Effect of glucose on polyphosphoinositide metabolism in isolated rat islets of Langerhans

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The metabolism of inositol-containing phospholipids during insulin secretion was studied in rat islets of Langerhans preincubated with [^3H]inositol to label their phospholipids. Glucose (20 mM) caused a rapid breakdown of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate and an accumulation of inositol trisphosphate and inositol bisphosphate. This effect was maximal at 60 s, did not require the presence of extracellular Ca^{2+} , and was abolished by mannoheptulose (15 mM), but not by noradrenaline (1 μM). Mannose (20 mM) and DL-glyceraldehyde (10 mM) produced similar effects to those of glucose, but galactose (20 mM) and KCl (30 mM) were without effect. These results are compatible with the hypothesis that an early event in the stimulus–secretion coupling mechanism in the pancreatic B-cell is the rapid breakdown of polyphosphoinositides catalysed by phospholipase C. Moreover, they suggest that the breakdown of polyphosphoinositides is linked to sugar metabolism in the B-cell. This observation is important, since it demonstrates that events in a cell other than plasma-membrane receptor occupancy can promote polyphosphoinositide hydrolysis.

Elucidation of the biochemical events involved in the transduction of extracellular signals into intracellular events has long been a subject of great interest. Recent work in this area has shown that the breakdown of plasma-membrane-associated polyphosphoinositides, in particular $\text{PtdIns}(4,5)\text{P}_2$, is an early event that follows the interaction of a wide variety of physiologically active agents with their specific cell-surface receptors (Downes & Michell, 1982; Hawthorne, 1983; Berridge, 1984). This breakdown of $\text{PtdIns}(4,5)\text{P}_2$ is mediated by phospholipase C, and the initial products of the reaction are diacylglycerol and inositol 1,4,5-trisphosphate. The transient production of diacylglycerol is thought to be important in signal transduction, since diacylglycerol increases the affinity of protein kinase C for Ca^{2+} , thereby activating the enzyme and promoting phosphorylation and activity changes in proteins involved in the response mechanism (Nishizuka, 1984). In addition, the production of inositol 1,4,5-trisphos-

phate appears to play an important role in signal transduction, since this agent has been shown to mobilize Ca^{2+} from intracellular stores in various tissues (Streb *et al.*, 1983; Joseph *et al.*, 1984). This is likely to be important in the stimulus/response mechanism, since Ca^{2+} is thought to act as an important intracellular signal in some tissues linking events related to stimulus recognition at the cell surface to the cell response (Rasmussen & Tenenhouse, 1968; Berridge, 1975).

The stimulus–secretion-coupling mechanism of the pancreatic B-cell utilizes Ca^{2+} as an intracellular signal molecule (Wollheim & Sharp, 1981) and may involve the activation of protein kinase C (Tanigawa *et al.*, 1982; Brocklehurst & Hutton, 1984; Thams *et al.*, 1984). It was therefore important to establish whether the breakdown of polyphosphoinositides is an early event in the stimulus–secretion-coupling mechanism of this cell type, especially in view of previous studies, which indicated that the breakdown of phosphatidylinositol occurs in islet cells during glucose-stimulated insulin secretion (Freinkel *et al.*, 1975; Clements & Rhoten, 1976). Such studies are of particular interest, since the breakdown of polyphosphoinositides normally follows directly from the interaction of a stimulus with a plasma-membrane receptor,

Abbreviations used: PtdIns , phosphatidylinositol; $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-phosphate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; InsP , inositol monophosphate; InsP_2 , inositol bisphosphate; InsP_3 , inositol trisphosphate.

but in the pancreatic B-cell glucose promotes secretion by a mechanism which is related to its metabolism rather than to direct receptor activation (Ashcroft, 1980; Hedekov, 1980).

In this study we have used [^3H]inositol to label islet phospholipids, as this enables an assessment of both the breakdown of individual inositol-containing phospholipids as well as the production of individual inositol phosphates. While this work was in progress, two reports appeared showing that glucose promotes the breakdown of [^{32}P]P_i-labelled polyphosphoinositides in islet cells (Laychock, 1983; Dunlop & Larkins, 1984). In addition, Best & Malaisse (1983) have demonstrated that glucose promotes the accumulation of total ^3H -labelled inositol phosphates in islet cells prelabelled with [^3H]inositol. However, in those studies the individual inositol phosphates were not characterized, and there was disagreement about the Ca^{2+} -dependency of the effect. A preliminary report of our findings has appeared (Prince *et al.*, 1984).

Experimental

Materials

myo-[2- ^3H (n)]Inositol was purchased from New England Nuclear, Southampton, Hants., U.K. Dowex AG1-X8 ion-exchange resin (formate form; 100–200 mesh) and polypropylene Econo-Columns were from Bio-Rad, Watford, Herts., U.K. The following reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K.: EDTA, EGTA, LiCl, *myo*-inositol, PtdIns, PtdIns4P, PtdIns(4,5)P₂, (–)-noradrenaline bitartrate, D-mannoheptulose, DL-glyceraldehyde. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K., and collagenase was from Boehringer, Lewes, Sussex, U.K. Merck precoated silica-gel-60 t.l.c. plates were purchased from BDH Chemicals, Poole, Dorset, U.K. All other reagents were of analytical grade or were the purest available commercially.

Methods

Isolation and incubation of islets of Langerhans. Islets of Langerhans were isolated from pancreatic tissue obtained from male Wistar rats (300–350 g) by collagenase digestion, as described in detail by Montague & Taylor (1968). All incubations were performed at 37°C in a bicarbonate-buffered medium (Gey & Gey, 1936), gassed with O₂/CO₂ (19:1) to pH 7.4 and containing glucose (4 mM), CaCl₂ (2 mM) and bovine serum albumin (3 mg/ml). This medium was supplemented with

test reagents as described for individual experiments.

After isolation, the islets were incubated in groups of 40 in 1.5 ml conical centrifuge tubes containing 20 μl of incubation medium supplemented with 8 μCi of *myo*-[2- ^3H (n)]inositol (sp. radioactivity 15.8 Ci/mmol). After 90 min incubation at 37°C the radioactive medium was removed from the islets, and they were washed twice with 0.4 ml of medium containing 1 mM-*myo*-inositol (non-radioactive) and 10 mM-LiCl (experimental medium). The islets were then incubated at 37°C for a further 10 min in 50 μl of the experimental medium to enable them to recover from the washing procedure. At the end of this incubation period, 50 μl of experimental medium containing test reagents (at twice the final concentration required) was added to the islets, and the incubation was continued at 37°C for the appropriate time. The incubation was terminated by the addition of 0.4 ml of acid methanol (0.1 M-HCl) at –20°C to the islets, which were then placed on solid CO₂.

The islets were disrupted ultrasonically (MSE ultrasonicator; 70 W for 10 s) in the acid methanol, after which 0.4 ml of chloroform followed by 0.3 ml of 2 M-KCl/5 mM-EDTA were added. This resulted in the formation of two phases, an upper aqueous phase containing the water-soluble inositol metabolites and a lower organic phase containing the inositol phospholipids. The two phases were separated after centrifugation at 9000g for 1 min and processed immediately. The upper phase was mixed with 5 ml of water and freeze-dried, and the lower phase was washed once with upper phase and dried under N₂.

Separation of water-soluble inositol metabolites. The freeze-dried upper phase was reconstituted in 2 ml of water and applied to a polypropylene Econo-Column containing 1 ml of Dowex AG1-X8 resin (formate form). The column was washed with 10 ml of water and the inositol metabolites were eluted by the stepwise addition of solutions containing increasing concentrations of formate, as described in detail by Berridge *et al.* (1983). Fractions corresponding to InsP, InsP₂ and InsP₃ were collected, and a 2 ml sample of each fraction was taken for determination of radioactivity by liquid-scintillation spectrometry after addition of 16 ml of Fisofluor 2 scintillation fluid.

The demonstration of the formation of inositol 1,3,4-trisphosphate as well as inositol 1,4,5-trisphosphate in parotid cells (Irvine *et al.*, 1984) raises the possibility that both isomers may also be formed in islet cells. However, the chromatographic method used in the present study to separate the individual inositol phosphates does not permit the identification of their precise isomeric form,

and it is for this reason that we have omitted positional locants when describing the inositol phosphates measured in this study.

Separation of inositol-containing phospholipids. The dried lower phase was reconstituted in 50 μ l of chloroform/methanol (9:1, v/v) and applied to precoated silica-gel-60 t.l.c. plates (Merck). Standard solutions of PtdIns, PtdIns4P and PtdIns(4,5)P₂ were also applied and the plate was developed in a solvent system containing chloroform/methanol/conc. NH₃ (sp.gr. 0.880)/water (90:90:7:20, by vol.) as described by Schacht (1978). The lipid spots were identified with iodine vapour, scraped into scintillation vials, and the radioactivity was determined by liquid-scintillation spectrometry after addition of 0.4 ml of methanol/water/HCl (80:20:1, by vol.), followed by 3.2 ml of FisoFluor 2 scintillation fluid.

Results

Initial experiments were performed to determine the incubation time and conditions that were necessary to ensure that the incorporation of [³H]inositol into islet-cell phospholipids had reached a steady state. In these studies the incubation conditions described in the Experimental section were established, and under these conditions an incubation period of 90 min was necessary to ensure that the incorporation of [³H]inositol into phospholipids had reached a steady state (Fig. 1). Fig. 2 shows that the labelling of the various inositol phosphates also appeared to reach a steady state within this time.

In many studies on phosphoinositide metabolism LiCl is included in the incubation medium, since this agent has been shown to prevent the hydrolysis of InsP to free inositol (Allison & Stewart, 1971) and can therefore be used to increase the sensitivity of methods used for monitoring the accumulation of inositol phosphates produced by the hydrolysis of phosphoinositides (Berridge *et al.*, 1982). The results in Table 1 show that 10 mM-LiCl after the [³H]inositol-labelling period increased the radioactivity associated with InsP and InsP₃ and decreased the radioactivity associated with PtdIns and PtdIns4P. These results presumably reflect the ability of Li⁺ to inhibit the hydrolysis of inositol phosphate to free inositol, thereby preventing the recycling of labelled inositol through the inositol phospholipids. The inclusion of 10 mM-LiCl in the incubation medium had no effect on either basal or stimulated insulin secretion, even during incubation periods of up to 60 min (results not shown). In view of these findings, it was decided to include 10 mM-LiCl in

Fig. 1. Time course of [³H]inositol incorporation into phosphoinositides in rat islets of Langerhans

Groups of 40 islets were incubated in medium containing [³H]inositol as described in the text. After various periods of incubation, the medium was removed from the islets and 0.4 ml of acid methanol at -20°C was added. The phosphoinositides were separated from inositol phosphates and from each other as described in the text and their radioactivities determined. Each point is the mean $\pm$ S.E.M. for six observations: Δ , PtdIns (left-hand scale); $\circ$, PtdIns4P (right-hand scale); $\square$, PtdIns(4,5)P₂ (right-hand scale).

Fig. 2. Time course of [³H]inositol incorporation into inositol phosphates in rat islets of Langerhans

Groups of 40 islets were incubated as described in Fig. 1, and at the end of the incubation the inositol phosphates were separated as described in the text and their radioactivities determined. Each point is the mean $\pm$ S.E.M. for six observations: $\blacktriangle$, InsP; $\bullet$, InsP₂; $\blacksquare$, InsP₃.

Table 1. Effect of LiCl on phosphoinositide and inositol phosphate metabolism in rat islets of Langerhans

Groups of 40 islets were incubated with medium containing [^3H]inositol for 90 min as described in the text. At the end of this period, the medium was removed and replaced by fresh medium containing inositol (1 mM) with or without the addition of LiCl (10 mM). The incubation was continued for a further 10 min, and then acid methanol at -20°C was added to the islets and the inositol phosphates and phospholipids were separated and their radioactivity was determined as described in the text. Each value is the mean $\pm$ S.E.M. for nine observations. Levels of significance: * $P < 0.01$ relative to control islets incubated in the absence of LiCl.

Phosphoinositide	Phosphoinositide radioactivity (c.p.m./40 islets)	
	- LiCl	+ LiCl
PtdIns	10727 $\pm$ 500	8584 $\pm$ 300*
PtdIns4P	310 $\pm$ 20	207 $\pm$ 15*
PtdIns(4,5) P_2	208 $\pm$ 20	197 $\pm$ 16
InsP	975 $\pm$ 50	4500 $\pm$ 200*
InsP $_2$	395 $\pm$ 20	430 $\pm$ 25
InsP $_3$	290 $\pm$ 10	526 $\pm$ 20*

the experimental medium in subsequent experiments.

The results in Figs. 3 and 4 show the time course of the effect of 20 mM-glucose on islet-cell phosphoinositide and inositol phosphate metabolism. A rise in glucose concentration to 20 mM resulted in a rapid loss of radioactivity from the phospholipids, which was recovered in the inositol phosphates. Since the effect of glucose was almost maximal at 1 min, this time point was chosen for further studies. The results in Tables 2 and 3 show that the effects of glucose on both inositol phosphate and phospholipid radioactivity were unaffected by the removal of extracellular Ca^{2+} with 4 mM-EGTA. In addition, the glucose effect was not altered by 1 μM -noradrenaline, but was abolished by 15 mM-mannoheptulose (Table 2). KCl (30 mM) had no effect on inositol phosphate labelling (Table 2). The results in Table 4 show that mannose (20 mM) and DL-glyceraldehyde (10 mM) were as effective as glucose in promoting the accumulation of inositol phosphates, whereas galactose (20 mM) was without effect.

Discussion

Elucidation of the mechanism whereby glucose controls the secretion of insulin from the pancreatic B-cell is an important objective, since diabetes may be related, at least in part, to a defective insulin secretory mechanism (Hellerstrom, 1984).

Fig. 3. Time course of the effect of 20 mM-glucose on phosphoinositide metabolism in rat islets of Langerhans. Groups of 40 islets were incubated in medium containing [^3H]inositol as described in the text. After 90 min incubation the medium was replaced by 50 μl of medium containing LiCl (10 mM) and *myo*-inositol (1 mM), and the incubation was continued for a further 10 min. At this point 50 μl of medium containing either 4 mM- or 35 mM-glucose was added to appropriate groups of islets and the incubation continued for various time periods. The incubation was terminated by the addition of acid methanol at -20°C to the islets, and the phosphoinositides were separated and their radioactivities determined as described in the text. Each point is the mean $\pm$ S.E.M. for eight observations: Δ , PtdIns (left-hand scale); $\circ$, PtdIns4P (right-hand scale); $\square$, PtnIns(4,5) P_2 (right-hand scale). The results are expressed as the loss of radioactivity of islets incubated in 20 mM-glucose after correction for the loss of radioactivity of islets incubated with 4 mM-glucose.

Evidence suggests that the response of a variety of tissues to stimuli in their environment involves the hydrolysis of plasma-membrane-associated PtdIns(4,5) P_2 catalysed by phospholipase C, with the release intracellularly of diacylglycerol and inositol 1,4,5-trisphosphate (Berridge, 1984).

In the present study we have shown that there is a rapid production of InsP $_3$ in islet cells after stimulation by glucose. Although previous work has suggested that glucose might have such an effect (Best & Malaisse, 1983; Laychock, 1983; Dunlop & Larkins, 1984), the present study provides the first direct evidence for the effect. In addition, we have shown that mannose and glyceraldehyde, agents that, like glucose, stimulate insulin secretion as a consequence of their metabo-

lism in the B-cell (Zawalich, 1979), also increase islet-cell InsP_3 . Galactose, however, an agent that is not rapidly metabolized by islet cells and does not stimulate insulin secretion, was without effect. Furthermore, the effect of glucose was abolished by mannoheptulose, a sugar that inhibits the

Fig. 4. Time course of the effect of 20mM-glucose on inositol phosphate metabolism in rat islets of Langerhans

Groups of 40 islets were incubated as described in Fig. 3, and at the end of the incubation the inositol phosphates were separated and their radioactivities determined as described in the text. Each point is the mean $\pm$ S.E.M. for eight observations: $\blacktriangle$, InsP_1 (left-hand scale); $\bullet$, InsP_2 (right-hand scale); $\blacksquare$, InsP_3 (right-hand scale). The results are expressed as the increase in radioactivity of islets incubated in 20mM-glucose after correction for the increase in radioactivity of islets incubated with 4mM-glucose.

insulin-secretory response of the B-cell by preventing glucose metabolism (Ashcroft & Randle, 1970). These observations suggest that the ability of glucose to increase islet InsP_3 may be consequential to metabolism of glucose within the islet cells and may therefore be an important component of the stimulus/response mechanism. However, they do not exclude the possibility that the effect is simply a consequence of the secretory process itself rather than a necessary component of the control mechanism. The results of the experiments with noradrenaline (Table 2) suggest that the production of InsP_3 does not occur as a consequence of insulin secretion, since under these conditions insulin secretion is inhibited (Morgan & Montague, 1984) but glucose is still able to increase InsP_3 . In addition, KCl at a concentration known to promote insulin secretion (Hedekov, 1980) was without effect on InsP_3 concentrations, supporting the suggestion that changes in InsP_3 are not simply a consequence of secretion. In many tissues the accumulation of inositol 1,4,5-trisphosphate during cellular activation is independent of extracellular Ca^{2+} and in this study we have shown that the ability of glucose to increase intracellular InsP_3 is also unimpaired by the removal of Ca^{2+} from the incubation medium. These results are in agreement with those of Best & Malaisse (1983) and Dunlop & Larkins (1984), although they disagree with those of Laychock (1983). This discrepancy may be related to the fact that in the studies by Laychock (1983) islets were preincubated with EGTA before stimulation. This may have depleted intracellular Ca^{2+} and thereby prevented phospholipase C activation, since the enzyme requires Ca^{2+} for activity (Schrey & Montague, 1983). The ability of glucose to increase intracellular InsP_3 independently of extracellular Ca^{2+} suggests that

Table 2. Effect of various incubation conditions on inositol phosphate metabolism in rat islets of Langerhans

Groups of 40 islets were incubated for 90 min in medium containing $[^3\text{H}]$ inositol as described in the text. The radioactive medium was then replaced by 50 μl of medium containing LiCl (10 mM) and *myo*-inositol (1 mM), and the incubation was continued for a further 10 min. At this time 50 μl of medium containing the agents under study at twice their final concentration was added and the incubation continued for a further 1 min. The incubation was terminated by the addition of 0.4 ml of acid methanol to the islets, and the inositol phosphates were separated as described in the text. Each value is the mean $\pm$ S.E.M. for ten observations. Levels of significance: * $P < 0.01$ relative to control islets incubated in 4mM-glucose.

Incubation conditions	Inositol phosphate radioactivity (c.p.m./40 islets)		
	InsP_1	InsP_2	InsP_3
4mM-Glucose	4330 $\pm$ 150	296 $\pm$ 15	357 $\pm$ 20
20mM-Glucose	5178 $\pm$ 200*	385 $\pm$ 26*	491 $\pm$ 30*
20mM-Glucose + 4mM-EGTA	5494 $\pm$ 250*	392 $\pm$ 28*	480 $\pm$ 29*
20mM-Glucose + 1 μM -noradrenaline	5200 $\pm$ 150*	390 $\pm$ 20*	490 $\pm$ 30*
20mM-Glucose + 15mM-mannoheptulose	4300 $\pm$ 180	290 $\pm$ 20	340 $\pm$ 24
30mM-KCl	3900 $\pm$ 200	260 $\pm$ 20	300 $\pm$ 30

Table 3. *Effect of EGTA on phosphoinositide metabolism in rat islets of Langerhans*

Groups of 40 islets were incubated as described in Table 2 and the phospholipid-associated radioactivity was determined as described in the text. Each result is the mean $\pm$ S.E.M. for ten observations. Levels of significance: * $P < 0.01$ relative to control islets incubated in 4 mM-glucose.

Incubation conditions	Phosphoinositide radioactivity (c.p.m./40 islets)		
	PtdIns	PtdIns4P	PtdIns(4,5)P ₂
4 mM-Glucose	8800 $\pm$ 300	230 $\pm$ 15	200 $\pm$ 10
20 mM-Glucose	7568 $\pm$ 350*	182 $\pm$ 15*	136 $\pm$ 14*
20 mM-Glucose + 4 mM-EGTA	7480 $\pm$ 320*	184 $\pm$ 14*	140 $\pm$ 13*

Table 4. *Effect of various sugars on inositol phosphate metabolism in rat islets of Langerhans*

Groups of 40 islets were incubated as described in Table 2, and the inositol phosphates were separated and their radioactivities determined as described in the text. Each result is the mean $\pm$ S.E.M. for nine observations. Levels of significance: * $P < 0.01$ relative to control islets incubated in 4 mM-glucose.

Sugar	Inositol phosphate radioactivity (c.p.m./40 islets)		
	InsP	InsP ₂	InsP ₃
4 mM-Glucose	4091 $\pm$ 150	301 $\pm$ 15	353 $\pm$ 20
20 mM-Glucose	5178 $\pm$ 200*	398 $\pm$ 15*	478 $\pm$ 30*
20 mM-Mannose	5113 $\pm$ 180*	390 $\pm$ 20*	459 $\pm$ 32*
20 mM-Galactose	4150 $\pm$ 130	298 $\pm$ 20	340 $\pm$ 30
10 mM-DL-Glyceraldehyde	4690 $\pm$ 130*	366 $\pm$ 15*	466 $\pm$ 30*

InsP₃ accumulation is not dependent on entry of Ca²⁺ into the B-cell. The results of the experiments using KCl (Table 2) also support this conclusion, since KCl had no effect on InsP₃ concentrations under conditions where the entry of extracellular Ca²⁺ into the B-cell is enhanced (Rorsman *et al.*, 1984).

The cytosolic concentration of free Ca²⁺ is thought to play an important role in stimulus-secretion coupling in the B-cell, and many agents which stimulate secretion are thought to do so by raising the cytosolic Ca²⁺ concentration (Hedekov, 1980). However, although it is generally accepted that much of this Ca²⁺ comes from outside the cell, there is evidence that the rapid release of Ca²⁺ from an organelle-bound pool inside the cell may be important, at least in the early phase of the secretory response (Wollheim & Sharp, 1981). The demonstration that inositol 1,4,5-trisphosphate can mobilize Ca²⁺ from a microsomal fraction of a rat insulinoma (Prentki *et al.*, 1984) is of obvious importance in this context, especially when considered in the light of our demonstration that the InsP₃ concentration is rapidly elevated in islet cells after glucose stimulation. Thus the release of Ca²⁺ from intracellular storage pools could contribute initially to the

mechanism whereby glucose promotes a rise in cytosolic Ca²⁺ concentration and insulin secretion.

In a previous study (Montague & Parkin, 1980) we demonstrated that glucose stimulation increases the diacylglycerol concentration in islet cells. The present results support this observation, since the products of phospholipase C action on polyphosphoinositides include diacylglycerol as well as the inositol phosphates. The importance of an increase in diacylglycerol has become more apparent with the demonstration that islet cells contain protein kinase C, a Ca²⁺- and phospholipid-dependent protein kinase which is activated by diacylglycerol (Tanigawa *et al.*, 1982; Hubinont *et al.*, 1984) and which may phosphorylate a number of islet-cell proteins (Thams *et al.*, 1984). Phosphorylation and hence activation of islet proteins involved in the secretory response may be an important aspect of the regulation of the insulin-secretory response (Howell, 1984), and the results of the present study suggest a mechanism whereby glucose could promote such phosphorylation.

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