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# Glucose-stimulated sequestration of $Ca^{2+}$ in clonal insulin-releasing cells

## Evidence for an opposing effect of muscarinic-receptor activation

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Net fluxes of $Ca^{2+}$ and acid production were studied in clonal insulin-releasing cells (RINm5F) by using colour indicators and dual-wavelength spectrophotometry. After equilibration with a medium containing 10–20 $\mu$M-$Ca^{2+}$, only minimal amounts of $Ca^{2+}$ (0.08 mmol/kg of protein) were released from the cells by subsequent additions of the respiratory blocker antimycin A and the $Ca^{2+}$ ionophore A23187. The presence of 20 mM-glucose resulted in an almost 5-fold increase of the acid production and in a stimulated net uptake of $Ca^{2+}$. The latter process was independent of the extracellular $Ca^{2+}$ concentration and reached saturation after $20 \pm 1$ min, when it corresponded to $1.18 \pm 0.07$ mmol of calcium/kg of protein. Whereas the thiol reagent iodoacetamide suppressed the acid production, interference with mitochondrial function by using antimycin A or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone had the opposite effect. The latter two drugs induced a selective release of $Ca^{2+}$ from a pool containing 35% of that taken up during glucose exposure. Most of the remaining $Ca^{2+}$ was liberated by A23187 or iodoacetamide. Carbamoylcholine was also selective in mobilizing glucose-stimulated calcium, but this calcium (17%) appeared to originate from the pool insensitive to mitochondrial poisons. The action of carbamoylcholine was blocked by atropine and did not depend on the presence of extracellular $Na^+$. The opposite effects of glucose and muscarinic-receptor activation on a non-mitochondrial calcium pool are consistent with participation of the endoplasmic reticulum in the glucose-induced sequestration of $Ca^{2+}$ in pancreatic $\beta$-cells.

## INTRODUCTION

Glucose induces a net uptake of $Ca^{2+}$ when clonal insulin-releasing RINm5F cells are incubated in a medium containing micromolar concentrations of extracellular $Ca^{2+}$ (Gylfe *et al.*, 1983). Since the sugar does not appear to depolarize these cells, and depolarization with $K^+$ had no detectable effect on the net fluxes of $Ca^{2+}$, it was concluded that the uptake was due to intracellular calcium sequestration. This concept was further substantiated by the observation that the rate of uptake was independent of the extracellular $Ca^{2+}$ concentration and that glucose lowered the cytoplasmic $Ca^{2+}$ activity (Rorsman *et al.*, 1983).

An intricate problem in the attempts to understand how glucose affects the intracellular sequestration of calcium in the pancreatic $\beta$-cells is the stimulatory action of the sugar on phosphoinositide turnover (Fex & Lernmark, 1972; Freinkel *et al.*, 1975; Clements & Rhoten, 1976; Best & Malaisse, 1983, 1984; Laychock, 1983; Montague *et al.*, 1985). In view of the proposed analogies between the effects of glucose and cholinergic agonists in promoting the formation of inositol 1,4,5-trisphosphate, it is pertinent that this water-soluble compound has been found to mobilize intracellular calcium from permeabilized RINm5F cells (Biden *et al.*, 1984; Joseph *et al.*, 1984). However, despite the reported similarities between the effects of nutrients and cholinergic agents on phospholipid metabolism, several studies have revealed that the two types of insulin secretagogues differ considerably with regard to their effects on the fluxes of various ions, including $Ca^{2+}$ (Nenquin *et al.*, 1984; Matthias *et al.*, 1984; Hellman & Gylfe, 1986). It is therefore likely that promotion by glucose of phospholipid breakdown represents a phenomenon not sufficiently pronounced to overcome the intracellular sequestration of calcium induced by the sugar.

In the present study the interactions between glucose and the acetylcholine analogue carbamoylcholine have been analysed in $Ca^{2+}$-depleted RINm5F cells by measuring the net fluxes of $Ca^{2+}$ under conditions allowing simultaneous recording of the acid production. We show that the sugar-stimulated uptake of $Ca^{2+}$ is a saturable process and that muscarinic-receptor activation results in mobilization of $Ca^{2+}$ incorporated in response to glucose.

## MATERIALS AND METHODS

### Materials

Reagents of analytical grade and deionized water were used. The $Ca^{2+}$ ionophore A23187 was from Boehringer Mannheim G.m.b.H., Mannheim, Germany, Phenol Red from E. Merck, Darmstadt, Germany, digitonin from Calbiochem-Behring, La Jolla, CA, U.S.A., and arsenazo III from Fluka A.G., Buchs, Switzerland. Sigma Chemical Co., St. Louis, MO, U.S.A., provided Hepes, EDTA, EGTA, antimycin A, oligomycin, CCCP and carbamoylcholine. Statens Veterinärmedicinska Anstalt, Uppsala, Sweden, supplied RPMI 1640 culture medium, and Hanks' balanced salt solution and trypsin were

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

obtained from Gibco, Paisley, Scotland, U.K. NU-SERUM was supplied by Collaborative Research Inc., Lexington, MA, U.S.A., and foetal bovine serum, penicillin and streptomycin were from Flow Laboratories, Irvine, Scotland, U.K. Nunclon culture flasks (200 ml) were bought from Nunc A/S, Kamstrup, Denmark.

### Cell culture

The studies were performed with the insulin-producing rat cell line RINm5F (Oie *et al.*, 1983). The cells were grown in 200 ml Nunclon flasks containing 25 ml of RPMI 1640 medium supplemented with 10% NU-SERUM, 1% foetal bovine serum, 100 units of penicillin/ml and 100 $\mu$g of streptomycin/ml. Only flasks with a confluent cell layer were accepted for the experiments. The cells were detached by incubation for 3 min at 37 °C with 2 ml of Hanks' balanced salt solution (pH 7.4) supplemented with 2.5 mg of trypsin/ml and 10 mg of EDTA/ml. At the end of the enzyme treatment the cells were suspended by gentle shaking, and 10 ml of the test medium used for the subsequent incubation was added to dilute the enzyme. After pelleting and two successive washes, the cells were resuspended in 1 ml of test medium.

### Measurement of net $Ca^{2+}$ fluxes and $H^+$ production

Net fluxes of $Ca^{2+}$ in the RINm5F cells were estimated from changes of the concentration of $Ca^{2+}$ in the test medium containing 125 mM-NaCl, 5.9 mM-KCl, 25 mM-Hepes, 20 $\mu$M-arsenazo III, 20 $\mu$M-Phenol Red, as well as approx. 12 mM-$Na^+$ added as NaOH to give a final pH

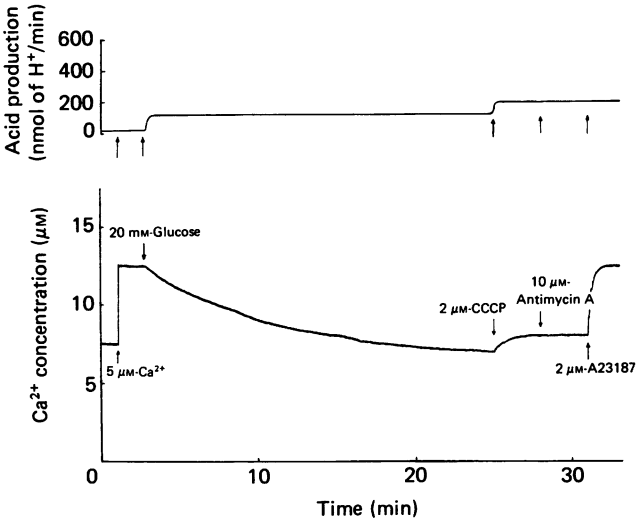


**Fig. 1. Glucose stimulation of net uptake of $Ca^{2+}$ and acid production in RINm5F cells**

The cells (4.0 mg of protein) were suspended in 1 ml of medium containing 20 $\mu$M-arsenazo III and 20 $\mu$M-Phenol Red. The presence of these indicators enabled the simultaneous monitoring of $Ca^{2+}$ activity and pH by recording the absorbance differences $A_{675}-A_{685}$ and $A_{499}-A_{525}$ respectively. Acid production was calculated from the amounts of NaOH required to keep pH constant at 7.40. The absolute concentration of $Ca^{2+}$ was determined by EGTA titration (results not shown). The increases in concentrations obtained after the additions of the different test substances are indicated.

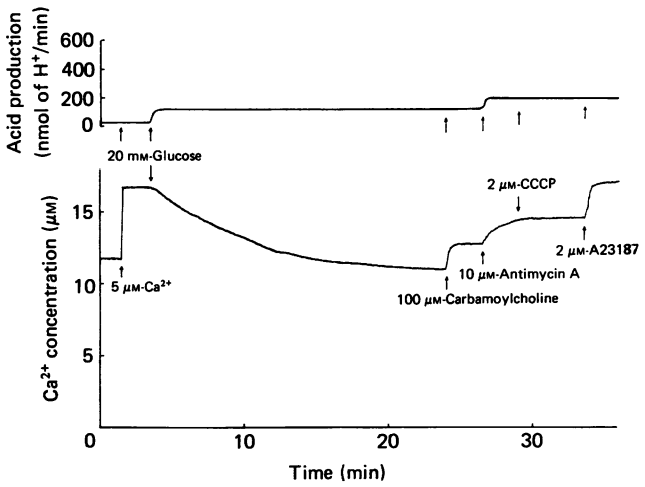


**Fig. 2. Carbamoylcholine-induced release of glucose-incorporated $Ca^{2+}$ from RINm5F cells**

The experiments were performed as described in the legend to Fig. 1, but with instead a cell suspension equivalent to 6.0 mg of protein/ml.

of 7.4. A $Na^+$-free version of this medium was obtained by replacing $Na^+$ with $K^+$ (Fig. 6). The suspension of cells (4–10 mg of protein/ml) was transferred to a disposable standard cuvette (10 mm path length) placed in the thermostatically controlled (37 °C) cuvette holder of a time-sharing multichannel spectrophotometer (Chance *et al.*, 1975). The $Ca^{2+}$ concentration and pH were monitored simultaneously by measuring the absorbance differences $A_{675}-A_{685}$ of the metallochromic indicator arsenazo III and $A_{499}-A_{525}$ of the pH indicator Phenol Red respectively. The presence of the $Ca^{2+}$-insensitive pH indicator made it possible to compensate for and quantify the production of $H^+$ equivalents by the cells by automated 5 nl additions of NaOH. It was thus possible to measure net fluxes of $Ca^{2+}$ without the interference of a changing pH, which varied by less than $\pm 0.01$ unit. To minimize dilution of the cell suspension, additions were made as concentrated solutions. Glucose (2 M) was dissolved in the same type of medium as used for the incubation. NaOH (2 M), $Ca^{2+}$ (10 mM) and EGTA (10 mM) were dissolved in water, whereas the other test substances were 1000-fold concentrated in dimethyl sulphoxide. Details about the experimental procedure are given in the Figure legends. After the experiments the cell suspensions were frozen and retained for measurements of protein (Udenfriend *et al.*, 1972).

### Evaluation of results

Statistical significances were calculated from the differences between paired test and control data by using Student's *t* test. Results are given as means $\pm$ S.E.M.

## RESULTS

Each experiment was preceded by a titration of extracellular $Ca^{2+}$ with EGTA, followed by additions of known amounts of $Ca^{2+}$. In Figs. 1–6 the last of these additions are shown. During this initial period of 5–10 min, the cells lost $Ca^{2+}$ and reached equilibrium, with no net fluxes of the ion. At this time the cells contained only small amounts of calcium, since sequential

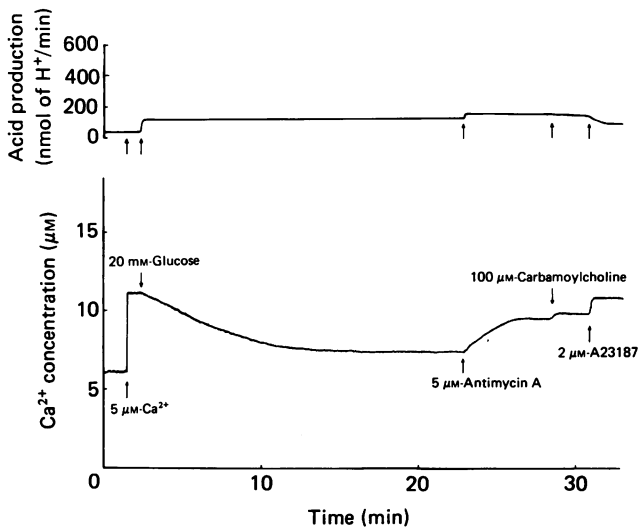


**Fig. 3. Carbamoylcholine-induced release of glucose-incorporated $Ca^{2+}$ from RINm5F cells after respiratory arrest**

The experiments were performed as described in the legend to Fig. 1, but with instead a cell suspension equivalent to 6.2 mg of protein/ml.

additions of the mitochondrial poison antimycin A and the $Ca^{2+}$ ionophore A23187 released as little as $0.03 \pm 0.02$ and $0.05 \pm 0.01$ mmol of calcium/kg of protein ($n = 7$) respectively, and digitonin permeabilization had no further effect.

Addition of 20 mM-glucose stimulated $Ca^{2+}$ uptake (Figs. 1–4). Independently of the extracellular $Ca^{2+}$ concentration (4–20 μM), this process reached saturation after $20 \pm 1$ min ($n = 24$), when it corresponded to $1.18 \pm 0.07$ mmol of calcium/kg of protein ($n = 24$). Some of this calcium ($34.7 \pm 4.5\%$; $n = 6$) was released in the presence of the uncoupler CCCP or antimycin A, without additive effects when the drugs were combined. Most of the remaining calcium incorporated in response to glucose ($57.3 \pm 9.0\%$; $n = 6$) was lost after exposure to A23187. The thiol reagent iodoacetamide had a similar but considerably slower effect (Fig. 4).

In a series of experiments like that in Fig. 2, carbamoylcholine was found to release calcium equivalent to $16.7 \pm 2.7\%$ ($n = 14$) of that taken up in response to glucose. This calcium appeared to originate essentially from pools insensitive to mitochondrial poisons. Subsequent interference with mitochondrial function still liberated $31.3 \pm 3.5\%$ ($n = 14$), whereas the fraction lost after the final exposure to A23187 was now decreased to $33.0 \pm 3.5\%$ ($n = 14$). Further evidence for a non-mitochondrial origin of the carbamoylcholine-sensitive calcium was forthcoming from the observation that there was some mobilization by muscarinic-receptor activation even after exposure to antimycin A (Fig. 3). Carbamoylcholine had no effect on the net fluxes of $Ca^{2+}$ before glucose exposure (Fig. 4), or when the concentration of the drug was doubled by a second addition after the subsequent glucose effect had reached saturation. The carbamoylcholine-induced mobilization of glucose-incorporated calcium was blocked by atropine (Fig. 5), but it was not affected when the $Na^+$ of the medium was replaced with $K^+$ ( Fig. 6). In the latter situation glucose still stimulated the net uptake of $Ca^{2+}$.

The production of $H^+$ equivalents increased from $8.0 \pm 1.0$ to $37.6 \pm 2.7$ mmol/min per kg of protein ($n = 22$; $P < 0.001$), when 20 mM-glucose was added to the RINm5F cells. On addition of antimycin A, there was a further increase, to $47.0 \pm 3.5$ mmol of $H^+$/min per kg of protein ($n = 17$; $P < 0.005$), whereas CCCP had no additional effect. However, CCCP also increased the acid production when added before antimycin A (Fig. 1). In the presence of iodoacetamide the $H^+$ production gradually decreased, being virtually abolished after 10 min (Fig. 4). Exposure to carbamoylcholine in the presence of glucose did not affect the acid production in six experiments, where the observation period was sufficient to allow accurate measurements.

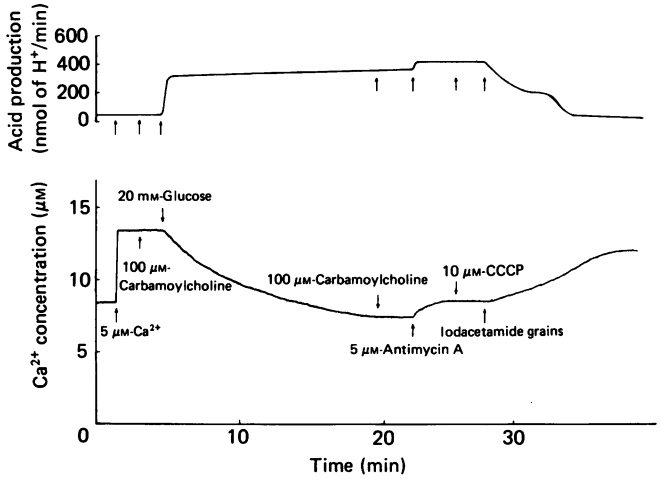


**Fig. 4. Lack of carbamoylcholine effect on net fluxes of $Ca^{2+}$ in RINm5F cells before glucose exposure**

The experiments were performed as described in the legend to Fig. 1, but with instead a cell suspension equivalent to 6.4 mg of protein/ml.

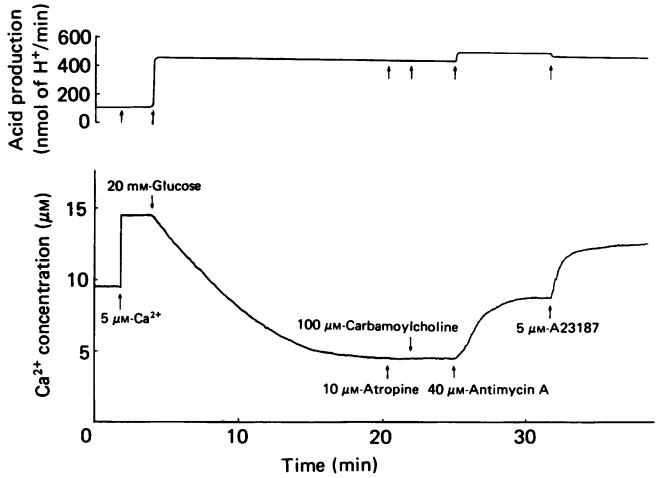


**Fig. 5. Atropine blockade of carbamoylcholine-induced release of glucose-incorporated $Ca^{2+}$ from RINm5F cells**

The experiments were performed as described in the legend to Fig. 1, but with instead a cell suspension equivalent to 6.5 mg of protein/ml.

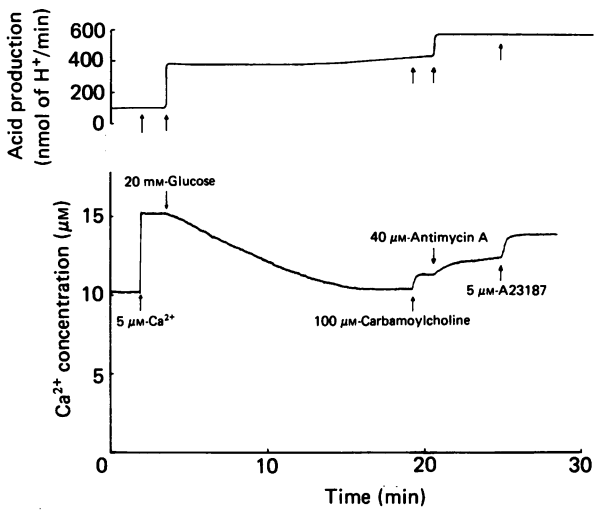


**Fig. 6. Carbamoylcholine-induced release of glucose-incorporated $Ca^{2+}$ from RINm5F cells incubated in the absence of extracellular $Na^+$**

The experiments were performed as described in the legend to Fig. 1, but with instead a cell suspension equivalent to 9.5 mg of protein/ml.

## DISCUSSION

The cell line RINm, which originates from a transplantable rat islet-cell tumour, secretes both insulin and somatostatin (Gazdar *et al.*, 1980). However, among the subclones it has been possible to isolate one (RINm5F) that secretes almost exclusively insulin (Oie *et al.*, 1983). The RINm5F cells exhibit typical tumour features, such as a high glycolytic flux and a glucose oxidation/utilization ratio as low as 0.05–0.15 (Gylfe *et al.*, 1983). As indicated from the present measurements of acid production in 20 mM-glucose, glycolysis was even higher than in the previous studies. Recent measurements of glucose utilization have confirmed the enhanced glycolytic flux (E. Gylfe, P.-O. Berggren & P. Rorsman, unpublished work). The increase in glucose-stimulated acid production obtained with antimycin A exposure cannot only be due to enhanced lactate output after blocking oxidative degradation of the sugar, since the uncoupler CCCP had a similar effect. An alternative explanation is that the inhibited ATP production stimulates glycolysis via the Pasteur effect (Krebs, 1972). It has been suggested that $HCO_3^-/Cl^-$ and $Na^+/H^+$ antiporters are important for the regulation of intracellular pH in $\beta$-cells (Pace *et al.*, 1983). However, there are probably additional mechanisms, since the RINm5F cells were able to expel protons in the absence of both $HCO_3^-$ and $Na^+$.

The RINm5F cells have been found to release insulin in response to $K^+$ depolarization and a number of secretagogues (Gylfe *et al.*, 1983; Praz *et al.*, 1983). Despite the rapid metabolism of glucose, the sugar had little or no effect on insulin release. Studies of $^{45}Ca$ movements indicate that the deficient secretory response to glucose reflects an inability of the sugar to induce opening of the voltage-dependent $Ca^{2+}$ channels (Abrahamsson *et al.*, 1984). However, as in normal $\beta$-cells, there is an inhibitory component in the action of glucose on the $^{45}Ca$ efflux. This inhibition can be attributed to intracellular sequestration of calcium, since the sugar decreases the cytoplasmic $Ca^{2+}$ activity (Rorsman *et al.*, 1983) and increases the net uptake of the ion (Gylfe *et al.*, 1983).

In the present study it was possible to deplete the cells of most calcium, by lowering the $Ca^{2+}$ concentration in a phosphate-free medium to approx. 10 $\mu$M, as judged from the amounts releasable with antimycin A and A23187 before glucose exposure. With the $Ca^{2+}$ deprivation a situation developed which favoured the analysis of the action of glucose on the sequestration of the ion. An important aspect of the glucose-stimulated net uptake of $Ca^{2+}$ is that it reaches saturation after 20 min. Since there seems to be a similar time-course of this depolarization-independent phenomenon in normal $\beta$-cells (Gylfe, 1982), there may be a causal relationship between decreasing $Ca^{2+}$ sequestration and the slowly increasing second phase of glucose-stimulated insulin release. The relatively small magnitude of the net uptake explains why it is difficult to demonstrate effects of glucose on the total content of $\beta$-cell calcium (Andersson *et al.*, 1982; Wolters *et al.*, 1982).

$Ca^{2+}$ release by uncouplers and A23187 has been used to differentiate between mitochondrial and total cellular pools of calcium (Bellomo *et al.*, 1982; Joseph *et al.*, 1983). However, uncouplers such as CCCP are protonophores and should obliterate $H^+$ gradients also in organelles such as secretory granules and lysosomes. The $\beta$-cell granules are rich in calcium, and, as in chromaffin granules (Burger, 1984), the $Ca^{2+}$-accumulating capacity has been suggested to be related to the presence of a proton gradient (Hellman *et al.*, 1979). To ascertain selectivity for the mitochondria, we have therefore also used the respiratory blocker antimycin A. However, it was not possible to enhance the maximal effects of either antimycin A or CCCP by combining the drugs, possibly because the RINm5F cells are sparsely granulated (Gylfe *et al.*, 1983).

After labelling *in situ* of $\beta$-cell-rich mouse islets with $^{45}Ca$, most of the glucose-incorporated radioactivity could be recovered from fractions containing secretory granules and mitochondria (Kohnert *et al.*, 1979; Andersson, 1983). The present data confirm the importance of the mitochondria. However, despite the few secretory granules in the RINm5F cells, glucose stimulated the net sequestration of $Ca^{2+}$ preferentially in non-mitochondrial pools. It is easy to underestimate the participation of pools other than those in mitochondria and granules, if the organelles involved are particularly susceptible to damage during homogenization. Since the RINm5F cells are incubated at micromolar concentrations of $Ca^{2+}$, and the uptake rate is independent of the extracellular concentration of the ion (Gylfe *et al.*, 1983), it can be expected that the sequestration represents high-affinity uptake by the organelles. Introduction of glucose after initial exposure to antimycin A caused only little uptake of $Ca^{2+}$ (results not shown), indicating that sequestration in non-mitochondrial pools also depends on mitochondrial function. Nevertheless, ATP production from glycolysis was apparently sufficient to maintain the calcium already present in the non-mitochondrial pools, since the mitochondrial poisons only released approx. 35% of the $Ca^{2+}$ taken up in response to glucose. Iodoacetamide, however, which also inhibits glycolysis, liberated all $Ca^{2+}$.

Using $\beta$-cell-rich mouse islet perifused with $Ca^{2+}$-deficient medium, we have demonstrated that muscarinic-receptor activation results in a pronounced initial, and then a delayed and more protracted, mobilization of $^{45}Ca$ (Hellman & Gylfe, 1986). The second phase of $^{45}Ca$ efflux, which followed from $Na^+$ influx, was inhibited by glucose. However, pre-exposure to glucose was a prerequisite for the initial efflux, which was probably accounted for by formation of inositol 1,4,5-trisphosphate. It was not possible to decide from those studies whether glucose increased the availability of the inositol 1,4,5-trisphosphate precursor or if the released radioactivity had been incorporated in response to the sugar.

As in mouse $\beta$-cells, the carbamoylcholine-induced release of $Ca^{2+}$ from the RINm5F cells could be attributed to muscarinic-receptor activation inhibited by atropine. Being rapid and insensitive to omission of extracellular $Na^+$, this net loss of $Ca^{2+}$ corresponded to the initial phase of $^{45}Ca$ efflux from the mouse islets. Moreover, the presence of glucose was necessary for the effect. Although it can be expected that glucose is important also for the function of the muscarinic-receptor activation pathway, the present data clearly demonstrate that carbamoylcholine releases $Ca^{2+}$ incorporated in response to the sugar. The size of the carbamoylcholine-sensitive pool thus corresponded to twice the total amount of calcium in the RINm5F cells before exposure to glucose. The observation that glucose and carbamoylcholine have opposite actions on a common intracellular pool of calcium is difficult to reconcile with the idea that intracellular mobilization of $Ca^{2+}$ from the inositol 1,4,5-trisphosphate-sensitive compartment is a factor also in nutrient-stimulated insulin release (Biden *et al.*, 1984). An interesting possibility is that the glucose-induced phospholipid breakdown results in formation of inositol 1,3,4-triphosphate (Berridge & Irvine, 1984). The measurements made so far do not discriminate between this isomer and the $Ca^{2+}$-mobilizing inositol 1,4,5-trisphosphate.

Cholinergic-receptor activation increases insulin release from both normal $\beta$-cells (Gagerman *et al.*, 1978; Nenquin *et al.*, 1984) and RINm5F cells (Prentki *et al.*, 1984). Whereas cholinergic drugs alone have little effect on the secretory process, the stimulatory action of glucose is considerably potentiated (Gagerman *et al.*, 1978; Nenquin *et al.*, 1984). From the concept that glucose stimulates the incorporation of the carbamoylcholine-sensitive $Ca^{2+}$, it follows that pre-exposure to the sugar is sufficient for a subsequent cholinergic mobilization of $Ca^{2+}$ (Hellman & Gylfe, 1986). In fact acetylcholine was found to induce initial release of insulin in the presence of only 2.8 mM-glucose when rat pancreatic islets had been previously incubated in a culture medium containing 8.3 mM of the sugar (Wollheim *et al.*, 1980).

$Ca^{2+}$ released by carbamoylcholine appeared to be of non-mitochondrial origin. The fact that pre-exposure to antimycin A decreased the action of carbamoylcholine should not be taken as indicating that this respiratory blocker had liberated $Ca^{2+}$ from the same pool. The inositol 1,4,5-trisphosphate signal-transduction mechanism is very sensitive to metabolic inhibitors (Berridge & Irvine, 1984). In rat insulinoma (Biden *et al.*, 1984) as well as in other cells (Berridge & Irvine, 1984) the endoplasmic reticulum is believed to be the source of calcium sensitive to inositol 1,4,5-trisphosphate. The significance of this pool is apparent from the fact that it corresponds to almost 17% of the total glucose-stimulated net uptake of calcium. However, a considerable fraction of the non-mitochondrial calcium taken up in response to glucose was insensitive to carbamoylcholine. Since it seems unlikely that the few secretory granules can account for all this calcium, further studies are needed to clarify both the location of the pools involved and how they are regulated by glucose.

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