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Characterization of the inositol 1,4,5-trisphosphate-induced Ca^{2+} release in pancreatic β-cells

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Pancreatic β-cells isolated from obese-hyperglycaemic mice released intracellular Ca^{2+} in response to carbamoylcholine, an effect dependent on the presence of glucose. The effective Ca^{2+} concentration reached was sufficient to evoke a transient release of insulin. When the cells were deficient in Ca^{2+}, the Ca^{2+} pool sensitive to carbamoylcholine stimulation was equivalent to that released by ionomycin. Unlike intact cells, cells permeabilized by high-voltage discharges failed to generate either inositol 1,4,5-trisphosphate ($InsP_3$) or to release Ca^{2+} after exposure to carbamoylcholine. However, the permeabilized cells released insulin sigmoidally in response to increasing concentrations of Ca^{2+}. Also in the absence of functional mitochondria these cells exhibited a large ATP-dependent buffering of Ca^{2+}, enabling the maintenance of an ambient Ca^{2+} concentration corresponding to about 150 nM even after several additional pulses of Ca^{2+}. $InsP_3$, maximally effective at 6 μM, promoted a rapid and pronounced release of Ca^{2+}. The $InsP_3$-sensitive Ca^{2+} pool was rapidly filled and lost its Ca^{2+} late after ATP depletion. The transient nature of the Ca^{2+} signal was not overcome by repetitive additions of $InsP_3$. It was possible to restore the response to $InsP_3$ after a delay of approx. 20 min, an effect which had less latency after the addition of Ca^{2+}. These latter findings argue against degradation and/or desensitization as factors responsible for the transiency in $InsP_3$ response. It is suggested that Ca^{2+} released by $InsP_3$ is taken up by a part of the endoplasmic reticulum (ER) not sensitive to $InsP_3$. On metabolism of $InsP_3$, Ca^{2+} recycles to the $InsP_3$-sensitive pool, implying that this pool indeed has a very high affinity for the ion. The presence of functional mitochondria did not interfere with the recycling process. The ER in pancreatic β-cells is of major importance in buffering Ca^{2+}, but $InsP_3$ only modulates Ca^{2+} transport for a restricted period of time following immediately upon its formation. Thereafter the non-sensitive part of the ER takes over the continuous regulation of Ca^{2+} cycling.

INTRODUCTION

It is well established that hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is the primary event subsequent to activation of a wide variety of cell-surface receptors (Berridge & Irvine, 1984). Such a hydrolysis leads to the formation of diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$), the latter mobilizing Ca^{2+} from an intracellular pool, most likely the endoplasmic reticulum (ER) (Berridge & Irvine, 1984). Until recently it has been thought that $InsP_3$ is dephosphorylated in various steps, eventually resulting in free inositol reacting with a metabolite of diacylglycerol to form phosphatidylinositol, a precursor of PtdIns(4,5)P_2 (Michell, 1975; Berridge & Irvine, 1984). However, with the demonstration of cyclic inositol phosphates as well as the inositol tris-/tetrakis-phosphate pathway, it is evident that phosphoinositide turnover represents a much more complicated series of events (Wilson *et al.*, 1985*a*,*b*; Biden & Wollheim, 1986; Irvine *et al.*, 1986). The ultimate physiological implication of these findings is not clear, and so far only the biological role for $InsP_3$ has been studied in more detail.

In a number of publications dealing with permeabilized clonal insulin-producing RINm5F cells as well as microsomal fractions from a rat insulinoma, the $InsP_3$-induced Ca^{2+} release has been thoroughly characterized (Biden *et al.*, 1984; Prentki *et al.*, 1984*a*, 1985). However, owing to limited amounts of tissue, it has not been possible to perform a similar characterization in a suspension of mouse β-cells. In the present study this problem was overcome by taking advantage of the Uppsala colony of obese–hyperglycaemic mice, containing hyperplastic islets comprising up to 90% β-cells (Hellman, 1965). Thus it was possible to characterize the $InsP_3$-induced Ca^{2+} release in a suspension of electrically permeabilized β-cells under conditions where their insulin-secretory machinery remained intact.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, and redistilled deionized water was used. Boehringer Mannheim supplied collagenase, and ionomycin was from Calbiochem. $InsP_3$ and [2-^{3}H]inositol were obtained from Amersham. Crystalline rat insulin was a gift from Novo A/S, and ^{125}I-labelled insulin was a gift from Farbwerke Hoechst. Bio-Gel P4 polyacrylamide beads were from Bio-Rad, Richmond, CA, U.S.A., and NU-serum® was purchased from Collaborative Research, Bedford, MA, U.S.A. The capillary tubing used for making the electrodes was obtained from Federick Haer and Co., Brunswick, ME, U.S.A., and the calcium cocktail was supplied by Fluka. All other chemicals were from Sigma.

Abbreviations used: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; $InsP_3$, inositol trisphosphate; ER, endoplasmic reticulum.

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Animals and preparation of cells

Adult obese-hyperglycaemic mice (*ob/ob*) of both sexes were taken from a local non-inbred colony (Hellman, 1965) and starved overnight. The animals were killed by decapitation, and the islets were isolated by a collagenase technique. A cell suspension was prepared essentially as described by Lernmark (1974). Briefly, the islets were dissociated into single cells and small clusters by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium supplemented with EGTA. Thereafter the cells were incubated at 37 °C, pH 7.4, overnight in RPMI 1640 medium supplemented with 10% (v/v) NU-serum®, 100 i.u. of penicillin/ml, 60 μg of gentamycin/ml and 100 μg of streptomycin/ml. To avoid attachment of the cells to the culture flask during incubation, the suspension was gently shaken.

Media

The medium used for isolation of islets, studies of cytoplasmic Ca^{2+} concentration, insulin release and formation of inositol phosphates in intact cells was a Hepes buffer, pH 7.4, physiologically balanced in cations with Cl^- as the sole anion (Hellman, 1975) and supplemented with 1 mg of bovine serum albumin/ml. For the studies of permeabilized cells a Hepes buffer, pH 7.0 (adjusted with KOH), was used; this contained 110 mM-KCl, 10 mM-NaCl, 2 mM-KH_2PO_4, 1 mM-$MgCl_2$ and 0.5 mg of bovine serum albumin/ml. All additions to the media are stated under their respective sections in the Materials and methods section or in the legends to Figures and Table 1.

Measurements of cytoplasmic free Ca^{2+} concentration

Cell suspensions were incubated for 45 min with 5 μM-quin 2 acetoxymethyl ester, which gave a quin 2 loading of approx. 0.40 ± 0.08 nmol/10^6 cells (mean ± S.E.M. for eight experiments). This value was obtained from calculations based on fluorescence maximum and extracellular quin 2 values at the beginning of each experiment, assuming that 1 mg dry wt. corresponds to 3.6×10^6 cells (Lernmark, 1974). The suspensions were washed twice in a Ca^{2+}-deficient buffer at 37 °C and resuspended in 1.5 ml of similar medium in a 1 cm-light-path polystyrene cuvette. To lower the Ca^{2+}concentration further, 0.5 mM-EGTA was added. Measurements were performed at 37 °C in an Aminco–Bowman spectrofluorimeter, slightly modified to allow constant stirring. The excitation and emission wavelengths were 340 and 490 nm respectively. Calibration was done essentially as described by Hesketh *et al.* (1983). The recordings shown are typical for experiments performed with at least three different cell preparations.

Cell permeabilization and measurements of the ambient Ca^{2+} concentration

After being washed twice in cold buffer, the cell suspension (0.3 ml) was permeabilized by exposure to high-voltage discharges, i.e. five pulses of 2.5 kV/cm (Knight & Baker, 1982). This treatment resulted in more than 99% permeabilized cells, as judged by Trypan Blue uptake. After permeabilization the cells were centrifuged, and the resulting pellet was suspended in 25 μl of the actual buffer supplemented with 2 mM-MgATP and an ATP-generating system consisting of 10 mM-phosphocreatine and 20 units of creatine kinase/ml. In the experiments represented by Fig. 7(*a*) the incubation medium was supplemented with 5 mM-succinate and mitochondrial inhibitors were omitted, whereas in all other experiments the mitochondrial Ca^{2+} transport was blocked by 0.2 μM-antimycin and 1 μg of oligomycin/ml. The cell suspension was stirred magnetically and the Ca^{2+} concentration in the medium measured with a Ca^{2+}-selective mini-electrode constructed and calibrated essentially as described by Tsien & Rink (1981). All experiments were performed at room temperature. Test substances were added with constant-volume pipettes, made in accordance with a previous description (Hellman *et al.*, 1967). The recordings shown are typical for experiments performed with at least three different cell preparations.

Production of inositol phosphates

The cell suspension was labelled with [2-^{3}H]inositol during a 24 h incubation period, washed twice in appropriate media and either permeabilized or not. In experiments with intact cells, the media were supplemented with 1.28 mM-Ca^{2+}, 20 mM-glucose and 1 mM-inositol. For the studies of permeabilized cells, the media were supplemented with 2 mM-MgATP, 10 mM-phosphocreatine, 20 units of creatine kinase/ml, 100 μM-GTP, 1 mM-inositol, 5 mM-EGTA (these values represent the total added concentration of each substance) and Ca^{2+} to reach a final Ca^{2+} concentration of 1 μM, as checked with the Ca^{2+} electrode before each experiment. The Ca^{2+} concentrations in the experiments with the intact and permeabilized cells were chosen in order to give optimal conditions for production of inositol phosphates. To ensure that the small proportion of intact cells did not contribute to formation of InsP_3, mitochondrial inhibitors were included. After that, the cell suspension was transferred to conical glass tubes (approx. 0.8×10^6 cells/100 μl of the respective buffer) and incubated for 1 min at 37 °C in the presence or absence of 100 μM-carbamoylcholine. The incubation was terminated by addition of 20 μl of trichloroacetic acid (60%, v/v), and, to separate water-soluble inositol phosphates from inositol-containing phospholipids, 2 ml of chloroform/methanol/HCl (200:100:1, by vol.) was added to each cell suspension. After addition of 0.5 ml of redistilled deionized water and mixing, the tubes were centrifuged at 670 *g* for 1 min, to allow separation of the aqueous phase from the lipid-containing chloroform phase. The aqueous phase (1 ml) containing the inositol phosphates was then transferred to new glass tubes and neutralized with 1 M-NaOH. Inositol phosphate, inositol bisphosphate and InsP_3 were separated by ion-exchange chromatography (1 ml column of Dowex 1 X 8; 200–400 mesh, formate form) as described by Berridge *et al.* (1983). The radioactivities of the fractions containing the different inositol phosphates were then determined by liquid-scintillation counting.

Insulin release

The kinetics of insulin release from intact cells were studied by perifusing approx. 10^6 cells, mixed with Bio-Gel P4 polyacrylamide beads (200–400 mesh), in a 0.5 ml column at 37 °C (Kanatsuna *et al.*, 1981). The flow rate was 0.3 ml/min, and 1 min fractions were collected. Insulin release from permeabilized cells were studied by adding the cells to solutions with different concentrations of Ca^{2+}, ranging from 10 nM to 0.1 mM. In this case the

actual buffers were supplemented with 2 mM-MgATP, 1 mg of bovine serum albumin/ml, 5 mM-EGTA and Ca^{2+} in appropriate amounts to give the desired Ca^{2+} concentrations. The equilibrium constants used for calculating these Ca^{2+} concentrations were selected from Martell & Sillén (1971), and the concentration was checked with the Ca^{2+} electrode before each experiment. The permeabilized cells were incubated with gentle agitation for 15 min at 37 °C. After incubation, the cell suspensions were centrifuged and samples were taken from the supernatant. All samples were assayed radioimmunologically for insulin, with crystalline rat insulin as the reference.

Staining of cell suspension

The cell suspensions were pelleted, fixed in Bouin and embedded in paraffin. Sections were stained with the unlabelled-antibody enzyme method for insulin (Sternberger *et al.*, 1970) and examined by light microscopy. It was thereby possible to establish that more than 90% of the cells in the suspensions used stained positively for insulin (Fig. 1).

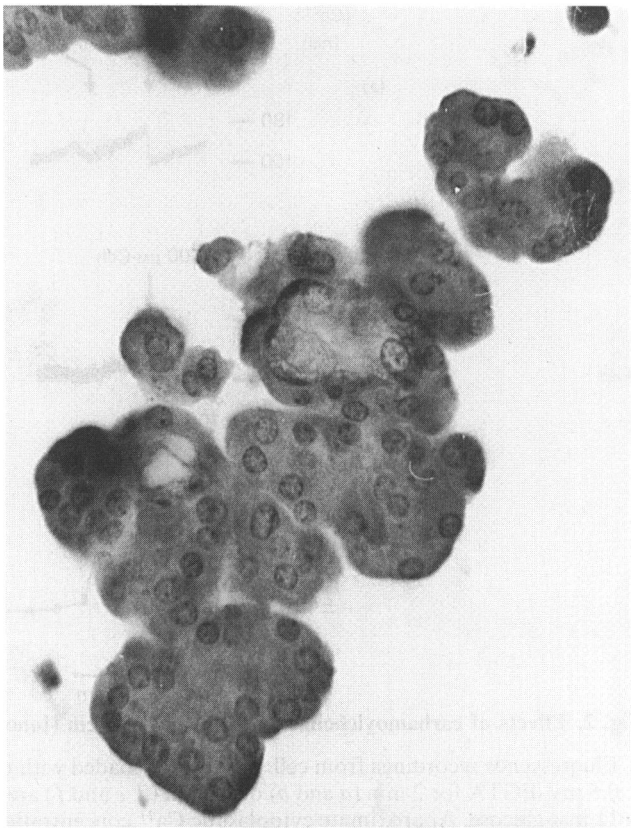

Fig. 1. Appearance of a β-cell suspension after fixation and staining for insulin (dark cells)

Magnification × 540.

RESULTS

Studies on intact cells

Carbamoylcholine stimulated the release of Ca^{2+} from an intracellular pool (Figs. 2*a*, *b* and *d*), as evident from measurements with the quin 2 technique in a Ca^{2+}-free medium. The effect of carbamoylcholine was critically dependent on the presence of the nutrient secretagogue glucose. Consequently, when carbamoylcholine was added after 2 min of incubation in the absence of glucose (Fig. 2*b*), the Ca^{2+} release was smaller than that observed in the presence of the sugar (Fig. 2*a*). After incubation of the cells for 10 min in the presence of glucose (Fig. 2*d*), carbamoylcholine still released Ca^{2+}, whereas the release was abolished if glucose was omitted (Fig. 2*f*). It was checked that the lack of response was not due to increased intracellular concentrations of quin 2, since, in the particular experiments represented by Fig. 2(*f*), the calculated loading was less than 0.40 nmol of quin $2/10^6$ cells. The releasable intracellular pool of Ca^{2+} decreased after prolonged incubation in the absence of extracellular Ca^{2+}. However, even when the preceding incubation period was only 2 min (Fig. 2*a*), ionomycin only evoked a minor Ca^{2+} release after carbamoylcholine stimulation, and there was no effect of the ionophore when the incubation period was extended to 10 min (Fig. 2*d*). In our hands the mouse β-cells released a greater percentage of their intracellular releasable Ca^{2+} pool than do RINm5F cells in response to carbamoylcholine (P. Arkhammar, T. Nilsson, & P.-O. Berggren, unpublished work). Accordingly, the β-cells responded less than the RINm5F cells to a subsequent stimulation by ionomycin (Wollheim & Biden, 1986). After 10 min of incubation in the absence of extracellular Ca^{2+}, ionomycin released similar amounts of Ca^{2+} as did carbamoylcholine, and in this case subsequent addition of carbamoylcholine had no effect (Fig. 2*e*). These data suggest that the Ca^{2+} pool releasable by carbamoylcholine has a very high affinity for the ion. Although the amount of Ca^{2+} released by carbamoylcholine was relatively modest, it was sufficient to evoke a transient release of insulin when the cells were perifused under experimental conditions similar to those in Fig. 2(*a*) (see Fig. 2*c*).

When measuring the formation of inositol phosphates in pancreatic β-cells in response to carbamoylcholine under optimal conditions, i.e. 1.28 mM-Ca^{2+} and 20 mM-glucose, there was a significant increase in all inositol phosphates. The values after stimulation corresponded to 136 (inositol phosphate), 226 (inositol bisphosphate) and 154 ($InsP_3$) % of those obtained in the absence of the agonist (Table 1). Although we did not separate the two isomers of inositol trisphosphate, it seems reasonable to conclude that also in pancreatic β-cells carbamoylcholine releases Ca^{2+} from the ER by the $InsP_3$ pathway. To gain further insight into the mechansims whereby the $InsP_3$-sensitive Ca^{2+} pool regulates Ca^{2+}, a more thorough investigation was performed with permeabilized pancreatic β-cells.

Studies on permeabilized cells

The release of insulin from permeabilized β-cells in response to increasing concentrations of Ca^{2+} was sigmoidal, reaching a maximum at about 100 μM of the ion (Fig. 3). When such cells were incubated at room temperature in the presence of mitochondrial inhibitors, ATP and an ATP-regenerating system, they buffered the ambient Ca^{2+} concentration to a steady-state value of about 140 nM; this differed somewhat between different cell preparations, and for no obvious reason values even as high as 300 nM could be obtained (Figs. 4, 6 and 7*b*). The mean ± S.E.M. for 15 cell preparations was 135 ± 7 nM. The amount of cells added was positively correlated

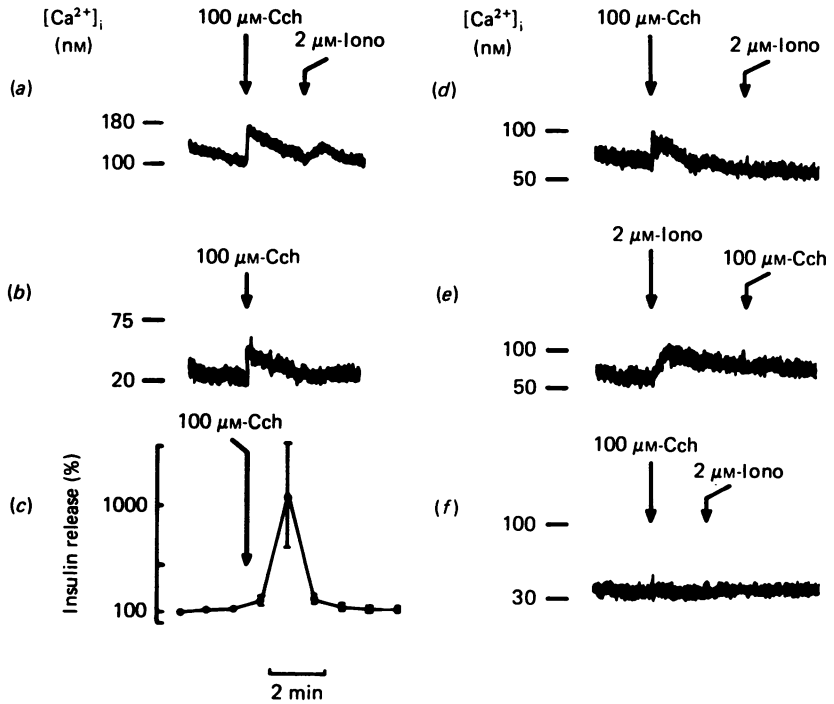

Fig. 2. Effects of carbamoylcholine (Cch) and ionomycin (Iono) on the cytoplasmic free Ca^{2+} concentration and insulin release

Fluorescence recordings from cell suspensions loaded with quin 2 and incubated in a Ca^{2+}-deficient medium supplemented with 0.5 mM-EGTA for 2 min (*a* and *b*) or 10 min (*d*, *e* and *f*) are shown. In (*a*), (*d*) and (*e*) the basal medium was supplemented with 11 mM-glucose. Approximate cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and additions of test substances are given to the left and above each recording, respectively. In (*c*) the effect of carbamoylcholine on insulin release is demonstrated on cells perifused in a Ca^{2+}-deficient medium supplemented with 11 mM-glucose for 10 min and with 0.5 mM-EGTA for a further 2 min before stimulation with carbamoylcholine. The release is expressed as percentage of basal release, the latter being defined as the mean value of the five fractions obtained before addition of carbamoylcholine in each experiment. Results are means ± S.E.M. for three experiments.

Table 1. Effects of carbamoylcholine on the production of inositol phosphates from intact or permeabilized β-cells

Data are expressed as means ± S.E.M. Statistical significances were evaluated by Student's *t*-test and based on paired observations: $*P < 0.05$, $**P < 0.01$.

		Radioactivity bound to inositol phosphates (d.p.m./ 0.8×10^6 cells)			
β-Cells	Incubation condition	Inositol phosphate	Inositol bisphosphate	$InsP_3$	*n*
Intact	Control	2966 ± 1321	353 ± 131	651 ± 215	5
	Carbamoylcholine (100 μM)	4030 ± 1583**	797 ± 210*	1004 ± 250**	5
Permeabilized	Control	1860 ± 179	452 ± 62	520 ± 72	7
	Carbamoylcholine (100 μM)	1836 ± 220	468 ± 50	588 ± 106	7

with the rate of Ca^{2+} uptake, but was of minor importance for the steady-state value, provided that the cell density exceeded approx. 4×10^6 cells/ml. The latter findings indicate that the steady-state value represents the true free Ca^{2+} concentration set by the intracellular buffering systems. Since mitochondrial inhibitors were included, the origin of this buffering system in mouse β-cells, as for example in RINm5F cells, is probably the ER (Prentki *et al.*, 1984*a*,*b*). As is evident from Fig. 4, the cells also buffered several pulses of added Ca^{2+}, indicating a large buffering capacity despite the absence of functional mitochondria. Indeed, the cells did not show any sign of being saturated even after the addition of up to ten 0.25 nmol pulses of Ca^{2+} (results not shown). It was beyond the scope of the present investigation to establish the maximal amounts of Ca^{2+} buffered and what this means in terms of increased total calcium content.

Irrespective of the Ca^{2+} concentration, carbamoylcholine failed to release Ca^{2+} in a suspension of permeabilized cells, even in the presence of up to 1 mM-GTP (results not shown). Since this lack of an effect might be accounted for by dilution of formed $InsP_3$, a

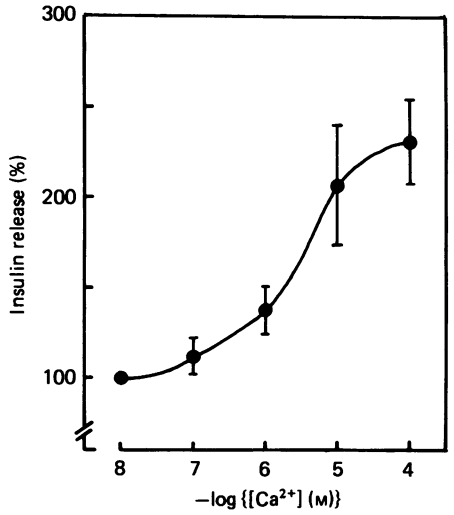

Fig. 3. Insulin release from suspensions of permeabilized β-cells in response to increasing concentrations of Ca^{2+}

The release is expressed as a percentage of that obtained at 10 nM-Ca^{2+}. Results are means ± S.E.M. for seven experiments.

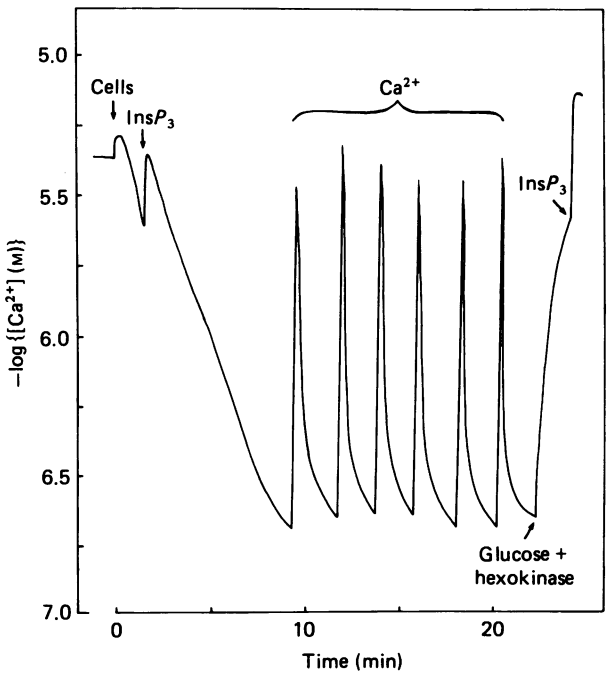

Fig. 4. Effects of $InsP_3$, Ca^{2+} and glucose + hexokinase on the ambient Ca^{2+} concentration in a suspension of permeabilized β-cells

Cells (0.2×10^6) were added at 0 min, and $InsP_3$ (6 μM), Ca^{2+} (0.25 nmol), and glucose (20 mM) + hexokinase (20 units/ml) were added as indicated.

relevant question concerning the permeabilized cells is the extent to which they maintain their capability of responding to agonists by the phospholipase-C-mediated hydrolysis of membrane (poly)phosphoinositides. As shown in Table 1, there was no significant increase in the production of inositol phosphates in the permeabilized β-cells in response to carbamoylcholine. The value after stimulation corresponded to 99 (inositol phosphate), 104 (inositol bisphosphate) and 113 ($InsP_3$) % of those obtained in the absence of the agonist.

Addition of $InsP_3$ to permeabilized cells produced a rapid and transient increase in the ambient Ca^{2+} concentration, as shown in Figs. 4, 6 and 7. This release was dependent on the amounts of $InsP_3$ given. By comparing the effects of different concentrations of $InsP_3$ with that of 6 μM-$InsP_3$ (the concentration giving the maximal effect), a dose–response curve was established. As shown in Fig. 5, this curve was a hyperbola, with a half-maximum at approx. 1 μM. The maximal release corresponded to 2.4 ± 0.2 nmol of Ca^{2+}/mg dry wt. (mean ± S.E.M. for six experiments), as determined by the addition of known amounts of Ca^{2+} giving the same peak height at an ambient Ca^{2+} concentration of 300 nM. The sensitivity to $InsP_3$ is comparable with what has previously been demonstrated in insulin-producing cells and rat islets (Biden *et al.*, 1984; Wolf *et al.*, 1985). To obtain maximal effects, 6 μM-$InsP_3$ was used throughout the present study. Since $InsP_3$ releases a major part of the Ca^{2+} taken up initially (Fig. 4), it is obvious that this Ca^{2+} pool is rapidly filled. Furthermore, cells that had lost most of their accumulated Ca^{2+} after depletion of ATP, by addition of glucose and hexokinase, still released the remaining Ca^{2+} in response to $InsP_3$. These data therefore give further support to the concept that the $InsP_3$-sensitive Ca^{2+} pool has a very high affinity for this ion and is strictly dependent on the presence of ATP. Whether using solutions buffered or not with EGTA, the actual Ca^{2+} concentration ranging between 300 and 800 nM under the latter conditions, we were unable to demonstrate a stimulatory effect of $InsP_3$ on insulin release (results not shown). In the presence of EGTA such a lack of response is obvious, but it is less obvious why $InsP_3$ failed to promote secretion in the absence of chelator. However, in view of the transiency in Ca^{2+} signal induced by $InsP_3$, it might be technically difficult to demonstrate a significant effect on insulin secretion.

Wolf *et al.* (1986) reported that glucose 6-phosphate increased the ATP-dependent ^{45}Ca content of the ER and inhibited the $InsP_3$-induced ^{45}Ca release in digitonin-

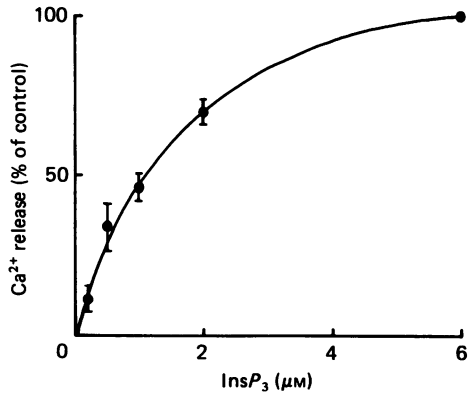

Fig. 5. Dose/response curve for $InsP_3$-induced Ca^{2+} release

The effects of different concentrations of $InsP_3$ are expressed as percentages of that obtained with 6 μM-$InsP_3$. Results are means ± S.E.M. for four to six experiments.

permeabilized rat islets. Furthermore. it was demonstrated that the effect on ^{45}Ca content was mediated by glucose 6-phosphatase localized in the ER. Since glucose-6-phosphatase activity is inhibited by vanadate (Singh *et al.*, 1981), we used vanadate-free MgATP (Sigma). However, even so there was no effect of glucose 6-phosphate on either Ca^{2+} uptake or $InsP_3$-induced Ca^{2+} release under our experimental conditions (results not shown).

Fig. 6 shows that repetitive pulses of $InsP_3$, to compensate for possible $InsP_3$ degradation, failed to maintain high Ca^{2+} values. Hence it is not plausible that the transiency in Ca^{2+} signal is simply due to $InsP_3$ degradation, but rather is of a more complex nature, reflecting desensitization of the relevant $InsP_3$ receptor and/or an uptake of Ca^{2+} into an $InsP_3$-insensitive pool. In accord with this concept, inhibition of $InsP_3$ phosphatase, by Mg^{2+} deficiency or addition of 2,3-bisphosphoglycerate (Downes *et al.*, 1982), was without major effect on the duration of the $InsP_3$-induced Ca^{2+} signal (results not shown). It was possible to recover a Ca^{2+} release if there was a delay of several minutes before addition of a new pulse of $InsP_3$, an effect that can be explained in terms of both increased sensitivity to $InsP_3$ and a recycling of Ca^{2+} into the $InsP_3$-sensitive pool.

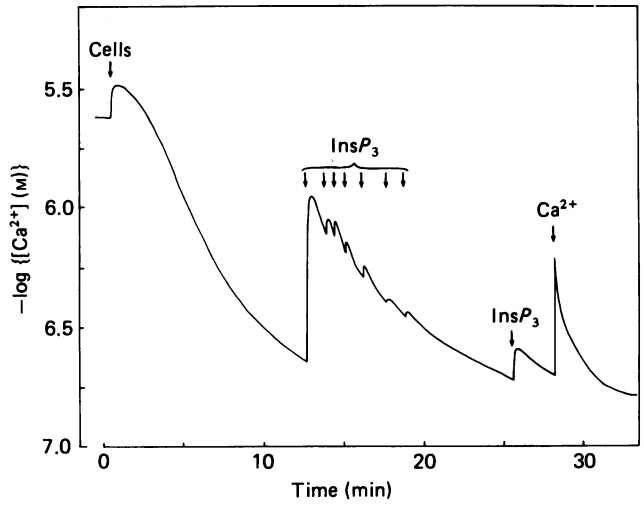

Fig. 6. Effects of repetitive pulses of $InsP_3$ and a pulse of Ca^{2+} on the ambient Ca^{2+} concentration maintained by permeabilized β-cells

Cells (0.16×10^6) were added at zero time, and $InsP_3$(6 μM) and Ca^{2+}(0.125 nmol) were added as indicated.

In Fig. 7 the uptake of Ca^{2+} as well as effects of two consecutive pulses of $InsP_3$ were studied in cell suspensions exposed or not to mitochondrial inhibitors. It is noteworthy that there were no major differences in either buffering capacity or effects of $InsP_3$ under the two conditions. Furthermore, it was possible to recover the $InsP_3$ effect after approx. 20 min also with functional mitochondria, indicating that a possible mitochondrial uptake of Ca^{2+} did not interfere with the Ca^{2+} pool sensitive to $InsP_3$. It is noteworthy that the $InsP_3$-sensitive Ca^{2+} pool could be filled even faster if an additional pulse of Ca^{2+} were given (results not shown).

DISCUSSION

Pancreatic β-cells isolated from obese–hyperglycaemic mice of the Uppsala colony (Hellman, 1965) were found to respond to activation of the muscarinic receptor by carbamoylcholine. This resulted in the formation of $InsP_3$, the mobilization of intracellular Ca^{2+} and a transient release of insulin. In accord with previous studies employing both isolated pancreatic islets and

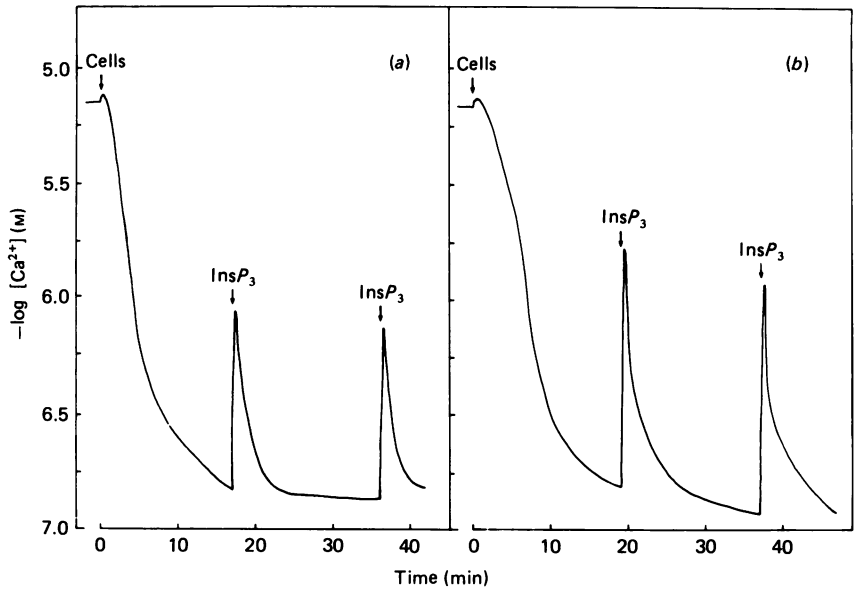

Fig. 7. Effects of $InsP_3$ on the ambient Ca^{2+} concentration maintained by permeabilized β-cells in the absence (*a*) or presence (*b*) of mitochondrial inhibitors

Cells (0.19×10^6 and 0.18×10^6 respectively) were added at zero time, and $InsP_3$ (6 μM) was added as indicated.

clonal insulin-producing RINm5F cells (Gylfe & Hellman, 1986; Hellman & Gylfe, 1986*a*; Hellman *et al.*, 1986*a*), the present study indicates that the Ca^{2+} pool released in the presence of carbamoylcholine was not only labile but also conditional on the presence of glucose, probably reflecting the ultimate need for adequate amounts of ATP. The origin of this Ca^{2+} pool is most likely the ER, and the release of Ca^{2+} is then supposed to be mediated by InsP_3 (Streb *et al.*, 1983; Berridge & Irvine, 1984; Biden *et al.*, 1984; Joseph *et al.*, 1984; Morgan *et al.*, 1985; Wolf *et al.*, 1985; Hellman *et al.*, 1986*b*; Wollheim & Biden, 1986).

In a number of studies, detergents such as digitonin or saponin have been used to permeabilize cells and thereby effectively to by-pass the plasma membrane. However, with these substances not only the plasma membrane but also intracellular membranes may be destroyed. To avoid this type of possible error we used high-voltage discharges, which only permeabilize the plasma membrane (Knight & Baker, 1982). β-Cells permeabilized by this procedure maintained their ability to release insulin in response to increasing concentrations of Ca^{2+}. The dose/response curve for insulin release was sigmoidal and resembled that previously obtained in permeabilized pancreatic islets (Yaseen *et al.*, 1982; Colca *et al.*, 1985; Jones *et al.*, 1985; Tamagawa *et al.*, 1985). The fact that maximal release was obtained at the fairly high Ca^{2+} concentration of 100 μM seems somewhat strange, since such a concentration is probably never reached within the cytoplasm during physiological conditions. However, it cannot be ruled out that there is a decrease in Ca^{2+} sensitivity after permeabilization, owing to loss of a modulating intracellular factor(s). Such a loss might also explain why the permeabilized β-cells were unable to form InsP_3 in response to carbamoylcholine. Under conditions where the Ca^{2+}-sensitivity is increased, for example by activating protein kinase C, insulin release was obtained at lower Ca^{2+} concentrations (Jones *et al.*, 1985; Tamagawa *et al.*, 1985).

When incubated in a medium containing mitochondrial inhibitors, ATP and an ATP-regenerating system, the permeabilized β-cells rapidly lowered the ambient Ca^{2+} concentration to a steady-state value of about 140 nM, which is in good agreement with what has previously been reported for other cells including clonal insulin-producing RINm5F cells (Biden *et al.*, 1984; Prentki *et al.*, 1985). Since microsomal fractions from rat insulinomas were able to lower the ambient Ca^{2+} concentration to the same extent as permeabilized RINm5F cells (Prentki *et al.*, 1984*a*,*b*), it is most likely that the ER is responsible for the actual buffering of Ca^{2+}. As in other cells, the uptake of Ca^{2+} in the β-cells was strictly dependent on the presence of ATP, and a shortage of this nucleotide led to a rapid loss of the ion. On the other hand, the presence of ATP enabled the β-cells to buffer large quantities of added Ca^{2+}.

From observations that glucose 6-phosphate increased the ATP-dependent ^{45}Ca content of the ER and abolished the InsP_3-induced ^{45}Ca release, Wolf *et al.* (1986) have suggested that glucose 6-phosphate can serve as an 'off' signal for insulin release by lowering the intracellular Ca^{2+} concentration in pancreatic β-cells. In the present study we were unable to confirm this report. In addition, if decreased ^{45}Ca efflux reflects a lowering of cytoplasmic Ca^{2+} concentration, it should be noted that not only glucose but also other nutrients elicit such an effect (Hellman & Gylfe, 1986*b*). Moreover, it is unlikely that glucose 6-phosphate impairs the response to InsP_3, since the presence of glucose was a prerequisite for maintaining the Ca^{2+} pool releasable by carbamoylcholine.

The InsP_3-induced Ca^{2+} release resembled that obtained in, for example, RINm5F cells in terms of both rapidity and size (Biden *et al.*, 1984; Prentki *et al.*, 1985). The fact that Ca^{2+} release evoked by InsP_3 was faster than that evoked by ATP depletion and occurred also in the presence of an ATP trap suggests that InsP_3 does not interfere with the influx component of Ca^{2+}. It is noteworthy that continuous presence of InsP_3 has been reported to maintain high Ca^{2+} values in saponin-permeabilized RINm5F cells, under conditions where mitochondrial Ca^{2+} uptake was inhibited (Prentki *et al.*, 1985). These results were interpreted as suggesting that InsP_3 degradation, rather than desensitization to InsP_3 or the presence of an InsP_3-insensitive compartment, might account for the transient nature of the InsP_3-induced Ca^{2+} response. This pattern was not observed in mouse β-cells, which failed to maintain high Ca^{2+} values in response to repetitive pulses of InsP_3. The fact that neither Mg^{2+} deficiency nor the presence of 2,3-bisphosphoglycerate, conditions known to block InsP_3 phosphatase (Downes *et al.*, 1982), affected the InsP_3-induced Ca^{2+} release further supports the view that InsP_3 degradation is not responsible for the transiency of the Ca^{2+} signal. After a certain delay there was a complete restoration of the InsP_3 response, an unexpected effect if desensitization to InsP_3 actually occurred. However, since the InsP_3 response was restored even faster after the addition of Ca^{2+}, it is more likely that the lack of response to an immediate additional pulse of InsP_3 reflects an uptake of Ca^{2+} into a pool not sensitive to InsP_3. After InsP_3 metabolism, Ca^{2+} is then recycled to the InsP_3-sensitive pool, a process taking approx. 20 min at an ambient Ca^{2+} concentration of about 150 nM. The InsP_3-insensitive Ca^{2+} pool might not necessarily be restricted to the ER, but might also be accommodated in other organelles. In this context, it should be recalled that β-cells contain a large number of secretory granules. Although it has been reported that secretory granules isolated from rat insulinomas failed to regulate the Ca^{2+} concentration in the medium (Prentki *et al.*, 1984*b*), we still do not know to what extent results from experiments with such tumour granules reflect the situation in normal β-cells. Since there was virtually no difference in the InsP_3 effect and Ca^{2+} buffering whether or not mitochondrial inhibitors were present, it is not plausible that the mitochondria interfere with the β-cell Ca^{2+} pool sensitive to InsP_3.

It is evident that the ER in pancreatic β-cells serves as a high-capacity Ca^{2+} pool, having a major role in regulating the cytoplasmic Ca^{2+} concentration. To maintain the integrity of the InsP_3-sensitive part of the ER, it is of the utmost importance that this Ca^{2+} pool has a very high affinity for the ion, which indeed was found to be the case. However, InsP_3 is only of direct importance in the modulation of Ca^{2+} transport for a restricted period of time after hydrolysis of PtdIns(4,5)P_2, and then the part of the ER not sensitive to InsP_3, possibly in co-operation with other organelles, takes over the continuous regulation of Ca^{2+} cycling. After InsP_3 degradation, the part sensitive to InsP_3 is re-filled with Ca^{2+}. Future studies should define the extent to which other organelles regulate cytoplasmic Ca^{2+} and conse-

quently also identify possible new modulators of intracellular Ca^{2+} cycling.

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