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Effect of Various Concentrations of Calcium on Arginine-Induced Insulin and Glucagon Release *in vitro* *

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The effect of extracellular calcium concentration on the insulin and glucagon release in response to arginine from the isolated perfused rat pancreas, has been studied. All the experiments were carried out in the absense of glucose in the perfusate. Arginine 10 mM elicited a biphasic glucagon release, and a monophasic insulin response. In the presence of calcium 2.5 mM an increase of total insulin and glucagon in response to arginine were obtained. The increase in glucagon release was only detectable during the second phase, while first phase was not modified by the concentration of calcium present in the perfusate. The results of this study show that extracellular calcium concentration influences positively insulin and glucagon responses to arginine *in vitro*.

Calcium plays as essential role in most exocrine and endocrine secretory processes (36). It has been well established that the presence of this cation is an absolute requirement for insulin secretion to occur in response to an insulinotropic agent (12, 29, 35). However, such an effect of extracellular calcium on glucagon secretion from the pancreatic alpha-cells remains controversial: although it has been reported a calcium dependency for glucagon secretion (10, 16, 22) other investigators have, on the contrary, observed that calcium deprivation resulted in an enhancement of glucagon release (8, 18, 19). Also conflicting results exist concerning the effect of raised calcium levels, which have been reported not to affect (4, 5, 17) or to inhibit (31) glucagon

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release. Further discrepancies have been reported by using ionophores which supposedly transport divalent cations across biological membranes (2, 39, 40).

This work was undertaken to further investigate the effect of calcium on glucagon and insulin release by using the isolated perfused rat pancreas. Hormonal secretion was evoked by arginine 10 mM in the presence of fixed concentrations of calcium ions (0.5, 1.0 and 2.5 mM) prevailing throughout the experiment. Since glucose is the most potent physiologic glucagon suppressor (38) all the perfusions were carried out in the absence of glucose in the perfusate.

Materials and Methods

Male Wistar rats, fed *ad libitum* with commercially available food, weighing 250-300 g were utilized in all experiments. After an overnight fast the animals were anesthetized with sodium pentobarbital (45 mg/kg i.p. Nembutal, Abbot). Pancreases were isolated as previously described by SUSSMAN *et al.* (37) with minor modifications (25, 33). The preparation consisted of the rat pancreas without the stomach and spleen and only including a small part of duodenum. After removal, the preparation was immediately transferred to the perfusion chamber of an Ambec Perfusion Apparatus (Beck Industries, Boulder Colorado, USA) and immersed in a bath of normal saline maintained at a constant temperature of 38° C. Pancreases were perfused *via* a canula inserted into the aorta, the effluent being collected from the canulated portal vein without recycling. A canula was inserted into the duodenum to allow evacuation of secretions. The flow rate was maintained constant at 2 ml/min which resulted in a perfusion pressure of 20-40 mm Hg.

The perfusate was a modified Krebs-Ringer bicarbonate buffer containing the following components: NaCl 120 mM; KCl 4 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 0.7 mM; $NaHCO_3$ 25 mM and $CaCl_2$ at concentrations 0.5, 1.0 and 2.5 mM for the various experimental conditions. The medium was supplemented with 2% (w/v) of bovine crystallized albumin (Armour Phamaceutical Co. Estbourne, England) and 2.5% (w/v) of dextran T-70 (Poviet, A. Christiaens, Bruxelles, Belgium). The medium was warmed at 38° C and continuously gassed with a mixture of O_2 and CO_2 (95:5); the resulting pH was 7.5.

After removal, the pancreas was perfused for 30 min before exposure to arginine stimulus. L-Arginine HCl (Fluka A.G., Buchs, Switzerland) was dissolved in the perfusion medium and infused into the circuit immediately above the pancreas through a side-arm perfusion pump (Braun Melsungen, Germany) working at a flow rate of 0.1 ml/min. The period of stimulation lasted 30 min and was followed by a 10 min perfusion in the absence of the amino acid. Two ml samples were collected from the canula inserted in the portal vein into tubes containing 1000 U Trasylol (Bayer, Leverkusen, Germany) immediately cooled on ice and frozen for storage at —20° C until assayed. Twenty samples were taken in each experiment; of these, four were taken during the prestimulatory period. After the switch to the medium containing arginine, the samples were collected every minute for 10 min and then every 5 min until the end of the perfusion.

Insulin (Immunoreactive Insulin, IRI) was measured in duplicate by the double-antibody procedure of HALES and RANDLE (13) with rat insulin as standard (Novo Research Institute, Copenhagen, Denmark). Glucagon (Immunoreactive Glucagon, IRG) was assayed in duplicate according to AGUILAR-PARADA *et al.* (1) using 125-I-glucagon (New England Nuclear, Boston, Mass., USA) as a tracer and a rabbit antiglucagon S6. This antiserum cross reacts with glucagon-like-immunoreactivity (GLI) of enteric origin,

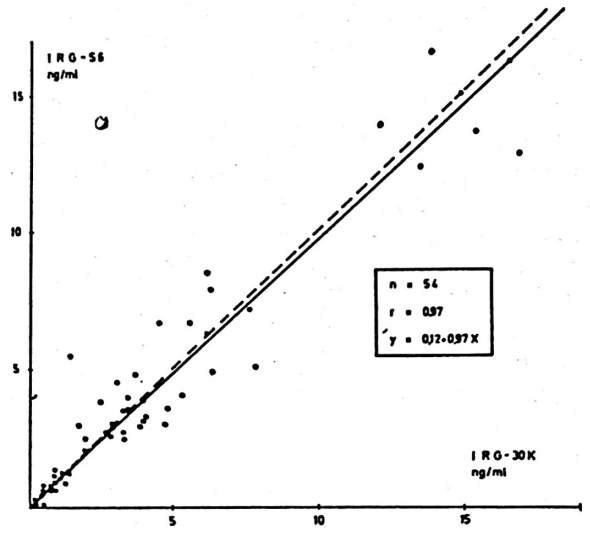

Fig. 1. *Measurement of immunoreactive glucagon (IRG) using antiserum S6 and antiserum 30 K.*
The identity line is indicated by the solid line; the experimental regression line is indicated by the dotted line.

but it gives similar values for perfusion samples to those obtained with Unger's clasical antibody 30 K (fig. 1). This could be expected since the GLI content of the duodenum is known to be low (9, 30). In our hands the GLI of duodenal origin in the rat was found to be 5.6 ng/g (w/w) with 30 K antiserum and 24.0 ng/g (w/w) with S6 antiserum. Porcine glucagon was used as standard (Novo Research Institute, Copenhagen, Denmark). Insulin and glucagon standard curves were performed in the perfusion medium to obtain the same conditions as in the sample tubes.

The rates of insulin (IRI) and glucagon (IRG) were calculated by multiplying the concentration measured in the respective samples by the flow rate and expressed as ng/min. Total release during the stimulation period were obtained by planimetry of the individual perfusion profiles and by calculating the mean of the respective areas. All results are expressed as mean ± SEM. Statistical analysis was performed using the Student t test for non-paired data.

Results

Secretion of glucagon (IRG) and insulin (IRI) were low in the absence of arginine during the pre-stimulatory period and no significant differences were found in the basal secretion of both hormones at the three concentrations of calcium tested (figs. 2 and 3).

Arginine 10 mM elicited biphasic glucagon (IRG) release at 2.5 mM calcium concentration (fig. 2). The peak response for the first phase occured always within one minute. The glucagon (IRG) release decreased after 3 min, remaining however higher than the basal levels. The second phase of release was characterized by the reascension of glucagon (IRG) secretion to attain a maximal rate at 8-10 min of the stimulatory period. After that, the glucagon (IRG) response tended to dimin-

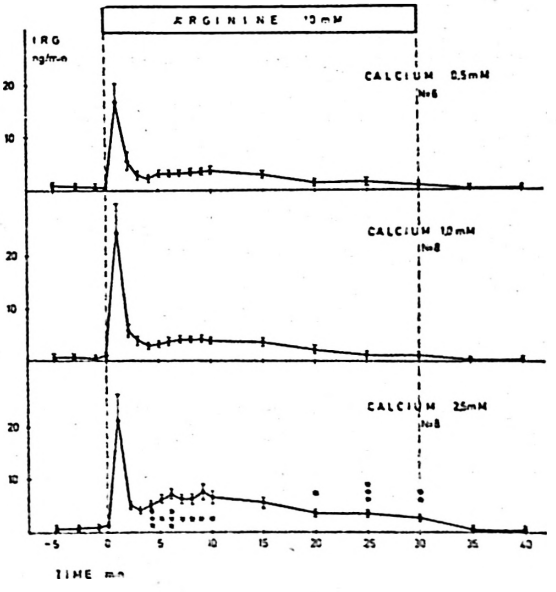

Fig. 2. *Effect of calcium on arginine-induced glucagon (Immunoreactive glucagon, IRG) release.*
Results are expressed as mean ± SEM. Statistical comparison has been made between calcium 1.0 and 0.5 mM and between calcium 2.5 and 1.0 mM. The number of perfusions is indicated in parenthesis. * correspond to $p < 0.05$, ** to $p < 0.02$ and *** to $p < 0.01$.

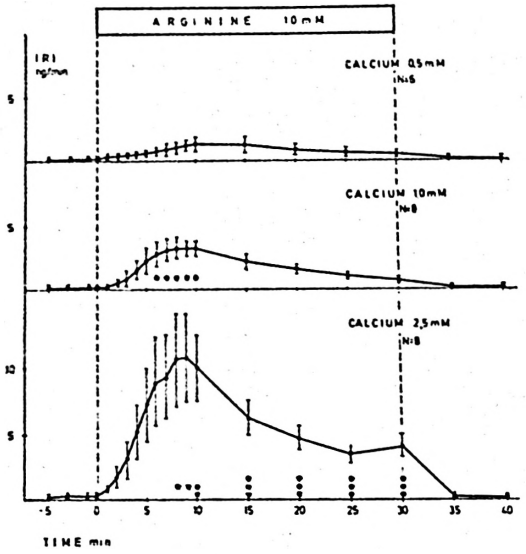

Fig. 3. *Effect of calcium on arginine-induced insulin (Immunoreactive insulin, IRI) release.* Results are expressed as mean ± SEM. Statistical comparison has been made between calcium 1.0 and 0.5 mM and between calcium 2.5 and 1.0 mM. The number of perfusions is indicated in parenthesis. * corresponds to $p < 0.05$, ** to $p < 0.02$ and *** to $p < 0.01$.

ish slowly. A prompt return to the basal levels was observed when the arginine infusion was stopped.

Total glucagon (IRG) released in response to the amino acid was 103.3 ± 8.6 ng/30 min at calcium 0.5 mM, 117.0 ± 14.1 ng/30 min at calcium 1.0 mM and 172.7 ± 21.3 ng/30 min at calcium 2.5 mM. The glucagon (IRG) released during the first phase was 25.6 ± 3.8 ng/3 min at calcium 0.5 mM, 35.2 ± 7.0 ng/3 min at calcium 1.0 mM and 31.1 ± 6.9 ng/30 min at calcium 2.5 mM. The total glucagon (IRG) released during the second phase was 77.7 ± 6.8 ng/27 min at calcium 0.5 mM, 81.8 ± 8.5 ng/27 min at calcium 1.0 mM and 141.5 ± 17.9 ng/27 min at calcium 2.5 mM. A significant increase ($p < 0.05$) in the total glucagon (IRG) released during the period of stimulation was found at calcium 2.5 mM. This increase was entirely due to the glucagon released during the second phase ($p < 0.01$), while the first phase release was not affected by the calcium concentration of the perfusate.

Insulin (IRI) response to arginine 10 mM was monophasic at all calcium concentrations tested, and was characterized by a progresive increase of the insulin secretion rate to attain a maximum at the 8th to the 10th minute of the stimulation period (figure 3). As for glucagon, insulin secretion rate tended to diminish toward the end of the stimulatory period. The decay of insulin release after removal of the arginine stimulus, was as rapid as that seen for glucagon.

Total insulin (IRI) released in response to the amino acid was 30.6 ± 9.0 ng/30 min at calcium 0.5 mM, 59.2 ± 12.5 ng/30 min at calcium 1.0 mM and 177.4 ± 42.3 ng/30 min at calcium 2.5 mM. A significant ($p < 0.025$) increase in the total amount of insulin (IRI) released during the stimulation period was observed when calcium 2.5 mM was present in the perfusion medium.

Discussion

Of the amino acid influencing glucagon and insulin release, arginine has been the most frequently studied (11, 34). This amino acid, in the absense of glucose directly stimulates glucagon secretion *in vitro*. In dynamic studies, perfusion (11, 34) or perifusion (32), a biphasic pattern of glucagon secretion is induced by a square-wave arginine stimulation. A similar effect of arginine on insulin secretion has not been consistently observed (7, 23, 28, 32). However the present study confirms that arginine is capable of stimulating insulin (IRI) release in the absence of glucose (3, 11). A monophasic pattern of insulin release was regularly observed.

The effect of extracellular calcium concentration on arginine-induced insulin (IRI) release, is in good agreement with the well established calcium dependency of insulin secretion (12, 29, 35) and show

that pancreatic beta-cell requirement for calcium is not limited to or specific for glucose-induced insulin release (10).

Total glucagon (IRG) released during the second phase was increased at calcium 2.5 mM, while the first phase was not modified by the extracellular calcium concentration. This might correspond to a differential sensitivity to calcium for the first and second phases of glucagon response to arginine. In fact, in the perfused rat pancreas, it has been found that in conditions of calcium deprivation, the first phase of glucagon response to arginine was more difficult to inhibit than the second phase (10, 22). These results confirm that glucagon secretion in response to arginine is modified by the extracellular calcium concentration (10, 16, 22).

It has been reported that glucagon secretion in the presence of glucose is augmented during calcium deprivation (18, 19). These results may appear at variance with ours, however the observation that glucose-inhibition of glucagon release is a calcium requiring process (24) suggests that the increased glucagon release occuring after calcium depletion might result from a lesser calcium-dependent inhibition by glucose. From these observations, LECLERQ-MEYER *et al.* (20-22) have postulated a dual role of calcium on glucagon secretion: first calcium would play a triggering role which is essential in the case of arginine-induced glucagon release; second, extracellular calcium would be required by the alpha-cell for the identification of glucose as an inhibitor of glucagon secretion.

As far as the effect of calcium on arginine-induced glucagon secretion is concerned, the possibility has been suggested, that calcium might increase the glucagon response by modification of an arginine-membrane receptor interaction (10). Three evidences support this hypothesis: calcium does not influence amino acids transport into pancreatic islets (14); arginine does not appear to be metabolized by mammalian islets (15) and non metabolizable analogues of arginine can elicit glucagon release (6). However the presented results show that the effect of arginine on glucagon release was elicited at the first minute after the switch of arginine infusion, while calcium-increased glucagon response to arginine was found only later.

The mechanism by which calcium affects secretory processes has not been established. It is known that glucose-induced insulin release is not only dependent upon the presence of calcium, but also is accompanied by the accumulation of calcium within pancreatic islets (27). Based on these observations, it has been postulated that calcium modulates insulin secretion by acting on a beta-cell microfilamentous-microtubular system that is involved in the transport of insulin secretory granules to membrane for subsequent release (26). Calcium may act similarly in regulating glucagon secretion from the pancreatic alpha-cell; however the presented results and the data so far available in the literature suggest, that alternative or additional modes of action cannot be excluded.

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Resumen

Se estudia el efecto de la concentración de calcio extracelular sobre la secreción de insulina y glucagón, en respuesta a la infusión continua de arginina 10 mM, *in vitro*, por el pancreas aislado y perfundido de rata. Todos los experimentos se realizaron en ausencia de glucosa en el medio de perfusión. La arginina induce una secreción bifásica de glucagón y una respuesta monofásica de secreción de insulina. En presencia de calcio 2,5 mM, se observa un aumento de la secreción total de insulina y glucagón en respuesta a la arginina. El incremento

en la secreción de glucagón se debe fundamentalmente al aumento en la cantidad de hormona liberada durante la segunda fase de secreción, mientras que la primera fase no experimenta modificación alguna en relación a la concentración de calcio presente en el medio de perfusión.

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