Calcium, Glucose and Glucagon Release*

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Summary. Using the isolated, perfused canine pancreas the importance of calcium for the normal secretory function of the pancreatic alpha cell was investigated. It was found that 1. increases in perfusate Ca^{++} from 1.3 to 4.8 mM and from 1.3 to 8.2 mM during perfusion with glucose concentrations of 25 and 150 mg/100 ml stimulate the release of both glucagon and insulin in a dose-related and a glucose-dependent fashion. The hormone responses to increases in calcium were, with few exceptions, biphasic 2. a 'Ca^{++} free' medium inhibited release of both hormones, and increases in perfusate glucose from 25 to 150 mg/100 ml were unable to suppress glucagon or to stimulate insulin. Addition of calcium (8.2 mM) resulted in re-establishment of the normal regulatory role of glucose upon release of both hormones, now being in a hyperactivated state by the high Ca^{++} concentration; 3. sudden Ca^{++} depletion of the perfusate from 2 mM at a glucose concentration of 200 mg/100 ml inhibited immediately the release of both hormones to very low levels, which remained low until the addition of Ca^{++} (2 mM). Ca^{++} is therefore an essential requirement for the normal secretory process of pancreatic glucagon, possibly involving uptake and accumulation within the A cell, as established for the B cell. It is suggested that Ca^{++} exerts its effect on the microtubular microfilamentous system.

Key words: Isolated, perfused pancreas, glucagon release, calcium, glucose.

It has long been known that the presence of calcium is essential for the function of various exocrine and endocrine processes, including the pancreatic B cell [1–4]. However, controversy exists on the importance of this cation for glucagon secretion from the pancreatic A cell. Rather unexpectedly, Leclercq-Meyer et al. [5] reported findings suggesting that glucagon release was enhanced during calcium deprivation in a preparation of incubated pieces of pancreas. Glucagon release would thus appear to belong to the rare endocrine processes, together with parathormone and possibly renin secretion [6], characterized by an increase in the absence of calcium. Gerich et al. [7] reported inhibition of arginine stimulated glucagon secretion during Ca^{++} deprivation in experiments with an in vitro perfused rat pancreas. Leclercq-Meyer et al. have later [8] and most recently [9] confirmed their earlier observations of a rise in glucagon output during calcium deprivation [5], now using an in situ perfused rat pancreas. Moreover, they found that calcium deprivation was associated with a stimulating effect of glucose upon glucagon output and suggested that these results are 'reminiscent of the situation encountered in human diabetes', because glucose does not suppress plasma glucagon in diabetic patients, 'and may occasionally induce a paradoxical hyperglucagonaemia'. Using a monolayer cell culture of fetal rat pancreas Wollheim et al. [10] have also reported results consistent with an enhancing effect of calcium deprivation upon glucagon release.

We thought it of interest to reinvestigate the basal postulate of a 'paradoxical' relationship between calcium and alpha cell function, and to characterize the effect of calcium upon glucagon release, using another isolated pancreas system, the perfused canine pancreas.
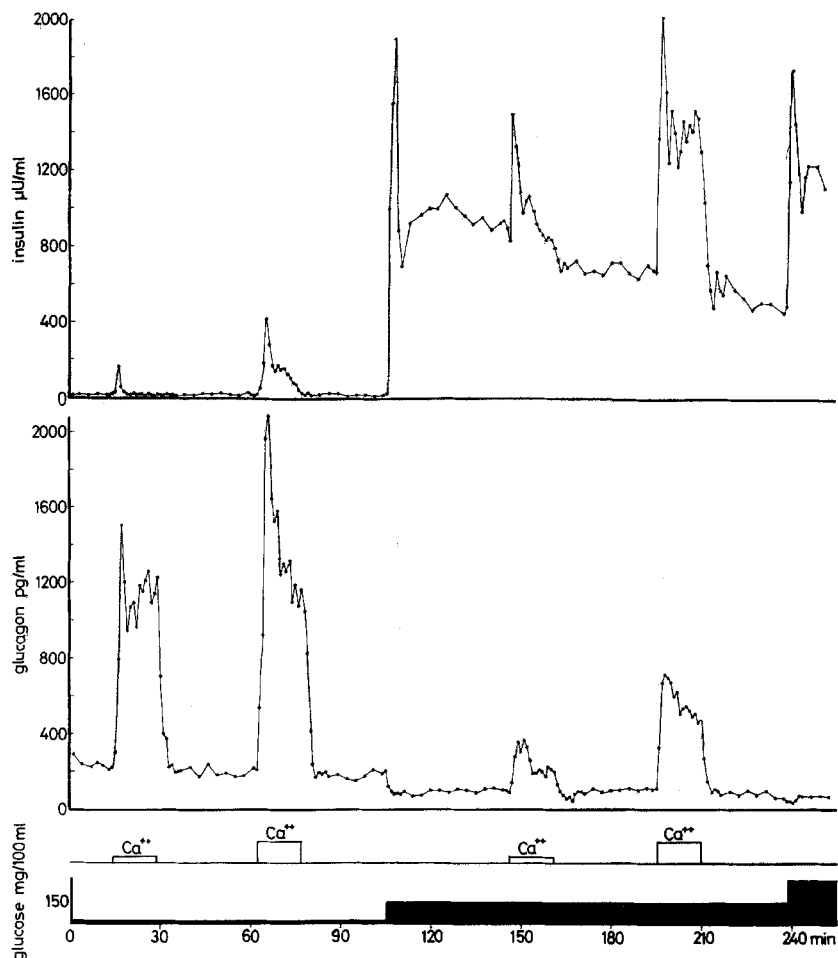
Fig. 1. Effect of a high extracellular Ca^{++} (4.8 and 8.2 mM) upon the secretion of glucagon and insulin during perfusion with low (25 mg/100 ml) and high (150 mg/100 ml) glucose concentrations. The Ca^{++} concentration in the basal perfusing medium was 1.3 mM. A representative experiment out of 3

Material and Methods

Male mongrel dogs, fasted overnight, weighing 18 to 28 kg were used as pancreas donors. The techniques for isolation of the canine pancreas and the perfusion system have been previously described in detail [11, 12]. In brief, the preparation consisted of the pancreas and the proximal 10 cm of the attached duodenum. A nonrecirculating medium consisting of a Krebs-Ringer buffer containing 4% dextran (MW 75000), 0.2% bovine albumin, glutamate, fumarate and pyruvate, each at a concentration of 5 mM, was introduced through the splenic and the coeliac arteries, and the total portal effluent was collected every 60 sec. The perfusion flow was 18–20 ml/min and the perfusion pressure was 30–40 mm Hg. Both parameters were constantly monitored and remained unchanged throughout the experiments. During the experiment, substances to be examined were added to the basic perfusion medium by means of constant side-arm infusion syringes containing appropriate high concentrations. This was also the case in experiments where the effect of glutamate, pyru-

vate and fumarate was investigated. The basic perfusion medium was composed without calcium. $CaCl_2$ was added to result in theoretical final concentrations of Ca^{++} of 1.3, 4.8 and 8.2 mM. Upon assay during calcium deprivation the total calcium concentration (albumin only 0.2%) averaged 0.18 mM, during calcium addition 1.3, 4.7 and 8.3 mM (SEM = 0.01 for all concentrations, n = 10). The effluent was collected in tubes containing EDTA (30%) 250 µl, resulting in a final concentration of 4 mg/ml.

The pancreas was perfused for an equilibration period of at least 30 minutes before samples were measured for their hormone content. The effect of Ca^{++} upon the release of glucagon and insulin was investigated employing 3 different experimental protocols: 1. The effect of sequential increases in perfusate Ca^{++} from the normal values of 1.3 mM to 4.8 and to 8.2 mM during perfusion with glucose concentrations of 25 and 150 mg/100 ml. In one experiment the order of stimulation was reversed, i. e. in this experiment perfusion was started at a high glucose concentration and the calcium concentration of 8.2 mM preceded that of 4.8 mM at both glucose

concentrations. Similar results were obtained. 2. The effect of a Ca^{++} depleted perfusion medium and a medium supplemented with Ca^{++} at a concentration of 8.2 mM during perfusion with glucose concentrations of 25 and 150 mg/100 ml. 3. The effect of sudden Ca^{++} depletion and repletion with Ca^{++} at a concentration of 2 mM during perfusion with a constant glucose concentration of 200 mg/100 ml in the presence and absence of glutamate, pyruvate and fumarate.

Glucagon and insulin were measured by a single antibody radioimmunoassay [14]. In the glucagon assay a highly specific antiserum (K 964, a gift from Lise Heding, NOVO Research Lab., Copenhagen) was used. The sensitivity of the glucagon assay allowed differences in concentrations of 10 pg/ml to be distinguished with 95% confidence. The separation of free from bound hormone was performed according to Ørskov by a wick-chromatography method (14).

Results

1. Effect of Increases in Perfusate Ca^{++} at Low and at High Concentrations of Glucose

Figure 1 shows the results of varying the Ca^{++} concentration of the medium at low and high glucose concentrations. In the basal state, with a calcium concentration of 1.3 mM, basal glucagon release was high and basal insulin release was low during perfusion with a glucose concentration of 25 mg/100 ml. When the glucose concentration in the perfusing medium was increased from 25 to 150 mg/100 ml, glucagon release was suppressed to low levels and insulin release followed the typical biphasic response patterns as previously described [12] (Fig. 1).

Infusion of Ca^{++} at concentrations of 3.5 mM and 6.9 mM for 15 minute periods to result in final concentrations of 4.8 mM and 8.2 mM stimulated both glucagon and insulin release in a dose-related fashion. This occurred during perfusion with low (25 mg/100 ml) as well as with high (150 mg/100 ml) glucose concentrations. The responses obtained were modified by the glucose concentrations present, as previously described for other bihormonal stimuli in this preparation [13, 15]: higher glucagon and lower insulin responses obtained at low than at high glucose concentrations (Figs. 1 and 2). In itself it is noteworthy that insulin release is stimulated by calcium at the very low glucose concentration of 25 mg/100 ml. The hormone responses to increases in calcium were biphasic, however, with a few exceptions. Thus the calcium concentration of
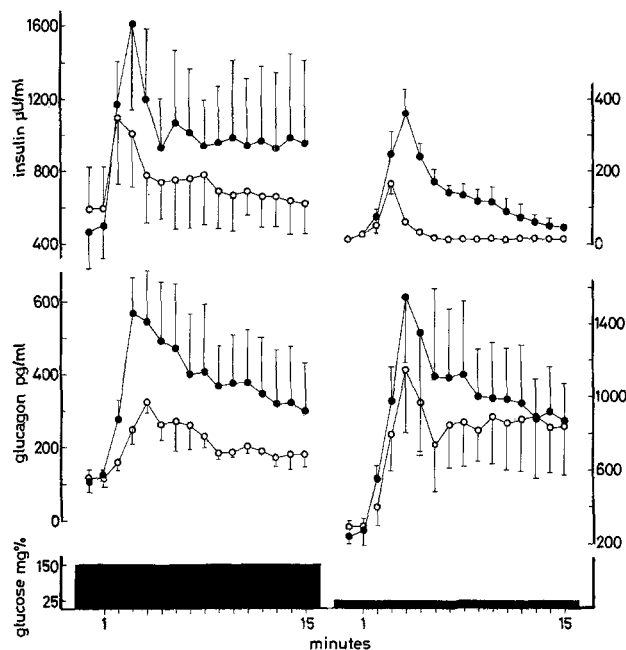

Fig. 2. Mean values (± SD) of insulin and glucagon concentrations during the stimulation periods of the individual experiments, described in legend to Figure 1. o——o Ca^{++} 4.8 mM, •——• Ca^{++} 8.2 mM

4.8 mM, and in one experiment out of three also the calcium concentration of 8.2 mM, elicited insulin release in a more uniphasic pattern at the low glucose concentration; the same was also seen in one experiment out of three with the calcium concentration of 4.8 mM, at glucose concentrations of 150 mg/100 ml. Glucagon responses to high Ca^{++} concentration appear to be biphasic, although less pronounced than the insulin responses. Mean values (± SD) of the stimulation periods of the individual experiments are given in Figure 2. Because of the uniformity and the magnitude of the individual responses n only equalled three.

2. Effect of a Ca^{++} Rich Perfusate upon Hormone Responses to Changes in Perfusate Glucose

To study the importance of Ca^{++} for the normal responses of glucagon and insulin to changes in perfusate glucose from 25 mg/100 ml to 150 mg/100 ml and back, as outlined above, the effect of the extreme situations of a 'calcium free' medium (0.18 mM ± 0.01 (SEM), n = 10) and a Ca^{++} rich medium (8.2 mM) was investigated for periods of 1–2 hours respectively (Fig. 3). A 'Ca^{++} free' medium inhibited release of both hormones. The high basal levels of glucagon, normally encountered during perfusion with a low glucose concentration of
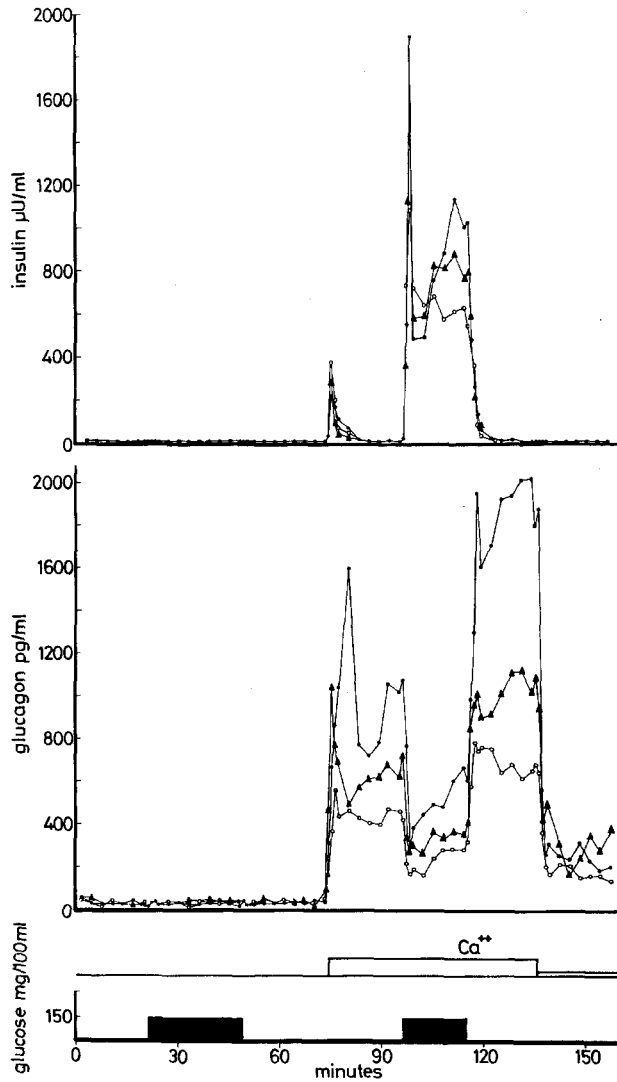
Fig. 3. Effect of a 'Ca^{++} free' and a Ca^{++} rich (8.2 mM) perfusing medium upon the regulatory role of glucose upon glucagon and insulin secretion. At the end of the experiment the Ca^{++} concentration was changed to 1.3 mM. The curves depict 3 experiments. When insulin values are identical only one curve has been shown
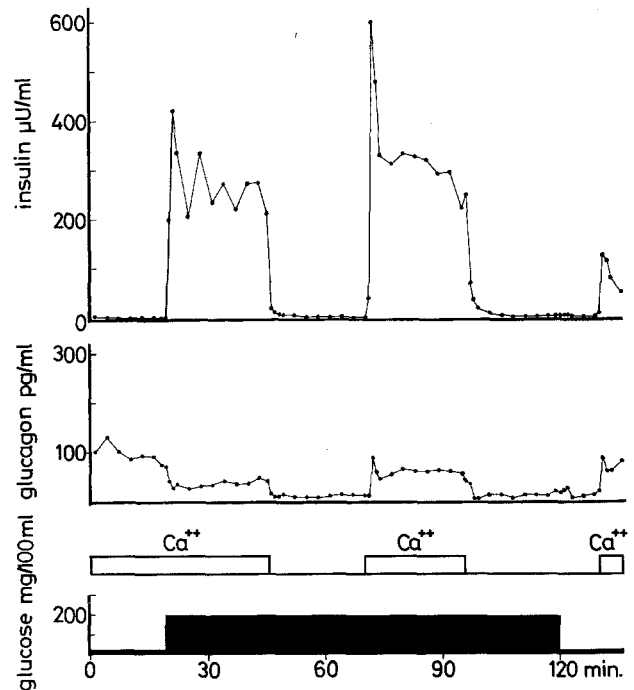

Fig. 4. Effect of depletion and repletion of calcium (2 mM) upon the secretion of glucagon and insulin during perfusion with a glucose concentration of 200 mg/100 ml. A representative experiment out of 3. In two other experiments the effect of calcium depletion was investigated three times in each experiment
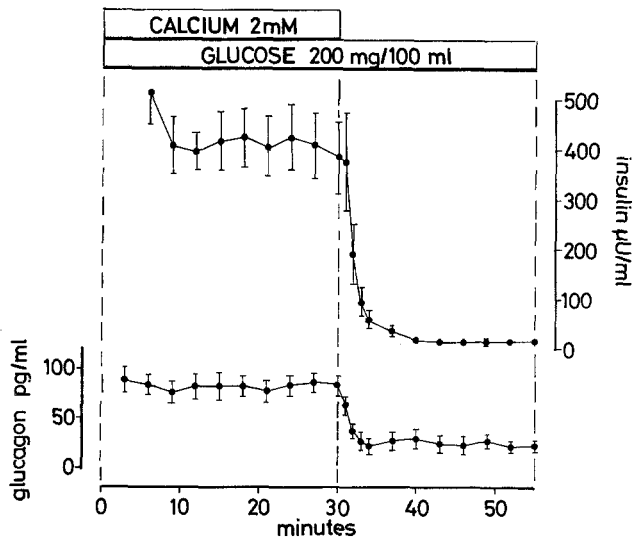

Fig. 5. Effect of depletion of Ca^{++} (2 mM) upon the secretion of glucagon and insulin during perfusion with a glucose concentration of 200 mg/100 ml. Mean values ± SEM of 8 experimental situations obtained from 3 perfusion experiments, twice in one experiment (Fig. 4) and thrice in two other experiments

25 mg/100 ml, were absent. In addition no further inhibition could be detected when the glucose concentration was increased to 150 mg/100 ml, which would normally inhibit release. Although the glucagon values were low, the detection limit of our assay would easily allow lower values to be detected. Changing the glucose concentration back to 25 mg/100 ml still had no effect on the A cell function. Thus the extremely low glucagon output during Ca^{++} deprivation appeared autonomous, under no control by its main signal, glucose. In agreement with the known effect of glucose and Ca^{++} upon the pancreatic B cell, glucose induced insulin release

was completely absent during Ca^{++} deprivation. Addition of Ca^{++} to the perfusion medium to a final concentration of 8.2 mM revived the function of both pancreatic cell types. At the low glucose concentration of 25 mg/100 ml both hormones were
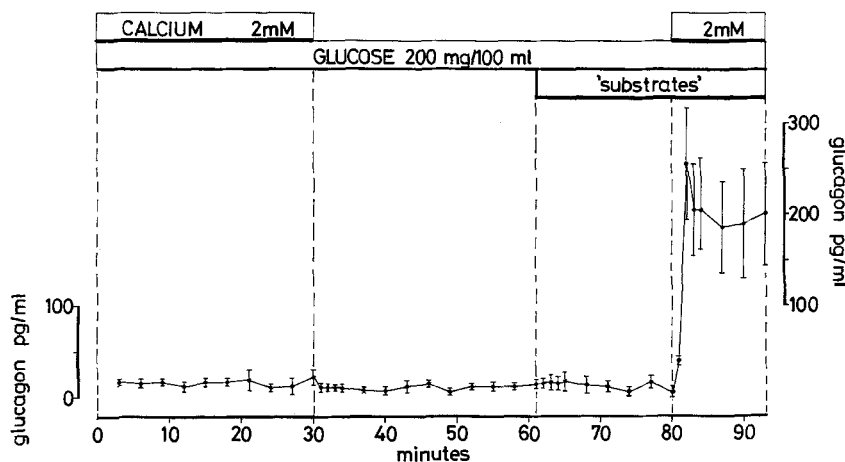
Fig. 6. Effect of depletion of Ca^{++} (2 mM) upon the secretion of glucagon during perfusion with a glucose concentration of 200 mg/100 ml in the absence of the substrates, glutamate, fumarate and pyruvate. Later in the experiments the effect of these substrates during Ca^{++} depletion and finally the effect of Ca^{++} repletion in the presence of these substrates. Mean values ± SEM of 3 experiments

stimulated; glucagon release increasing in a biphasic pattern to levels above the ones seen at normal Ca^{++} concentrations (1.3–2.6 mM), insulin release exhibiting a single phase release, even at this non-stimulatory concentration of glucose. When the glucose concentration was increased to 150 mg/100 ml, glucagon release was suppressed; however, the levels obtained were higher than the levels obtained at low concentrations of glucose at a normal Ca^{++} concentration and much higher than the levels obtained with a 'Ca^{++} free' medium. Insulin release was enhanced in a typical biphasic pattern. Changing the glucose concentration back to 25 mg/100 ml stimulated glucagon to high levels and decreased insulin to levels attained prior to the high glucose infusion. Changing the calcium concentration to 1.3 mM abruptly decreased the release of glucagon to a level normally observed at a low, non-inhibitory glucose concentration of 25 mg/100 ml. Data from the individual experiments are recorded in Figure 3.

3. Effect of Sudden Ca^{++} Depletion and Repletion During Perfusion with a Constant Glucose Concentration

To study the interrelationship of calcium and glucose for the release of glucagon another experimental protocol was pursued. A representative experiment is given in Figure 4. During perfusion with a constant concentration of glucose of 200 mg/100 ml at a Ca^{++} concentration of 2.0 mM the effect of depletion and repletion of this Ca^{++} concentration upon glucagon and insulin release was investigated for sequential periods of about 30 minutes. Upon discontinuation of the Ca^{++} infusion at this high glucose concentration release of both glucagon and insulin ceased abruptly, with very low levels, which remained low until readdition of the same concentration of Ca^{++} to the perfusion medium. Glucagon

was thereupon released in a biphasic pattern to reach levels characteristic of those observed at inhibitory glucose concentrations, and insulin was stimulated following a typical biphasic release pattern. The protocol of sudden Ca^{++} depletion, using the same infusion periods as outlined in Figure 3, was repeated 8 times, twice in one experiment (Fig. 4) and thrice in two other experiments. Mean results of the individual test situations are depicted in Figure 5.

The effect of sudden calcium depletion was also studied in three experiments in the absence of glutamate, pyruvate and fumarate, at a glucose concentration of 200 mg/100 ml. Upon discontinuation of the calcium infusion release of glucagon fell in one experiment ($+Ca^{++}$ 26.0 ± 3.3 (n = 8) versus $-Ca^{++}$ 15.8 ± 1.4 (n = 13)), and was slightly, although insignificantly, reduced in two experiments (11.1 ± 2.7 (n = 9) versus 8.5 ± 1.3 (n = 13), 11.9 ± 2.1 (n = 9) versus 8.8 ± 1.2 (n = 14)). Addition of glutamate, fumarate and pyruvate did not change release significantly. However, when calcium was again added glucagon release increased abruptly. At no time did glucose at a concentration of 200 mg/100 ml cause an increase in glucagon release during perfusion with a calcium depleted medium. Mean results of the experiments are illustrated in Figure 6.

Discussion

In the present investigation results are reported on the importance of Ca^{++} for normal secretory function of the pancreatic A cell. The study has shown 1. that supraphysiological concentrations of Ca^{++} stimulate a dose-related secretion of both glucagon and insulin, both being modified by the glucose concentration of the medium 2. that the presence of

Ca^{++} is necessary for the normal regulatory role of glucose for glucagon release and 3. that glucagon release, inhibited by high concentrations of glucose, will be instantaneously further suppressed, when the perfusion medium is suddenly depleted of Ca^{++}. Thus calcium is an essential requirement for the normal secretory process of pancreatic glucagon, as already established for the release of insulin.

Some divergence in results has been reported concerning the stimulatory effect of calcium upon the release of glucagon. Boettger et al. [15], using intraduodenal infusion of calcium in dogs, and Kuzuya et al. [16], using injection in the pancreatic artery of calcium, also in dogs, reported failure to affect glucagon release. Such studies are not necessarily at variance with those of the present study, since the increases in ionized serum calcium were small and variable — about 10% — in the first mentioned study and may have been too short lived in the last mentioned one. In recent reports Lundquist et al. [17], using the isolated, perfused rat pancreas and Fujimoto et al. [18], using a monolayer cell culture of newborn rat pancreas, have reported enhanced glucagon release in response to increases in the calcium concentration, findings which corroborate the results of the present study.

More controversy exists concerning the importance of the presence of calcium for the normal release of glucagon and for the normal regulatory role of glucose for glucagon release. Using islets from the Syrian hamster, Nonaka et al. [19] reported that lack of calcium did not affect glucagon release in response to glucose. However, since glucagon release in these collagenase treated islets failed to be modified by quite a number of substances which have later been shown to influence release, the negative results may suggest an altered responsiveness of this in vitro system during the isolation procedure.

Leclercq-Meyer et al. [5] reported that lack of calcium enhanced glucagon release from incubated slices of rat pancreas and, in more recent studies using an isolated, perfused rat pancreas [8–9] they confirmed this result and further, noted a paradoxical stimulation of glucagon to glucose when the perfusion medium was depleted of calcium. Also Wollheim et al. [10], apart from showing enhanced glucagon release to raised calcium levels, reported results indicating an enhanced glucagon release during calcium deprivation in a monolayer cell culture of fetal rat pancreas. The results of these studies are in marked contradiction to the findings of the present study.

The reason for the controversial results is unknown. In the present study we have shown that insulin release in the absence of Ca^{++} decreased immediately to almost zero values and that it could not be stimulated by even high glucose concentrations. Readdition of Ca^{++} revived normal function of both the B and the A cell. In the first paper, Leclercq-Meyer et al. [5] reported that the inhibition of insulin output during calcium deprivation was very slight. This may mean that the time required to deplete the tissue of Ca^{++} is very long in this preparation, as pointed out by Gerich et al. [7]. In the 1975 paper [8] the insulin effect of Ca^{++} depletion was more marked, though a considerable insulin output did occur, and it is interesting to note that this output greatly surpassed that obtained in the presence of a normal Ca^{++} concentration at a glucose concentration of 150 mg/100 ml, where insulin output was almost zero. Since Gerich et al. [7], also using a perfusion of the rat pancreas, reported that glucagon release in response to arginine was dependent upon the presence of Ca^{++}, it appears that a difference in species could hardly be an explanation for the contrasting results.

The results of Wollheim et al. [10] are also at variance with ours. Since our normal perfusion medium contains glutamate, fumarate and pyruvate, and since that of Malaisse et al. and that of Wollheim et al. does not, this difference in perfusion medium might be an explanation. However, in the absence of these substrates no inverse relationship between glucagon and glucose could be demonstrated. Ca^{++} depletion and high glucose thus did not stimulate glucagon release, but rather, although insignificantly, slightly reduced the output. The addition of glutamate, pyruvate and fumarate, which are thought to supply the cells with more easily available substrates of oxygenation, did not stimulate glucagon release by itself but appeared to have a permissive effect upon the A cell. The reason for the lower glucagon levels in the experiments where glutamate, fumarate and pyruvate were absent is thought to be due to the length of time during which the pancreas has been deprived of more easily available energy. The same explanation applies for the higher levels of glucagon obtained in response to Ca^{++} 2 mM, when glutamate, fumarate and pyruvate are added late in the experiments. The higher level thus reflects the response of cells whose energy requirements have been more 'optimally' provided. Our findings demonstrate that the presence or absence of these substrates in our perfusion medium also cannot account for the different results.

The site and the mode of action of calcium in secretory processes have not been established. However, it is known that glucose-stimulated insulin release is associated with accumulation of calcium within the pancreatic islets [4, 20]. Based on the

analogy between stimulus secretion coupling in the B cell and excitation contraction coupling in muscle, Malaisse et al. [21] postulated that calcium could trigger insulin release by activating a contractile, microtubular microfilamentous system involved in the migration and extrusion of secretory granules [21]. In the present study we have shown that the presence of Ca^{++} is essential also for the normal secretory process of pancreatic glucagon, and it may be anticipated that this involves calcium uptake and accumulation as established for the B cell, calcium thereupon acting similarly in regulating glucagon secretion. Our recent findings that the calcium antagonist Verapamil inhibits glucagon release [22] and that a high calcium concentration can overcome somatostatin inhibited glucagon release (submitted for publication), as previously shown for somatostatin induced inhibition of insulin release [23], are further evidence in favour of a calcium dependency of glucagon release.

Since Ca^{++} affects glucagon and insulin release in the same direction as shown in the present study, it is unlikely that the pertubations of glucagon and insulin encountered in human diabetes mellitus of the juvenile type, which is characterized by high or relatively high glucagon and low insulin levels, can be accounted for by defects in the calcium activation of the microtubular microfilamentous system of these two types of cells.

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