Identification and metabolism of polyphosphoinositides in isolated islets of Langerhans

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Isolated islets were incubated with [^{32}P]P_i and radiolabelling of polyphosphoinositides were determined. Labelling equilibrium was approached after 45 min, with a half-time of 15 min. D-Glucose decreased the amount of [^{32}P]PO₄ in phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] and phosphatidylinositol 4-phosphate (PtdIns4P) within 0.5 min, and loss of radiolabel was still evident at 1 min. [^{32}P]PO₄ levels in polyphosphoinositides returned to basal levels within 5 min. Neither D-galactose nor D-glucose after pretreatment of islets with mannoheptulose elicited the polyphosphoinositide effect. The glucose-stimulated breakdown of polyphosphoinositides was inhibited by EGTA; re-addition of Ca²⁺ partially restored the glucose effect. Ionomycin and tolbutamide promoted the rapid breakdown of PtdIns(4,5)P₂, whereas the breakdown of PtdIns4P was less rapid and of a lesser magnitude. The results suggest that the Ca²⁺-dependent breakdown of polyphosphoinositides is an early metabolic event during the initiation of insulin release.

The metabolism of phosphoinositides in cell membranes receptive to a variety of stimuli has been linked to initiation of physiological responses, including stimulus–secretion coupling (Michell, 1975; Downes & Michell, 1982). PtdIns turnover is often associated with alterations in cation transport and/or availability (Dawson & Hauser, 1970; Michell *et al.*, 1981), which potentially affect cell secretory mechanisms. The polyphosphoinositides PtdIns4P and PtdIns(4,5)P₂ have also been described as associated with plasma membranes of cells where they may be involved in cell-cation mobilization, especially of Ca²⁺, due to their strong ionic nature (Hendrickson & Fullington, 1965; Dawson & Hauser, 1970; Buckley & Hawthorne, 1972; Griffin *et al.*, 1979). Diminution of membrane polyphosphoinositide levels by either phosphatase or phosphodiesterase activity (Griffin *et al.*, 1979) could lead to increased cellular Ca²⁺ availability, membrane fluidity and fusogenicity (Clements *et al.*, 1978). In pancreatic islets, it has been hypothesized that breakdown of phosphoinositides is more integrally related to initiation of secretion than is

phospholipid resynthesis (Clements & Rhoten, 1976). The dependency of the early phase of insulin release on intracellular Ca²⁺ reserves (Wollheim & Sharp, 1981) may reflect a breakdown of phosphoinositides with resultant cation mobilization. The purpose of the present study was to identify PtdIns4P and PtdIns(4,5)P₂ in isolated pancreatic islets and to characterize the turnover of these membrane moieties in response to physiological and pharmacological secretagogues. The results show that catabolism of islet polyphosphoinositides is responsive to various stimuli of insulin secretion.

Experimental

Adult male Sprague–Dawley rats (150–200 g) were decapitated and the pancreata excised and digested with collagenase to isolate islets, as described previously (Laychock, 1982). For each experiment, isolated islets from three rats were pooled and then incubated in batches of 40–60 islets per plastic conical Microfuge tube. Islet incubation medium was modified Krebs–Ringer bicarbonate buffer containing (mM): NaCl (119.8); KCl (4.8); MgSO₄·7H₂O (1.2); CaCl₂·2H₂O (2.5); NaHCO₃ (24.6); Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate] (16); glucose (2.8); and 0.01% bovine serum albumin. The pH was adjusted to 7.4

Abbreviations used: PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

with HCl after equilibration with a gas phase of O_2/CO_2 (19:1). In Ca^{2+} -deprivation experiments 5 mM-EGTA was added to the medium before pH adjustment. Islets were pre-incubated for 20 min, and then the medium was replaced with fresh oxygenated buffer containing $10 \mu Ci$ of $[^{32}P]P_i/0.1 ml$. Islets were then exposed to 17 mM-glucose for 2 min, since a brief exposure to glucose enhances the physiological response of islets to later glucose stimulation. Immediately thereafter, reduction in the glucose concentration to 4 mM and the radioactivity to $4.2 \mu Ci/0.1 ml$ was achieved by volume addition up to 0.6 ml. Islets were exposed to $[^{32}P]P_i$ for up to 60 min in time-dependency experiments, or for 45 min followed by vehicle or agent addition in other studies. For each experiment, the initial level of $[^{32}P]PO_4$ in islets after 45 min of labelling was quantified as a zero-time ($t = 0$) control. Paired batches of islets were subsequently exposed to various agents for 0.5–10 min intervals. In specified experiments paired control islets for each time point after exposure of islets to drugs were evaluated for levels of radiolabel, whereas in other experiments the level of $[^{32}P]PO_4$ in each phospholipid within each experiment is expressed as a percentage change from the paired zero-time control.

The incubations were stopped by addition of 2.25 ml of chloroform/methanol (1:2, v/v) plus $20 \mu l$ of 6 M-HCl and the phospholipids were extracted by the Bligh & Dyer (1959) method as modified by Lapetina & Michell (1972) using 2 M-KCl as the ionic contributor. PtdIns4P and PtdIns(4,5) P_2 were separated from other radiolabelled phospholipids by t.l.c. in one dimension as described by Weiss *et al.* (1982) and visually detected by autoradiography. Radiolabelled phospholipids co-migrate with PtdIns4P and PtdIns(4,5) P_2 standards identified with I_2 vapour staining. The spots containing the polyphosphoinositides remained as single spots and retained the level of radioactivity obtained after one-dimensional development when the plates were subsequently developed in a second-dimension using the solvent system chloroform/methanol/acetic acid/water (120:46:19:13, by vol.). Phosphatidic acid and a combined PtdIns/PtdSer fraction were similarly identified and quantified in certain experiments. The PtdIns/PtdSer fraction was demonstrated by a second t.l.c. system (Laychock, 1982) to contain $79 \pm 2\%$ $[^{32}P]$ PtdIns.

The areas on t.l.c. corresponding to autoradiographic exposure sites for known phospholipids were scraped into scintillation vials containing 5 ml of scintillation cocktail and radioactivity was quantified by liquid-scintillation spectrometry.

Values are expressed as means $\pm$ S.E.M. for the stated number (n) of different experimental determinations. All observed effects of drugs were evaluated using a two-way analysis of variance

(ANOVA) or Student's t -test with a statistical significance level of $P < 0.05$.

Authentic phospholipid standards were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (type III) was from Worthington Biochemicals, Freehold, NJ, U.S.A. $[^{32}P]P_i$ (sp. radioactivity 50–100 Ci/mol) was from New England Nuclear, Boston, MA, U.S.A. All other chemicals were reagent grade.

Results

The time course of $[^{32}P]PO_4$ incorporation into islet PtdIns4P, PtdIns(4,5) P_2 as well as PtdIns/PtdSer and phosphatidic acid is shown in Fig. 1. PtdIns(4,5) P_2 - and PtdIns4P-labelling kinetics are similar, although absolute levels of radiolabel in PtdIns(4,5) P_2 often exceeded those found in PtdIns4P and the ratio of PtdIns(4,5) P_2 to PtdIns4P after 45 min was $1.5 (\pm 0.1)$ ($n = 8$). Both phospholipids approach labelling equilibrium by 45 min, with a half-time of 15 min. Phosphatidic acid-labelling kinetics were similar to those of the polyphosphoinositides, but PtdIns/PtdSer labelling increased in a more linear fashion throughout 60 min (Fig. 1). The relative levels of incorporation of $[^{32}P]PO_4$ into PtdIns/PtdSer and phosphatidic acid after 45 min (4.0 ± 0.4 , $n = 17$) also agree with previously published data for islets (Freinkel *et al.*, 1975).

Glucose (17 mM), the physiological secretagogue that maximally stimulates insulin release (Laychock, 1981), caused a rapid decrease in the amount of $[^{32}P]PO_4$ in PtdIns(4,5) P_2 during 1 min of stimulation (Fig. 2a). When compared with paired batches of unstimulated islets, glucose (17 mM) reduced the amount of $[^{32}P]PO_4$ in PtdIns(4,5) P_2 at 0.5 min by $35 (\pm 8)\%$ ($P < 0.05$, $n = 5$). Within 5 min after glucose stimulation, $[^{32}P]PO_4$ incorporation in PtdIns(4,5) P_2 had returned to control levels (Fig. 2a). $[^{32}P]PO_4$ incorporation into PtdIns(4,5) P_2 reached $138 (\pm 10)\%$ of the time-zero value ($n = 5$) after 10 min of incubation.

Glucose stimulation affected the level of $[^{32}P]PO_4$ in PtdIns4P in a manner similar to that observed in PtdIns(4,5) P_2 . The level of $[^{32}P]PO_4$ in PtdIns4P decreased during 0.5 min of stimulation and remained depressed for at least 1 min (Fig. 2b). Glucose (17 mM) also reduced the level of $[^{32}P]PO_4$ in PtdIns4P at 0.5 min by $29 (\pm 5)\%$ ($P < 0.05$, $n = 5$) compared with paired batches of unstimulated islets at 0.5 min. The level of radiolabel returned to control levels within 5 min (Fig. 2b), and by 10 min the level of $[^{32}P]PO_4$ in PtdIns4P had risen to $143 (\pm 24)\%$ of initial label incorporation (results not shown).

Islets incubated with the non-metabolizable sugar

Fig. 1. Time-dependent phospholipid incorporation of $[^{32}\text{P}]\text{PO}_4$.

Islets were incubated with $[^{32}\text{P}]\text{P}_i$ and stimulated with glucose (17 mM) for 2 min. Levels of radiolabel in PtdIns/PtdSer ($\square$), PtdIns4P ($\circ$), PtdIns(4,5)P₂ ($\bullet$) and phosphatidic acid ($\blacksquare$) were determined beginning at 1 min after the glucose concentration was returned to 4 mM. Values represent the radioactivity (c.p.m.) per 50 islets (means $\pm$ S.E.M.) for three or four different experiments.

D-galactose, which is not a secretagogue in islets (Ashcroft, 1981), did not show the polyphosphoinositide effect characterized by glucose stimulation (Figs. 2a and 2b). Preincubation of islets for 10 min with mannoheptulose (30 mM), which inhibits glucose oxidation (Idahl, 1980), also inhibited the glucose effect on polyphosphoinositide metabolism (Table 1a).

The polyphosphoinositide effect of glucose was also investigated in islets deprived of Ca^{2+} by incubation in buffer containing EGTA. Ca^{2+} -deprived islets stimulated with 17 mM-glucose did not respond with polyphosphoinositide breakdown (Table 1b); however, the re-addition of an excess of 2.5 mM- Ca^{2+} plus 17 mM-glucose partially restored the glucose effect (Table 1b).

The cation ionophore ionomycin (Serhan *et al.*, 1982) was studied in islet incubations to determine the effect of elevated intracellular Ca^{2+} concentrations on the polyphosphoinositide effect. The data in Fig. 3 show that ionomycin (2 μM) stimulated the rapid loss of $[^{32}\text{P}]\text{PO}_4$ from PtdIns(4,5)P₂ within 5 min, but had a much less rapid effect on $[^{32}\text{P}]$ -

PtdIns4P levels. Tolbutamide, an insulin secretagogue (Laychock, 1983) that has been described as behaving like an ionophore in islets (Couturier & Malaisse, 1980; Malaisse, 1980), also stimulated the loss of $[^{32}\text{P}]\text{PO}_4$ from PtdIns(4,5)P₂ and PtdIns4P (Fig. 3) in a manner similar to that of ionomycin.

The response of the islet PtdIns/PtdSer fraction to stimulation was unlike the response of polyphosphoinositides. Although glucose modestly reduced $[^{32}\text{P}]\text{PtdIns/PtdSer}$ levels after 0.5 min ($87 \pm 6\%$ of $t=0$ value, $n=13$, $P<0.02$) compared with control values ($113 \pm 9\%$ of $t=0$ value), within 1 min $[^{32}\text{P}]\text{PO}_4$ levels in PtdIns/PtdSer were no different from control values ($109 \pm 7\%$ of $t=0$ value). Additionally, glucose-stimulated islets manifested increased biosynthesis of PtdIns/PtdSer after 10 min of stimulation ($145 \pm 9\%$ of $t=0$ value, $n=5$). However, the difference from control ($116 \pm 16\%$ of $t=0$ value) was not significant. Incubations for 1 h or longer appear necessary to detect enhanced PtdIns/PtdSer biosynthesis in islets (Fex & Lernmark, 1972, 1975).

Fig. 2. $[^{32}\text{P}]\text{PO}_4$ levels in polyphosphoinositides. Islets were pre-incubated with $[^{32}\text{P}]\text{P}_i$ for 45 min and then buffer (x) or buffer containing either glucose (17 mM final concentration) (●) or galactose (17 mM final concentration) (○) was added at $t=0$. $[^{32}\text{P}]\text{PO}_4$ levels in PtdIns(4,5) P_2 (a) and PtdIns4P (b) were determined at the indicated times. Values represent mean percentages of $[^{32}\text{P}]\text{PO}_4$ at $t=0$ ($\pm$ S.E.M.) for five to 15 different experiments. Glucose-treated-islet values were significantly different from controls in (a) ($P<0.05$) and (b) ($P<0.01$) at 0.5 min and 1 min as determined by two-way ANOVA.

Discussion

The incorporation of $[^{32}\text{P}]\text{PO}_4$ into phospholipids observed in the present study closely approximates the profile found in islet membranes by other investigators (Fex & Lernmark, 1975; Freinkel *et al.*, 1975). Labelling of polyphosphoinositides in islets is clearly illustrated using $[^{32}\text{P}]\text{P}_i$, whereas studies that utilized *myo*- $[^3\text{H}]$ inositol were unsuccessful (Clements & Rhoten, 1976). The islet membrane ratio of $[^{32}\text{P}]\text{PO}_4$ in PtdIns(4,5) P_2 and PtdIns4P is very similar to that in parotid acinar cells (Weiss *et al.*, 1982) and slightly lower than the

Fig. 3. $[^{32}\text{P}]\text{PO}_4$ levels in polyphosphoinositides of ionophore-treated islets. Islet phospholipids were prelabelled with $[^{32}\text{P}]\text{P}_i$ for 45 min, and then either ionomycin (2 μM) (x) or tolbutamide (1 mM) (●) was added at $t=0$. Levels of radiolabel in PtdIns(4,5) P_2 (—) and PtdIns4P (---) were determined at the indicated times. Values represent the mean percentages of $[^{32}\text{P}]\text{PO}_4$ at $t=0$ ($\pm$ S.E.M.) for four to six different experiments. Ionomycin- and tolbutamide-treated-islet PtdIns(4,5) P_2 and tolbutamide-treated-islet PtdIns4P values were significantly different from control $[^{32}\text{P}]\text{PO}_4$ levels ($P<0.01$) as determined by two-way ANOVA.

Table 1. $[^{32}\text{P}]\text{PO}_4$ incorporation into islet membrane polyphosphoinositides

Values represent percentages of $t=0$ control values for 0.5 min incubations; the numbers of different determinations are shown in parentheses. In (a) islets were pretreated with 30 mM-mannoheptulose and then incubated in the presence or in the absence of 17 mM-glucose. In (b), islets were pre-incubated in buffer containing an excess of 2.5 mM-EGTA; islets were then incubated in the presence or in the absence of glucose (17 mM) and/or an excess of 2.5 mM- Ca^{2+} . EGTA, and EGTA plus Ca^{2+} , are paired experiments; EGTA plus glucose, and EGTA plus Ca^{2+} plus glucose, are paired experiments. * indicates values significantly different ($P<0.05$) compared with paired controls (100% at $t=0$ value) using Student's *t*-test.

Treatment	$[^{32}\text{P}]\text{PO}_4$ levels (%)	
	PtdIns(4,5) P_2	PtdIns4P
(a) Mannoheptulose	106 $\pm$ 11 (9)	105 $\pm$ 6 (9)
Mannoheptulose plus glucose	99 $\pm$ 11 (9)	121 $\pm$ 14 (9)
(b) EGTA	95 $\pm$ 12 (3)	96 $\pm$ 12 (5)
EGTA plus Ca^{2+}	111 $\pm$ 11 (3)	108 $\pm$ 5 (4)
EGTA plus glucose	108 $\pm$ 6 (8)	111 $\pm$ 3 (8)
EGTA plus Ca^{2+} plus glucose	85 $\pm$ 5* (6)	92 $\pm$ 3* (6)

value for isolated pancreatic acini (Putney *et al.*, 1983).

The loss of [^{32}P]PO $_4$ from polyphosphoinositides within 1 min of glucose stimulation is similar in magnitude to the loss of [^{32}P]PO $_4$ from PtdIns(4,5)P $_2$ in vasopressin-stimulated hepatocytes (Kirk *et al.*, 1981) and methacholine-stimulated parotid acinar cells (Weiss *et al.*, 1982) and mimics the acetylcholine-induced loss of [^{32}P]PO $_4$ from polyphosphoinositides in smooth muscle (Abdel-Latif *et al.*, 1977; Akhtar & Abdel-Latif, 1978, 1982). On the other hand, galactose does not stimulate insulin release (Ashcroft, 1981) nor affect PtdIns turnover in pancreatic fragments (Farese *et al.*, 1981), and also did not release [^{32}P]PO $_4$ from islet polyphosphoinositides. Likewise, mannoheptulose inhibits glucose phosphorylation and insulin release, and prevented the glucose-induced breakdown of the polyphosphoinositides. Thus the evidence suggests that glucose metabolism and perhaps a glucose metabolite is required for activation of the mechanisms involved in rapid polyphosphoinositide breakdown. These findings are consistent with reports that sugars that fail to affect insulin secretion do not elicit phosphate ion efflux (Carpinelli & Malaisse, 1980; Freinkel *et al.*, 1974), *myo*-inositol release or [^{32}P]PO $_4$ labelling in PtdIns in islets (Freinkel *et al.*, 1975; Clements & Rhoten, 1976; Clements *et al.*, 1978).

In these studies, the effects of glucose on the breakdown of islet polyphosphoinositides were also modulated by Ca $^{2+}$, since the inhibition of lipid breakdown by the Ca $^{2+}$ -chelating agent EGTA was partially reversed by Ca $^{2+}$ addition. Consistent with the Ca $^{2+}$ requirement for breakdown of polyphosphoinositides is the finding that extracellular Ca $^{2+}$ and Ca $^{2+}$ -mobilized from intracellular pools are required for secretion (Wollheim & Sharp, 1981). Loss of [^{32}P]PO $_4$ from islet polyphosphoinositides was also observed during incubation of islets with ionophores that evoke insulin release and increase the level of free intracellular Ca $^{2+}$ in islets (Conaway *et al.*, 1976; Malaisse, 1980). Moreover, the secretagogue tolbutamide translocates Ca $^{2+}$ (Couturier & Malaisse, 1980) and activates Ca $^{2+}$ -dependent phospholipid fatty acid turnover in islets (Laychock, 1983), in addition to its effect on polyphosphoinositide breakdown. Thus, elevation of intracellular Ca $^{2+}$ levels in islets appears to affect enzymes responsible for polyphosphoinositide metabolism.

Other cells exposed to calcium ionophores also lose [^{32}P]PO $_4$ from polyphosphoinositides (Lang *et al.*, 1977; Akhtar & Abdel-Latif, 1978; Allan & Michell, 1978; Griffin & Hawthorne, 1978; Griffin *et al.*, 1979), which suggests that the metabolism of polyphosphoinositides by phosphodiesterases and phosphomonoesterases in certain tissues is Ca $^{2+}$ -dependent (Garrett *et al.*, 1976; Kai & Hawthorne,

1969). However, in other cell types the phosphodiesterase does not appear to be stimulated by Ca $^{2+}$ and ionophores do not provoke breakdown of these lipids (Kirk *et al.*, 1981; Michell *et al.*, 1981; Weiss *et al.*, 1982). Glucose and ionophores may stimulate islet phosphodiesterases, which metabolize lipids to 1,2-diacylglycerol (Clements & Rhoten, 1976; Montague & Parkin, 1980), and/or they may affect the phosphomonoesterases and enhance PtdIns production. However, since glucose and ionophores do not equally affect the breakdown of polyphosphoinositides, these stimulatory agents may have different effects on the metabolizing enzymes.

The present studies demonstrating that insulin secretagogues provoke the breakdown of polyphosphoinositides are reminiscent of a similar phospholipid effect in other secretory cells (Weiss *et al.*, 1982; Putney *et al.*, 1983). The polyphosphoinositide effect may mediate changes in Na $^+$, K $^+$ -permeability (Kai & Hawthorne, 1969; Akhtar & Abdel-Latif, 1978), Ca $^{2+}$ -dependent ATPase activity (Buckley & Hawthorne, 1972), proteolipid polarity (Redman, 1972), intracellular Ca $^{2+}$ levels (Buckley & Hawthorne, 1972; Pappu & Hauser, 1981) and glycoprotein mobility (Sheetz *et al.*, 1982). Owing to the negative valency of polyphosphoinositides (Hendrickson & Fullington, 1965), these phospholipids may also contribute to cation mobilization when their breakdown is initiated early in the initial phase of insulin secretion. Thus polyphosphoinositide metabolism is envisaged as an early event in agonist-induced insulin release, which may participate in the propagation of the stimulus signal.

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