

A role for calcium in the breakdown of inositol phospholipids in intact and digitonin-permeabilized pancreatic islets

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Glucose (20 mM) and 4-methyl-2-oxopentanoate (10 mM) both caused a pronounced stimulation of insulin release and of [³H]inositol phosphate production in rat pancreatic islets prelabelled with *myo*-[³H]inositol. Secretory responses to these nutrients were markedly impaired by lowering the Ca²⁺ concentration of the incubation medium to 10⁻⁴ M or less, whereas stimulated inositol phosphate production was sensitive to Ca²⁺ within the range 10⁻⁶–10⁻⁴ M. Inositol phosphate formation in response to carbamoylcholine was also found to be dependent on the presence of 10⁻⁵ M-Ca²⁺ or above. Raising the concentration of K⁺ in the medium resulted in a progressive, Ca²⁺-dependent stimulation of inositol phosphate production in islets, although no significant stimulation of insulin release was observed. In islets prelabelled with *myo*[³H]inositol, then permeabilized by exposure to digitonin, [³H]inositol phosphate production could be triggered by raising the Ca²⁺ concentration from 10⁻⁷ to 10⁻⁵ M. This effect was dependent on the concentration of ATP and the presence of Li⁺, and involved detectable increases in the levels of InsP₃ and InsP₂ as well as InsP. A potentiation of inositol phosphate production by carbamoylcholine was observed in permeabilized islets at lower Ca²⁺ concentrations, although nutrient stimuli were ineffective. No significant effects were observed with guanine nucleotides or with neomycin, although NADH produced a modest increase and adriamycin a small inhibition of inositol phosphate production in permeabilized islets. These results strongly suggest that Ca²⁺ ions play an important role in the stimulation of inositol lipid metabolism in islets in response to nutrient secretagogues, and that inositide breakdown may actually be triggered by Ca²⁺ entry into the islet cells.

INTRODUCTION

The stimulation of pancreatic islets with certain types of secretagogue results in enhanced metabolism of inositol-containing phospholipids (for a review, see Best & Malaisse, 1984). In common with a wide variety of tissues which display stimulated phosphoinositide metabolism (Berridge, 1984), the initial event in pancreatic islets appears to be breakdown of the polyphosphoinositides PtdIns4,5P₂ and PtdIns4P (Best & Malaisse, 1985; Laychock, 1983) with the resultant formation of inositol phosphates (Best & Malaisse, 1983*b*) including InsP₃ (Best & Malaisse, 1985; Montague *et al.*, 1985). It has been furthermore reported that InsP₃ is able to mobilize Ca²⁺ sequestered in a preparation of rat insulinoma microsomes (Prentki *et al.*, 1984) and in saponin-permeabilized insulin-secreting tumour cells (Joseph *et al.*, 1984), raising the possibility that stimulated hydrolysis of inositol lipids in islets might, as in a number of other tissues, lead to the mobilization of intracellular calcium stores, particularly from the endoplasmic reticulum (see Berridge, 1984, for review). If this were the case, it might be expected that inositol lipid hydrolysis in islets would not itself be induced by, or dependent upon, increased uptake or mobilization of calcium in the islet cells. Of the agents so far investigated, the principal types of secretagogue known to induce inositol lipid breakdown are nutrients, such as glucose (Best & Malaisse, 1983*b,c*, 1985; Montague *et al.*, 1985; Laychock, 1983) and

4-methyl-2-oxopentanoate (Best & Malaisse, 1983*b,c*, 1985), and neurotransmitter-type stimuli including carbamoylcholine (Best & Malaisse, 1983*a*, 1985; Morgan *et al.*, 1985). The calcium-dependency of these responses have remained poorly defined. It has been reported that stimulated inositol lipid metabolism in islets persists under conditions of calcium-depletion (Montague *et al.*, 1985; Morgan *et al.*, 1985) though at a reduced level (Best & Malaisse, 1983*b*), whereas Laychock (1983), Axen *et al.* (1983) and Rana *et al.* (1985) have reported that glucose-induced breakdown of islet inositol lipids is abolished in the absence of calcium. It seems likely that the above discrepancies may be largely attributable to poorly-defined conditions of calcium-depletion in the incubation media.

In order to define more closely the calcium requirements for stimulated inositol lipid hydrolysis in islets, the latter has been investigated in response to both nutrient (glucose and 4-methyl-2-oxopentanoate) and neurotransmitter (carbamoylcholine) stimuli under conditions where the Ca²⁺ concentration in the incubation media was controlled by the use of Ca²⁺-EGTA buffers. For comparative purposes, insulin secretory responses have been monitored under similar conditions.

In addition, a digitonin-permeabilized islet preparation has been adapted for the study of inositol lipid breakdown in response to increasing concentrations of free Ca²⁺. These two distinct approaches suggest that increased inositol lipid hydrolysis upon stimulation of

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns4,5P₂, phosphatidylinositol 4,5-bisphosphate; InsP, inositol phosphate; InsP₂, inositol bisphosphate; InsP₃, inositol trisphosphate.

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islets is markedly dependent upon the presence of Ca^{2+} in the medium, and that inositol lipid breakdown in permeabilized islets can be induced by concentrations of the cation which might be expected in the cytosol of stimulated islet cells. Thus, increased Ca^{2+} uptake into islet cells, particularly in response to nutrient stimuli, may be a prerequisite for phosphoinositide hydrolysis.

EXPERIMENTAL

Materials

myo-[2- ^3H]inositol and ^{125}I -insulin were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and anti-insulin antibody and human insulin standards from Wellcome. Collagenase (*Clostridium histolyticum*), ATP, NADH, NAD^+ , GTP, guanosine 5'-[γ -thio]triphosphate and guanosine 5'-[β,γ -imido]triphosphate were purchased from Boehringer Mannheim, whilst PtIns, PtdInsP, PtInsP₂, carbamoylcholine, neomycin sulphate, adriamycin hydrochloride, 4-methyl-2-oxopentanoate and *myo*-inositol were supplied by Sigma.

Islet incubations

Pancreatic islets were prepared by collagenase digestion of four pancreases obtained from 200–250 g male or female Wistar rats (Lacy & Kostianovsky, 1967). For measurements of insulin secretion, groups of ten islets were incubated in 1 ml of bicarbonate-buffered medium (Malaisse *et al.*, 1970) containing 0.25% (w/v) bovine serum albumin gassed with O_2/CO_2 (19:1) for 60 min. In some experiments, the Ca^{2+} concentration of the medium was buffered by using Ca^{2+} -EGTA buffers at pH 7.4 (4 mM-EGTA with varying concentrations of CaCl_2 , assuming the dissociation constant of Ca -EGTA to be 7). At the end of this incubation period, 100 μl aliquots of medium were removed and analysed for immunoreactive insulin content by radioimmunoassay (Malaisse *et al.*, 1970). For measurement of inositol lipid hydrolysis in intact islets, groups of 100 islets were preincubated for 2 h at 37 °C in 1 ml of the above bicarbonate medium containing 6 μCi of *myo*-[^3H]inositol. The medium was then removed and the islets washed once in 1.0 ml of medium containing 1 mM-*myo*-inositol (unlabelled) prior to incubation in 0.9 ml of medium containing 5 mM-LiCl and 1 mM-*myo*-inositol. Incubations were started by the addition of 0.1 ml of medium containing test substances. Incubations were terminated by the addition of 1.0 ml of ice-cold trichloroacetic acid (20%, w/v) followed by centrifugation. The supernatant was removed, extracted four times with 5 vol. of water-saturated diethyl ether, mixed with 4 ml of water and applied to a 0.5 ml column of Dowex AG1X8 (200–400 mesh; formate form). The columns were washed with 15 ml of water and the [^3H]inositol phosphates were eluted together with 4 ml of 1.0 M-ammonium formate/0.1 M-formic acid. The samples were counted for radioactivity after the addition of 10 ml of scintillation fluid (Unisolve E; Koch-Light).

For studies of inositol lipid metabolism in permeabilized islets, groups of 100 islets were preincubated with *myo*-[^3H]inositol and washed as described above, then incubated at room temperature for 20 min in 1 ml of bicarbonate medium containing 20 μg of digitonin/ml and 10^{-7} M- Ca^{2+} . In preliminary experiments, it was found that such treatment resulted in the loss of 65–70% of islet lactate dehydrogenase content. This permeabiliz-

ation medium was then removed and replaced by 1 ml of medium containing 120 mM-KCl, 1 mM- MgCl_2 , 21 mM- NaHCO_3 , 5 mM- ATP^{4-} , 5 mM-LiCl, 1 mM unlabelled *myo*-inositol, and varying concentrations of Ca^{2+} (using the Ca^{2+} -EGTA buffer system described above) and/or test substances. The incubations were terminated and labelled inositol phosphates measured as described above. In certain experiments, the inositol phosphates were fractionated with increasing concentrations of ammonium formate as described by Berridge *et al.* (1983), and the lipid-bound radioactivity was extracted from the trichloroacetic acid precipitated pellet with 3 ml of chloroform/methanol/5 M-HCl (200:100:1, by vol.). The extracts were washed twice with 1 ml of 0.1 M-KCl, dried under a stream of N_2 , and re-dissolved in 200 μl of extraction solvent. The different phosphoinositide classes were separated by t.l.c. as described previously (Best & Malaisse, 1985).

A limited number of experiments were performed to investigate insulin release from permeabilized islets. Groups of 20 islets were permeabilized with digitonin as described above, the digitonin-containing medium was removed and replaced with 1 ml of medium containing 120 mM-KCl, 1 mM- MgCl_2 , 21 mM- NaHCO_3 , 5 mM- ATP^{4-} and varying concentrations of Ca^{2+} . Incubations were carried out at either room temperature or 37 °C for 30 min. Aliquots (100 μl) of medium were then taken and assayed for immunoreactive insulin content.

Analysis of the data was essentially similar to that described previously (Best & Malaisse, 1983a). Briefly, the control values for each individual experiment were pooled, and the test values were expressed with respect to the mean of the control values. Statistical significance was ascribed by using Student's *t*-test.

RESULTS

The incubation of islets in the presence of 20 mM-glucose and 10^{-3} M- Ca^{2+} resulted in an approx. 6-fold stimulation of insulin secretion compared with the control values (2.8 mM-glucose, 10^{-3} M- Ca^{2+} ; Fig. 1a). The secretory response was accompanied by a marked enhancement of [^3H]inositol phosphate production in islets prelabelled with [^3H]inositol. The stimulation of insulin secretion by glucose was severely impaired when the Ca^{2+} concentration of the medium was reduced to or below 10^{-4} M (Fig. 1a). Glucose-stimulated [^3H]inositol phosphate production was also impaired by lowering the Ca^{2+} concentration of the medium, although a significant inhibition was only apparent at Ca^{2+} concentrations of 10^{-5} M or less.

A marked stimulation of insulin secretion and [^3H]inositol phosphate production was also observed in islets incubated with 10 mM-4-methyl-2-oxopentanoate (Fig. 1b). Both responses could be severely impaired by lowering the Ca^{2+} concentration of the medium, and again secretion showed a greater dependency on this cation than did stimulated [^3H]inositol phosphate production.

In islets treated with 1 mM-carbamoylcholine, no significant stimulation of insulin release was found with the present 'batch' incubation technique. However, carbamoylcholine was an effective stimulus for [^3H]inositol phosphate production in islets prelabelled with [^3H]inositol (Fig. 1c). This response could be significantly impaired by lowering the Ca^{2+} concentration of the

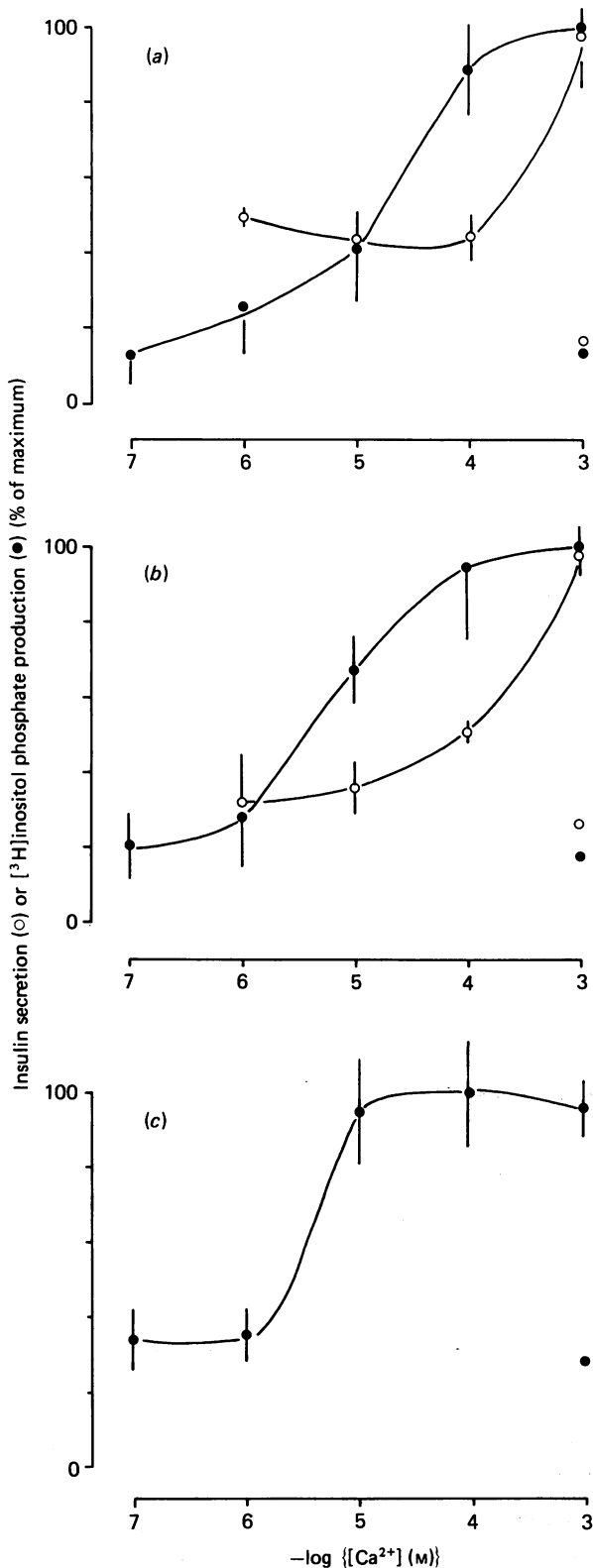


Fig. 1. Dependency on Ca²⁺ concentration of insulin secretion (60 min incubations; ○) and [³H]inositol phosphate production (20 min incubations; ●) in intact pancreatic islets in response to 20 mM-glucose (a), 10 mM-4-methyl-2-oxopentanoate (b) and 1 mM-carbamoylcholine (c)

The isolated points represent the corresponding basal values (2.8 mM-glucose) at 10⁻³ M-Ca²⁺. Each point represents the mean ± S.E.M. of three (inositol phosphates) or six (insulin secretion) determinations.

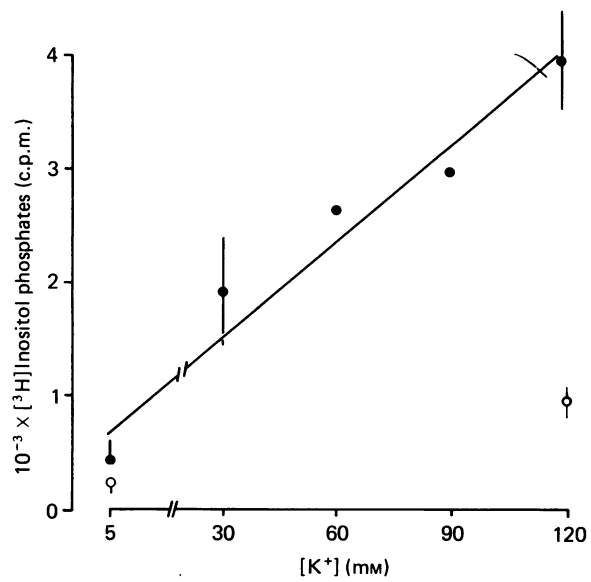


Fig. 2. [³H]inositol phosphate production in intact pancreatic islets in response to increasing concentrations of K⁺ in the presence of 10⁻³ M-Ca²⁺

Incubations were for 20 min. Each point represents the mean ± S.E.M. of two or three separate determinations. ○ represents the corresponding values at 10⁻⁶ M-Ca²⁺.

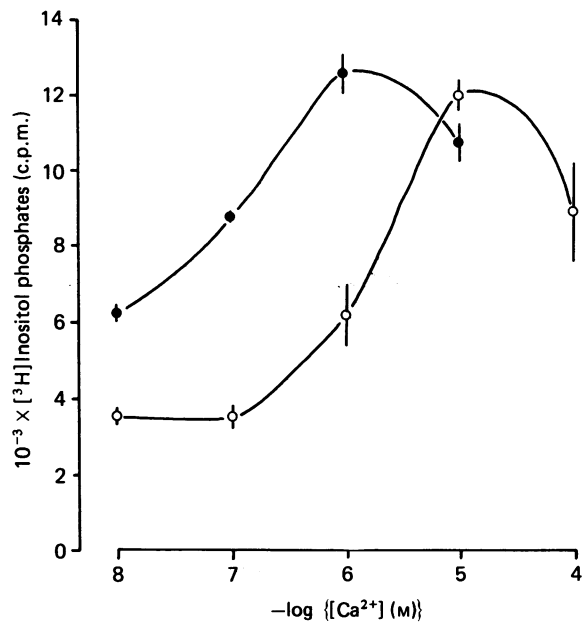


Fig. 3. Effect of increasing concentrations of Ca²⁺ on [³H]inositol phosphate production in digitonin-permeabilized islets in the absence (○) and presence (●) of 1 mM-carbamoylcholine

Incubations were for 20 min. Each point represents the mean ± S.E.M. of three or four separate determinations.

medium to 10⁻⁶ M or below, in contrast with [³H]inositol phosphate production in response to the nutrient secretagogues which was significantly impaired at 10⁻⁵ M-Ca²⁺.

Raising the K⁺ concentration of the incubation medium by substituting for Na⁺ resulted in a concentra-

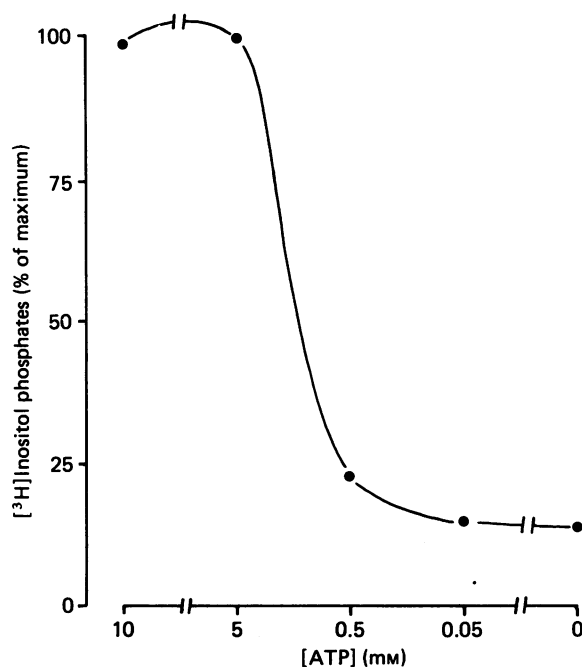


Fig. 4. Dependency on ATP concentration of $[^3\text{H}]$ inositol phosphate production in digitonin-permeabilized pancreatic islets in response to 10^{-5}M-Ca^{2+}

Incubations were for 20 min. Each point represents the mean of duplicate determinations.

tion-dependent stimulation of $[^3\text{H}]$ inositol phosphate production in $[^3\text{H}]$ inositol-labelled islets (Fig. 2). This effect was dependent on the presence of Ca^{2+} (10^{-3}M ; Fig. 2) but was not affected by $10\ \mu\text{M}$ -atropine (results not shown). Treatment with K^+ concentrations up to $125\ \text{mM}$ failed to stimulate significantly insulin secretion from islets during a 60 min incubation.

When islets were prelabelled with $[^3\text{H}]$ inositol and subsequently permeabilized by exposure to $20\ \mu\text{g}$ of digitonin/ml for 20 min, $[^3\text{H}]$ inositol phosphate production was found to be markedly sensitive to Ca^{2+} concentrations. Thus, raising the Ca^{2+} concentrations of the medium from 10^{-7}M to 10^{-5}M resulted in an approx. 3-fold stimulation of $[^3\text{H}]$ inositol phosphate production (Fig. 3). This effect was dependent on the presence of Li^+ in the incubation medium (results not shown). In $[^3\text{H}]$ inositol-labelled permeabilized islets, $1\ \text{mM}$ -carbamoylcholine caused a significant stimulation of $[^3\text{H}]$ inositol phosphate production at lower (10^{-8} – 10^{-6}M) Ca^{2+} concentrations (Fig. 3). However, no additivity was observed with carbamoylcholine at higher concentrations of Ca^{2+} (10^{-5}M) and, in general, the effects of carbamoylcholine in permeabilized islets were of a smaller magnitude than those achieved with intact islets. Neither glucose nor 4-methyl-2-oxopentanoate induced a significant stimulation of $[^3\text{H}]$ inositol phosphate production in permeabilized islets (Table 1).

$[^3\text{H}]$ inositol phosphate production in permeabilized islets incubated in 10^{-5}M-Ca^{2+} showed a striking dependency on the concentration of ATP in the incubation medium (Fig. 4). The separation of $[^3\text{H}]$ inositol phosphates formed during incubation of permeabilized islets at 10^{-5}M-Ca^{2+} revealed a stimulation of formation of InsP_3 and InsP_2 in addition to InsP (Fig. 5). In the

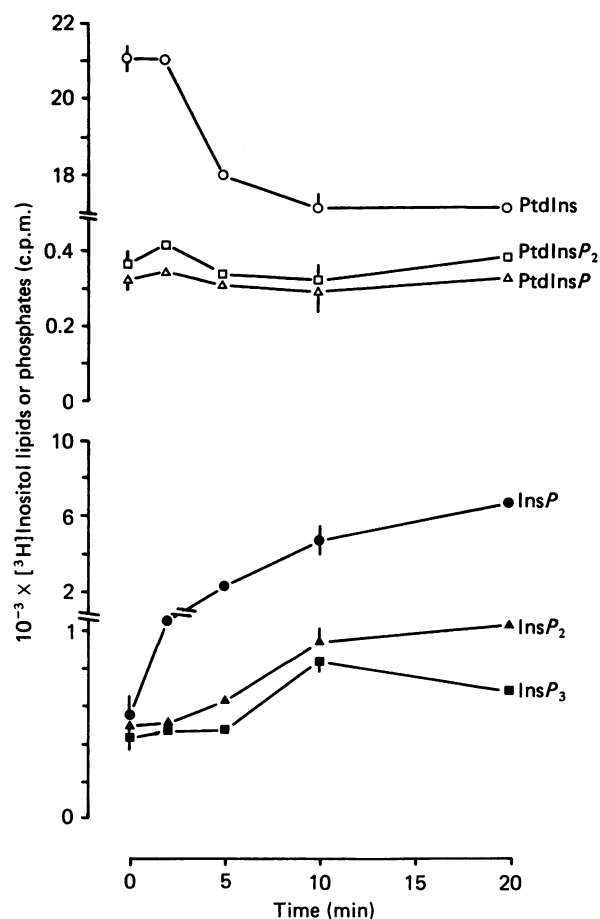


Fig. 5. Effect of 10^{-5}M-Ca^{2+} on levels of $[^3\text{H}]$ inositol phospholipids and $[^3\text{H}]$ inositol phosphates in digitonin-permeabilized islets

Each point represents the mean \pm S.E.M. of two or three separate determinations. Note the split axes.

same experiments, a corresponding depletion of radioactivity associated with PtdIns was noted, there being no major changes in the levels of labelled PtdIns 4P or PtdIns $4,5\text{P}_2$ (Fig. 5).

The effect of guanine nucleotides was examined on $[^3\text{H}]$ inositol phosphate production in permeabilized islets at 10^{-7}M - and at 10^{-5}M-Ca^{2+} (Table 1). Neither GTP ($100\ \mu\text{M}$) nor its non-hydrolysable analogues, guanosine 5'- $[\beta,\gamma\text{-imido}]$ triphosphate and guanosine 5'- $[\gamma\text{-thio}]$ -triphosphate, showed any significant enhancement of $[^3\text{H}]$ inositol phosphate production in either the presence or absence of $1\ \text{mM}$ -carbamoylcholine at either concentration of Ca^{2+} . However, a modest, though statistically significant stimulation of $[^3\text{H}]$ inositol phosphate production was observed in the presence of NADH ($100\ \mu\text{M}$; Table 1). No such effect was found with NAD $^+$.

Adriamycin ($1\ \text{mM}$) was found to inhibit $[^3\text{H}]$ inositol phosphate production in permeabilized islets incubated in 10^{-5}M-Ca^{2+} by approx. 30% (Table 1). A lower concentration of this drug ($100\ \mu\text{M}$) was ineffective, as was neomycin.

The release of insulin from digitonin-permeabilized islets incubated in the presence of 10^{-7}M-Ca^{2+} was found to be $306 \pm 3\ \mu\text{-unit}/30\ \text{min}$ per islet ($n = 3$). This value was not significantly different from that obtained when

Table 1. Effects of various agents on [³H]inositol phosphate production in permeabilized pancreatic islets

Groups of 100 islets were incubated for 2 h with *myo*-[³H]inositol, then permeabilized by exposure to digitonin (20 µg/ml) for 20 min at 10⁻⁷ M-Ca²⁺. The islets were resuspended in high-K⁺ medium containing 5 mM-LiCl, 1 mM-inositol and either 10⁻⁷ M- or 10⁻⁵ M-Ca²⁺. Incubations were for 20 min. Abbreviations: p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate; GTPγS, guanosine 5'-[γ-thio]triphosphate.

Conditions		[³ H]Inositol phosphates (c.p.m.)	<i>n</i>	<i>P</i>
10 ⁻⁷ M-Ca ²⁺				
1	Control	3344 ± 266	16	—
2	10 ⁻³ M-Carbamoylcholine	6262 ± 556	7	< 0.001 versus line 1
3	20 mM-Glucose	3764 ± 459	3	—
4	10 mM-4-Methyl-2-oxopentanoate	3963 ± 515	3	—
5	10 ⁻⁴ M-NADH	5055 ± 967	6	< 0.05 versus line 1
6	10 ⁻⁴ M-NAD ⁺	3222 ± 197	3	—
7	10 ⁻⁴ M-GTP	4166 ± 704	4	—
8	10 ⁻⁴ M-p[NH]ppG	3433 ± 312	8	—
9	10 ⁻⁴ M-GTPγS	2146 ± 404	3	—
10	10 ⁻³ M-Carbamoylcholine + 10 ⁻⁴ M-GTP	6072 ± 104	3	—
11	10 ⁻³ M-Carbamoylcholine + 10 ⁻⁴ M-p[NH]ppG	4972 ± 213	3	—
10 ⁻⁵ M-Ca ²⁺				
12	Control	8875 ± 932	11	< 0.001 versus line 1
13	10 ⁻⁴ M-GTP	7321 ± 1221	3	—
14	10 ⁻⁴ M-p[NH]ppG	9954 ± 1895	5	—
15	10 ⁻⁴ M-GTPγS	8024 ± 1519	3	—
16	10 ⁻³ M-Adriamycin	6024 ± 274	3	—
17	10 ⁻⁴ M-Adriamycin	8077 ± 504	3	—
18	10 ⁻³ M-Neomycin	9775 ± 479	3	—

the Ca²⁺ concentration of the incubation medium was raised to 10⁻⁶ M (251 ± 33; *n* = 3) or 10⁻⁵ M (263 ± 15; *n* = 3).

DISCUSSION

It has been well documented that the secretion of insulin from pancreatic islets, in common with a wide variety of secretory processes, is highly sensitive to Ca²⁺ concentrations in the bathing medium, and is severely impaired when the concentration of the cation is reduced below the physiological level (Grodsky & Bennett, 1966; Milner & Hales, 1967; Malaisse *et al.*, 1978). The present findings concerning stimulated insulin secretion under conditions of controlled Ca²⁺ concentrations are entirely consistent with these earlier results. However, no conclusive data have been published until now concerning the dependency for Ca²⁺ of stimulated inositol lipid hydrolysis in pancreatic islets. When this subject has been investigated in other tissues, at least two types of patterns emerge. Thus, in a number of tissues, including parotid gland (Weiss *et al.*, 1982), exocrine pancreas (Putney *et al.*, 1983) and liver (Creba *et al.*, 1983), stimulated inositol lipid breakdown appears to persist in media depleted of Ca²⁺. In contrast, this process appears to be conspicuously dependent on Ca²⁺ concentration in neutrophils (Cockcroft *et al.*, 1980), mast cells (Cockcroft & Gomperts, 1980) and visceral smooth muscle (Best *et al.*, 1985). The present study provides evidence that stimulated inositol lipid hydrolysis in pancreatic islets is

also sensitive to the concentration of extracellular calcium, though it should be noted that this calcium sensitivity is less than that of stimulated insulin secretion. Thus, glucose-induced insulin secretion was markedly inhibited when the Ca²⁺ concentration of the medium was lowered to 10⁻⁴ M, whereas inositol lipid hydrolysis in response to glucose was only inhibited at Ca²⁺ concentrations below 10⁻⁴ M. A similar pattern was observed using 4-methyl-2-oxopentanoate as stimulus. In contrast, it is interesting to note that inositol lipid hydrolysis stimulated by carbamoylcholine showed a reduced Ca²⁺ sensitivity compared with the above nutrient-type stimuli, being only inhibited below 10⁻⁵ M-Ca²⁺, suggesting that nutrients and neurotransmitter stimuli probably induce inositol lipid hydrolysis by different mechanisms, and might even mobilize distinct pools of phosphoinositide.

This suggestion that Ca²⁺ may play an important role in stimulated inositol lipid metabolism in pancreatic islets in response to nutrient stimuli is substantiated by the demonstration that depolarization with high concentrations of K⁺ also caused a Ca²⁺-dependent, concentration-related stimulation of phosphoinositide breakdown. This finding is at variance with that of Montague *et al.* (1985) who found no effect of a relatively low concentration of K⁺ (30 mM) on inositol phosphate production in islets, but is consistent with the reports of Axen *et al.* (1983) and Mathias *et al.* (1985) who found that exposure of [³H]inositol-labelled perfused islets in 50 mM-K⁺ resulted in a marked increase in effluent radioactivity. It could be

argued that K^+ -depolarization could affect inositol lipid breakdown in islets by causing the release of local neurotransmitters from cholinergic nerve endings known to be present in islets (Coupland, 1958). However, this seems unlikely in view of the fact that the K^+ effect persisted in the presence of atropine, and K^+ was able to potentiate the effects on inositol lipid breakdown of a maximal concentration of carbamoylcholine. Incidentally, both carbamoylcholine and high K^+ failed to influence significantly the rate of insulin secretion, despite causing a dramatic stimulation of inositol lipid hydrolysis. It has previously been demonstrated that these two stimuli provoke an early, transient and Ca^{2+} -dependent stimulation of insulin release from perfused islets (Loubatieres-Mariani *et al.*, 1973; Henquin & Lambert, 1974; Mathias *et al.*, 1985) which would have presumably not have been apparent with the present batch-incubation technique. These observations suggest that neither the mobilization of extracellular Ca^{2+} by K^+ depolarization (Herchuelz *et al.*, 1980) nor of intracellular Ca^{2+} by carbamoylcholine (Morgan *et al.*, 1985) are sufficient to cause insulin release in a manner comparable to that evoked by 20 mM-glucose, despite comparable stimulation of inositol lipid breakdown by these agents.

The second line of evidence suggesting an important role for Ca^{2+} in the nutrient-stimulated inositol lipid breakdown in islets arises from experiments using islets permeabilized with digitonin. Permeabilization of the islet cells' plasma membrane, either with digitonin or electrical discharge, has been employed to investigate Ca^{2+} -induced protein phosphorylation (Colca *et al.*, 1985) and insulin release (Yaseen *et al.*, 1983) respectively. The present study suggests that islets made permeable by exposure to digitonin may also provide a useful model to study the mechanism and determinants of inositol lipid metabolism, by virtue of the ability to control directly the immediate environment of the phosphoinositides, presumably on the inner face of the plasma membrane (Downes & Michell, 1982) and the enzymes responsible for their metabolism.

Permeabilization may thus prove applicable to the study of lipid metabolism in a wide variety of cells and tissues, providing a compromise between intact cells and cell-free systems which have been utilized for this purpose (Lin & Fain, 1981; Harrington & Eichberg, 1983; Cockcroft & Gomperts, 1985).

The most striking result to emerge from the present investigation of inositol lipid hydrolysis in permeabilized islets was that this response could be triggered by increasing concentrations of Ca^{2+} within the range that might be expected to exist in the cytosol of stimulated islets. It is interesting to note that a similar increase in Ca^{2+} was found to activate polyphosphoinositide phosphodiesterase extracted from rat brain, when the substrate was presented in a form comparable with its native environment in the plasma membrane (Irvine *et al.*, 1984a). However, the same authors reported that the Ca^{2+} -sensitivity of this enzyme was considerably reduced when the incubation medium was supplemented with K^+ and Mg^{2+} , conditions used in the present study. The reason for this discrepancy is unclear, but could arise from differences in the tissue and preparation. Separation of the inositol phosphates formed in permeabilized islets upon raising the Ca^{2+} concentration from 10^{-7} M to 10^{-5} M revealed significant quantities of $InsP_3$ and $InsP_2$ as well as $InsP$. The pattern of formation of these

compounds was qualitatively similar to that observed in intact islets in response to either nutrient or neurotransmitter stimuli (Best & Malaisse, 1985) and suggested accelerated breakdown of polyphosphoinositides at high Ca^{2+} concentrations. The fact that the detected loss of lipid-bound radioactivity was in the phosphatidylinositol fraction probably reflected rapid repletion of the polyphosphoinositides by ATP-dependent phosphorylation of phosphatidylinositol. Indeed, Ca^{2+} -activated phosphoinositide hydrolysis in permeabilized islets was markedly dependent on the presence of ATP at concentrations expected in the cytosol, and was virtually undetectable in the absence of ATP.

The stimulation of inositol lipid metabolism in permeabilized islets by Ca^{2+} was associated with a loss of the response to the nutrient stimuli glucose and 4-methyl-2-oxopentanoate, presumably either because metabolism of these substances was impaired in permeabilized islets, or due to a perturbation of the ionic fluxes normally elicited by such stimuli (Malaisse *et al.*, 1981). The possibility that increased levels of reduced nicotinamide nucleotides might provide a direct link between nutrient metabolism in islets and inositol lipid hydrolysis (Best & Malaisse, 1983c) seems unlikely in view of the extremely modest influence upon the latter of high concentrations of NADH. It seems more likely that the increased uptake of Ca^{2+} known to accompany nutrient-induced insulin release might provide the trigger for accelerated phosphoinositide breakdown. This is probably not the case, however, for neurotransmitter-stimulated hydrolysis of islet inositol lipids, particularly since enhanced production of inositol phosphates in response to carbamoylcholine persisted in permeabilized islets, albeit at a reduced level compared with the response observed in intact islets. One possible mechanism linking cholinergic receptor occupancy with inositol lipid breakdown involves a putative guanine nucleotide-binding protein, and some evidence has recently been provided that antagonist-induced phosphoinositide breakdown in subcellular preparations can be potentiated by guanine nucleotides (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985). However, in permeabilized islets, no modification of inositol lipid metabolism either in response to Ca^{2+} or carbamoylcholine was apparent with either GTP or its non-hydrolysable analogues. It is of course possible that sufficient concentrations of guanine nucleotides may persist in permeabilized tissues to activate sufficiently a nucleotide-binding protein regulating phosphoinositide metabolism.

A further point of dissimilarity between inositol lipid metabolism in certain subcellular membrane preparations and permeabilized islets is the greatly reduced susceptibility of inositol lipid metabolism in the latter to inhibition by neomycin and adriamycin, which have previously been shown to block phosphoinositide breakdown in kidney homogenates (Schibeci & Schacht, 1977) and erythrocytes (Hickman *et al.*, 1986) respectively. The reasons for this apparent discrepancy are unclear, although it is possible that the preparation of cell membranes, with the accompanying loss of integrity of cellular structure, may result in an altered conformation of certain membrane lipids, which facilitates the binding of drugs such as neomycin. For similar reasons, it may be that the investigation of phospholipid metabolism in permeabilized cells or tissues may provide a closer approximation to the situation in the intact cell than the

use of isolated membrane preparations. Comparative studies involving both types of preparation from the same tissue are required to clarify this issue.

In conclusion, the present study provides strong evidence that Ca^{2+} , in particular increased uptake of the cation into islets cells, may play an essential role in the accelerated breakdown of inositol lipids upon stimulation of pancreatic islets by nutrient secretagogues. This might seem an unlikely mechanism, particularly in view of the fact that $\text{Ins}1,4,5\text{P}_3$, the immediate product of $\text{PtdIns}4,5\text{P}_2$ breakdown, has been itself shown to cause Ca^{2+} mobilization from intracellular sources in a number of systems, including insulin-secreting cells (Biden *et al.*, 1984) and microsomes from insulinoma cells (Prentki *et al.*, 1984). However, recent studies (Irvine *et al.*, 1984b) have revealed that considerable amounts of $\text{Ins}1,3,4\text{P}_3$ are produced in parotid glands in addition to the $\text{Ins}1,4,5\text{P}_3$ isomer, particularly upon prolonged stimulation. It is at present unknown whether $\text{Ins}1,3,4\text{P}_3$ shares the ability of $\text{Ins}1,4,5\text{P}_3$ to release Ca^{2+} from intracellular stores, and clearly each isomer of InsP_3 may be found to act via distinct cellular signalling systems. The question thus arises as to which form(s) of InsP_3 are generated in pancreatic islets upon stimulation with various types of agonist.

I should like to express my appreciation to Drs. J. D. McGivan and J. G. Schofield and Professor W. J. Malaisse for invaluable discussion during this study, to Angela Bufton for technical assistance and to Anne Hepburn for typing the manuscript. This work was supported by the Medical Research Council.

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