Ca²⁺-sensitive phosphatidylinositol 4-phosphate metabolism in a rat β -cell tumour

Nigel E. TOOKE, C. Nicholas HALES and John C. HUTTON*

Department of Clinical Biochemistry, University of Cambridge, New Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

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1. Subcellular fractions were isolated from a rat β -cell tumour by centrifugation of homogenates on Percoll and Urografin density gradients. Fractions were incubated with [γ -³²P]ATP, and labelling of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate was used to measure phosphatidylinositol kinase and phosphatidylinositol 4-phosphate kinase activities, respectively. 2. The distribution of enzyme markers in density gradients indicated that phosphatidylinositol kinase was located in both the plasma membrane and the secretory-granule membrane. Phosphatidylinositol 4-phosphate kinase activity was low in all fractions. 3. Phosphatidylinositol kinase activity of secretory granules and plasma membranes was decreased to 10–20% of its initial value by raising the free [Ca²⁺] from 1 μ M to 5 μ M. The enzyme had a K_m (apparent) for ATP of 110 μ M (secretory granule) or 120 μ M (plasma membrane) and a K_a for Mg²⁺ of 7 mM (secretory granule) or 6 mM (plasma membrane). Ca²⁺-sensitivity of phosphatidylinositol kinase in calmodulin-depleted secretory granules and plasma membranes was not affected by addition of exogenous calmodulin, although activity was stimulated by trifluoperazine in the presence of 0.1 μ M or 40 μ M-Ca²⁺. Trifluoperazine oxide had no effect on the enzyme activity of secretory granules. 4. Plasma membranes had a phosphatidylinositol 4-phosphate phosphatase activity which was stimulated by raising the free [Ca²⁺] from 0.1 to 40 μ M. The secretory granule showed no phosphatidylinositol 4-phosphate-degrading activity. 5. These results suggest the presence in the tumour β -cell of Ca²⁺-sensitive mechanisms responsible for the metabolism of polyphosphoinositides in the secretory granule and plasma membrane.

Stimulated secretion by a number of tissues has been shown to be accompanied by an increase in the turnover of phosphatidylinositol (see reviews by Berridge, 1981; Putney, 1981; Best & Malaisse, 1983a). In the pancreatic β -cell, phosphatidylinositol turnover may be promoted by the secretagogues glucose (Fex & Lernmark, 1975; Freinkel *et al.*, 1975; Clements *et al.*, 1977; Best & Malaisse, 1982), leucine and arginine (Fex & Lernmark, 1975), and 4-methyl-2-oxopentanoate (Best & Malaisse, 1983a) and the potentiators carbamoylcholine and cholecystokinin/pancreozymin (Best & Malaisse, 1983b).

The pathways of breakdown and resynthesis of

Abbreviations used: PtdIns4P, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; PtdIns(4,5)P₂, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate.

* To whom reprint requests should be addressed.

phosphatidylinositol and the cellular localization of the reactions are, however, unclear in any of the tissues so far studied. It has been proposed that breakdown of phosphatidylinositol during cell stimulation either occurs directly or proceeds via its phosphorylated derivatives, PtdIns4P and PtdIns(4,5)P₂ (Kirk *et al.*, 1981; Michell, 1982; Weiss *et al.*, 1982; Putney *et al.*, 1983). The initial reaction whose activation results in increased turnover appears to occur within the plasma membrane, at least in those cells responding to stimuli that act on plasma-membrane receptors.

Phosphatidylinositol and the polyphosphoinositides may be degraded by phosphodiesterases to form 1,2-diacylglycerol. This neutral lipid may promote membrane fusion (Allan & Michell, 1978, 1979); thus turnover of polyphosphoinositides in the secretory granule and the plasma membrane

may provide a mechanism by which the 'fusibility' of these membranes might be controlled. 1,2-Diacylglycerol has also been recognized as an activator of protein kinase C, thus providing another mechanism by which changes in this metabolic pathway may mediate the intracellular response to extracellular messengers (Nishizuka & Takai, 1981).

The present paper investigates the properties of enzymes responsible for the metabolism of polyphosphoinositides in the secretory granule and plasma membrane of the β -cell. The study was carried out on a transplantable rat insulinoma, a tissue which contains predominantly β -cells and which is capable of secreting insulin by a process possibly identical with that in pancreatic islets (Sopwith *et al.*, 1981).

Materials and methods

Preparation of membrane fractions

The rats and tumours used were derived from material supplied by Professor W. L. Chick, New England Deaconess Hospital, Boston, MA, U.S.A. Homogenates of the tumour were prepared by the method of Hutton & Peshavaria (1982) in a medium containing 0.27 M-sucrose, 10 mM-Mes (4-morpholine-ethanesulphonic acid) and 2.8 mM-EGTA adjusted to pH 6.5 with Tris base. The homogenate was centrifuged for 10 min at 1700g, and 10 ml portions of the supernatants were layered on 25 ml of homogenizing medium containing 27% (v/v) Percoll (Pharmacia, Uppsala, Sweden) in centrifuge tubes (8.5 cm $\times$ 2.5 cm). The tubes were centrifuged for 45 min at 35000g in a Beckman type 30 rotor. The gradients thus formed were fractionated into 22 tubes. Purified granules were prepared from the zone at the tube base (fraction B, Fig. 1) by the method of Hutton *et al.* (1982), then resuspended at 5–10 mg of protein/ml in EGTA-free homogenizing medium and stored at -70°C .

To prepare plasma-membrane-enriched fractions, 5 ml portions of fraction C (Fig. 1) were diluted to 10 ml with 10 mM-Tris adjusted to pH 7.4 with HCl and layered on a 26 ml pre-formed linear density gradient of 20–53% (v/v) Urografin 325 (Schering Chemicals, Burgess Hill, West Sussex, U.K.) in 10 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose. The tubes (8.5 cm $\times$ 2.5 cm) containing these samples were centrifuged for 3 h at 100000g (r_{av} , 11.8 cm) at 4°C in a Beckman type SW-27 rotor. The pink zone near to the top of the gradient (fraction D, Fig. 2) was diluted 5-fold with sucrose-free buffer and centrifuged for 1 h at 100000g in a SW-27 rotor. The pellet was resuspended in buffer at 5–10 mg of protein/ml and stored at -70°C .

Secretory-granule membranes were prepared by osmotic lysis. Isolated secretory granules (1–2 mg of protein) were suspended in 1.5 ml of medium containing 10 mM- NH_4HCO_3 and 1 mM-EDTA adjusted to pH 9.0 with NH_3 . The suspension was layered on 25 μl of 60% (w/v) sucrose in a 1.5 ml-capacity polypropylene tube (Sarstedt, Leicester, Leics., U.K.) and placed inside a water-filled centrifuge tube (9.5 cm $\times$ 1.5 cm). The samples were centrifuged for 30 min at 110000g in a MSE 6 $\times$ 16.5 ml rotor (r_{av} , 11.0 cm). The turbid zone immediately above the sucrose layer was removed, diluted with 1.5 ml of NH_4HCO_3 /EDTA buffer and centrifuged on to a sucrose cushion as before. The washed membranes were diluted with buffer containing 10 mM-Mes and 2.8 mM-EGTA (pH 6.5) and stored at 2 mg of protein/ml at -70°C .

Phospholipid phosphorylation assays

Membrane fractions (20 μg of protein) were incubated for 1 min at 37°C in 30 μl of buffer, pH 6.9, containing 50 mM-Mes/Tris base, 2 mM- MgCl_2 , 5 mM-EGTA and 0–5 mM- CaCl_2 (all final concns. in assay). The free Ca^{2+} concentration in these media (1 nM–40 μM) was calculated by the method of Storer & Cornish-Bowden (1976). Other additions, where indicated, were: 10–100 μM -trifluoperazine or trifluoperazine oxide added as 5-fold concentrated solutions in 5% (v/v) dimethyl sulphoxide; 1.2 μM bovine brain calmodulin (prepared as in Hutton *et al.*, 1981). Dimethyl sulphoxide alone had no detectable effect on phospholipid phosphorylation at these concentrations. The reaction was started by adding 20 μl of 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5–10 Ci/mmol). The reaction was stopped usually after incubation for 1 min at 37°C by adding 1 ml of ice-cold chloroform/methanol (2:1, v/v) containing 0.25% HCl and left at 4°C for 10 min. Aqueous 1 M-HCl (200 μl) was then added, the samples were mixed vigorously and the two phases were separated by centrifuging at room temperature for 5 min at 900g (r_{av} , 12 cm) in a MSE bench centrifuge. The lower, phospholipid-containing, phase was washed twice with 750 μl of upper-phase solvent (chloroform/methanol/0.1 M-HCl, 3:48:47, by vol.).

T.l.c. of phospholipids

The method of Gonzalez-Sastre & Folch-Pi (1968) was used to resolve polyphosphoinositides. Samples were reduced in a stream of O_2 -free N_2 and applied to t.l.c. plates (20 cm $\times$ 20 cm, pre-coated with 0.25 mm-thick silica gel 60 F₂₅₄; E. Merck, Darmstadt, West Germany) which had been impregnated with 1% potassium oxalate and air-dried at 110°C for 1 h. T.l.c. plates were developed with chloroform/methanol/aq. 7.5% (w/v) NH_3 (9:7:2, by vol.). Radioactive areas were

located by autoradiography with Cronex 4 X-ray film [Dupont (U.K.), St. Neots, Cambs., U.K.]. The silica gel was then transferred to scintillation vials, 10 ml of water was added and the radioactivity (Cerenkov radiation) was determined in a Packard liquid-scintillation spectrometer.

Identification of phosphorylated phospholipids

Fractionation of lipid extracts of various membrane fractions of the β -cell tumour tissue by the method of Gonzalez-Sastre & Folch-Pi (1968) resolved three major radiolabelled phospholipid components with mobilities identical with those of authentic PtdIns(4,5) P_2 , PtdIns4P and phosphatidylinositol + phosphatidic acid. Where present, labelling of PtdIns(4,5) P_2 represented less than 5% of total incorporation of ^{32}P into polyphosphoinositides. The separation of phospholipids by t.l.c. on silica gel 60 F₂₅₄ with chloroform/methanol/acetic acid/water (45:25:10:1, by vol.) as developing solvent resolved ^{32}P -labelled phosphatidylinositol and phosphatidic acid.

Radiolabelled phospholipids were also analysed by ion-exchange chromatography of their deacylated forms (Downes & Michell, 1981). Phospholipid extracts of phosphorylated membranes were deacylated by mild alkaline hydrolysis and applied to 1.5 cm $\times$ 0.5 cm Bio-Rad AG 1-X10 (50–100 mesh) columns (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and eluted by the procedure of Downes & Michell (1981). Radioactivity of the eluates was determined from their Cerenkov radiation. Most of the radiolabel associated with polyphosphoinositides was recovered in the fraction containing glycerophosphoinositol 4-phosphate, which is the expected deacylation product of PtdIns4P. Only a minor proportion of ^{32}P (<5%) was associated with glycerophosphoinositol 4,5-bisphosphate, the deacylated derivative of PtdIns(4,5) P_2 .

Phospholipid phosphatase assay

Membrane fractions (20 μ g of protein) were incubated in the presence of 1 μ M- Ca^{2+} with [γ - ^{32}P]-ATP for 10 min as described above. Ice-cold 50 mM-Mes buffered to pH 6.9 with Tris base (70 μ l), was then added. Samples were centrifuged for 5 min at 8000g (r_{av} , 5 cm) in an Eppendorf centrifuge. The pelleted granules were then resuspended in 50 μ l of buffer containing 2 mM-ATP and 0.1 or 40 μ M free Ca^{2+} . After incubation for 1–10 min at 37°C, the reaction was stopped by solvent extraction as above.

Marker proteins

Subcellular fractions were assayed for NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967), cytochrome *c* oxidase (Cooperstein & Lazarow,

1951) and arylsulphatase (Roy, 1953). The latter assay included a centrifugation step to remove Percoll precipitated by the final colour reagent. 5'-Nucleotidase activity was measured in the presence of 0.1% Lubrol 12A9 by a radiometric method (Avruch & Wallach, 1971).

Protein was determined in samples after precipitation with ice-cold 5% (w/v) trichloroacetic acid. Samples free of Percoll or Urografin were assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Other samples were assayed by a fluorimetric procedure (Udenfriend *et al.*, 1972). The blank contributed by Percoll or Urografin was determined by using density media as standards in the assay.

Insulin was determined by a modification (Hutton & Peshavaria, 1982) of the immunoassay of Wright *et al.* (1968).

The densities of gradient fractions were determined by comparison of their refractive indices with those of media of similar composition and known density.

Materials

Organic solvents and reagents were of AnalaR grade and were obtained from BDH Chemicals, Poole, Dorset, U.K. O₂-free N₂ was obtained from BOC Ltd, Ipswich, Suffolk, U.K. [γ - ^{32}P]-ATP was prepared by the method of Walsh *et al.* (1971) from carrier-free [^{32}P] P_i (Amersham International, Amersham, Bucks., U.K.) and adjusted to pH 6.9 with Tris base. Trifluoperazine dihydrochloride was obtained from Smith, Kline and French, Welwyn Garden City, Herts., U.K., and trifluoperazine oxide was a gift from Dr. D. Harrison, Nuffield Department of Clinical Biochemistry, University of Oxford. PtdIns4P and PtdIns(4,5) P_2 standards were gifts from Dr. R. F. Irvine, A.R.C. Institute of Animal Physiology, Babraham, Cambs. Other phospholipid standards were supplied by Sigma (London) Chemical Co., Poole, Dorset, U.K.

Results

Subcellular distribution of phospholipid-phosphorylation activity

The subcellular localization of phospholipid-phosphorylation activity was defined by assaying activity in individual fractions prepared from tumour homogenates by isopycnic density-gradient centrifugation. Labelling of phosphatidylinositol + phosphatidic acid in fractions prepared by using Percoll gradients was coincident with the distribution of the endoplasmic-reticulum marker, NADPH-cytochrome *c* reductase (Fig. 1). Labelling of PtdIns4P, on the other hand, occurred

Fig. 1. *Percoll-density-gradient centrifugation of a tumour homogenate*

A nuclei-free tumour homogenate was layered on to 27% (v/v) Percoll and centrifuged. Material in zone A was pelleted to yield a lysosome-rich fraction, and zone B was further fractionated on 40% (v/v) Percoll to prepare a secretory-granule-rich fraction. Zone C was used to prepare plasma membranes.

principally in those gradient fractions which also showed maximal insulin content (secretory granule) or 5'-nucleotidase activity (plasma membrane).

Subfractionation of the plasma-membrane-rich zone (fraction C, Fig. 1) on a linear Urografin gradient enabled further resolution of plasma membrane from contaminating mitochondria and endoplasmic reticulum (Fig. 2). Again labelling of PtdIns4P was coincident with the distribution of the activity of 5'-nucleotidase, the plasma-membrane marker. The plasma-membrane-rich fraction obtained from the Urografin gradient (fraction D) was purified approx. 15-fold relative to that of the homogenate and showed a low contamination with respect to mitochondria, lysosomes and secretory granules, as judged by relative specific

activities of their respective marker proteins (Table 1).

Membranes prepared from granules by lysis had a specific activity for phosphatidylinositol kinase which was comparable with that of plasma membranes, but also had a high specific activity for the plasma-membrane marker, 5'-nucleotidase. Granule membranes, however, had a density of 1.08 g/ml on Urografin density gradients and could therefore be clearly distinguished from plasma membranes which, when analysed under the same conditions, had a density of 1.12 g/ml (Fig. 3). 5'-Nucleotidase activity co-migrated with phosphatidylinositol-phosphorylating activity of both plasma membranes and granule membranes, suggesting that 5'-nucleotidase is a genuine component of the secretory granule.

Fig. 2. Urografin-density-gradient centrifugation of plasma-membrane-enriched fractions

Material enriched in 5'-nucleotidase (zone C; Fig. 1) from the initial density gradient was layered on to a linear 20–53% (v/v) Urografin density gradient and centrifuged as described in the Materials and methods section. Material in zone D was recovered as the plasma-membrane-rich fraction and pelleted by centrifugation.

Table 1. *Measurement of marker proteins in subcellular fractions prepared from tumour homogenates*

Subcellular fractions were prepared from tumour homogenates by differential and density-gradient centrifugation and were assayed for membrane marker-protein activity. Units: immunoreactive insulin, units/mg of protein; phosphatidylinositol kinase, nmol/min per mg of protein; others, $\mu\text{mol/min}$ per mg of protein. Each value is the mean $\pm$ S.E.M. of results obtained with three different tissue preparations. Values in parentheses are relative specific activities, calculated as the protein specific activity in the fraction divided by that of the initial homogenate. Abbreviations: ND, not determined; NDA, no detectable activity. Total activities of marker proteins recovered in the various fractions represented 85–110% of the activities present in the initial homogenate.

Marker protein	Activity in subcellular fractions				
	Homogenate	Plasma membranes	Secretory granules	Granule membranes	Lysosomes
Immunoreactive insulin	0.55 ± 0.06	0.50 ± 0.13 (0.9)	7.2 ± 0.03 (13.1)	0.29 ± 0.049 (0.5)	1.16 ± 0.65 (2.1)
5'-Nucleotidase	0.026 ± 0.001	0.40 ± 0.07 (15.3)	0.076 ± 0.003 (2.9)	0.20 ± 0.01 (7.8)	0.09 ± 0.003 (3.4)
Phosphatidylinositol kinase	0.042 ± 0.001	0.19 ± 0.05 (4.5)	0.10 ± 0.009 (2.5)	0.15 ± 0.008 (3.7)	0.008 ± 0.001 (0.2)
NADPH-cytochrome <i>c</i> reductase	0.20 ± 0.01	0.35 ± 0.046 (1.8)	0.015 ± 0.002 (0.08)	ND	NDA
Arylsulphatase	0.045 ± 0.007	0.041 ± 0.017 (0.9)	1.18 ± 0.16 (26.2)	ND	2.91 ± 0.75 (64.7)
Cytochrome <i>c</i> oxidase	0.19 ± 0.039	0.058 ± 0.001 (0.3)	0.094 ± 0.022 (0.5)	ND	0.021 ± 0.01 (0.1)

Fig. 3. *Urografin-density-gradient centrifugation of ^{32}P -labelled membranes*

Secretory-granule (a) and plasma-membrane (b) fractions were each incubated for 10 min at 37°C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in buffer containing $0.1 \mu\text{M-Ca}^{2+}$. After pelleting by centrifugation, membranes were prepared from each fraction by incubation in buffer containing $10\text{mM-NH}_4\text{HCO}_3$ and 1mM-EDTA , pH 9.0. Membranes were then layered on to separate linear 15–53% (v/v) Urografin gradients and centrifuged for 3 h at $100000g$ (r_{av} 11.8 cm). Gradients were fractionated and assayed for 5'-nucleotidase activity and ^{32}P radioactivity. Zones I and II, which showed greatest ^{32}P radioactivity, were extracted with acid chloroform/methanol and the ^{32}P in $\text{PtdIns}4\text{P}$ was measured. Values were 55% and 58% of total radioactivity for secretory granules (zone I) and plasma membranes (zone II) respectively. The remainder was associated with non-lipid material.

Phosphorylation of phosphatidylinositol in secretory granules

The apparent K_m for ATP for the phosphatidylinositol-phosphorylating system of the secretory granule was $110\ \mu\text{M}$, and phosphorylation was markedly stimulated by increasing the Mg^{2+} concentration in the incubation medium, the K_a value being $7.3\ \text{mM}$.

The rate of phosphorylation of phosphatidylinositol in the secretory granule was initially linear, but rapidly decreased after a few minutes of incubation (Fig. 4). When pre-labelled secretory granules were further incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, additional incorporation was seen, suggesting that the fall in incorporation rate might be due to ATP depletion rather than a decreased availability of endogenous phosphatidylinositol substrate.

Phosphorylation of phosphatidylinositol was markedly decreased by raising the free $[\text{Ca}^{2+}]$ in the incubation medium from $0.1\ \mu\text{M}$ to $40\ \mu\text{M}$, although the time course of the reaction was largely unaffected (Fig. 4).

Incubation of pre-labelled secretory granules with unlabelled ATP in the presence of high or low

Fig. 4. Time course of phosphate incorporation from ATP into PtdIns4P of secretory granules

(a) Secretory granules ($20\ \mu\text{g}$ of protein) were incubated at 37°C with $0.2\ \text{mM}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in $50\ \mu\text{l}$ of buffer containing $0.1\ \mu\text{M}$, $1.0\ \mu\text{M}$ ($\circ$) or $40\ \mu\text{M}$ ($\bullet$) free Ca^{2+} . (b) Granules were incubated for 10 min at 37°C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of $0.1\ \mu\text{M}$ Ca^{2+} as in (a), followed by centrifugation at $8000g$ for 5 min. Pelleted granules were resuspended in buffer containing $0.2\ \text{mM}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $0.1\ \mu\text{M}$ free Ca^{2+} and incubated at 37°C . In each case phospholipids were extracted at the times shown, and the incorporation of ^{32}P into PtdIns4P was measured by methods described in the Materials and methods section. Results are means $\pm$ S.D. for three experiments.

$[\text{Ca}^{2+}]$ (Fig. 5) did not lead to a significant decrease in the labelling of PtdIns4P, indicating that a phosphatase was not present in the secretory granules. This suggested that the Ca^{2+} -induced changes in activity were due to the presence of a Ca^{2+} -sensitive phosphatidylinositol kinase.

Measurement of the activity of the phosphatidylinositol kinase of the secretory granule over a range of free Ca^{2+} concentrations showed a 10-fold decrease in activity when the free Ca^{2+} concentration was raised from 1 to $5\ \mu\text{M}$ (Fig. 6). The Ca^{2+} -sensitivity appeared to be unaffected by the addition of micromolar exogenous calmodulin to the incubation medium (results not shown).

Phosphatidylinositol phosphorylation was also assayed in the presence of the calmodulin anta-

Fig. 5. Measurement of PtdIns4P phosphatase activity in secretory granules and plasma membranes

Secretory granules (a) and plasma membranes (b) ($20\ \mu\text{g}$ of membrane protein) were incubated for 10 min at 37°C with $0.2\ \text{mM}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in $50\ \mu\text{l}$ of buffer containing $0.1\ \mu\text{M}$ free Ca^{2+} . The medium was diluted with $70\ \mu\text{l}$ of ice-cold $50\ \text{mM}$ Mes, pH 6.9, and the membranes were pelleted by centrifugation. Membranes were resuspended in $50\ \mu\text{l}$ of buffer containing $2\ \text{mM}$ unlabelled ATP and $0.1\ \mu\text{M}$ ($\circ$) or $40\ \mu\text{M}$ ($\bullet$) free Ca^{2+} , and incubated at 37°C . PtdIns4P was extracted at the times indicated and assayed for incorporation of ^{32}P . Results are means $\pm$ S.D. for four determinations; * significantly different by Student's t test ($P < 0.025$).

gonist trifluoperazine and its analogue trifluoperazine oxide (Fig. 7). In the presence of $0.1 \mu\text{M}$ Ca^{2+} an increase in the phosphatidylinositol kinase activity of the secretory granule was observed at concentrations of trifluoperazine greater than $10 \mu\text{M}$, with a maximal effect at $50 \mu\text{M}$. Activity was also increased in the presence of $40 \mu\text{M}$ free Ca^{2+} and concentrations of trifluoperazine greater than $20 \mu\text{M}$. At concentrations of trifluoperazine of $50 \mu\text{M}$ or more, the activity of phosphatidylinositol kinase approached that seen at $0.1 \mu\text{M}$ free Ca^{2+} in the absence of trifluoperazine. Incubation of secretory granules with trifluoperazine oxide under identical conditions of high and low $[\text{Ca}^{2+}]$ resulted in no significant alteration in phosphatidylinositol kinase activity (results not shown).

Phosphorylation of phosphatidylinositol in plasma membranes

The properties and kinetics of the plasma-membrane phosphatidylinositol-phosphorylating system were, in most respects, essentially identical with those of the secretory granule. Phosphatidylinositol phosphorylation showed a comparable sensitivity to free $[\text{Ca}^{2+}]$ (Fig. 6), which was not affected by the addition of exogenous calmodulin (results not shown). The phosphatidylinositol

kinase had a K_m (apparent) for ATP of $120 \mu\text{M}$ and a K_a for Mg^{2+} of 7mM .

The plasma-membrane phosphatidylinositol kinase showed a response to trifluoperazine com-

Fig. 6. Inhibition of phosphatidylinositol phosphorylation by free Ca^{2+}

Secretory granules (○) or plasma membranes (●) ($20 \mu\text{g}$ of protein) were incubated for 1 min at 37°C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of various concentrations of free Ca^{2+} . PtdIns4P was isolated and assayed for incorporation of ^{32}P .

Fig. 7. Effect of trifluoperazine on phosphatidylinositol phosphorylation

Secretory granules (a) or plasma membranes (b) ($20 \mu\text{g}$ of protein) were incubated for 1 min at 37°C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of $0.1 \mu\text{M}$ (○) or $40 \mu\text{M}$ (●) free Ca^{2+} and with various concentrations of trifluoperazine added as a solution in dimethyl sulphoxide. PtdIns4P was isolated and the incorporation of ^{32}P was measured. Results are means $\pm$ S.D. for three experiments.

parable with that noted for the secretory granule, except that activity in the presence of $0.1 \mu\text{M-Ca}^{2+}$ continued to rise with trifluoperazine concentrations greater than $50 \mu\text{M}$ (Fig. 7).

In contrast with secretory granules, phosphatase activity was detected in preparations of plasma membranes, although the rate of degradation of $\text{PtdIns}4\text{P}$ was slow (Fig. 5). Activity appeared to be stimulated by raising the free $[\text{Ca}^{2+}]$ from 0.1 to $40 \mu\text{M}$.

Discussion

The presence of the polyphosphoinositides, $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, or the kinases responsible for their synthesis has been reported in subcellular fractions of various secretory tissues, including the adrenal medulla (Buckley *et al.*, 1971; Phillips, 1973; Lefebvre *et al.*, 1976) and parotid gland (Oron *et al.*, 1978). The presence of polyphosphoinositide-degrading enzymes in the chromaffin cell has also been demonstrated (Lefebvre *et al.*, 1976). Little, however, is known of the localization of enzymes and pathway intermediates in islets of Langerhans, since most studies have been performed in intact tissue.

In the present study we report phosphatidylinositol kinase and $\text{PtdIns}4\text{P}$ phosphatase activities in membrane fractions of an insulin-secreting β -cell tumour. The activity of $\text{PtdIns}4\text{P}$ kinase was apparently low in these fractions; however, this may have been related to the availability of endogenous substrate or lack of cofactors in these preparations. This was indicated by the finding that intact cells incubated for 90 min in the presence of $[\text{}^3\text{P}]\text{P}_i$ had incorporated the radioisotope into $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ to an equivalent extent in all membrane fractions later prepared from the tissue (N. E. Tooke & J. C. Hutton, unpublished work).

The intracellular distribution of phosphatidylinositol kinase closely followed that of 5'-nucleotidase, initially suggesting that the kinase was localized solely within the plasma membrane. It became apparent in this study, however, that 5'-nucleotidase was itself distributed bimodally, with a second component in the secretory-granule membrane. This conclusion was substantiated by the results of experiments in which the activity of the ectoenzyme 5'-nucleotidase was assayed in intact and Triton X-100-lysed tumour cells. These studies indicated that 30% of 5'-nucleotidase is present in an internal pool, possibly in the secretory granules (N. E. Tooke, unpublished work). Phosphorylated membranes prepared from plasma-membrane and secretory-granule fractions had different buoyant densities. Furthermore the plasma-membrane phosphatidylinositol kinase appeared to be accompanied by a Ca^{2+} -sensitive phosphatase, which

was absent from the secretory-granule fractions. Thus the activity in the secretory-granule fraction could not be attributed to contamination by plasma membranes.

The kinetic properties of the phosphatidylinositol kinase in the plasma membrane were indistinguishable from those of the granule enzyme, and were in many ways comparable with those of the enzyme in other secretory tissues. The secretory granule has a K_m (apparent) for ATP of $110 \mu\text{M}$, which is comparable with values determined for the secretory-granule enzyme in the parotid gland [$70 \mu\text{M}$ (Oron *et al.*, 1978)] and chromaffin cell [$250 \mu\text{M}$ (Lefebvre *et al.*, 1976); but cf. $5 \mu\text{M}$ (Phillips, 1973)]. The enzyme is also markedly stimulated by Mg^{2+} .

Unlike those previous studies, however, the phosphatidylinositol kinase of both the secretory granule and the plasma membrane was sensitive to small changes in free $[\text{Ca}^{2+}]$. Inhibition of the enzyme in other tissues has been reported for free Ca^{2+} concentrations of greater than $100 \mu\text{M}$ (Kai *et al.*, 1966; Buckley & Hawthorne, 1972; Lefebvre *et al.*, 1976; Oron *et al.*, 1978). The phosphatidylinositol kinase of the insulin secretory granule and plasma membrane, in contrast, appears to be sensitive to changes in free $[\text{Ca}^{2+}]$ in the low micromolar range. The nature of the mechanism responsible for the Ca^{2+} -sensitivity of the phosphatidylinositol kinase is unclear at present. High concentrations of the Ca^{2+} -dependent regulator protein calmodulin are present in the insulinoma (Hutton *et al.*, 1981) and other insulin-secreting tissues (Sugden *et al.*, 1979), and insulin secretion can be inhibited by calmodulin antagonists (Gagliardino *et al.*, 1980; Sopwith *et al.*, 1981; Niki *et al.*, 1981). Addition of exogenous calmodulin to granules and plasma membranes appeared to have no effect on the Ca^{2+} -sensitivity of the phosphatidylinositol kinase activity. This does not, however, exclude the possible presence of tightly bound calmodulin not removed by exposure to EGTA during the preparation of these fractions. Incubation of both secretory granules and plasma membranes with the calmodulin antagonist trifluoperazine stimulated phosphatidylinositol kinase activity, which is consistent with this possibility. The analogue trifluoperazine oxide, which is a poor calmodulin antagonist and, unlike trifluoperazine, has no significant effect on glucose-stimulated insulin secretion in islets (Gagliardino *et al.*, 1980), was likewise without effect on phosphatidylinositol kinase activity. If trifluoperazine was acting purely as a calmodulin antagonist, then one would expect, as observed here, a relief of Ca^{2+} -inhibition brought about by high ($40 \mu\text{M}$) Ca^{2+} . One might also expect, however, little effect on trifluoperazine at low ($0.1 \mu\text{M}$) free Ca^{2+} concentrations,

which was clearly not the case. The effects of trifluoperazine may be complicated by non-specific membrane effects brought about by the hydrophobic nature of this molecule (see Landry *et al.*, 1981) or may be due to its interaction, as a cation, with anionic lipids (Allan & Michell, 1975). In light of these findings, the role of calmodulin in the control of the tumour phosphatidylinositol kinase must be considered equivocal at present.

The fact that Ca^{2+} at micromolar concentrations can regulate the synthesis and degradation of $\text{PtdIns}4\text{P}$ in a system *in vitro* as demonstrated here suggests a possible role for polyphosphoinositides in the stimulus–secretion coupling of insulin release. Islet phosphatidylinositol turnover in response to insulinotropic stimuli is thought to be at least partially Ca^{2+} -dependent (Clements *et al.*, 1977; Laychock, 1983), although initiation of turnover may be a Ca^{2+} -independent process (Best & Malaisse, 1983a). The results presented here suggest that stimulation could be accompanied by enhanced phosphatidylinositol turnover through an activation of $\text{PtdIns}4\text{P}$ phosphatase located in the plasma membrane. On the other hand, turnover may be decreased if it proceeded via the polyphosphoinositides, since both plasma-membrane and secretory-granule kinases would be inhibited. These observations emphasize the need for further information about the metabolic and cellular pathways of these reactions, together with the steady-state concentrations of the intermediates, before any conclusions can be reached concerning the importance of these reactions to stimulus–secretion coupling in the β -cell.

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