Paradoxical Glucagon Response after Stimulation with Glucose and Arginine in Isolated Pancreatic Sand Rat Islets

M. Ziegler, H.-J. Hahn, B. Ziegler, E. Köhler, and H. Fiedler

Central Institute for Diabetes "G. Katsch", Karlsburg, German Democratic Republic

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Summary. Isolated pancreatic islets of normoglycemic sand rats do not respond to 2.5 mM glucose with an enhanced glucagon secretion, which could be observed in normal Wistar rats. Arginine stimulates glucagon release in the presence of 2.5 mM glucose in Wistar rats as well as in sand rats. The secretion pattern is not caused by insulin deficiency since sand rat islets are characterized by an increased insulin secretion rate *in vitro*. This paradoxical glucagon se-

cretion is not caused by a changed glucagon content but might be related to this species which is able to develop a diabetic syndrome spontaneously.

Key words: Pancreatic islets, insulin secretion, insulin content, glucagon secretion, glucagon content, wistar rats, sand rats, glucose, arginine.

In vivo as well as *in vitro* it is well established that pancreatic glucagon release is normally stimulated by hypoglycemia, amino acids and intestinal hormones. For example, *in vivo* it has been observed that glucagon secretion is stimulated in different species [1–6], by hypoglycemia induced by starvation, phlorrhizin or insulin suggesting that glucagon plays an important role in mobilizing stored substrates. Glucagon release in response to different amino acids is stimulated only in the state of normoglycemia or hypoglycemia [5, 7–11], whereas the effect of amino acids is abolished by hyperglycemia [2, 12]. In perfused pancreas and duct-ligated pancreas slices it is possible to demonstrate an enhancement of glucagon secretion by low glucose and high amino acids concentration in the incubation medium [10, 13–15], whereas with collagenase isolated islets a significant stimulatory action of low glucose levels in contrast to that of high amino acids levels could not be observed uniformly [16–21]. Using a careful isolation technique the glucagon and insulin releasing effect of low and high glucose concentrations and of arginine (in the presence of 2.5 mM glucose) on isolated islets of Wistar rats and sand rats was investigated. In addition to this the glucagon recovery rate as well as the insulin/glucagon ratio was calculated under different experimental situations. The sand rat was selected as a species, which develops spontaneously a diabetic state under special dietetic conditions [22].

Material and Methods

Animals

Sand rats (*Psammomys obesus*) captured in the United Arab Republic were adapted for 2 weeks on

captivity in Cairo. After a further adaptation period of 8 weeks in the animal house of our institute the experiments were carried out within 10 days. The animals were fed with a fresh vegetable diet consisting of green cabbage, chinese cabbage, meal worms and vitamins (about 30 Cal/day, percentage of dry food: 57% carbohydrate, 32% protein, 11% fat). The "clinical" parameters characterizing the investigated animals are summarized in table 2.

Wistar rats, inbred more than 30 generations in our laboratory, were fed with commercial rat pellets chow (VEB Mischfutterwerk Altglienicke, GDR).

Experimental

The islets of adult animals of both sexes after 12 h starvation (water ad libitum) were isolated by means of a modified collagenase technique [23]. Immediately after isolation groups of 10 islets were collected for measurement of insulin and glucagon content. 40 islets were preincubated in 12 ml Krebs-Ringer bicarbonate buffer [24], supplemented with 2.5 mM D-glucose and 5 mg/ml bovine serum albumin equilibrated with 95% O₂ and 5% CO₂ (pH 7.4) at 37° C for 30 min. Then the islets were divided into four equal groups of 10. Three of the groups were incubated for 120 min at 37° C in the same type of buffer with addition of 2.5 or 16.5 mM glucose or, 10 mM L-arginine in the presence of 2.5 mM glucose. The remaining 10 islets were used for the determination of insulin and glucagon content, after preincubation.

After incubation the islets were separated from buffer under the stereomicroscope. The buffer was

quickly frozen and stored (-20°C) until the hormones were measured. For the determination of the islet content of hormones they were transferred after isolation, preincubation or incubation into 1 mM HCl containing 0.3% (w/v) human serum albumin (HSA) and 0.9% NaCl, quickly frozen to -80°C and freeze dried. The residue was redissolved and diluted in 40 mM phosphate buffer (pH 7.4) containing 0.3% HSA, 0.024% thiomersal, and 80 ATrU/ml Contrycal^R (RIA-buffer). Glucagon and insulin were assayed radioimmunologically.

Analytical

Blood samples for glucose and insulin were collected from a tail vein or after decapitation. Blood glucose was determined by a modification of the hexokinase method [25], plasma insulin (immuno-reactive insulin activity, IRI) was measured by radioimmunoassay (RIA) using a modification of back-titration method [26]. For determination of glucagon (IRG, immunoreactive glucagon activity) a modification of RIA-technique according to Heding [27] was used. To enhance the immunogenicity glucagon was conjugated with water soluble 1-ethyl-3-(3-diethylaminopropyl) carbodiimide hydrochloride [28]. Glucagon antibodies were raised in rabbits by weekly injections of coupled glucagon (1 mg) emulsified in equal volume of Freund's complete adjuvant. Twice crystallized pork glucagon was used as standard and for iodination with ^{125}I using the chloramine-T-method [29].

The specific activity of ^{125}I -glucagon was enhanced to 110 mCi/mg by anion exchange chromatography on QAE-Sephadex A-25 according to Jørgensen [30]. Anti-glucagon serum, standard dilution samples and ^{125}I -glucagon were stored at -20°C and thawed immediately before use. All reagents were diluted in RIA-buffer. For determination of immunoreactive glucagon 200 μl of anti-glucagon serum were added to triplicates of 100 μl of standard or unknown sample. After 16 h of preincubation at 4°C , 100 μl of ^{125}I -glucagon was added and after a further incubation period at 4°C for 4 h, the antibody-bound ^{125}I -glucagon was precipitated with 2.1 ml ethanol (91% v/v). The index of precision was 0.0211 calculated as the ratio between the standard deviation of % bound ^{125}I -glucagon and the slope of the regression line. The sensitivity was 18.53 ± 4.13 pg IRG/tube.

The lyophilized islets were assayed in several dilutions to check the cross-reactions of used anti-beef-pork glucagon serum R₁₆ with IRG of Wistar rats and sand rats.

The content of insulin (IRI) was determined in a

modification of the backtitration method already described [31], which also gave evidence for the suitability of rat insulin standards in our antibody system.

For testing the protective effect of protease inhibitors on secreted glucagon [16, 17, 20, 32] we incubated standard glucagon in Krebs-Ringer bicarbonate buffer at 37°C up to 6 h with or without addition of Contrycal (80 ATrU/ml). The calculated glucagon recovery was used to check glucagon degradation.

Statistical Calculations

The hormone content, as well as the secretion rate were calculated in ng/islet. The influence of the islet size was excluded by selecting islets of similar size for the investigations (between 150–250 μm in diameter [33]). The statistical calculations were made from the mean values $\pm$ S.E.M. between test and control incubations carried out in parallel, in a series of 8 experiments on Wistar rats and 9 investigations on sand rats. The glucagon recovery was estimated as the sum of glucagon secretion (after 120 min) and hormone content after incubation referred to the tissue glucagon content before incubation (100%).

Chemicals

Collagenase (141 U/mg) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Glucagon (for clinical use) for immunization, monocomponent pig glucagon (Lot Nr. MC 9969), and proinsulin free rat insulin (Lot Nr. R 170) for iodination and preparation of standard solutions were donated by NOVO A/S, Copenhagen, Denmark. ^{125}I was from Isocommerz GmbH, Dresden, GDR. VEB Arzneimittelwerke, Dresden, GDR, supplied Contrycal[®]. Bovine serum albumin was bought from Behring-Werke, Marburg, FRG; human serum albumin from Institut für Impfstoffe, Dessau, GDR. Thiomersal was purchased from Serva-Feinchemikalien, Heidelberg, FRG; QAE Sephadex A-25, from Pharmacia Fine Chemicals, Uppsala, Sweden and 1-ethyl-3-(d-diethylaminopropyl) carbodiimide hydrochloride from Schuchardt, München, FRG. D-glucose and L-arginine hydrochloride were obtained from Ferak, Berlin-West. All other substances and salts were of analytical grade.

Results

RIA of IRG

In Fig. 1 the standard curve together with the curves for different dilutions of lyophilized islet extracts

from sand rats and Wistar rats are shown to be linear and parallel to one another. Since the standard curves are independent of dilution, it is possible to use porcine glucagon as standard and therefore all values are expressed as porcine glucagon equivalents. Standard glucagon preparations from Wistar or sand rats were not available.

The linear slope of the standard curve is expressed by the coefficient of regression ($r = 0.9954 \pm 0.0008$, $n = 11$). The index of precision ($\lambda = 0.0412 \pm 0.0064$) demonstrates the low standard deviation of the assay procedure.

Fig. 1. Comparison of standard curve (—) of pig glucagon and dilution curves of pancreatic glucagon from islets of Wistar rats (◇—◇) and sand rats (○—○). r represented the index of correlation, λ the index of precision of the standard curve

Glucagon Degradation

The absence of any fall in IRG concentration in the KRB buffer without or with addition of Contrycal is shown in Table 1. Therefore all incubations for measurement of glucagon secretion could be carried out without added enzyme inhibitors. This conclusion is also supported by the calculated glucagon recovery (see Table 4).

Content and Secretion of Insulin and Glucagon

Both species used in this study are normoglycemic and without any signs of hyperinsulinism (Table 2). The insulin as well as the glucagon content of isolated islets and the molar insulin/glucagon ratio do not differ between the species (Table 2).

The insulin secretion of isolated pancreatic islets from Wistar rats was significantly stimulated by 16.5 mM glucose or 10 mM arginine in the presence of 2.5 mM glucose (Table 3). The sand rat islets also re-

Table 1. Stability of glucagon in buffer. A Standard solution of glucagon was incubated without or with addition of Contrycal for up to 6 h in Krebs-Ringer bicarbonate at 37° C. At the indicated time samples were removed for measuring the glucagon concentration. The results are expressed in ng/ml as mean $\pm$ S.E.M. of 6 different experiments

medium	Incubation time (h)				
	0	1	2	4	6
KRB-buffer	4.51	4.78	4.63	4.39	4.61
without contrycal	± 0.03	± 0.04	± 0.09	± 0.06	± 0.07
KRB-buffer	4.44	4.27	4.44	4.20	4.32
with contrycal (80 ATrU/ml)	± 0.01	± 0.13	± 0.18	± 0.08	± 0.06

Table 2. Characterization of investigated animals. Blood samples for determination of blood glucose as well as plasma insulin concentration were collected from the tail vein or after decapitation. The insulin and glucagon content of isolated islets were assayed immediately after isolation as described in "materials and methods". The results are expressed as mean $\pm$ S.E.M. from 8 experiments using Wistar rats and 9 investigations on sand rats, respectively. For calculation of the molar insulin/glucagon ratio the following molecular weights were used: insulin — 5777, glucagon — 3485

	Wistar rats	sand rats
Body weight (g)	174 $\pm$ 4	155 $\pm$ 9
Blood glucose (mg/100 ml)	68 $\pm$ 4	64 $\pm$ 6
Plasma insulin (μ U/ml)	54 $\pm$ 4	63 $\pm$ 5
Insulin content (ng/islet)	74.5 $\pm$ 4.5	72.1 $\pm$ 2.9
Glucagon content (ng/islet)	3.04 $\pm$ 0.08	2.67 $\pm$ 0.13
Insulin/glucagon ratio	19.2 $\pm$ 1.4	17.2 $\pm$ 0.9

sponded under identical conditions with an increased rate of insulin secretion. But since the hormone output at low glucose was higher, the relative efficiency of the stimulus was not so pronounced. Furthermore the response of the B-cells was not as uniform as that seen in islets of Wistar rats.

Glucagon response of the islets of Wistar rats was characterized by a significant stimulus of IRG secretion by low glucose concentration in the presence or absence of arginine when compared to 16.5 mM glu-

cose. The effect of 10 mM arginine + 2.5 mM glucose was significantly ($P < 0.05$) higher than 2.5 mM glucose alone.

In contrast to the glucagon response of Wistar rats

glucagon release. This ratio was exaggerated in all investigations on sand rat islets *in vitro*. This was a result of the enormous amount of secreted insulin and absent A₂-cell response with 2.5 mM glucose alone,

Table 3. Insulin and glucagon release of isolated pancreatic islets in response to glucose and arginine. Islets were preincubated for 30 min in Krebs-Ringer bicarbonate buffer with 2.5 mM glucose. 10 islets per vessel were then incubated for 120 min in 3.0 ml KRBB medium under the indicated test situations. Mean values $\pm$ S.E.M. are given for each group

	Wistar rat			Sand rat		
	glucose			glucose		
	2.5 mM	16.5 mM	10 mM arginine 2.5 mM glucose	2.5 mM	16.5 mM	10 mM arginine 2.5 mM glucose
Insulin release (ng/islet/120 min)	3.11 $\pm$ 0.60	14.55 $\pm$ 1.17	7.11 $\pm$ 1.08	8.50 $\pm$ 1.25	20.80 $\pm$ 2.62	16.40 $\pm$ 2.33
<i>P</i> (compared to 2.5 mM glucose)		< 0.001	< 0.001		< 0.005	< 0.05
Glucagon release (ng/islet/120 min)	0.39 $\pm$ 0.07	0.22 $\pm$ 0.04	0.58 $\pm$ 0.08	0.09 $\pm$ 0.02 (0.48)	0.33 $\pm$ 0.07 (0.70)	0.20 $\pm$ 0.05 (0.37)
<i>P</i> (compared to 2.5 mM glucose)		< 0.02	< 0.05		< 0.01	< 0.05
Molar insulin/ glucagon ratio	7.4 $\pm$ 2.1	65.8 $\pm$ 16.1	8.1 $\pm$ 1.1	125.1 $\pm$ 56.9	106.8 $\pm$ 42.8	163.6 $\pm$ 37.3
<i>P</i> (compared to 2.5 mM glucose)		< 0.01	n.s.		n.s.	n.s.
n.s. not significant						

Table 4. Glucagon recovery from isolated pancreatic islets. The glucagon content of isolated islets was determined immediately after isolation, after 30 min of preincubation in Krebs-Ringer bicarbonate buffer (2.5 mM glucose), corresponding to 100% for the calculation of the recovery rate, and after 120 min test incubation under the indicated situations. The recovery (ng/islet) was calculated as the sum of the amounts of IRG in the islets and in the buffer. The statistical significances, judged from the mean values $\pm$ S.E.M. of differences between parallel incubations, are indicated for effects of different stimuli on glucagon content compared to the same parameter after the prestimulatory period (^a $P < 0.05$, ^b $P < 0.01$)

	Wistar rats (n = 8)			Sand rats (n = 9)		
	Content (ng/islet)	Secretion (ng/islet/120 min)	recovery (%)	Content (ng/islet)	Secretion (ng/islet/120 min)	recovery (%)
Isolation	3.04 $\pm$ 0.08			2.67 $\pm$ 0.13		
Preincubation	3.03 $\pm$ 0.08		100	2.32 $\pm$ 0.16		100
Incubation with						
2.5 mM glucose	2.70 $\pm$ 0.10 ^a	0.39 $\pm$ 0.27	102.1 $\pm$ 2.2	2.09 $\pm$ 0.13	0.13 $\pm$ 0.04	95.5 $\pm$ 3.6
16.5 mM glucose	2.90 $\pm$ 0.11	0.22 $\pm$ 0.04	103.2 $\pm$ 5.3	1.84 $\pm$ 0.25	0.36 $\pm$ 0.07	94.6 $\pm$ 4.5
10.0 mM arginine and 2.5 mM glucose	2.44 $\pm$ 0.12 ^b	0.58 $\pm$ 0.08	99.6 $\pm$ 2.7	2.30 $\pm$ 0.34	0.20 $\pm$ 0.07	107.8 $\pm$ 6.1

we observed paradoxical glucagon secretion of isolated sand rat islets. With increased glucose concentrations the glucagon secretion was enhanced ($P < 0.025$).

The molar insulin/glucagon ratio of secreted hormones in the Wistar rats was increased under conditions of stimulated insulin secretion and inhibited

and also in the presence of 10 mM arginine, to stimulate IRG secretion.

As Table 4 shows, there was no loss of glucagon in our *in vitro* system. The calculated glucagon recovery (the sum of hormone content of islets and of buffer after incubation) did not differ from the values fol-

lowing preincubation. The effectiveness of stimulation of Wistar rat islets was supported by decreased glucagon content under conditions of increased IRG release. A similar decrease of glucagon content, could be observed in the sand rat islets after incubation with 16.5 mM glucose.

Discussion

The sand rat is a desert rodent with an alleged high risk of developing a diabetic syndrome. It was hypothesized that it is possible to alter this metabolic response by controlled nutrition [22]. Because one of the metabolic lesions could be connected with the pancreatic islets [34–36], we investigated the insulin and glucagon secretion as well as the hormone content of pancreatic islets *in vitro*. We must assume on the basis of the values depicted in Table 2 that the animals principally do not differ significantly from other species with normal metabolism. To exclude methodological influences during the isolation and incubation procedure, the same experimental protocol was used simultaneously on Wistar rats.

During 120 min incubation the insulin secretion of Wistar rat islets was increased about 5-fold by 16.5 mM glucose when compared to 2.5 mM glucose.

Conflicting results concerning the stimulatory action of arginine on insulin secretion in the presence of low glucose concentration have been reported [37–42].

Although we did not find a significant increase in insulin secretion in Wistar islets incubated for 30 min (43), a stimulatory action of arginine was observed after prolonged incubation of 120 min in the presence of low glucose. Possibly differences in species, incubation time and the experimental situations used, could play an important role in this connection.

Our results and those described earlier [18, 19] demonstrate that collagenase isolated islet of rats are a useful model for investigation of glucagon release. This suggests that the A-cells are not altered significantly by our isolation procedure [44]. As Table 3 shows, 10 mM arginine in presence of 2.5 mM glucose significantly stimulated the glucagon release. It seems important to stress that all incubations were carried out without addition of protease inhibitor. The calculated recovery underlined the suitability of the *in vitro* conditions used for investigations of insulin and glucagon release. The calculated insulin/glucagon ratio is also in concordance with *in vivo* results [45] and experiments on perfused pancreas [46].

In contrast to the described glucagon recovery we omitted doing the same of insulin. Two reasons for this may be mentioned, (i) insulin balance during an

incubation time of 120 min is influenced by insulin biosynthesis [47], whereas the rate of glucagon biosynthesis is lower [48], and (ii) Kohnert *et al.* [49] described an insulin degrading enzyme system, which could be involved in the insulin metabolism of B-cells, influencing the insulin content. Cellular glucagon breakdown cannot be excluded. Using the described *in vitro* system the results from pancreatic islets of normoglycemic and normoinsulinemic sand rats are in part controversial. As has been established in other species, 16.5 mM glucose stimulated insulin secretion. The relative magnitude of the response compared with 2.5 mM glucose was lower because a large amount of insulin was released in the presence of 2.5 mM glucose. Probably, the glucose threshold was lowered in this species (as preliminary studies have shown [50]). An altered reactivity of sand rat insulin in RIA has been excluded [31]. The calculated recovery from dilution curves rather suggested a diminished binding of sand rat insulin compared with rat insulin.

The sand rat islets did not only fail to exhibit a stimulation of glucagon secretion in response to 2.5 mM glucose, they responded paradoxically. They showed an increased glucagon secretion at 16.5 mM glucose. At present we cannot decide, whether this fact really represents a stimulation with 16.5 mM glucose or a loss of inhibition.

Unger and coworkers [51] reported a relative hyperglucagonemia in patients with diabetes mellitus. Because such a disorder is also seen in experimental diabetic animals with insulin deficiency [20, 21, 45, 52] it has been hypothesized that the inappropriate stimulation of glucagon secretion is the consequence of the insulin-deficient state. In confirmation of these suggestions an inhibitory effect of insulin on glucagon release was demonstrated [21, 52, 53], but only with high doses of insulin.

In contrast to the quoted observations, Unger *et al.* [51] were not able to demonstrate a normalizing effect of insulin administration in adult diabetics with hyperinsulinism. Similar results were reported by Gerich *et al.* [54] in juvenile diabetics even in the presence of large amounts of exogenously administered insulin. Studies on diabetic animals with hyperglycemia and hyperinsulinemia [55, 56] have shown a similar abnormal A-cell response *in vitro* and the absence of an inhibitory effect of the endogenously secreted insulin. In view of these studies it would be attractive to speculate that the results reported here reflect an early dysfunction of A-cells in connection with the development of diabetes. The paradoxical glucagon secretion seen in the isolated sand rat islets could be observed despite the fact that insulin secretion is rather high while the animals are in a normoglycemic state.

Although the observations could support the hypothesis of an intrinsic defect of A-cells [54, 57] in normoglycemic sand rats, we cannot exclude that this behavior reflected a species-dependent diminished adaptation on captivity in connection with a response to the "artificially prepared" diet. This disturbance of the insulin/glucagon interaction may be a basis for the development of further alterations in the metabolism of this species.

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Dr. H.-J. Hahn
 Dept. of Experimental
 Diabetes Research
 Central Institute for Diabetes
 DDR-2201 Karlsburg
 German Democratic Republic