansion are calculated.
Membrane thermodynamic characteristics are derived using path integral technique. We expressed the pressure and order parameter profiles via the eigenfunctions and their derivatives of the self-adjoint operator of chain energy density. At room temperature only a few discrete lowest energy eigenfunctions contribute to membrane properties.

P-438

C-terminal Gg-protein farnesylated peptide and DEPE membrane interactions

F. Barcelo[1], J. Prades[1], S. S. Funari[2], P. V. Escriba[1]
[1]Molecular and Cellular Biomedicine Laboratory, Fundamental Biology Department, University of Balearic Islands, [2]HASYLAB, DESY, Hamburg

The isoprenyl group of the G protein γ subunit is involved in protein-protein and lipid-lipid interactions (A.I. Magee, Biochem. J. *376*(2003) e3-4). On the other hand, the physical properties of the membrane can be modified by the presence and binding of large amounts of G proteins and vice versa, the membrane structure influences G protein-membrane interactions. In the present study, we sought for the potential role that lipid polymorphism has in G protein-membrane interactions. Focusing the interest on PE model membranes, two peptides, $P_{\gamma\text{-}FN}$ and P_γ were synthetised with a defined sequence (PLLTPVPASENPFREKKFFCAILacetylated) which is a copy of the C-terminal region of the bovine Gγ protein subunit (P16874). The peptide $P_{\gamma\text{-}FN}$ has the cysteine residue derivatised with a farnesyl group similarly to the G_γ-subunit. We report the interactions of $P_{\gamma\text{-}FN}$ and P_γ with DEPE membranes using X-ray diffraction, DSC and FTIR techniques. The data presented supports that $P_{\gamma\text{-}FN}$ interacts with DEPE creating microdomains with increased nonlamellar forming propensity. This study suggests that the farnesyl moiety of the Gγ subunit could mediate the protein anchorage into membrane domains.

P-439

Interaction and lipid-induced conformation of two cecropin-melittin hybrid peptides

M. Bastos[1], F. Abrunhosa[1], G. Bai[1], P. Gomes[1], D. Andreu[2], M. Prieto[3]
[1]Dep. of Chemistry, Fac. Sciences, P-4169-007 Porto, Portugal, [2]Dep. of Experimental and Health Sciences, Univ. Pompeu Fabra, Barcelona, Spain, [3]Centro de Química Estrutural / I.S.T., (UTL) Lisboa, Portugal

Eukaryotic antibiotic peptides (EAPs) have been studied for the past years as they may become an alternative to conventional antibiotic therapy. Efforts have been directed to increase their potency and specificity for pathogenic microbes while minimising their cytotoxic effect towards eukaryotic cells. One successful approach is based on the synthesis of hybrid sequences derived from naturally occurring EAPs. CA(1-8)M(1-18) showed improved antimicrobial activity relative to parent Cecropin A and greatly reduced the undesirable haemolytic effect of Melittin. Taking this peptide as lead, a subsequent approach was to reduce its size while retaining antimicrobial activity, as in CA(1-7)M(2-9). Both have been extensively studied in terms of antimicrobial activity, but detailed biophysical studies are needed to fully understand their mechanism of action. Therefore we have been studying their interaction with liposomes by a variety of techniques, namely calorimetry (DSC and ITC), CD, light scattering, SPR and Fluorescence spectroscopy. The peptides were synthesized by Fmoc/^{t}Bu solid phase methods. LUV's from DMPC, DMPG and their 3:1 mixture were used as model membranes. The results obtained from the various techniques will be discussed together in an attempt to further understand the mechanism of action of these peptides.

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– Membrane Microdomains –

P-440

A spin label structural study of salt and cholesterol on cationic liposomes

C. R. Benatti, M. T. Lamy
Instituto de Física, Universidade de São Paulo, São Paulo, SP, Brazil

The presence of cholesterol in cationic amphiphile/DNA complexes was found to facilitate the lipoplex interaction with membrane cells. Considering the possible relevance of the cationic amphiphile/cholesterol bilayer structure, in the present work, thermostructural properties of dioctadecyldimethylammonium bromide (DODAB) liposomes are investigated, in low and high ionic strength, with different cholesterol contents. DODAB bilayers were monitored by lipid spin labels, both close to the lipid/water interface, and at the bilayer core. As expected, the cationic lipid gel phase is stabilized by the presence of ions, the bilayer presenting a higher gel/fluid transition temperature (T_m) in high ionic strength. As found for low ionic strength (Benatti, Feitosa, Fernandez, Lamy-Freund, Chem. Phys. Lipids 111, 93, 2001), high salt DODAB membranes also present a clear coexistence of the two phases around T_m, and a thermal hysteresis of about 3°C. Salt was found to somewhat increase the bilayer packing, both at the gel and liquid phases. Cholesterol solubility in DODAB bilayers seems to be rather low, as the presence of pure cholesterol domains can be clearly detected by spin labels, for cholesterol/DODAB ratios as low as 1/6, both in low and high ionic strength dispersions.

P-441

Structural study of pulmonary surfactant membranes using X-ray scattering

J. Bernardino de la Serna[1], R. Vargas[2], J. Pérez-Gil[1], L. Mateu[2]
[1]Dpto. Bioquímica, Facultad de Biologia, Universidad Complutense de Madrid, Spain, [2]Laboratorio de Estructura Molecular. Dpto. Biología Estructural, Instituto Venezolano de Investigaciones Cientificas (IVIC), Caracas, Venezuela

Mamalian alveolar surface is lined with a highly surface-active film named pulmonary surfactant (PS). This complex is synthesized in pneumocyte II cells in the form of lipid-protein bilayers that finally have to reach the air-water interface where adsorbing in the form of a monolayer with associated bilayers.This multilayered film has the ability to prevent the collapse of alveoli during the successive compression-expansion breathing cycles. PS is a complex lipoproteic mixture containing aprox. 90% lipids and 10% proteins (weight) Neither the structural organization of this multi-membrane complexes nor that of the interfacial film are known. We have performed x-ray scattering studies of purified membranes of porcine pulmonary surfactant and some of its isolated fractions in the absence or presence of the hydrophobic proteins and cholesterol, as a function of water content and temperature, in order to elucidate the structure of the arrangements. Some of our results show the presence of lamellar and hexagonal phases in a wide range of concentration and temperature. The paraffin chain conformation has been also studied.

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Host membrane cholesterol widens the fusion pore created by influenza hemagglutinin

S. Biswas[1], P. S. Blank[2], J. Zimmerberg[2]
[1]Johns Hopkins University School of Medicine, [2]National Institute of child Health & Human Development/NIH

Membrane fusion is thought to proceed through a number of nonlamellar intermediates, one of which is a pauci-molecular lipidic connection between the two contacting (outer) leaflets of the fusing bilayers, termed a 'modified stalk', followed by opening and then expansion of a fusion pore connecting the two distal (inner) leaflets. Here we tested for cholesterol-specific interactions near or in the modified stalk or fusion pore that may affect fusion by taking advantage of the naturally low cholesterol levels (<4 mol %) of Sf9 insect cells engineered (HAS cells) to express the hemagglutinin of influenza virus. Enrichment of HAS cells with cholesterol reduced the delay of lipid dye diffusion time after triggering, indicating that cholesterol facilitates faster membrane lipid mixing prior to pore opening. Increased cholesterol also increased aqueous content mixing between HAS cells fusing to human red blood cells (RBC), over a broad distribution of HA expression levels. This suggested that fusion pore expansion was favored by cholesterol, an idea substantiated both by fluorescent microscopy data on transcell dye diffusion and by electrical data on fusion pore conductivity in cholesterol-enriched cells. Also cholesterol analogues with modified head group inhibit fusion. Overall, these results support the idea that host cell cholesterol requires intact head group and, cholesterol acts at early before opening of the fusion pore and, late in fusion pore expansion.

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Theoretical analysis of configuration of phospholipid vesicles with isotropic inclusions

B. Bozic, S. Svetina, V. Kralj-Iglic
Institute of Biophysics, Faculty of Medicine, University of Ljubljana, Slovenia

We study theoretically shapes of phospholipid vesicles with isotropic membrane inclusions. It is considered that the inclusion energetically prefers certain local membrane curvature since a mismatch between its intrinsic curvature and the local membrane curvature rises an energy. The equilibrium shapes of the vesicles are obtained by minimization of the membrane energy at fixed membrane area, fixed enclosed volume and fixed number of inclusions. The variation for axisymmetric vesicle shapes leads to a system of Euler-Lagrange equations that are solved numerically. It is shown that the presence of inclusions in the membrane can induce transitions between classes of shapes with different symmetry. At certain values of parameters, a singularity in the derivative of the meridian curvature occurs. Most shapes below this singularity are well approximated by the area-difference elasticity model with renormalized model constants.

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Influence of antihypertensive drugs on elasticity parameters of synthetic and biological membranes

T. Breitenbach[1], A. Rosenkranz[1], H. Lob[1], R. Berkels[1], T. Sottmann[2], R. Strey[2], R. Roesen[1]
[1]Dept. of Pharmacology, University Hospital Cologne, Germany, [2]Institut für physikalische Chemie, Universität zu Koeln, Germany

Loss of vascular endothelial function due to oxidative damage promotes the development and progression of atherosclerotic diseases. Recently, we could show protection of cultured endothelial cells (EC) by lipophilic dihydropyridine-type calcium antagonists (DHP-CA) decreasing the formation of reactive oxygen species (ROS).
Hence we studied the change in membranous behaviour of synthetical bilayers (DMPC) after incubation with several DHP-CA in comparison to angiotensin-conversion-enzyme inhibitors (ACE-Inh.) using pipette aspiration technique to calculate bending rigidity (κ) and area expansion modulus (K_a). Moreover, EC were investigated under control and conditions of enhanced ROS-formation (c[Glucose]=5mM and 30mM, respectively).
Micromolar concentrations of DHP-CA tended to increase physical resistance of the DMPC-membranes in contrast to the addition of ACE-Inh., identified by appropriate values of κ and K_a. In EC ROS-induced cellular damage was indicated by destabilising of the cell-membrane, which was abolished in the presence of DHP-CA and only in part by ACE-Inh., in spite of nearly equal inhibition of ROS-formation.
Thus, in addition to the well known antioxidative effects of the DHP-CA a membrane stabilising component may contribute to the pleiotropic mechanism of cell protection and strengthening of endothelium in atherosclerotic plaques.

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Glycosphingolipids and domain organization on model membranes: a multi-technique approach

P. Brocca, L. Cantù, M. Corti, E. del Favero, S. Motta
University of Milan, Dept. of Chemistry, Biochemistry and Biotechnology

Membrane ordered microdomains, or rafts, are one of the cool topics in biochemistry. These domains, so compact and structured to be resistant to detergent treatment, are enriched in sphingolipids and cholesterol. We have taken up a multi-technique investigation on phospholipid/sphingolipid mixed aggregates aimed to assess the effects on the physical and structural properties of simple model membranes brought about by two typical raft components: gangliosides (glycosphingolipids) and sphingomyelin (SM). Mixed systems of DMPC containing minority amounts (2%-10%) of gangliosides or SM have been studied in the form of dry bilayers or of extruded vesicles in water solutions.
All findings agree in proving that the presence of gangliosides, even in very low molar ratio, modifies the structure of the membrane over extended regions: *a)* organizes islets on the surface (SANS and SAXS) *b)* determines new periodicities in the lamellar phase (Small Momentum Transfer Diffractometry) c*)* modifies the out-of-plane vs in-plane dynamics in the bilayer (Incoherent Elastic Neutron Scattering) *d)* yields striking new features to the surface local organization (Wide Angle X-ray Scattering) *e)* settle on a multiplicity in the chain melting transition (Densitometry) *f)* soften the bilayer (Dynamic Laser Light Scattering).

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A home built W-band EPR spectrometer and its application to spin labelled membrane proteins

H. Brutlach, E. Bordignon, H.-J. Reyher, H.-J. Steinhoff
Universitaet Osnabrueck, 49076 Osnabrueck, Germany

Electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL) and molecular dynamics (MD) simulations has emerged as a powerful method to study the structure and conformational dynamics of membrane proteins (1). By combining SDSL techniques with high-field/high-frequency EPR, however, a new dimension in the SDSL applicability has been achieved recently concerning sensitivity and selectivity to molecular motion, to polarity and proticity of the microenvironment of the spin label, and to conformational changes of spin labelled protein segments (2). We report on the setup of a home built EPR-Spectrometer working from 94.95 to 95.05 GHz in pulse- and cw-mode and illustrate its capabilities with applications to spin labelled NpSRII-NpHtrII complex variants reconstituted in purple membrane lipids. Due to the enhanced Zeeman splitting the EPR spectra performed at 290K and at 170K reveal site-specific, i.e., local polarity and proticity in the protein system.
1. Steinhoff, H.-J. (2004) Multi-frequency EPR spectroscopy studies of the structure and conformational changes of site-directed spin labelled membrane proteins, *in:* Supramolecular structure and function 8, (ed. G. Pifat-Mrzljak) Kluwer Academic/Plenum Publisher, New York: 157-177.
2. Savitsky, A., Kühn, M., Duché, D., Möbius, K., and Steinhoff, H.-J. (2004) Spontaneous refolding of the pore-forming colicin A toxin upon membrane association as studied by X- band and W-band high-field EPR spectroscopy. J. Phys. Chem. B. 108:9541-ff

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Spontaneous formation of detergent micelles around the outer membrane protein OmpX

R. A. Böckmann[1], A. Caflisch[2]
[1]Theoretical & Computational Membrane Biology, Center for Bioinformatics Saar, Universität des Saarlandes, Germany, [2]Department of Biochemistry, University of Zurich, Switzerland

The structure and flexibility of the outer membrane protein X (OmpX) in a water-detergent solution and in pure water are investigated by molecular dynamics simulations on the 100 ns timescale and compared with NMR data. The simulations allow for an unbiased determination of the structure of detergent micelles and the protein−detergent mixed micelle. The short-chain lipid dihexanoylphosphatidylcholine (DHPC) as a detergent aggregates into pure micelles of about 18 molecules and on the protein surface. The detergent binds in the form of a monolayer ring around the hydrophobic β-barrel of OmpX rather than in a micellar-like oblate. About 40 DHPC lipids are sufficient for an effective suppression of water from the surface of the β-barrel region. The phospholipids bind also on the extracellular, protruding β-sheet. Here, polar interactions between charged amino acids and phosphatidylcholine headgroups act as condensation seed for detergent micelle formation [1]. The polar protein surface remains accessible to water molecules. In total, about 90−100 detergent molecules associate within the protein-detergent mixed micelle, in agreement with experimental estimates. The simulation results indicate that OmpX is not a water pore and support the proposed role of the protruding β-sheet as a 'fishing rod'.
[1] R.A. Böckmann & A. Caflisch, Biophys.J.(2005), *in press*

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Ceramide chain asymmetry: effects in biomembranes

D. C. Carrer[1], S. Schreier[2], B. Maggio[1]
[1]Dpto. Química Biológica, Fac. Cs. Químicas, UNC, Córdoba, Argentina, [2]Instituto de Química, Univ. of Sao Paulo, Sao Paulo, Brazil

An important part of natural ceramides have very long fatty acyl chains, which make ceramide molecules very asymmetric. We have used calorimetry and EPR to study the effect of ceramide chain asymmetry on mixtures of C8Cer with DMPC. A phase diagram is provided along with information on the mobility of the chains at different temperatures both below and above the phase transition temperature of the mixtures. The results indicate a partial interdigitation of C8Cer chains in the gel phase, producing a correlation between the organization of both hemilayers. Based on our data, we propose that the effects of asymmetric ceramides on biomembranes are bimodal and similar to those of cholesterol.

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Dynamic confinement of NK2 receptors in the plasma membrane of HEK cells : a vrFRAP analysis

L. Cezanne[1], S. Lecat[2], B. Lagane[1], C. Millot[1], J. Vollmer[2], H. Matthes[2], J.-L. Galzi[2], A. Lopez[2]
[1]IPBS/CNRS, 205 route de Narbonne, 31062, Toulouse, France, [2]CNRS UPR9050, ISBS, Illkirch, France

The dynamical organization of membrane components is of great interest for the functional and structural study of biological membranes. In the case of the signal transduction chain via GPCRs, this is essential since numerous proteic partners have to meet and to interact in the membrane before the cellular response occurs. The technic used in this study (vrFRAP or Fluorescence Recovery After Photobleaching at variable radii) has allowed us to analyse the membrane compartmentalization of EGFP-NK2 receptors as well of lipids probed by C_6-NBD-PC in HEK plasma membranes. Results showed that, before activation by NKA, NK2R were confined in domains (radius 420 $\pm$ 80 nm) with a lateral diffusion coefficient of 0.4 $\pm$ 0.1 10^{-9} cm^2/s. After activation, the same proportion of receptors (30 $\pm$ 7%) were confined in smaller domains (radius 170 $\pm$ 50 nm) where they were immobilized in the time scale of FRAP experiments. In both experimental conditions, 70% of NK2R were free to diffuse between domains with a lateral diffusion coefficient $\approx 10^{-9}$ cm^2/s. Co-localization experiments with various fluorescent proteins demonstrated that the membrane structures where NK2R were confined are pre-coated pits not yet invaginated in the membrane. The hypothesis currently under investigation in our laboratory is that " these domains could be signalization platforms where various proteic partners could be distributed in order to optimize their interactions in time and in the membrane plane ".
L.Cézanne et al. (2004) J.Biol.Chem. *279*, 45057-45067.

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Sphingomyelin/cholesterol membranes studied by multinuclear solid state NMR and X-ray diffraction

J. A. Clarke, J. M. Seddon, R. V. Law
Department of Chemistry, Imperial College London, South Kensington Campus, London SW7 2AZ, UK.

We present the phase diagram for egg-yolk sphingomyelin (egg SM) and cholesterol ($\leq$60 mol%) in excess water, determined using multinuclear solid state NMR spectroscopy and X-ray diffraction. We have established an empirical relationship between the ^{13}C MAS γ-*gauche* effect, and chain order parameters from ^{2}H-NMR, allowing an average order parameter to be estimated without the use of isotopic substitution. From this data we can estimate the lipid chain length, and find that increasing the cholesterol concentration above the T_m of the SM leads to a limiting chain extension of ca. 17.5 Å by 30 mol% cholesterol. Above10-20 mol% cholesterol there is disruption of the gel phase packing, as seen by the distribution of isotropic chemical shifts in the ^{31}P-NMR MAS data. Above the T_m, we find no direct evidence of L_α - L_o phase coexistence, only a continuous transition between the two phases, with the L_α phase becoming progressively more restricted conformationally, eventually forming an L_o phase. SM forms the L_o phase at lower cholesterol concentrations compared to dipalmitoylphosphatidylcholine (DPPC), which has a similar T_m. This is due to the fact that the SM chains (in the absence of cholesterol) are intrinsically more ordered than phosphatidylcholine (PC) chains, which is reflected in the ^{13}C-NMR MAS data, and some previous ^{2}H-NMR data. Study of the motion and ordering of deuterated cholesterol by ^{2}H-NMR shows that the ordering effect of cholesterol on the SM chains saturates at lower cholesterol concentrations than for PC.

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Measuring lipid asymmetry in supported bilayers by fluorescence interference contrast microscopy

J. M. Crane, V. Kiessling, L. K. Tamm
Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, USA

There is substantial scientific and practical interest in engineering supported lipid bilayers with asymmetric lipid distributions as models for biological cell membranes. In principle, it should be possible to make asymmetric supported lipid bilayers by either the Langmuir-Blodgett/Schäfer (LB/LS) or Langmuir-Blodgett/vesicle fusion (LB/VF) techniques (Kalb et al., *Biochim. Biophys. Acta* **1103**:307-316, 1992). In the present work, we developed a technique that is based on fluorescence interference contrast (FLIC) microscopy to measure lipid asymmetry in supported bilayers. We compared the final degree of lipid asymmetry in LB/LS and LB/VF bilayers with and without cholesterol in liquid-ordered (l_o) and liquid-disordered (l_d) phases. An asymmetrically labeled bilayer made by the LB/LS method was found to be at best 70-80% asymmetric once completed. In LB/LS bilayers of either l_o or l_d phase, cholesterol increased the degree of lipid mixing between the opposing monolayers. The use of a tethered-polymer support for the initial monolayer did not improve lipid asymmetry in the resulting bilayer. However, asymmetric LB/VF bilayers retained nearly 100% asymmetric label, with or without the use of a tethered polymer support. Finally, lipid mixing across the center of LB/LS bilayers was found to have drastic effects on the appearance of l_d-l_o phase coexistence as shown by epifluorescence microscopy.

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Fluorescence lifetime imaging microscopy (FLIM) of membrane lipid domains and rafts

R. F. M. de Almeida[1], J. W. Borst[1], A. J. W. G. Visser[1], M. Prieto[2]
[1]MicroSpectroscopy Centre, Laboratory of Biochemistry, Wageningen University, [2]Centro de Química-Física Molecular, Instituto Superior Técnico da Universidade Técnica de Lisboa

Characterization of lipid domains is a major topic in membrane biophysics. Recently, observation of giant unilamellar vesicles (GUV's) by confocal or two-photon microscopy allows direct visualization of gel/fluid and liquid ordered/liquid disordered phases. The phase assignment is based on the relative signal intensity from the observed domains in combination with the expected partition behaviour of the fluorescent lipid (or lipid analogue) in use. Localisation studies give insight into lipid domain formation, but partitioning cannot be resolved with this approach. These and other factors can lead to ambiguous phase assignment. It is known that the quantum yield of specific fluorescent lipids is dependent on the host lipid phase. For most fluorescent lipids, the fluorescence lifetime is not influenced by concentration and the unambiguous assignment of the phase corresponding to a particular domain in a two-phase mixture can be resolved by FLIM. With this technique the fluorescence lifetime in each pure phase can easily be determined. The fluorescent dye "Fast DiO" has a fluorescence lifetime of about 800 ps in the fluid phase, whereas a fluorescence lifetime of about 1400 ps is encountered in the gel phase. In the absence of clear domains, the recovered fluorescence lifetime distribution of "Fast DiO" can also be an indicator of heterogeneity. This methodology currently is applied to the characterization of gel/fluid domains and liquid disordered/liquid ordered rafts in multi-component GUV's.

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Rafts big and small. Their size determination on a ternary membrane phase diagram

R. F. M. de Almeida[2], L. M. S. Loura[3], A. Fedorov[1], M. Prieto[1]
[1]Centro de Química-Física Molecular, IST, Lisboa, Portugal, [2]Centro Química e Bioquímica, Faculdade de Ciências de Lisboa, Portugal, [3]Departamento e Centro de Química, Universidade de Évora, Portugal

The ternary lipid system palmitoylsphingomyelin (PSM)/palmitoyloleoylphosphatidylcholine (POPC)/cholesterol is a model for lipid rafts. Previously (de Almeida, R. F. M., Fedorov, A. and Prieto, M. (2003) *Biophys. J.* **85**, 2406-2416), the phase diagram for that mixture was obtained, establishing the composition and boundaries for lipid rafts. In the present work, this system is further studied, in order to characterize the size of the rafts. For this purpose, a time-resolved fluorescence resonance energy transfer (FRET) methodology, is used. It is concluded that: 1) the rafts on the low raft fraction of the raft region are small (below 20 nm), whereas on the other side the domains are larger; 2) on the large domain region, the domains are larger in the ternary system (> 75 $\sim$100 nm) than in binary systems phosphatidylcholine/cholesterol (between $\sim$20 and $\sim$75-100 nm); 3) the raft marker ganglioside G_{M1} in small amounts (and excess cholera toxin subunit B) does not affect the general phase behavior of the lipid system, but can increase the size of the rafts on the small domain region. In sum, lipid-lipid interactions alone can originate lipid rafts on very different length scales.

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Biophysical characterization of Liposomes entrapped in a Sol-Gel matrix

R. Esquembre[1], M. Ferrer[2], R. Mallavia[1], F. del Monte[2], C. R. Mateo[1]
[1]IBMC. Universidad Miguel Hernández. 03202-Elche (Spain), [2]Instituto de Ciencia de Materiales, CSIC. 28049-Madrid (Spain)

Immobilization of liposomes shows interesting applications in protein biology, membrane biophysics and biosensor technology. A number of different immobilization methods have been described and the dynamical and physical properties of such systems have been explored using several biophysical techniques. These studies have shown that most of immobilization process greatly affects the stability of the membrane. During the last decade sol-gel technology has been widely employed to immobilize biomolecules while membrane immobilization by this method has been scarcely reported. In this work we have entrapped liposomes composed of zwitterionic and anionic lipids (DMPC and DMPG, respectively) into sol-gel matrix prepared from tetramethyl orthosilicate using an alcohol-free sol-gel route. Physical and dynamical properties of the immobilized systems have been analysed from steady-state and time-resolved fluorescence spectroscopy using the membrane probes DPH, BODIPY, laurdan and trans-parinaric acid. Stability and phase transition behaviour was also explored from DSC experiments. Results indicate that DMPG liposomes practically preserve their physical properties upon immobilization. However, immobilization of DMPC largely perturbs the structural order of the lipid bilayer as well as the cooperativity of the phase transition.

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The bending elasticity of charged lipid bilayers

O. A. Finogenova[1], V. Vitkova[2], J. Genova[2], M. D. Mitov[2], I. Bivas[2], Y. A. Ermakov[2]
[1]A.N.Frumkin Institute of Electrochemistry RAS, [2]Institute of Solid State Physics BAS

The bending rigidity of lipid bilayers was investigated via micromanipulation of giant lipid vesicles (GV), made from the mixture of phosphatidylserine (PS) and phosphatidylcholine (PC). A presence of sucrose (>0.1 M) was used for GV preparation to facilitate the swelling of lipids. The elasticity of membrane was found to increase with the saturation at PS content more than 15mol%. As a control the electrokinetic measurements were used to evaluate the surface charge density in the presence of 0.17 M sucrose and 0.01 M KCl in comparison to same electrolyte with no sucrose. Surface potential calculated from electrokinetic data, also the boundary potential measured with planar lipid membranes demonstrate the minimal effect of sucrose: their changes does not exceed 10-20 mV of different sign for PC and PS membranes. The quantitative analysis was done according to the theory of Winterhalter,M., Helfrich,W. (1992) and May,S. (1996). The principal parameter to fit the theory to the experiment is the position of neutral surfaces for both monolayers. In the case if these surfaces are placed at outer surface of the membrane the theories predict the saturation level about 8 times below the experimental data. The better agreement was found when the neutral surface immersed at some depth into membrane. The depth was assumed proportional to PS/PC ratio due to different incorporation of sucrose into PC and PS membranes.

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Polyelectrolyte nanorings

H. Flores[1], J.-L. Menchaca[2], E. Perez[2], C. Gergely[4], F. J. Cuisinier[3]
[1]Facultad de Estomatologıca, UASLP, Alvaro Obregon 64, 78000 San Luis Potosı, Mexico, [2]Instituto de Fısica, UASLP, Alvaro Obregon 64, 78000 San Luis Potosı, Mexico, [3]INSERM U 595, Odontologie, Universite Louis Pasteur, Strasbourg, France, [4]GES, Univ. Montpellier II, 34095 Montpellier Cedex 05

Nanoring structures formed by self-assembly of poly(ethylenimine) and poly(sodium 4-styrenesulfonate) during sequential adsorption were observed by AFM microscope in liquid cell technique. Filter pore size and carbonate ion concentration are identified as critical parameters for their formation. We show how these two parameters modulate the nanorings formation as a consequence of the ydrophobic polyelectrolyte domains formed into the polyelectrolyte solutions and the screening effect produced by the divalent carbonate ions present also in solutions. The nanorings size is controlled through competition between electrostatic and hydrophobic interactions where the charge density of the substrate plays an important role.

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The interaction of ebola fusion peptide with cellular membranes: the role of lipids rafts

M. S. Freitas, F. Almeida, A. P. Valente, J. L. Silva
Universidade Federal do Rio de Janeiro, Instituto de Bioquímica Médica, Programa de Biologia Estrutural, Brazil

Ebola viruses are filamentous and enveloped that belongs *Filoviridae* family. The lipid envelope contains one type of protein (Ebola GP) arranged into oligomers. The Ebola GP is responsible for both receptor binding and membrane fusion. It is proteolytically cleaved into disulfide-linked GP1 and GP2 subunits. The N-terminal domain of glycoprotein (GP2) is the fusion peptide region, which is thought to insert directly into the target membrane. The main goal of this work is to characterize the interaction between the fusion peptide and cellular membranes. To obtain the structural information on peptide-lipid bilayer interaction, we used spectroscopic techniques with living cells. The interaction between Ebola fusion peptide with VERO or BHK-21 cells induced lipid mixing of cellular membranes. However, the Ebola peptide was not able to induce lipid mixing of cholesterol-depleted VERO or BHK-21 cells indicating that cholesterol is playing a crucial role in membrane fusion. To understand the importance of cholesterol, we isolated membrane microdomains, named lipid rafts. First over all, we analyzed the peptide-lipid interaction by NMR. The chemical shift of ^{1}H resonances suggest that the fusion peptide interact with lipid rafts. Besides, the peptide induced lipid rafts aggregation, indicating fusion among rafts. This work is the first indicative of direct interaction between Ebola fusion peptide and lipid rafts, a crucial information since the fusion peptide is a potential target to the development of antiviral drugs.

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Effect of organotin compounds and their mixture with flavovols on model membrane

J. Gabrielska, M. Soczynska-Kordala, R. Zylka, S. Przestalski
Agricultural University Wroclaw, Poland

Our investigations showed that diphenyltin (DPhT) and triphenyltin chlorides (TPhT) in thepresence of UV increased the phosphatidylocholine liposome oxidation. EPR results confirmed the sugestion that UV can induce the free radical form of phenyltins. The work investigates the possibility of the protective action of quercetin (Quer) on liposomes exposed to phenyltins and UV. The concentrations of Quer and its mixtures with phenyltins were so determined (and compared with trolox and BHT) so that they induce 50% inhibition of oxidation. They were 11.6, 10.0 and 4.5 μM/L, which constitutes the following sequence of action: Quer:TPhT>Quer:DPhT>Quer. This relation is confirmed by the results on the antiradical ability of the compounds towards the free radical diphenylpricrylhydrazil. Similar sequences obtained in both studies suggest the action of the mixtures as radical scavenges. The Quer's ability to form complexes with phenyltins indicates (i) a possible way to inhibit peroxidation, (ii) the stabilizing role of chelating in the antioxidative action of the Quer:phenyltins. The differentiation in the action of the compounds studied may result from different localizations in the membrane, which is indicated by the results of the fluorimetric and IR studies. *[Work sponsored by KBN no 2 PO4 087 29]*

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Cholesterol content targets different active conformations and signalisation of μ-opioid receptor in CHO cells

G. Gaibelet, B. Lagane, A. Saulière, S. Ravault, C. Lebrun, A. Lopez, L. Cézanne
IPBS/CNRS, 205 route de Narbonne, 31062 Toulouse, France

On native membranes of Saccharomyces cerevisiae which surexpressed μ-opioid receptor and where ergosterol was found specifically, we have demonstrated that, ergosterol constrained the μ-opioid receptor in a low affinity conformation (1). Conversely cholesterol restored high affinity state in modified [ergosterol-depleted - cholesterol-complemented] yeast membranes. The present study, on mammalian plasma membranes of CHO cells, analyse the effects of ergosterol and cholesterol contents both on the ligand binding and on the G-protein coupling of μ-opioid receptor. It shows: (i) After G-protein decoupling by means of Gpp(NH)p, only agonist binding with Bmax decreased and Kd increased, seems to be affected by the cholesterol content. (ii) In absence of Gpp(NH)p and in cholesterol-depleted membranes, G-protein coupling efficiency (Emax) was affected while agonist potency (EC_{50}) and Bmax were unchanged.
This results suggest that the cholesterol content as well as G-protein stabilise different high affinity receptor conformations in equilibrium in the membranes, and that after cholesterol depletion a population of receptors in high affinity conformation does not couple no more to one G-protein subtype of Gαi or Gαo which couple to μ-opioid receptor. Thus, cholesterol seems to target a specific activated conformation. Accounting to the lateral distribution of membrane components (cholesterol, receptor and G-protein) we propose a cholesterol-dependent model for μ-opioid signalisation.

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Interaction of the N-t segment of surfactant protein SPC with phospholipid bilayers: a ^{2}H-NMR study

A. González-Horta[1], M. Morrow[2], J. Pérez-Gil[1]
[1]Dept Bioquimica, Fac Biología, Universidad Complutense, Madrid, Spain, [2]Dept Physics, Memorial University of Newfoundland, St. John´s, Canada

Pulmonary surfactant forms a lipid-protein surface active film at the respiratory air-aqueous interface to prevent alveolar collapse. Surfactant SP-C is an small peptide of 35 amino acids, containing a transmembrane α-helix and a cationic N-terminal segment with palmitoylated cysteines. Two peptides with sequence corresponding to the N-terminal segment of SP-C have been synthesized, either with free or palmitoylated cysteines, reconstituted in bilayers of perdeuteraded or choline-labeled dipalmitoylphosphatidylcholine and egg phosphatidylglycerol (DPPC/PG, 7:3 w/w), and studied by ^{2}H-NMR. We have also studied a peptide sterified with deuterated palmitic chains. We did not detect specific interaction between the peptides and either lipid component of the mixture. The effect of both peptides on the acyl chain order was negligible in the liquid crystalline phase, as deduced from the first spectral moment (M_1), while palmitoylated peptide had a little ordering effect on the bilayers in the gel phase. Both peptides produced tilting of the headgroup toward the bilayer surface while decreasing the headgroup orientational order. The spectra of peptide with deuterated palmitics indicated that protein-attached chains have more intrinsic mobility than the chains of the lipids themselves.

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Do domains form in asymmetric membranes? A fluorescence and atomic force microscopic study

S. O. Hagge, A. Böhling, T. Gutsmann, U. Seydel
Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Division of Biophysics, D-23845 Borstel, Germany

The concept of lipid rafts (cholesterol-rich domains) is generally accepted for eukaryotic cells, however, not much is known about the formation of domains in asymmetric membranes lacking cholesterol and the mutual interaction of the two monolayers. We studied the outer membrane (OM) of Gram-negative bacteria which is extremely asymmetric with respect to its lipid composition: The lipid matrix of the inner leaflet is composed of a phospholipid mixture (PL), whereas the outer leaflet is composed solely of lipopolysaccharides (LPS). In lipid monolayers of natural LPS prepared on an aqueous subphase the formation of domains could be observed, thus, a formation of domains in the OM seems likely. We focused on the determination of domains formed in asymmetric planar bilayers prepared by the Montal-Mueller technique using fluorescent microscopy. Furthermore, we imaged asymmetric solid supported bilayers prepared by the Langmuir-Blogdett technique using combined fluorescent and atomic force microscopy. We found that domains, which are clearly visible in LPS monolayers, were not found in asymmetric bilayers prepared on the side of LPS and on the other of PL, indicating a strong interaction between the two monolayers.

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Effect of propylene glycol on bilayers formed by equimolar mixtures of phospholipid and cholesterol

R. D. Harvey, N. Azad, P. J. Quinn, M. J. Lawrence
Molecular Biophysics Group, PSRD, King´s College London, 150 Stamford Street, London SE1 9NH, UK

The effects increasing concentrations of propylene glycol (PG), upon aggregates formed from equimolar mixtures of distearoylphosphatidylcholine (DSPC) and cholesterol (Chol), were investigated using freeze-fracture electron microscopy (FFEM), SANS and SAXS. FFEM replicates of sonicated 1 mg/ml dispersions of DSPC:Chol mixtures in 20-100% w/w PG, revealed the presence of multilamellar vesicles of ∼150 nm diameter in up to 80% PG, with little lamellar phase present in 100% PG. SANS measurements were performed on 1 mg/ml hydrogenous DSPC:Chol mixtures sonicated in deuterated PG (0 – 100% w/w with D_2O) at 25^{o}C. Samples in 0-60% PG were readily modelled as mixtures of single bilayers and multibilayer stacks, showing increasing *d*-spacing and a slight effect on layer thickness, with increasing PG concentration. At 80% PG, a marked decrease in layer thickness was modelled. Modelling of SANS data for samples dispersed in 100% PG, as lamellae, was unsuccessful. For SAXS studies at 25^{o}C, DSPC:Chol mixtures were prepared in 0-100% PG at a total lipid concentration of 30% w/w. SAXS data analysis for DSPC:Chol mixtures in up to 80% PG showed increasing *d*-spacing with increasing PG concentration; correlating well with the SANS data. Above 80% PG, a phase transition occurs, resulting in a Pn3m type cubic phase with a lattice constant of 12.75 nm.

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Effect of lipid headgroup size on pore formation induced by antimicrobial peptides

W. C. Hung[1], M. T. Lee[2], F. Y. Chen[2], H. W. Huang[3]
[1]Department of Physics, Chinese Military Academy, Taiwan, [2]Department of Physics, National Central University, Jhongli, Taiwan, [3]Department of Physics & Astronomy, Rice University, Houston, Texas

Antimicrobial peptides are known to create pores in cell membranes. We used alamethicin and melittin, the best-studied peptides that create so-called barrel-stave and toroidal pores respectively, to study the effect of lipid headgroup size on pore formation. The membranes of different averaged headgroup sizes were represented by DOPC, DOPC:PE(3:1), DOPC:PE(2:1) and DPhPC, DPhPC:PE(9:1), DPhPC:PE(6:1) that are different in PE lipid component ratio. The method of oriented circular dichroism (OCD) was used to monitor the peptide orientation in bilayers as a function of the peptide-to-lipid molar ratio (P/L). The same samples were scanned by x-ray diffraction to measure the change of bilayer thickness with (P/L). Our result shows that the pore is easier to create by antimicrobial peptides in the membranes of larger lipid headgroup size. We further discuss our result by the theory we proposed previously (Huang, et al. 2004).
* Huey W. Huang, Fang-Yu Chen and Ming-Tao Lee (2004) Molecular mechanism of peptide-induced pores in membranes, *Phys. Rev. Lett.* 92, 198304.

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– Membrane Microdomains –

P-465

Fluorescence study into lysozyme effect on the structure of model membranes

V. Ioffe, Y. A. Domanov, G. P. Gorbenko
Dept of Biological and Medical Physics, V.N.Karazin Kharkiv National University

Steady-state measurements of pyrene and anthrylvinyl-phosphatidylcholine (AV-PC) fluorescence in the model bilayer membranes composed of phosphatidylcholine (PC) and its mixtures with cardiolipin (CL), cholesterol (Chol) and cetyltrimetylammonium bromide have been performed to gain insight into the effect of lysozyme on molecular organization of lipid bilayer. Analysis of the vibronic structure of the probe emission spectra showed that transverse distribution of pyrene monomers does not change on the lysozyme binding to liposomes. Excimer-to-monomer fluorescence intensity ratio was found to reduce on the lysozyme association with PC/CL liposomes. The magnitude of this effect increased with increasing CL content. The results obtained have been interpreted as indicating decrease in the membrane free volume on the formation of both electrostatic and hydrophobic protein-lipid contacts. The sign and magnitude of this effect was altered on incorporation of Chol into PC/CL bilayer. Modification of the bilayer was also displayed in the changes of AV-PC fluorescence. Lysozyme association with PC/CL membranes resulted in gradual decrease of fluorescence with protein concentration. Ionic strength dependence of this effect suggested substantial contribution of electrostatic interactions to AV-PC-detected bilayer modifications.

P-466

The Hofmeister effect of anions on the insertion of hypericin in lipid bilayers

D. Ionescu, C. Ganea
Department of Biophysics, Carol Davila Medical University, Eroii Sanitari Blvd. 8, 050474, Bucharest, Romania

The ions belonging to Hofmeister series are frequently encountered in various food products and drugs. It is already known that they can have stabilizing or destabilizing effect on cell membrane structure. Therefore, the study of their effects in membrane structures could help to understand the mechanisms underlying the function of these structures and to develop some applicative domains such as pharmacology. Our study concerns the effects of some of the anions of the Hofmeister series, especially the nitrate and acetate ions on the electrical characteristics of lipid bilayers and of lipid bilayers which contain the naphtodiantrone hypericin which is clinically used due to its antidepressant properties. It has been shown that hypericin can insert itself in the artificial bilayers depending on its concentration and on the bilayer composition, influencing the electric profile of the bilayer. It was suggested that the efficacity of hypericin as an antidepressant is related to its capacity to incorporate and orient itself in the lipid plasma membrane. Our target is to study the insertion of hypericin in artificial lipid bilayers and the way it is affected by some Hofmeister anions.

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Dynamic organization of putative raft-targeted membrane proteins in biomimetic membranes

N. Kahya[1], L. Kalvodova[2], D. A. Brown[3], K. Simons[2], P. Schwille[1]
[1]Biotechnological Centre, Dresden University of Technology, [2]Max-Planck-Institute for Molecular Cell Biology and Genetics, Dresden, Germany, [3]Dept. of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York, USA

The mechanisms and structural determinants responsible for targeting membrane proteins to rafts are not fully understood yet. In particular, the surface roughness of bulky transmembrane domains could drive most of the transmembrane proteins out of cholesterol-rich domains. We propose a biophysical tool for correlating the differential packing of diverse membrane proteins to their structural properties. The dynamic self-organization of proteins functionally reconstituted into Giant Unilamellar Vesicles (GUVs) was investigated by confocal imaging and Fluorescence Correlation Spectroscopy. The partitioning into liquid-ordered phases of α-helical peptides, single- and multi-span proteins was compared to that of GPI-anchored and membrane-bound proteins in GUVs composed of natural and synthetic lipid mixtures. Finally, we explored the effect of protein and lipid cross-linking and/or other stimuli on raft-targeting behavior to identify possible structural changes that could inhibit or enhance the association with raft-like domains. This will give us elements of prediction for some proteins, e.g. receptors, to take advantage of rafts during events such as signaling.

P-468

A quantitative model describing the selective solubilisation of membrane domains

S. Keller[1], A. Tsamaloukas[2], H. Heerklotz[2]
[1]Research Institute of Molecular Pharmacology FMP, Berlin, [2]Biocentre of the University of Basel, Basel

The classical three-stage model of membrane solubilisation (mixed membranes, membrane-micelle coexistence, micelles) is not applicable to demixed, domain-forming membranes and must therefore fail to describe the phenomenon of detergent-resistant membranes (DRMs). In lack of a quantitative model, it has often been believed that ordered, detergent-depleted domains are inert, whereas fluid domains are solubilised. We establish a quantitative model based on equilibrium thermodynamics, analogous to the three-stage model but including three components (two lipids and the detergent) in four phases (two membrane phases such as more ordered and fluid, mixed micelles, detergent also in aqueous solution). For a given set of total concentrations and input parameters (initial abundance of ordered domains, solubilisation boundaries of the pure lipids, etc.), it serves to calculate the phase boundaries and partial concentrations of all components in all phases. The results imply that the abundance and composition of ordered domains may vary substantially upon addition of detergent, both before and during solubilisation of the fluid phase. It seems that all gel-phase or order-preferring lipids are thermodynamically resistant regardless of the presence of a second, fluid phase. However, thermodynamic or kinetic resistance is not sufficient for obtaining DRMs since the resistant particles may be too small to be isolated.

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Lipid preferences of SP-B and SP-C in saturated and unsaturated lipid surfactant model layers

U. Klenz, D. Breitenstein, H. J. Galla
Institute of Biochemistry, Universtity of Münster, Wilhelm Klemm Str. 2, 48149 Münster, Germany

Breathing in lung is enabled by lung surfactant. It prevents alveoli collapse by reducing surface tension.
In this complex lipid protein mixture key components have been identified like dipalmitoylphosphatidylcholine (DPPC), unsaturated phospholipids, phosphatidylglycerols and the hydrophobic surfactant proteins SP-B and SP-C.
Although interaction of SP-B and SP-C with lipids are already well known, details still have to be elucidated. Thus we explored the miscibility of DPPC with negative charged Dipalmitoylphosphatidylglycerol (DPPG), unsaturated Palmitoyloleoylphosphatidylcholine (POPC), Palmitoyloleoylphasphatidylglycerol (POPG) and two hydrophobic surfactant proteins SP-B and SP-C and their kinetics in mixtures by film balance and fluorescence microscopy. The lateral distribution of SP-B, DPPC and DPPG was probed by TOF-SIMS.
Against the widespread opinion that SP-B only interacts with negatively charged DPPG, we detected no preference of SP-B and SP-C for DPPC or DPPG on Ca^{2+} containing subphase at 20 °C. On water SP-B likely prefers to interact with DPPC. The le/lc phase transition of saturated and unsaturated lipid monolayers is not affected by the two proteins. The unsaturated lipids fluidize the monolayer and unmix with saturated DPPC. SP-B and SP-C probably act like a mediator between the solid DPPC and the liquid POPC or POPG phase.

P-470

Changes of platelet membrane organization under microwave irradiation

E. Kovacs[1], T. Savopol[1], D. Martin[2], N. Iacob[2], E. Carstea[2]
[1]Biophysics and Cell Biotechnology Dept., « Carol Davila » Medical University, P.O. Box 35-43, 050461 Bucharest, Romania, [2]National Institute for Lasers, Plasma and Radiation Physics

Changes in membrane order are known to alter the function of integral proteins such as ATP-ase as well as a series of membrane receptors. Consequently, microwave induced membrane order could have physiological implications. The detailed biophysical interaction mechanisms for these effects are not currently available; experiments on simple in vitro models are expected to offer the keys to understanding the nature of non-thermal and windowed effects.
We studied the changes in membrane fluidity of human platelets extracted from freshly donated blood, induced by irradiation with pulse modulated 2.45 GHz microwaves, SAR from 136 W/kg to 684W/kg. The experimental arrangement consists mainly in a 2.45 GHz generator of adjustable output power in the range of 0 – 50 W and a modified conventional coaxial probe used for two functions: as impedance matching device and as MW irradiating antenna. The antenna is placed into a spectrofluorometer cuvette containing the biological sample. Contrary to sham exposed samples, the irradiated ones regularly showed a sudden drop of the membrane fluidity at approximately 2 min after irradiation start. Since there was no such sharp modification of temperature during irradiation (30 min), the observed fluidity discontinuity was attributed to a non-thermal effect. Parallel studies of membrane phase-transition temperature and other physical and chemical parameters are performed in order to explain the observed effects.

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Monte Carlo study on the energetic state of lipid membrane in electric field

K. Kubica[1], M. Kotulska[2]
[1]Agricultural University, Department of Physics and Biophysics, 50375 Wroclaw, Poland, [2]Wroclaw University of Technology, Division of Biomedical and Measurement Engineering, 50370 Wroclaw, Poland

Electrical field of high intensity applied to lipid membranes creates electropores. Induced electroporation is considered as a safe method for controlled application of drugs and nanomolecules into cells. However, the question why creating electropores is energetically favourable and what phenomena occur in the membrane on molecular level has not been answered. Sensitivity of biological membranes to electric field is studied by means of Monte-Carlo method based on modified Pink model. Total energy of the system is calculated as a sum of electrostatic interactions between polar heads, conformational energy of the chains and energy of van der Waals interactions between chains. Additionally, some of the lattice nodes are left void which accounts for spots, which are not occupied by lipids due to the membrane irregularity. The energetic state of the membrane with concentrated and dispersed vacancies are compared with fully occupied membrane model. We investigate how electrical field change the energetic preference between these situations and study interactions between molecules as a function of the field.

P-472

Transverse phospholipid imbalance in plasma membrane recruits lipid rafts and induces cellular extensions

R. Larive, L. Baisamy, N. Bettache
CNRS-UMR 5539, Université Montpellier 2, cc107, place Eugène Bataillon, 34095 Montpellier cedex 5, France

We have previously shown that a physical membrane constraint, generated by addition of a phospholipid excess in the outer leaflet of the plasma membrane of platelets and L929 fibroblasts, induces actin polymerisation and cellular extensions via phosphoinositide 3-kinase activation[1]. In this work, we investigate the implication of lipid rafts in this process. We demonstrate that phospholipid excess triggers protein enrichment in lipid rafts. Consequently to their recruitment to plasma membrane, after lipid analogue incorporation, several protein tyrosine kinases are activated and phosphorylate a number of signalling proteins. To reinforce such results, we have depleted cholesterol in lipid rafts by methyl-β-cyclodextrin treatment. This depletion prevents actin polymerisation and consequently cellular extension. In addition, we observe that lipid raft disruption inhibits PtdIns-3-kinase activation in resting platelets submitted to a phospholipid excess.
Our results suggest that phospholipid addition modulates the lateral organization of plasma membrane, demonstrating that lipid raft organisation is crucial for cell shape changes in response to a constraint applied to the plasma membrane.
[1]Bettache et al. (2003) J. Cell Sci., 116, 2277-2284.

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P-473

Transverse phospholipid imbalance in plasma membrane recruits lipid rafts and induces cellular extensions

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P-474

Effect of lipid headgroup size on pore formation induced by antimicrobial peptides

M.-T. Lee[1], W.-C. Hung[2], F.-Y. Chen[1], H. W. Huang[3]
[1]Department of Physics, National Central University, Jhongli, Taiwan, [2]Department of Physics, Chinese Military Academy, Taiwan, [3]Department of Physics & Astronomy, Rice University, Houston, Texas

Antimicrobial peptides are known to create pores in cell membranes. We used alamethicin and melittin, the best-studied peptides that create so-called barrel-stave and toroidal pores respectively, to study the effect of lipid headgroup size on pore formation. The membranes of different averaged headgroup sizes were represented by DOPC, DOPC:PE(3:1), DOPC:PE(2:1) and DPhPC, DPhPC:PE(9:1), DPhPC:PE(6:1) that are different in PE lipid component ratio. The method of oriented circular dichroism (OCD) was used to monitor the peptide orientation in bilayers as a function of the peptide-to-lipid molar ratio (P/L). The same samples were scanned by x-ray diffraction to measure the change of bilayer thickness with (P/L). Our result shows that the pore is easier to create by antimicrobial peptides in the membranes of larger lipid headgroup size. We further discuss our result by the theory we proposed previously (Huang, et al. 2004).
* Huey W. Huang, Fang-Yu Chen and Ming-Tao Lee (2004) Molecular mechanism of peptide-induced pores in membranes, *Phys. Rev. Lett.* 92, 198304.

P-475

The submicroscopic organisation of live cell membranes viewed as a source of molecular confinement

P.-F. Lenne[1], L. Wawrezinieck[2], H. Rigneault[1], D. Marguet[2]
[1]Institut Fresnel- CNRS and University Paul Cézanne Aix Marseille III - Domaine Universitaire de Saint Jerôme - 13397 Marseille cedex 20 - France, [2]Centre Immunologie Marseille Luminy - CNRS, INSERM and Univ. Aix-Marseille II - Parc scientifique de Luminy - 13009 Marseille cedex 9 - France

Our present description of the plasma membrane includes heterogeneities or mechanisms that may hinder or even confine the diffusion of membrane molecules. Indeed, the diffusion of proteins may be impeded by the presence of "corrals" formed by the actin-based membrane skeleton, whereas lipid microdomains are thought to transiently sequester molecules which are involved in signaling pathways and cell sorting.
We show that fluorescence correlation spectroscopy observations at various spatial scales enable to quantify an average confinement time within a microdomain and a related effective diffusion coefficient. Using this strategy on live cells, we report that microdomain-associated molecules exhibit different temporal confinements, which range from a few to tens of milliseconds. Variation of the cholesterol content results in drastic changes of confinement times. Hindrance by the cytoskeleton leads to diffusion laws having a different shape. Our method enables to distinguish different mechanisms responsible for the confinement of molecules diffusing in the plasma membrane.

P-476

The structure and function of molecular films of lung surfactant: the role of cholesterol and concentration

Z. Leonenko[1], L. Gunasekara[1], M. Schoel[1], K. Nag[2], S. Schürch[1], M. Amrein[1]
[1]Faculty of Medicine, University of Calgary, Canada, [2]Department of Physics, Memorial University of Newfoundland, Canada

Neonates often lack mature pulmonary surfactant (PS) and need treatment with exogenous surfactant. PS is a defined lipid-protein mixture that covers the air-lung interface. It reduces the surface tension to a very low value, which is required for the structural and functional integrity of the lung. Cholesterol is removed from surfactant replacement formulations obtained from animal lung extracts or not added to artificial systems, because it has earlier been found to deteriorate surface activity by *in vitro*-biophysical studies. We show that cholesterol has no such detrimental effects but is beneficial for surfactant function as long as a physiological molar ratio is observed. Surface tension – area isoterms show that molecular films produced by high concentration of BLES lower surface tension more effectively than that of low concentration. Surfactant function is observed in a *captive bubble surfactometer* and related to the molecular architecture of surfactant obtained by *atomic force- and fluorescence light microscopy*.

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Enzymatic modification of lateral domain sizes can induce lipid scrambling in unilamellar vesicle

I. Lopez-Montero[1], M. Velez[2], P. F. Devaux[1]

[1]Institut de Biologie Physico Chimique UMR 7099 Paris France, [2]Universidad Autonoma de Madrid Spain

In eukaryotic cells the two leaflets of the plasma membrane have a different lipid composition. Lipid scrambling triggered by cytosolic calcium, which takes place particularly during apoptosis, has been attributed to a putative calcium dependent lipid "scramblase". We propose that an endogenous calcium dependent sphingomyelinase (SMase) could be a scramblase. Experiments with giant vesicles containing various amounts of SM show that the formation of ceramide by SMase modifies the vesicles integrity. Low proportions of SM (2%) lead to a single stable membrane invagination, which we attribute to the excess surface on the inner monolayer. The addition of a small percentage of ceramide (1%) to the external surface on the contrary triggers a bud, with a short life time (5min) indicating the rapid diffusion of ceramides between the two monolayers. When SM is above 5% of the lipid composition, SMase triggers pore formation or vesicle collapse. However if high percentage of SM and cholesterol amounts are present, membrane domains can be seen and a line tension between both phases prevents membrane collapse. Vesicles are prevented also from membrane rupture if lyso-PC (10%) is added. However, in the latter case volume decrease suggests the presence of nanopores. Pore formation permits transmembrane lipid scrambling. Thus, if a cell membrane has a small percentage of SM in the inner leaflet, a calcium dependent sphingomyelinase may behave like a lipid scramblase

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Enzymatic modification of sphingomyelin domains can induce lipid scrambling in giant vesicles

I. Lopez-Montero[1], M. Vélez[2], P. F. Devaux[1]

[1]Institut de Biologie Physico-Chimique, UMR CNRS-Université Paris7-7099, [2]Instituto de Ciencia de los Materiales -Universitad Autonoma de Madrid

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P-479

New bicelle membranes: synthesis and characterization by solid state NMR

C. Loudet[1], S. Gineste[2], M.-F. Achard[2], E. J. Dufourc[1]

[1]UMR 5144 CNRS-UBx1, IECB, Pessac, France, [2]UPR 8641 CNRS, CRPP, Pessac, France

A new type of bicelles, that orient such as their membrane plane is perpendicular to the magnetic field Bo, is being developed. This study is complementary to that of DMPC/DCPC bicelles that orient with their plane parallel to B_0 (Raffard et al, *Langmuir*, 2000).
These new systems are composed of a mixture of long-chain lipids (1-Tetradecanoyl-2-(4-(4-Biphenyl)Butanoyl)-sn-glycero-3-PhosphatidylCholine, TBBPC), and short chain lipids (DCPC). The TBBPC has been synthesized by an esterification reaction between a lyso PC and the 4-(4-biphenyl)butanoïc acid; its specificity resides in its positive anisotropic susceptibility due to the presence of two phenyl rings. TBBPC/DCPC bicelles hence orient with their plane perpendicular to B_0.
Partial temperature-composition-hydration diagrams of these new bicelles have been established using ^{31}P and ^{14}N solid state NMR and compared to the DMPC/DCPC system. TBBPC/DCPC form bicelles on a narrow compositional range but over a large temperature span, whereas the DMPC/DCPC bicelles exhibit the reverse situation.
The dynamics of TBBPC/DCPC bicelles has been probed by ^{2}H solid state NMR, using deuterated TBBPC-d27 that has been newly synthesized.
Finally, these new bicelles are shown to be very promising to study the orientation of hydrophobic helices in membranes using wide line ^{15}N-NMR.

P-480

Spectroscopic characterization of a new membrane probe: the hexadecyl-amine-o-aminobenzoate

C. A. Marquezin[1], I. Y. Hirata[2], L. Juliano[1], A. S. Ito[1]

[1]Faculdade de Filosofia, ciencias e letras de ribeirao preto, universidade de sao paulo, [2]escola paulista de medicina, universidade federal de sao paulo

We report the results of investigation performed on the spectroscopic properties of a new fluorescent probe. The fluorophore *o*-aminobenzoic acid was covalently bound to the hydrocarbon chain hexadecylamine, producing the lipophilic probe hexadecyl-amine-*o*-aminobenzoate. The behavior of the probe was dependent on the polarity of the medium: fluorescence spectral position, quantum yield and lifetime decay indicate distinct behavior in water compared to ethanol and ciclohexane. Steady state fluorescence anisotropy was very low in the organic solvents, where the probe presented high rotational diffusion, as demonstrated by the short correlation times obtained from fluorescence anisotropy decay measurements. The whole fluorescence properties of the probe indicate that it has low solubility in water, leading to the formation of aggregates in aqueous medium, and its dissolution in organic solvents. In the presence of sodium dodecyl sulphate micelles, the probe aggregates disappear, due to dissolution in the non polar phase of the amphiphilic aggregates. Experimental results demonstrated also the incorporation of the probe in the lipid phase of DMPG vesicles, where it acts as a monitor of surface potential and phase transitions.

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Effect of positively-charged short peptides on stability of cubic phases of DOPA/MO membranes

S. M. Masum, S. J. Li, T. S. Awad, M. Yamazaki
Dept. Physics, Fac. Science, Shizuoka University, Shizuoka, 422-8529, Japan

To elucidate the stability of IPMS cubic phases of biomembranes is indispensable from biological and physicochemical aspects. In this report, we investigated the effect of positively-charged peptide-3K (LLKKK) and poly (L-lysine) on the phase stability of monoolein (MO) membranes containing negatively charged dioleoylphosphatidic acid (DOPA) (i.e., DOPA/MO membranes) using SAXS. At first, the effect of peptide-3K on 10%DOPA/90%MO membrane in excess water, which is in the Q^{229} phase, was investigated. At 3.4 mM peptide-3K, a Q^{229} to Q^{230} phase transition occurred, and at > 3.4 mM peptide-3K, the membrane was in the Q^{230} phase. Poly (L-lysine) (Mw:1K$\sim$4K) also induced the Q^{230} phase in the same membrane. We also investigated the effect of peptide-3K on 25%DOPA/75%MO-MLV, which is in L_α phase. In the absence of peptide, the spacing of MLV was very large (11.3 nm), but at $\geq$ 8 mM peptide-3K, it greatly decreased to a constant value (5.2 nm), irrespective of the peptide concentration, indicating that peptide-3K and the membranes form an electrostatically-stabilized aggregation with low water content. On the basis of these results, we discuss the mechanism of the effects of the positively-charged short peptides (peptide-3K) and poly (L-lysine) on the structure and phase stability of DOPA/MO membranes.

[1] Masum et al., Langmuir, in press, 2005

P-482

Variable radii FRAP and anomalous diffusion: Monte-Carlo simulations and experimental data on cells

J.-L. Meunier[1], N. Olivi-Tran[1], C. Favard[2]
[1]Institut Non Lineaire de Nice, UMR 6618 CNRS , 1361 route des Lucioles 06560 Valbonne - France, [2]Institut de Pharmacologie et Biologie Structurale, UMR 5089 CNRS, 205, route de Narbonne, 31077 Toulouse Cedex 4 France

FRAP technique have been used for decades to measure movements of molecules in 2D. Data obtained by FRAP experiments are assumed to be described through means of two parameters, a diffusion coefficient (as defined in a pure Brownian model) and a mobile fraction. Recently, FRAP at variable radii has been developped using the same diffusion model, allowing therefore access to geometrical characteristics of the surrounding landscape of the molecule. Nevertheless, it has already been shown that recoveries data can also been described using anomalous subdiffusion (through means of α and Γ as parameters), this without significant difference in the quality of the fit. In order to discriminate between these two models, numerical simulation of FRAP recoveries at variable radii were performed using anomalous diffusion in a landscape without barriers. If theory predicts that α is spatially invariant, our data suggest a linear regression with a negative slope of α with 1/R (R being the photobleaching radius). Finally these simulations were compared to experimental data obtained at variable radii on living cells using the PH domain of a peripheral protein (EFA6, exchange factor for ARF6). A positive slope linear regression of α with 1/R was observed suggesting a non anomalous behaviour of the diffusion in our experimental time-scale and length-scale.

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Lipid-phase specificity of rotational diffusion and molecular orientation in biomembranes

D. J. Mudaliar, R. P. Walvick, A. A. Heikal
Department of Bioengneering, Pennsylvania State University, University Park, PA 16802

Cellular plasma membranes are heterogeneous, dynamic and complex systems that regulate numerous biological processes such as signal transduction and protein trafficking. Lipid phase (i.e., order) affects the fluidity and mechanical properties of the biomembranes as well as the transport characteristics of membrane proteins and biomolecules. Recent studies suggest the existence of specialized lipid microdomains (or rafts) that serve as portals of entry for various pathogens and toxins. Very limited effort has been devoted to imaging rotational diffusion of phase-specific markers in biomembranes as a probe of lipid domains. Here, we present a novel and quantitative approach for monitoring the spatio-temporal fluctuation of lipid phases in giant unilamellar vesicles (GUVs) as a model system that mimics biomembranes. Our preliminary results provide a molecular perspective of the dynamics of lipid phases and will ultimately help our understanding of their structure-function relationship in living cells.

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Microscopic imaging of membrane fluidity by laurdan at video rate and aplication to observing rafts

K. Muto, T. Ohba, T. Kiuchi, T. Tanaka, K. Ohki
Department of Physics, Tohoku University, Sendai, Japan

Introduction: The biomembranes maintain heterogeneity as microdomain in its dynamic structure. Existence of domains rich in sphingolipid and cholesterol is reported as phase-separated 'rafts'. The function of membrane protein is affected by physical property of the lipid bilayer membrane. Laurdan is able to monitor membrane fluidity, and we have developed an instrument to image spatial and temporal change in membrane fluidity by use of laurdan.
Methods: Fluorescence microscope was equipped with a home-build dual-view optical unit. Microscopic image of membranes stained with laurdan is separated into 440 nm-image and 490 nm-image by the combination of monochromatic filters and dichroic mirrors. Each image is focused on CCD camera side by side. And generalized polarization (G.P.) image was calculated according to "G.P.=(I440nm-I490nm)/I440nm+I490nm)". CHO cells were treated with sphingomyelinase (SMase) or methyl-beta-cyclodexstrin (MbCD) which is capable of removing cholesterols.
Results and discussion: The G.P. imaging at video rate was applied to a giant liposome of DMPC (Tm = 23°C) and DMPE (Tm = 49°C), and it showed temperature-induced phase separation at video rate. When CHO cells were treated with SMase, change of the membrane fluidity was hardly observed. However, when CHO cells were treated with 1 mM MbCD (no change occurred at this concentration), SMase treatment of these cells raised the membrane fluidity. The results indicate that specific interaction between sphingomyelin and cholesterol.

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Composition dependence of aggregation form and phase segregation in a bacterial model membran system

G. Pabst[1], B. Pozo Navas[1], G. Deutsch[1], E. Sevcsik[1], K. A. Riske[2], R. Dimova[2], P. Garidel[3], K. Lohner[1]
[1]Institute of Biophysics and X-ray Structure Research, Austrian Academy of Sciences, Schmiedlstraße 6, A-8042 Graz, Austria, [2]Max Planck Institute of Colloids and Interfaces, Theory Department, Am Mühlenberg 1, D-14476 Golm, Germany, [3]Martin-Luther-University Halle/Wittenberg, Institute of Physical Chemistry, Mühlpforte 1, D-06108 Halle/Saale, Germany

We have determined the mixing properties and the aggregation behavior of phospholipid mixtures composed of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and -phosphatidylglycerol (POPG) applying differential scanning calorimetry , X-ray diffraction and optical phase contrast microscopy. A phase diagram was constructed using experimental data in combination with a simulation of the solidus and liquidus line based on regular solution theory. The lipids show a thermodynamic non-ideal miscibility, both in the L_α and L_β phase, respectively. Further, the system exhibits a miscibility gap due to a phase segregation at high POPG concentrations in accordance with theoretical predictions. Pure POPE aggregates into multilamellar and pure POPG into unilamellar vesicles, respectively. PE/PG mixtures, however, form oligolamellar vesicles that consist of about 5 and less bilayers. The number of bilayers decreases with PG content. The layers within the oligolamellar liposomes are positionally correlated within the gel phase, but undergo an unbinding transition and become uncorrelated in the fluid phase.

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Can lipid domains control membrane permeabilization and DNA uptake in cells submitted to electric field?

E. Phez[1], L. Cezanne[1], A. Charpentier[1], B. Lagane[1], P. Pagniez[1], I. Tsoneva[2], J. Teissié[1], M.-P. Rols[1]
[1]IPBS-CNRS (UMR 5089), 205 route de narbonne toulouse France, [2]Institute of Biophysics, Bulgarian Academy of Sciences

We visualized the early events of membrane permeabilization and DNA transfer by electric field. A fast exchange of small molecules takes place across the membrane. In the case of DNA transfer, a complex process is present involving a key step of association of DNA with the membrane. This results in the formation of 0.2 μm membrane domains where DNA is trapped. This data is consistent with a multi step model of DNA transfer across the membrane and suggest the existence of competent membrane area for DNA transfer (1). The purpose of this work is to better understand the molecular state of the cell membrane where plasmid interacts and therefore to characterize the domains of the membrane/DNA interaction. Our hypothesis is that lipid rafts may control DNA electrotransfer. For that purpose, CHO cells have been depleted in cholesterol by using cyclodextrins. Incubations of cells with 5mM cyclodextrins, 15 min at 37°C, resulted in decreasing their cholesterol by 40%. Under these conditions, cell viability is preserved. Membrane permeabilization to electric pulses does not significantly change. DNA membrane interaction is not affected. Gene expression dramatically decreases tenfold. These results show a correlation between plasma membrane cholesterol content and DNA expression. They suggest the involvement of cholesterol depletion on nucleic acid traffic and the resulting protein expression.
1- Golzio, Teissié and Rols. *PNAS* 99: 1292-7 (2002)

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The magnetic resonance study of lipid-protein interactions in human plasma LDL

G. Pifat-Mrzljak, M. Kveder, Ž. Marinić, A. Kriško, D. Vikić-Topić
Ruđer Bošković Institute, Bijenička 54, Zagreb, Croatia

Various spectroscopic approaches are being used to study the extremely complex structural organization of the LDL particle with the single idea to provide the information at the atomic resolution about this protein-lipid assembly. In this context we aim to investigate the noncovalent interaction of the protein part (apo B with the molecular weight of cca. 500 kDa) with the lipid matrix (more than 3000 molecules). The approach is based on the combination of ^{1}H NMR (600 MHz) spectroscopy with the X-band CW-EPR and thiol specific spin labeling. In the ^{1}H spectra of LDL we concentrate on the composite spectral peak centered around 3.25 ppm which has been assigned to the methyl headgroups of phosphatidylcholine and sphingomyelin of LDL [Biochemistry 39(32) (2000) 9763]. We explore the surface solvation of these phospholipids via relaxation enhancement of a polar paramagnetic compound. In addition, we analyze the influence of methanethiosulphonate spin label covalently attached to the free thiol groups of apoB on the phospholipid spectral peak. This approach offers an insight to the regions of the protein sequence which are in contact with the lipid matrix of LDL.

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Examining the lipid raft hypothesis in living cells using targeted quantum dots and single molecule imaging

F. Pinaud[1], X. Michalet[1], E. Margeat[1], H.-P. Moore[2], S. Weiss[1]
[1]University of California Los Angeles, Biochemistry & Chemistry Dpt., [2]University of California Berkeley, Molecular & Cell Biology Dpt.

GPI-anchored proteins often associate with sphingolipid-sterol rich microdomains of the plasma membrane called rafts. These domains play important roles in signal transduction and protein sorting in cells. Understanding the formation, maintenance and disruption of rafts domains and their interaction with GPI-anchored proteins is of key importance to the fields of membrane biophysics and cell biology. The dynamic interactions of GPI-receptors with rafts can be addressed by single molecule fluorescence microscopy, unraveling events usually hidden in ensemble measurements. We use biocompatible peptide-coated luminescent quantum dots to study lipid rafts and their associated proteins. Quantum dots are specifically targeted and used for the imaging of raft associated GPI-anchored CD14-avidin chimeric receptors expressed in HeLa cells. The high photostability of the quantum dots allows tracking of single receptors over minutes with excellent time and spatial resolutions. Dual color imaging by total internal reflection microscopy reveals the dynamic interactions of the receptors with cholera toxin labeled sphingolipid-sterol microdomains. Various diffusion patterns and diffusion coefficients of the receptors are observed and correlated with the distribution of the microdomains in the cell membrane. Cholesterol depletion with mBCD and lovastatin treatments and actin depolymerization with latrunculin significantly affect the diffusive behavior of the receptors and their localization.

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Interaction of Gg-protein farnesylated peptide and DMPS model membranes

J. Prades[1], S. S. Funari[2], P. V. Escriba[1], F. Barcelo[1]
[1]Molecular and Cellular Biomedicine, Fundamental Biology Department, University of Balearic Islands, [2]HASYLAB, DESY, Hamburg

The membrane association of Gβγ dimmers seems to be assisted by the farnesyl group on the Gγ subunit and the electrostatic interactions between PS lipids and the protein. Here, we addressed this issue analyzing the interactions of the C-terminal G_γ-protein farnesylated peptide (PLLTPVPASENPFREKKFFC[Farnesyl]AIL; $P_{\gamma-FN}$) with DMPS model membranes, using X-ray diffraction, DSC, FTIR and fluorescence techniques. The same peptide without farnesylation (P_γ) and the isoprenoid farnesol were also studied. We demonstrate that $P_{\gamma-FN}$-DMPS membrane association is characterized by the appearance of domains with increased structural organization of the liquid lamellar phase. $P_{\gamma-FN}$-DMPS interactions are mediated by: (i) the formation of a peptide-lipid headgroup complex in which the electronic environments of the phosphate and the carbonyl groups are altered and (ii) the insertion of the farnesyl moiety into the bilayer. These model systems suggest that the farnesyl group would assist the association of $P_{\gamma-FN}$ to anionic membranes and induce the clustering of the peptide. These data explain in part the G protein clustering in the vicinity of G protein-coupled receptors.

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Critical point and virtual microdomains in lecithin membranes. Modeling and experiment

M. S. Prosandeeva[1], D. P. Kharakoz[1], E. I. Tiktopulo[2]
[1]Institute of theoretical and experimental biophysics RAS, [2]Institute of Protein Research RAS

We have analyzed the chain-melting transition in lipid membranes by comparing experimental (calorimetric, volumetric and acoustic) data with the Monte Carlo simulation of an Ising-like model with temperature-dependent Hamiltonian. The nearest-neighbor interaction was set by interfacial contact extra energy E whose value determines both the cooperative character of the transition and the value of critical temperature, $T_c = aE/k_B$, dividing the ranges of the continuous ($T>T_c$) and discontinuous ($T<T_c$) transitions (a=1.79 for the triangular lattice). Comparison of the results of modeling with the experimental data for saturated lecithins with chain length n<20 reveals that the chain-melting transition occurs at the temperature (T_m) above T_c. This explains why the transition is continuous in these lipids. Both the T_c and T_m temperatures increase with the chain length. However, T_c increases faster than T_m and, hence, there should be a chain length n* above which T_c is greater than T_m and thus the transition becomes discontinuous. Extrapolation shows that n* is about 22. Surprisingly, this estimate is similar to that observed earlier (Pubst et al.; 2004) when anomalous swelling in lipid multilayers disappears. This would mean that in lipids with n>22, the transition is of first order. (Supported by INTAS-01-0105 and RFBR-05-04-49206.)

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Monitoring the organization and dynamics of membrane-bound melittin utilizing NBD fluorescence

H. Raghuraman, A. Chattopadhyay
Centre for Cellular and Molecular biology (CCMB), Uppal Road, Hyderabad 500 007, INDIA

Melittin is a cationic hemolytic peptide isolated from the European honey bee, *Apis mellifera*. The organization of membrane-bound melittin has earlier been shown to be dependent on the physical state and composition of membranes. In this study, we covalently labeled the N-terminal end and Lys-7 of melittin with an environment-sensitive fluorescent group 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) to monitor the influence of negatively charged lipids and cholesterol on the organization and dynamics of membrane-bound melittin. Our results indicate that the NBD group of melittin labeled at its N-terminal end does not exhibit red edge excitation shift (REES) in DOPC and DOPC/DOPG membranes, whereas the NBD group of melittin labeled at Lys-7 exhibits REES of ∼8 nm. This could be attributed to different locations of these analogues in membranes. Interestingly, the membrane environment of these analogues is sensitive to the presence of cholesterol which is supported by time-resolved fluorescence measurements. In order to monitor the localization of NBD-labeled melittin analogues when bound to membranes, we plan to carry out membrane penetration depth measurements using the parallax method. The significance of our results in the overall context of the role of membrane lipids in the organization and function of membrane proteins and peptides will be discussed.

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Interaction of the pro-apoptotic protein tBid with the mitochondrial membrane: role of cardiolipins

C. Rameau[1], P. Dupaigne[1], F. Pariselli[2], P. X. Petit[2], S. Bernard[1]
[1]Laboratoire de Neuro-Physique Cellulaire, Université Paris 5, 45 rue des Saints-Pères 75006 Paris, [2]Institut Cochin INSERM U567, UMR 8104, 24 rue du Faubourg St-Jacques, 75014 Paris, France

Bid, a pro-apoptotic member of the Bcl-2 family, is activated through cleavage by caspase 8 in cytosol. The carboxy-terminal fragment (tBid) translocates to the mitochondria where it induces release of cytochrome c by a mechanism that is not yet understood. A major controversy is whether tBid acts by itself or through modulation of other pro-apoptotic members of the Bcl-2 family such as Bax and Bak. It has been suggested that cardiolipin, which is present in mitochondrial membranes, can mediate the initial targeting of tBid. We have investigated the interactions between tBid (and its hydrophobic helix H6 and H7) and different phospholipids using the monolayer technique. It is demonstrated that the presence of the negatively charged cardiolipins greatly enhances the insertion of tBid and H6 into the phospholipid monolayer. The modification of two charged amino acid residues of the H6 helix abolishes its insertion. Furthermore, the excess free energies of mixing were evaluated from the isotherms. The negative values obtained indicate the existence of attractive interactions between cardiolipin and H6 in the mixed monolayers. These results suggest that cardiolipins at the contact sites between the two mitochondrial membranes could mediate binding of tBid, thereby assuming interaction with other proteins. Grazing incidence X-ray diffraction experiments were also undertaken in order to obtain information on the molecular organization of the cardiolipins in the presence of the alpha-helix.

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Influence of sodium and calcium chloride on the fluidity of phosphatidylcholine membranes

M. Rappolt[1], J. Štrancar[2], A. Hodzic[1], P. Laggner[1], G. Pabst[1]
[1]Institute of Biophysics and X-Ray Structure Research, Austrian Academy of Sciences, Graz, Austria, [2]Laboratory for Biophysics - EPR Center, Jožef Stefan Institute, Ljubljana, Slovenia

Alkali chlorides play an important role in several biological processes. The specific binding of cations with biological membranes influences not only their fluidity and structure, but consequently also processes like endo- and exocytosis or membrane fusion. In this work we report about the effect of sodium and calcium chloride (0 - 330 mM) on the stability, structure and dynamics of palmitoyloleoylphosphatidylcholine (POPC) bilayers. Combining the techniques of small angle X-ray scattering and spin-label electron paramagnetic resonance spectroscopy, both the global and local parameters of the chosen model membrane systems are characterized. Both salts perturb the positional order within the multibilayer systems considerably. In the case of NaCl we find no significant changes to the overall bilayer structure at all concentrations, but simply a screening of the van der Waals interactions between adjacent bilayers. In contrast, $CaCl_2$ leads at low concentrations to a strong bilayer repulsion, which is completely screened at higher concentrations. In the latter concentration regime we also find a strong increase of the lipid acyl chain order and henceforth an increase of membrane thickness. Our results are confronted with recent molecular dynamics simulation studies, which were carried out on the same model system (see [1] and references therein).
[1] Böckmann, R.A. and H. Grubmüller. 2004. Angew. Chem. Int. Ed. 43: 1021-1024.

P-494

Formation of supported lipid membranes - mechanisms and the role of the solid support

R. Richter[1], A. Brisson[2]
[1]Department of Biophysical Chemistry, Institute for Physical Chemistry, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany, [2]Laboratory of Molecular Imaging and Nano-Bio-Technology, IECB, University of Bordeaux I, 2 Rue Robert Escarpit, 33607 Pessac Cedex, France

Supported lipid bilayers (SLBs) are popular models of cell membranes with potential bio-technological applications, yet the mechanism of SLB-formation is only partially understood. In this study, the process of vesicle deposition and SLB-formation was investigated by Quartz Crystal Microbalance with Dissipation Monitoring (QCMD) and Atomic Force Microscopy (AFM) [1]. The mechanism of SLB-formation on mica [2] is shown to differ substantially from SLB-formation on silica [3], which illustrates the importance of the solid support in this self-organization process. Furthermore, the role of the lipid charge and the presence of calcium as determinants of the lipid deposition pathway are highlighted. QCM-D and AFM are demonstrated to be highly complementary, providing global kinetic information and local structural information on the self-organization processes simultaneously.
[1] Richter, R. P.; Brisson, A. *Langmuir* (2004) *20*, 4609-4613.
[2] Richter, R. P.; Brisson, A. *Biophys. J.* in press.
[3] Richter, R. P.; Mukhopadhay, A.; Brisson, A. *Biophys. J.* (2003) *85*, 3035-3047.

P-495

Dynamics of viscoelastic filament-coated vesicles

S. Rochal[1], V. Lorman[2], G. Mennessier[2]
[1]Physical Faculty, Rostov State University, Rostov-on-Don, Russia, [2]Laboratoire de Physique Theorique et Astroparticules, UMR 5207 CNRS - Universite Montpellier II, France

Recent experimental progress resulted in the creation of *in vitro* composite systems like biofilament-coated vesicles which approach the cell membrane dynamics. New microrheology technique has shown striking difference of their behavior with respect to that of bare vesicles. We propose a model for the low-frequency dynamics of spherical composite vesicles (CVs) which takes into account 2D viscoelastic properties of the CVs surrounded by a viscous liquid. Full elasto-hydrodynamic equations of motion describe linearly coupled stretching and bending modes and an independent shear mode. The qualitative difference of these results with i) conventional hydrodynamic theory of fluid vesicles ; and ii) theory of the flat membrane dynamics is discussed. The shear elasticity of the CVs gives an essential contribution to the relaxation rate of the bending mode at small wave numbers. The notion of physical incompressibility of CVs is opposed to the usual surface area constraint. We show that even in the incompressible spherical CV with a finite shear modulus, the bending mode involves both radial and tangent displacements. For this reason the response of the viscoelastic CV is quite different from that of the fluid vesicle or of the flat membrane. To compare the theory with available data we calculate the power spectra of the actin-coated vesicles.

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Transient pores in giant vesicles solubilized in aqueous medium

N. Rodriguez[1], F. Pincet[2], S. Cribier[1]
[1]Laboratoire de Physico-Chimie Moléculaire des Membranes Biologiques, Institut de Biologie Physico-Chimique, 13 rue PM Curie, 75005 Paris, France, [2]Laboratoire de Physique Statistique, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris, France

The understanding of pores formation in lipidic membranes is important for several fields such as controled delivery of drugs encapsulated in liposomes, electropermeation or solubilization of membranes. We present here experiments with giant vesicles that are solubilized in a controled fashion in an aqueous medium. Large transient pores are evidenced with brigth field and fluorescence microscopy. The size of these pores can reach several micrometers and their lifetimes can be a few minutes. The pore formation theory in stretched vesicles has already been developed by the group of Françoise Brochard. This theory led them to the study of vesicles filled with a viscous medium to slow down the kinetic of pore closure. We have adapted their model to prove that the stabilization of pores in aqueous medium that we observed can be explained by the concomitant solubilization of the vesicle. Simulations performed with Mathematica® enable us to determine relevant biophysical parameters such as edges energy.

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Formation and characterization of poly (ethylene glycol) supported bilayers

C. Rossi[1], M. Odorico[2], A. Berquand[3], P. Parot[2], J. Chopineau[4]
[1]UMR 6022 CNRS, UTC, BP 20529, 60205 Compiègne. France, [2]CEA Valrhô DSV/DIEP/SBTN, Centre de Marcoule, 30207 Bagnols sur Cèze. France, [3]Institut Curie, UMR-CNRS 168, LRC-CEA 8, 75231 Paris. France, [4]CBS, Centre Universitaire de Nîmes, 30000 Nîmes. France

A biomimetic membrane was obtained from vesicles containing Egg-PC and DSPE-PEG_{3400}-NHS as anchoring molecules. Liposomes linked to the surface (cysteamine-coated gold or silanized glass) and disrupted leading to the formation of a bilayer.
The formation process depends on too many parameters to be able to predict the conditions leading to the formation of a continuous membrane. An experimental design, according to a four-parameter Doehlert matrix, was applied to the fluorescence experiments. This design allows modeling of the experimental responses by second order polynomial equations. Then mobile fraction and diffusion coefficient were optimum in the following experimental domain: DSPE-PEG abundance from 1% to 5% (w), EggPC concentration from 0.1 to 1 mg/ml, reaction time from 1 to 8 hours and resting time from 10 to 20h.
The bilayer thickness was determined by SPR onto gold substrate. Each step of the construction was followed by AFM imaging both on glass and gold supports. Altogether the results validated the optimum conditions domain. The tethered bilayer, designed here, is very versatile as it can be adapted easily to different types of support.

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Lipid microdomains driven by headgroup interactions at membrane surface

M. Roux[1], S. Moutard[3], C. Hocquelet[1], F. Djedaini-Pilard[2], B. Perly[1]
[1]URA CNRS 2096 - DBJC/SBFM & SCM/DRECAM CE Saclay, 91191 Gif sur Yvette, [2]Laboratoire des Glucides, Université de Picardie Jules Verne, 80039 Amiens, [3]OZ-Biosciences - Parc Scientifique de Luminy, BP13, 13273 Marseille

Amphiphilic cyclodextrins are obtained by grafting an hydrophobic anchor – cholesterol or acyl chains - onto the cyclodextrin molecular cage. These compounds are prone to segregate through interactions between cyclodextrin headgroups at the surface of phosphatidylcholine membranes, leading to lateral separation of a cyclodextrin-rich lipid phase within pure lipids. Cholesteryl cyclodextrin with a succinyl spacer inserted between a cholesterol moiety and a β-cyclodextrin headgroup segregates on a large range of concentrations (5-40%) and temperatures (37 to -12 ^{o}C) to give a cyclodextrin-rich phase containing ∼1.5 lipids per cyclodextrin. Restraining the cyclodextrin molecular space by removing the flexible spacer or increasing the cyclodextrin size, prevents the phase separation. The substitution of the sterol nucleus by two acyl chains allows to sequestrate three times more lipids (∼4-5) than the cholesteryl derivative. However, reducing the hydrophobic moiety to a single acyl chain, loosening the cyclodextrin insertion at the bilayer surface, prevents the formation of a cyclodextrin-rich phase. The amphiphilic cyclodextrin-containing membranes provide a straightforward example on the formation of lipid microdomains within bilayers, through finely-tuned intermolecular interactions of polar headgroups at membrane surface.

P-499

What controls the thickness of biological membranes?

J. N. Sachs[1], D. P. Tieleman[2], D. M. Engelman[1]
[1]Yale University, [2]University of Calgary

The recent interest in lipid microdomains has challenged the biophysical community to develop new approaches for constructing theoretical and physical models of these more complex, biologically relevant systems. Here, we aim to attempt this leap by making a set of computational and experimental measurements on the effect of protein concentration on the thickness profile of membranes. Such information, surprisingly unknown to date, will help in establishing an underlying framework for comparison of native and potentially pathological cellular membrane processes. A novel approach to molecular dynamics simulations of membrane protein systems is used to augment experimentally averaged thickness profiles obtained by small-angle X-ray scattering by providing detailed molecular-level information unavailable to the experiments. Specifically, we explore the role of membrane protein:lipid ratio, with the perspective that perhaps a very small fraction of biological membranes should be considered unperturbed.

P-500

Dynamics of lipid rafts in normal cells revealed by molecular imaging

K. Sakata-Sogawa[1], S. Yamasaki[2], T. Saito[2], M. Tokunaga[3]
[1]Res. unit for single molecule immunoimaging, RCAI, RIKEN, Japan, [2]Lab. for cell signaling, RCAI, RIKEN, Japan, [3]Natl. Inst. of Genetics, Japan, [4]Grad. Univ. for Advanced Studies, Japan

Lipid rafts are submicroscopic sphingolipid- and cholesterol-rich domains in which many signaling proteins are assembled. This assembly is supposed to enhance the interaction between signaling proteins and increase the efficiency of signal transduction cascades. Although accumulating evidence from microscopic studies shows the existence of lipid rafts, their dynamic features still remain to be elucidated. Aiming at visualization of the dynamics of lipid rafts *in vivo*, we constructed $LAT^{\Delta cp}$/GFP, a chimeric protein of GFP and $LAT^{\Delta cp}$, which is known as a raft-localized protein, and established transgenic mice expressing $LAT^{\Delta cp}$/GFP ubiquitously. This chimeric protein retains the consensus raft localization signal sequence but not the functional tyrosine residues. Indeed, $LAT^{\Delta cp}$/GFP localized mainly in biochemically-identified lipid rafts in various types of cells from the transgenic mice driven by CAG promoter. We prepared bone marrow derived mast cells from the mice and observed them at 37^{o}C using a single-molecule TIRF microscope. The lateral and vertical motions of the fluorescent clusters were imaged, possibly reflecting the movements of single rafts. Single molecule images of $LAT^{\Delta cp}$/GFP were visualized and their movements showed the similar behavior as single rafts. The results of the quantitative analysis of the dynamics of lipid rafts supported the existence of the rafts and revealed quick movements both lateral and transverse.

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Lateral diffusion of the mu opioid receptor in the SH-SY5Y membrane

A. Sauliere, G. Gaibelet, C. Millot, S. Mazeres, A. Lopez, L. Salome, L. Cezanne
IPBS/CNRS, UMR5089, 205 route de Narbonne, 31077, TOULOUSE cedex, France.

The mu opioïd receptor (hMOP) is a G-protein coupled receptor which mediates effects of opioïds ligands like morphine. The knowledge of the structural organization of the various proteic partners in the signal transduction chain via this receptor can give us essential information about its functioning. We are interested in its dynamic organisation in the plasmic membrane (Daumas et al. 2003) in relation with its pharmacological response, but also with the membrane environment. We address the question of the compartmentation of the mu opioid receptor in micro-domains in regard to its activation by ligands.
The neuroblastoma cell line SH-SY5Y was chosen because of its hMOP endogenous expression. The mu opioid receptor, in fusion with GFP and a T7 peptide in N-terminal, was stably expressed in these cells, and its membrane localisation checked. The pharmacological properties (binding, adenylate cyclase inhibition) of the receptor were also investigated and found to be preserved. The lateral diffusion of hMOP is studied with two dynamical approaches which are SPT (Single Particle Tracking) and vrFRAP (Fluorescence Recovery After Photobleaching at variable observation radii, Cézanne et al. 2004). First results will be discussed.
Daumas F et al. *Biohys.J.* (2003) 84:356.
Cézanne L et al. *J.Biol.Chem.* (2004). 279:45057

P-502

Interaction of proteins with lipid bilayers analysed by temperature controlled AFM

S. Schuy, A. Janshoff
University of Mainz, Department of Physical Chemistry

Domain formation in heterogeneous mixtures of lipid bilayers has recently attracted an immense attention and is believed to play a key role in molecular cell recognition, signal transduction and conformational changes in membrane proteins. The specificity of the binding of several proteins, such as Streptavidin to biotinylated heterogeneous lipid bilayers as well as the binding of and Annexin A1 to a mixture of POPC and POPS is presented on this poster. On the one hand the incorporation of biotinylated-DOPE in heterogeneous lipid bilayer mixtures composed of DOPC and C_{15}-PC will be determined by temperature controlled atomic force microscopy. The specific binding of streptavidin to the biotin receptors will be used as a marker to determine the distribution of the biotinylated DOPE within the homogeneous and heterogeneous lipid mixture. On the other hand the calcium dependent binding process of Annexin A1 to solid supported membranes consisting of POPC and POPS (4:1 mol%) was investigated by means of AFM. It could be shown that there is a strong influence of the calcium content on the size as well as the shape of the calcium induced POPS domains within a POPC matrix. We were able to record time elapsed AFM images that display the calcium induced phase separation process of POPS at the defect border of the POPC - POPS lipid mixture on a solid support.

P-503

Structure and membrane interactions of equinatoxin II, a pore-forming cytolysin

F. Separovic[1], G. Anderluh[2], A. Razpotnik[2], Z. Podlesek[2], Y.-H. Lam[3], A. Watts[3], R. S. Norton[4]
[1]School of Chemistry, University of Melbourne, 3010, Australia, [2]Biology Dept, University of Ljubljana, Slovenia, [3]Biochemistry Dept, University of Oxford, OX1 3QU, UK, [4]Walter & Eliza Hall Institute of Medical Research, Parkville 3050, Australia

Sea anemone cytolysins (actinoporins) are highly basic proteins of ∼20 kDa that generate pores in membranes containing sphingomyelin (SM). They share little sequence similarity with other pore-forming proteins, suggesting a unique structure and mechanism of action. The actinoporin equinatoxin II (EqtII) consists of two short helices packed against opposite faces of a β-sandwich structure formed by two 5-stranded β-sheets. ^{2}H and ^{31}P solid-state NMR shows that the toxin enhances slow motions in lipids and destabilizes the membrane in SM-containing lipid bilayers [Bonev *et al*. 2003, *Biophys J* 84:2382]. We carried out ^{19}F NMR studies of EqtII with all five Trp residues replaced by 5-F-Trp and assigned each peak by single-site mutations (to Phe) [Anderluh, *et al.* 2005, *J Mol Biol* 347:27]. Chemical shift changes observed upon binding indicate regions that interact with membrane lipids. The lack of change for W149 indicates that interaction with SM in model membranes is not sufficient to trigger dissociation of the N-terminal helix from the bulk of the protein. This dissociation is considered to be essential for pore formation. ^{19}F solid-state NMR studies are underway to characterise the subsequent steps towards pore formation in bilayer membranes. Our results emphasize the value of ^{19}F NMR for studying protein interactions with membranes.

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Solid State NMR of sterols and membrane proteins: assignment, H-bonds, folding, dynamics

O. Soubias[1], M. Renault[1], F. Jolibois[2], O. Saurel[1], V. Réat[1], A. Milon[1]
[1]IPBS CNRS, 205 rte de Narbonne, TOULOUSE, FRANCE, [2]IRSAMC, UPS, Toulouse

We will present results obtained with DMPC-cholesterol and DMPC-ergosterol (natural abundance, 10% and 100% ^{13}C-labelled) from various MAS experiments at spinning rate ranging from 8 to 15 kHz: 2D INEPT, CP based HETCOR, INADEQUATE. Combination of these experiments has allowed for complete assignment of both sterols in their membrane environment. The comparison of carbon chemical shifts in organic solvent and in membranes has revealed specific chemical shifts variations which were analysed in terms of hydrogen bonding and rotameric states of the hydroxyl group, using *ab initio* calculations of the isotropic chemical shifts(1). Using quantum mechanical calculations of static CSA tensors and the experimental motionally averaged CSAs, we could extract orientational constraints sufficient to characterize completely the sterol's dynamics(2).

Similar strategies using a combination of oriented bilayers and MAS experiments have been applied to bacteriorhodopsin and OMPA membrane domain in order to analyse folding and dynamics of these two membrane proteins(3).
(1) *Chemistry* **10**:5996-6014 (2004) (2) *Biophys. J.*, in press (2005) ; (3) *Magn. Reson. Chem.* **42**:212-7 (2004)

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Detergents induce lo-phase domains budding and fission from giant unilamellar heterogeneous vesicles

G. Staneva[1], M. Seigneuret[2], K. Koumanov[1], G. Trugnan[3], M. Angelova[3]
[1]Inst of Biophysics-BAS Sofia Bulgaria, [2]Inst Cochin U567-UMR8104 Paris France, [3]INSERM U538 UPMC Paris France

The behavior of GUV, containing liquid ordered phase (l_o) domains, was investigated with respect to the addition of detergents. GUV, made of PC, SM, and cholesterol, were prepared by electroformation. The detergents were: lysoPC, the product of phospholipase A_2, as well as Triton X100 and Brij98 - detergents used to isolate lipid rafts as DRM. Local external addition (by a micropipette) of each of the three detergents at subsolubilizing amounts promoted expulsion of l_o domains from the GUV as small vesicles. The vesiculation, taking several seconds, was followed directly by videomicroscopy. The budding and fission processes associated with such events were interpreted as due to two distinct effects of the added detergent. The budding is due to the initial incorporation of the detergent in the outer membrane leaflet which increases the spontaneous curvature of the bilayer. The fission is related to the inverted cone molecular shape of the detergent which stabilizes positively curved structures, e.g. pores involved in vesicle separation. We never observed in GUV neither domain formation, nor domain coalescence induced by the addition of detergents. The l_o-phase domains have been recently used as models for biological lipid rafts. Our work supports the idea that isolation of DRM from biomembranes by detergents is not an artifact. It also suggests that the physicochemical mechanisms involved in l_o domain budding and fission might play a role in raft-dependant endocytosis in cells.

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Separation of lipid vesicles using surface acoustic waves

D. M. Steppich, A. Wixforth, M. F. Schneider
Universität Augsburg - Germany

Phospholipid bilayers serve as model membranes for biological cells. In order to understand the basic principles, which trigger membrane phase transitions, we measure the thermodynamic (e.g. enthalpy or entropy change) and mechanic (e.g. compressibility) properties of lipid monolayers on a fluorescence film balance device.
In order to see the effects of phase state on the membrane polarisation we apply a novel technique designed in our institute. We use a SAW driven laminar shear flow to separate vesicles on a planar $LiNB0_3$-Chip ("flat fluidics") with an eight like structure made by photolithography. The vesicles can either be separated by charge in a homogenous electrical field or by different polarisation in an inhomogeneous field. For this so called dielectrophoresis the alternating polarisibility of vesicles in different phases or because of different composition is used. One of our future goals is to devide human melanoma cells from blood cells.

P-507

Motional/polarity heterogeneity in biomembranes as determined by SL EPR – GHOST

J. Strancar, Z. Arsov, T. Koklic, I. Dogsa
Laboratory of Biophysics – EPR center, Jozef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

After decades of intensive research on biological membranes, their complexity still remains one of the toughest problems to the experimental techniques. As the complexity originates from complex biochemical composition as well as from different short and long-range interactions that define the state of this complex system, it seems that even more effort is needed for experimental and theoretical consideration to come closer.
To provide one of the possible experimental attempt for characterization of the complexity of motional/polarity patterns in biological membranes that can be applied for both model and real membranes we will present the characterization based on spin labeling EPR (electron paramagnetic resonance), spectral simulations, multiple hybrid evolutionary optimizations and GHOST condensation. Major scheme of this approach will be shown consisting of schematic presentation of various stages of this approach together with some basic evaluation on model binary and complex lipid bilayers as well as on real membranes. In the model membranes the effects of the cholesterol/glycosphyngolipids will be discussed whereas in the case of real biomembranes the correlations between motional/polarity patterns and physiological responses of the living cells will be searched.

P-508

Coexisting domains in live cell plasma membranes characterized by spin label ESR spectroscopy

M. J. Swamy[1], L. Ciani[2], M. Ge[3], D. Holowka[3], B. Baird[3], J. H. Freed[3]
[1]School of Chemistry, University of Hyderabad, Hyderabad-500 046, India, [2]Department of Chemistry, University of Florence, Firenze, Italy, [3]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA

The importance of membrane-based compartmentalization in eukaryotic cell function has become broadly appreciated, and a number of studies indicate that these eukaryotic cell membranes contain coexisting liquid-ordered (L_o) and liquid-disordered (L_d) lipid domains. However, so far there has been no direct demonstration of differences in the order parameters for the lipids in these two types of regions or their relative population in the plasma membranes of live cells. In this study, we provide direct evidence for the presence of two different types of lipid populations in the plasma membranes of live cells from four different cell lines by ESR spectroscopy. Analysis of the ESR spectra recorded between 5^oC and 37^oC shows that the spin-labeled phospholipids incorporated experience two types of environments, liquid-ordered-like and liquid-disordered, with distinct order parameters and rotational diffusion rates, but with some differences amongst the four cell lines. These results suggest that coexistence of lipid domains that differ significantly in their dynamic order in the plasma membrane is a general phenomenon. The liquid-ordered-like region is typically found to be the major component, in contrast to a model in which small liquid-ordered lipid rafts exist in a "sea" of disordered lipids.

Posters
– Membrane Microdomains –

P-509

Shape changes and vesicle fission of GUVs of liquid- ordered phase membrane induced by Lyso-PC

T. Tanaka[2], R. Sano[1], Y. Yamashita[1], M. Yamazaki[1]
[1]Dept. of Physics, Fac. of Science, Shizuoka University, Shizuoka, 422-8529, Japan, [2](present address) Laboratoire PCC, Institut Curie CNRS 168, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

Liquid-ordered phase (*l*o phase) of lipid membranes and rafts have attracted much attention. Giant unilamellar vesicles (GUVs) of DPPC/cholesterol (chol) membranes in the *l*o phase and also GUVs of sphingomyelin(SM)/chol membranes in the *l*o phase were formed in water at 20−37 °C successfully. To elucidate the interaction of substances with a long hydrocarbon chain with the *l*o phase membrane, we investigated the interaction of low concentrations (less than CMC) of lysophosphatidylcholine (lyso-PC) with these GUVs. We found that lyso-PC induced several shape changes and vesicle fission of these GUVs above their threshold concentrations in water. The analysis of these shape changes indicates that lyso-PC can be partitioned into the external monolayer in the *l*o phase of the GUV from the aqueous solution. Threshold concentrations of lyso-PC in water to induce the shape changes and vesicle fission increased greatly with a decrease in chain length of lyso-PC. Thermodynamic analysis of this result indicates that shape changes and vesicle fission occur at threshold concentrations of lyso-PC in the membrane. We proposed a mechanism for the lyso-PC induced vesicle fission of GUVs.

P-510

Bicelles: disks or perforated lamellae?

M. N. Triba, D. E. Warschawski, P. F. Devaux
CNRS, UMR7099, IBPC, Paris, France

Mixtures of long-chain lipids (DMPC) and short-chain lipids (DHPC) in water form complexes with astonishing properties, which vary with temperature and with the molar ratio of the two lipids. For example, some of these objects spontaneously align in a magnetic field above the transition temperature Tm of DMPC. Various morphologies have been proposed to explain this behaviour. Using Phosphorous-31 NMR, we have been able to provide a new explanation that accounts for all observations (Triba *et al. Biophys. J.* **88**:1887–1901, 2005). We have shown that orientation depends on the formation of lipid disks, called *bicelles*, and the *increase* of their radius with temperature. We have also detected the occurrence of perforated vesicles above a second critical temperature, Tv, and the disappearance of these perforations above a third temperature Th. The driving force for these transformations is a long known but overlooked phenomenon: the miscibility of DHPC in the DMPC bilayer. Accordingly, we have named our new model the "*mixed bicelle*" and we have determined the conditions for which it aligns efficiently in the field of an NMR magnet.

P-511

Patterning complexity into supported planar bilayers

K. Vats, M. Kyoung, E. D. Sheets
Department of Chemistry, Pennsylvania State University

The relationship between the structure of biomembranes and their function remains elusive. We are exploiting a combination of microlithography and quantitative fluorescence microscopy to systematically probe the molecular interactions that lead to lipid domain formation as well as domain dynamics. Complexity is built into supported bilayers by pattering domains via a polymer lift-off approach. We find that the complex bilayers retain their patterns, and that multilayers do not form, as assessed by quantitative fluorescence microscopy. Fluorescence correlation spectroscopy (FCS) also shows that the fluid phase lipids retain typical diffusion coefficients, and atomic force microscopy is being used to further characterize these patterned bilayers. In addition to FCS, we are combining single particle tracking with imaging to probe the molecular dynamics associated with lipid domains and interfaces at high spatial and temporal resolution. Ultimately, we will follow the dynamics of domains and individual lipids within this milieux as a function of cholesterol content to understand how these physical interactions in cholesterol-rich rafts control biological function. Our approach will also lead to innovative and much improved biosensors.

P-512

Phase diagrams of ternary lipid bilayer mixtures by fluorescence microscopy and 2H NMR

S. L. Veatch[1], K. Gawrisch[2], S. L. Keller[1]
[1]University of Washington, Seattle WA, [2]National Institutes of Health, Rockville, MD

Phase diagrams for ternary membranes containing cholesterol are complex, and there is evidence for lipid organization on multiple length-scales. Greater than 10nm length-scale structure has been observed in many lipid mixtures containing cholesterol and at least one additional lipid species (including DPPC, POPC, or SM) by methods that probe short length scales such as FRET, fluorescence quenching, and NMR. A subset of these lipid mixtures also exhibit phase separation into large-scale liquid domains when observed by fluorescence microscopy (> 1 micron) or NMR. Here we combine the results of two separate experimental methods and present data for ternary lipid membranes probed by both fluorescence microscopy and ^{2}H NMR. Phase diagrams of DOPC, DPPCd62, and cholesterol show qualitative agreement, but differ in the exact location of the miscibility phase boundary. Possible explanations for discrepancies between results of experimental methods will be discussed, including sample type (multilamellar vesicles vs. unilamellar vesicles), the inclusion of fluorescent probes in microscopy experiments, and the intrinsic time and distance-scales of the two experimental methods. The resultant phase diagrams will be discussed in the context of recent and established findings from other laboratories.

Posters
– Membrane Microdomains –

P-513

Nanosecond dynamics at membrane-water interface observed by time-resolved stokes shift of laurdan

M. Vincent[1], B. de Foresta[2], J. Gallay[1]

[1]L.U.R.E. Laboratoire pour l´Utilisation du Rayonnement Electromagnétique, Université Paris-Sud, Bâtiment 209D, B. P. 34, F-91898 Orsay Cedex, France;, [2]Service de Biophysique des Fonctions Membranaires, CEA DSV/DBJC and URA CNRS 2096, Centre d´Etudes de Saclay, 91191 Gif sur Yvette Cedex, France.

We studied the dipolar relaxation at the surfactant/water interface in reverse micelles of AOT/water in isooctane, a mimicked membrane/water interface, with the amphipathic solvatochromic fluorescent probe LAURDAN. A negative component was observed in the fluorescence decays of the red edge emission spectrum – the signature of excited state reactions. The deconvolution of the transient reconstructed spectra of LAURDAN with three log-normal Gaussians made it possible to separate the specific dynamic solvent response from the intramolecular excited state reactions of the probe. The dynamic solvent response of LAURDAN was described by unimodal kinetics on the nanosecond time scale, in contrast to that reported by water-soluble probes, which was biphasic (on the subnanosecond and nanosecond time scales) due to the heterogeneous distribution of these probes in the water pool and at the surfactant/water interface. Most of this spectral shift probably resulted from water relaxation as it was highly sensitive to the water to surfactant molar ratio (w_0) (60-65 nm at w_0 = 20-30). A small part of this spectral shift (9 nm at $w_0 = 0$) probably resulted from dipolar interaction with the AOT polar head group. The measured relaxation time values were in the range of the rotational motion of the AOT polar head group region as assessed by LAURDAN and TOE fluorescence anisotropy.

P-514

Phospholipases caught in action at lipid interfaces: how lipid structure restricts PL activity

K. Wagner, G. Brezesinski

Max Planck Institute of Colloids and Interfaces, 14424 Potsdam, Germany

Langmuir monolayers of phospholipids are very suitable models to study enzymatic reactions at interfaces. Applying surface-sensitive methods like GIXD and IRRAS permits *in situ* observations of particular interactions occurring at biological membranes. Thus, phospholipase D was previously found to have maximum activity in the disordered liquid-expanded phase state of the monolayer, whereas PLA_2 requires both a pre-orientation of the substrate and membrane defects for effective hydrolysis. However, the crucial parameter of the substrate structure is still unknown.
Phosphatidic acid (PA), the reaction product of the PLD catalyzed hydrolysis, seems to inhibit the reaction through a modification of the substrate structure. To determine the structure parameter that restricts PLD activity, DPPC is mixed with different spacer molecules that decrease the tilt angle in the mixtures as shown by GIXD. Accordingly, IRRAS measurements reveal a reduced hydrolysis turnover for mixed compared to pure DPPC monolayers over a wide range of surface pressure, but it does not correlate with the decreased tilt angle. Obviously, the addition of spacer molecules rather changes the PC/PA miscibility behaviour, which affects the reaction. Preliminary data indicate the necessity of PC/PA demixing for complete hydrolysis. These results point once more to the vital aspect of phase separation in biological membranes as seen for PLA_2 before.

P-515

Integration of biological molecules and syntheic membranes to create excitable vesicles

D. Wendell, J. Patti, J. Isobe, C. Montemagno
University of California, Los Angeles

The neuron can be considered the brain's fundamental processing unit. Several strategies have been used to harness the potential of neural networks, including software algorithms, network imitation in silicon architecture, and the *in vitro* growth of neurons.
Our group has recently begun a project to utilize ion channel and gap junction proteins with engineered biomimetic copolymers to replicate some of the functionality of a neural network. This interdisciplinary project integrates contributions from molecular biology, synthetic chemistry, and mathematical modeling. The goal is the creation of an excitable vesicle (EV) capable of generating an action potential using trans-membrane ion flow, and transferring that potential to its neighbors.
Mathematical simulations have facilitated the choice and quantity of proteins for the system as well as the effects of volume on EV signal generation. Connexin, the principle protein of gap junctions, can be overexpressed and purified from insect cells and sodium and potassium ion channels can be obtained from *E. coli*. These proteins can then be reconstituted into sub-micron polymer vesicles which mimic the natural cell membrane to produce emergent functionality. Ultimately, EVs may be used to analyze ionic current behavior in very small volumes and may eventually be used to create networks which enable the study of emergent properties in complex systems. We will present recent progress in the construction and integration of these parts.

P-516

Investigation of glycolipid-domains in lipid monolayers by fluorescence & scanning force microscopy

B. M. Windschiegl, C. Steinem

Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Germany

Recent findings suggest that in plasma membranes a significant fraction of lipids is organized in structures called lipid *rafts*. These lipid *rafts* are rich in sphingolipids, cholesterol and glycolipids. Based on the observation that α-hydroxylation of glycosphingolipids is unusually high in human colonic cancer our emphasis was put on the influence of α-hydroxylation of galactocerebroside (GalCer) in the lateral organisation of membranes. Thus we investigated artificial lipid monolayers with defined concentrations of α-hydroxylated or non-hydroxylated GalCer, cholesterol, sphingomyelin and phosphocholine.
On the one hand we employed fluorescence microscopy at the air-water interface to visualize microdomain formation at various surface pressures. The comparison of several lipid mixtures revealed no difference in the phase behaviour of non-hydroxylated and α-hydroxylated GalCer containing lipid mixtures.
On the other hand we used scanning force microscopy to characterize lipid monolayers immobilized on mica. In both cases, in lipid mixtures with non-hydroxylated and α-hydroxylated GalCer, we detected microdomains with a height difference of (0.7 ± 0.1) nm. In case of the mixture with 10 % non-hydroxylated GalCer these domains with an average diameter of (19 ± 12) nm occupied around 10 % of the total area. In mixtures with 10 % α-hydroxylated GalCer an area of 20 % of the total area was covered, while the domains exhibited a much smaller size of only (4 ± 2) nm.

Posters

– Morphogenesis: from cell adhesion to organs –

P-517

Comparing creep and dynamical responses in microrheological experiments on living cells

M. Balland, N. Desprat, D. Icard, A. Asnacios, J. Browaeys, S. Hénon, F. Gallet
Matière et Systèmes Complexes - UMR 7057 associée au CNRS et à l´Université Paris 7

We compare the results of two microrheological experiments on the actin cytoskeleton of single cells. In the first one, the creep function J(t) of a cell stretched between two glass plates is measured after applying a constant force step (1). In the second one, a micrometric bead specifically bound to transmembrane receptors is driven by an oscillating optical trap, and the viscoelastic coefficient $G(\omega)=G'(\omega)+iG''(\omega)$ is retrieved (2).
Both J and G exhibit the same power law behavior $J(t) = At^{\alpha}$ and $|G(\omega)| = G_0\omega^{\alpha}$, with the same exponent $\alpha \sim 0.2$. This is consistent with the general relationship $s^2J^*(s)G^*(s) = 1$ (where * denotes the Laplace transform).
This power law behavior is very robust, since the average value of the exponent $<\alpha> = 0.22 \pm 0.05$ does not globally depend on the cell type, on the nature of complex transmitting the mechanical stress, nor on the typical length scale of the experiment (as confirmed by other AFM measurements).
The exponents α are normally distributed over the cell population, while the prefactors A and G_0 follow a log-normal distribution. We have developed a phenomenological model, involving an infinite number of time relaxation in the system, which accounts both for the power laws and for the distribution of mechanical parameters measured in the system.
(1) Balland et al. Eur. Biophys. J. *in press* (2005)
(2) Desprat et al. Biophys. J. **88** 2224-2233 (2005)

P-518

Dynamics of L1 contacts in neuronal growth cones

C. Dequidt[1], P. Alberts[2], T. Galli[2], D. Choquet[1], O. Thoumine[1]
[1]Physiologie cellulaire de la synapse, Institut Magendie, Université Bordeaux 2, [2]Membrane traffic and neuronal plasticity, Institut du fer à moulin, Paris

The neuronal adhesion molecule L1 is a major player in axonal guidance. The recycling of L1 at growth cones is documented, but little is known about the dynamic turnover of L1 adhesions. We used latex microspheres coated with L1-Fc chimera or anti-L1 antibodies which made specific contacts with hippocampal neurons transfected with L1-GFP. To measure the overall turnover of these contacts, we photobleached the L1-GFP signal around the beads. The fluorescence recovery shows a fast regime which corresponds to the rapid diffusion of unbound and intracellular receptors, and a slow regime only for L1-Fc coated beads, interpreted as the association and dissociation of L1-L1 homophilic adhesions. We calculated turnover rates of about 4.2 h^{-1} for L1-Fc coated beads and 10^{-4} h^{-1} for anti-L1 coated beads. We used thrombin treatment to cleave the N-terminal GFP tag and remove surface fluorescence, in order to distinguish the source of L1-GFP which contributes to fluorescence recovery. L1-GFP rich vesicles traffic in the neurite and cluster at bead contacts where they could exocyte. After washing the thrombin, the average fluorescence recovers with a turnover rate of about 0.4 h^{-1}, showing that exocytosis account for only 20% of the renewal rate of molecules at L1-dependent contacts. Altogether, these results show that the dynamics of L1 contacts is governed not only by trafficking events, but also by diffusion and transient trapping through ligand-receptor bonds.

P-519

Probing Single Cell Mechanics at High Strains

N. Desprat, J. Browaeys, A. Asnacios
Laboratoire Matière et Systèmes Complexes. Université Paris 7 - CNRS UMR 7057 - Case courrier 7056 - 2, place Jussieu - 75251 Paris Cedex 05 - France

Using a novel micro-rheometer, we stretched individual cells under a constant applied force (creep experiment) and measured, for the first time, the creep function J(t) of a single living cell [1]. J(t) appeared to be a weak power-law of time ($J(t)=At^{\alpha}$), indicating that living cells are materials with a large distribution of relaxation time constants.
However, this measurements were done at low strains (or, equivalently, at short times) where the stress experienced trough the cell could be considered as constant. We present here a simple geometrical analysis allowing to calculate the creep function J(t) from high (400%) strain data. We show, for C2.7 myoblasts, that J(t) behaves as power-law of the time from 0.1 to more than 1000 seconds.
[1] Desprat et al. Biophys. J. **88** 2224-2233 (2005).

P-520

FAT-like domains: a novel family of versatile signalling modules

M.-L. Garron[1], G. M. O´neill[2], A. Lerner[2], M. K. Höllerer[4], J. M. Werner[4], M. E. Noble[4], R. T. Premont[3], S. T. Arold[1]
[1]CBS, UMR 5048 CNRS – UMR 554 INSERM, Montpellier, France., [2]The children´s Hospital at Westmead, Westmead, Australia; Boston Medical Center, Boston, USA, [3]Duke University Medical Center, Durham, USA, [4]University of Oxford, UK

Focal adhesions (FAs) are multi-protein complexes that regulate important cellular processes and are involved in oncogenic transformation and metastasis. The FA kinase (FAK), a scaffolding protein central to assembly and disassembly of FAs, harbours at its C-terminus the FA targeting (FAT) domain. Despite its simple structure (a four-helix bundle, as shown by us and others previously), FAT fulfils a panoply of functions: it allows FAK localisation at FAs by interacting with so-called LD motifs of paxillin, contributes to FAK signalling via Grb2, and mediates FAK dissociation from FAs. For this, large conformational changes and phosphorylation of FAT are necessary. Using structure based sequence alignment we have revealed potential FAT-like domains within the C-termini of Cas and p85/GIT1 family proteins. Employing a pluridisciplinary approach including data from X-ray crystallography, Small Angle X-ray Scattering, NMR, isothermal titration calorimetry and cellular biology, we have characterised structure, function and regulation of FAT and these FAT-like domains. We show that even though 4-helix structure and FA localisation are conserved features of these domains, they have evolved individual molecular mechanisms for recruitment and regulation. Inhibitors for FAT-like domains might prove useful in blocking formation of metastases.