Phosphatidylinositol hydrolysis in isolated guinea-pig islets of Langerhans

Michael P. SCHREY and William MONTAGUE

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

(Received 8 May 1983/Accepted 10 August 1983)

Previous studies have reported an increased turnover of phospholipid in isolated islets of Langerhans in response to raised glucose concentrations. The present investigation was thus undertaken to determine the nature of any phospholipases that may be implicated in this phenomenon by employing various radiolabelled exogenous phospholipids. Hydrolysis of 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol by a sonicated preparation of islets optimally released radiolabelled lysophosphatidylinositol, arachidonic acid and 1,2-diacylglycerol at pH 5, 7 and 9 respectively. This indicates the presence of a phospholipase A_1 and a phospholipase C. However, the lack of any labelled lysophosphatidylinositol production when 2-acyl-1-[^{14}C]stearoylglycerophosphoinositol was hydrolysed argues against a role for phospholipase A_2 in the release of arachidonic acid. Phospholipase C activity as measured by phosphatidyl-*myo*-[^3H]inositol hydrolysis was optimal around pH 8, required Ca^{2+} for activity and was predominantly cytosolic in origin. The time course of phosphatidylinositol hydrolysis at pH 6 indicated a precursor-product relationship for 1,2-diacylglycerol and arachidonic acid respectively. The release of these two products when phosphatidylinositol was hydrolysed by either islet or acinar tissue was similar. However, phospholipase A_1 activity was 20-fold higher in acinar tissue. Substrate specificity studies with islet tissue revealed that arachidonic acid release from phosphatidylethanolamine and phosphatidylcholine was only 8% and 2.5% respectively of that from phosphatidylinositol. Diacylglycerol lipase was also demonstrated in islet tissue being predominantly membrane bound and stimulated by Ca^{2+} . The availability of non-esterified arachidonic acid in islet cells could be regulated by changes in the activity of a phosphatidylinositol-specific phospholipase C acting in concert with a diacylglycerol lipase.

The interaction and regulation of intracellular messengers such as Ca^{2+} and prostaglandins during stimulated insulin secretion is an area that remains poorly understood. However, the mobilization of Ca^{2+} within the β -cell by raised glucose levels plays an unequivocal role in triggering insulin release (Rubin, 1982). In addition, recent studies suggest that the oxygenation products of arachidonic acid metabolized either by the cyclo-oxygenase pathway (Metz, 1981) or the lipoxygenase pathway (Metz *et al.*, 1982) may also be involved in the regulation of insulin secretion.

In many tissues the availability of the eicosanoid precursor arachidonic acid is regulated by the turnover of membrane phospholipid to which it is esterified (Irvine, 1982). The liberation of this arachidonic acid is in turn determined by the action of certain phospholipases, which in many instances require Ca^{2+} for optimal activity. It is thus possible

that a functional link may exist between Ca^{2+} mobilization and phospholipase activity during stimulus-secretion coupling in the β -cell. A number of studies have observed an increased turnover of the polar head group of phosphatidylinositol in isolated rat pancreatic islets on exposure to high glucose concentrations (Freinkel *et al.*, 1975; Clements & Rhoten, 1976). This phenomenon occurs in many tissues in response to a variety of agonists (Michell & Kirk, 1981) and a mediatory role for phosphatidylinositol-specific phospholipase C is generally accepted, though it is becoming apparent that hydrolysis of the polyphosphoinositides is the initiating step. Recently we have observed an increase in 1,2-diacylglycerol concomitant with a fall in radiolabelled phospholipid in glucose-stimulated guinea-pig islets prelabelled with [^3H]glycerol (Montague & Parkin, 1980), which is also consistent with a role for phospholipase C during insulin secretion.

Furthermore, addition of phospholipase C to isolated islets mimicks both the phospholipid turnover and insulin secretion evoked by raised glucose levels (Tanigawa *et al.*, 1982). In many tissues 2-arachidonoyl-1-stearoylglycerophosphoinositol is the predominant species of this phospholipid (White, 1973) and in certain cell types it has been demonstrated that phosphatidylinositol is rapidly hydrolysed to 1,2-diacylglycerol, which is in turn hydrolysed by a diacylglycerol lipase to liberate non-esterified arachidonic acid (Rittenhouse-Simmons, 1979; Di Renzo *et al.*, 1981).

The purpose of the present investigation was to identify and characterize any phospholipase activity present in isolated guinea pig islet tissue by employing a variety of exogenous radiolabelled phospholipid substrates. Our findings indicate the presence of a phosphatidylinositol-specific phospholipase C that is soluble and Ca^{2+} -dependent. However, non-esterified arachidonic acid is a major product of phosphatidylinositol hydrolysis around pH 6 and evidence is presented for the sequential involvement of phospholipase C and a diacylglycerol lipase. A possible mediatory role for these islet enzymes in the regulation of insulin secretion is discussed.

Materials and methods

Preparation of islet tissue

Pancreatic islets were isolated from guinea pigs (800 g approx.) of the Duncan Hartley strain by tissue disaggregation with collagenase as previously described (Lacy & Kostianovsky, 1967). The isolated islets were then placed in ice-cold Tris/HCl buffer (10 mM), pH 7.2, containing KCl (90 mM), at a concentration of around 250 islets per ml. The islet suspension was disrupted by ultrasonication with 2×10 s bursts in an MSE ultrasonicator. Phospholipase activity was then measured in portions of this sonicated preparation under a variety of conditions. In experiments where enzyme activity was measured either in a high-speed pellet or supernatant, isolated islets were resuspended in 0.3 M sucrose containing 10 mM-Tris/HCl (pH 6.0) and homogenized in a ground-glass homogenizer. The homogenate was centrifuged at 1000 g for 5 min to remove nuclei and cellular debris. The resultant supernatant was then centrifuged for 90 min at 100 000 g at 4°C to provide a particulate and soluble fraction. The 100 000 g pellet was resuspended in Tris/HCl (10 mM), pH 7.2, containing KCl (90 mM) at a volume equal to that of the original homogenization volume. Protein in these various fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of substrates

1-Acyl-2-[^{14}C]arachidonoyl-glycerophosphoinositol, -glycerophosphoethanolamine and -glycerophosphocholine. These were synthesized by enzymic transfer of [^{14}C]arachidonic acid to the corresponding 1-acyl-lysophospholipid using a rat liver microsomal preparation as the source for acyltransferase. Lysophosphatidylethanolamine and lysophosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 1-Acyl-glycerophosphoinositol was obtained by hydrolysing phosphatidylinositol with phospholipase A_2 from *Crotalus durissus terrificus* venom (Sigma). The reaction mixture contained 2 mM- CaCl_2 , 20 mM- NaCl , deoxycholate (0.5 mg/ml), phosphatidylinositol (1.8 mM) sonicated in Tris/HCl (100 mM), pH 8.9, and phospholipase A_2 (9 units) in a final volume of 3 ml. Incubation was carried out for 1 h at 37°C and terminated by addition of 13.5 ml of chloroform/methanol (acidified with 0.1 M-HCl) (1:2, v/v). Lipids were extracted and fractionated by t.l.c. in chloroform/methanol/acetic acid/0.1 M-sodium borate (50:30:8:3, by vol.) and the 1-acylglycerophosphoinositol (R_f 0.16) was quantified by phosphorus determination (Chen *et al.*, 1956) after elution with acidified Bligh and Dyer (1959) solvent [chloroform/methanol (containing 0.1 M-HCl) (1:2, v/v)].

The incubation conditions for the biosynthetic reacylation of lysophospholipid with [^{14}C]arachidonic acid were similar to those described by Wassef & Horowitz (1981). The incubation mixture contained 1.6 mM-ATP, 100–150 μM -lysophospholipid, 0.1 mM-CoA (lithium salt), 2 mM- MgCl_2 , 10–15 μCi of [$1\text{-}^{14}\text{C}$]arachidonic acid (sp. radioactivity 58.4 Ci/mol), 60 mM-Tris/HCl buffer (pH 7.4) and 7 mg of rat liver microsomal protein in a final volume of 3 ml. The reaction was carried out for 45 min at 37°C under an atmosphere of N_2 . The individual phospholipids were extracted and purified by t.l.c. using two separate solvent systems as previously described (Schrey & Rubin, 1979). The specific radioactivities (c.p.m./ μmol) for phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine were 7.9×10^6 , 4.4×10^6 and 2.0×10^6 respectively. In each case at least 95% of the radiolabel was esterified to the *sn*-2-position as judged by examination of the hydrolysis products of snake venom phospholipase A_2 action.

2-Acyl-1-[^{14}C]stearoylglycerophosphoinositol. 2-Acylglycerophosphoinositol was obtained by incubating phosphatidylinositol with guinea-pig acinar-tissue sonicated material (0.38 mg of protein). This was used as the source of phospholipase A_1 since this species has been previously described to possess a highly active secretory pancreatic phospholipase A_1 (White *et al.*, 1971). The method for

the biosynthetic reacylation of 2-acylglycerophosphoinositol with [^{14}C]stearic acid was the same as that described above (Wassef & Horowitz, 1981) except that 30 μCi of [^{14}C]stearic acid (sp. radioactivity 55.7 Ci/mol) was used. The radio-labelled phosphatidylinositol was extracted and purified as before and had a specific radioactivity of 2.5×10^6 c.p.m./ μmol . At least 80% of the [^{14}C]stearate was esterified to the *sn*-1-position as evidenced by hydrolysis with snake venom phospholipase A_2 .

Phosphatidyl-*myo*-[^3H]inositol. Phosphatidyl-*myo*-[^3H]inositol was prepared by incubating 1 ml of a rat liver low-speed (1650g) supernatant [obtained by homogenizing 8.6 g of rat liver in 13 ml of Tris/HCl/KCl (50 mM), pH 7.4] with 200 μCi of *myo*-[2- ^3H]inositol (sp. radioactivity 6.6 Ci/mmol), cytidine (10 mM), hydroxybutyrate (5 mM) and MnCl_2 (1 mM) in Tris/HCl (50 mM), pH 7.4, in a final volume of 3 ml for 1 h at 37°C. Radiolabelled phosphatidylinositol was extracted with acidified methanol/chloroform (2:1, v/v) and purified by t.l.c. using two separate solvent systems (Schrey & Rubin, 1979). The specific radioactivity of the phosphatidyl-*myo*-[^3H]inositol was 3.9×10^7 c.p.m./ μmol . All radiolabelled phospholipid substrates were stored in chloroform at -20°C.

1-Acyl-2-[^{14}C]arachidonoylglycerol. This substrate was generated, for the assay of diacylglycerol lipase, by incubating phospholipase C (*Bacillus cereus*) purchased from Sigma with 1-acyl-2-[^{14}C]arachidonoylglycerophosphocholine. After extraction of the lipids 1,2-diacylglycerol was purified by t.l.c. using petroleum ether/diethyl ether/acetic acid (40:10:1) as the solvent system. The radiolabelled 1,2-diacylglycerol was eluted from the gel with methanol/chloroform (2:1, v/v) and the solvent removed under N_2 . The 1-acyl-2-[^{14}C]arachidonoylglycerol was then redissolved in a petroleum ether to minimize isomerization and stored at -20°C.

Incubation conditions. In general, unless otherwise stated, radiolabelled phosphatidylinositol substrate was always present at a final concentration of 5 μM , being resuspended in aqueous solution by brief vortex-mixing in water to minimize any non-enzymic degradation. The assay was performed in a final volume of 0.25 ml containing 50 mM-Tris/HCl (at an appropriate pH), 1 mM- CaCl_2 and sonicated islet protein (25–80 μg). Modifications of this protocol are indicated where appropriate. The reactions were initiated by addition of the enzyme and were performed at 37°C and terminated at the appropriate times. At pH values below 6 a sodium acetate buffer (50 mM) was employed. Background activity due to non-enzymic hydrolysis was always measured in control incubations without added enzyme and subtracted from overall enzyme activity. In some instances

hydrolysis is expressed in terms of the amount of product formed as a percentage of the total radioactivity present.

Extraction and analysis of reaction products. Enzyme incubations were terminated by addition of 6 vol. of chloroform/methanol (containing 0.1 M-HCl) (1:2, v/v) and the lipids extracted into chloroform by further addition of 2 vol. of chloroform and 3 vol. of KCl/EDTA (2 M/5 mM). Carrier standards of arachidonic acid, 1,2-diacylglycerol, 1,3-diacylglycerol and monoacylglycerol (obtained from Sigma Chemical Co.) were then added to each chloroform extract, which was then dried under N_2 . The lipid extract was then resuspended in 50 μl of chloroform/methanol (9:1, v/v) and applied to precoated silica gel 60 t.l.c. plates (Merck) and developed in a solvent system containing petroleum ether (bp. 40–60°C)/diethyl ether/acetic acid (40:10:1, by vol.). The plate was then dried and further developed in diethyl ether to 2 cm beyond the origin to separate monoacylglycerol from phospholipid, which remained at the origin. All the neutral lipid fractions were effectively separated from one another in this system. Each fraction was located by exposure to I_2 vapour and the radioactivity present in the gel was measured by liquid-scintillation counting in Fiso fluor 3 scintillation cocktail (Fisons). Separation of individual phospholipids and their respective lysophospholipids was achieved by t.l.c. as previously described (Schrey & Rubin, 1979). In experiments where lysophosphatidylinositol production was measured, incubations were terminated by heating in a boiling-water bath for 2 min and lipids were extracted with an equal volume of *n*-butanol (Hirasawa *et al.*, 1981), and analysed by t.l.c. as described above.

Phospholipase C activity was measured by determining the production of [^3H]phosphoinositol from phosphatidyl-*myo*-[^3H]inositol. Incubations were extracted with an equal volume of *n*-butanol and the amount of [^3H]phosphoinositol present in the aqueous phase was determined after separation from free inositol by t.l.c. in chloroform/methanol/water (6:4:1, by vol.) (Wightman *et al.*, 1981). Activity was expressed either in terms of the percentage of total radioactivity present as phosphoinositol or as pmol generated.

Results

Characteristics of phosphatidylinositol hydrolysis in islet tissue

Fig. 1 shows the effect of pH on the percentage distribution of product formed when either 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol (Fig. 1a) or 2-acyl-1-[^{14}C]stearoylglycerophosphoinositol (Fig. 1b) is incubated with islet sonicated material for 30 min in the presence of 1 mM- Ca^{2+} . The major

Fig. 1. The effect of pH on phosphatidylinositol hydrolysis by islet sonicated material

Reactions were performed in 50 mM-sodium acetate buffer (pH 4 and 5), Tris/HCl (pH 6–9) or Tris base (pH 10) containing Ca^{2+} (1 mM), 50 μg of islet protein and radiolabelled phosphatidylinositol (5 μM) at 37°C for 30 min. Curves show hydrolysis of (a) 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol and (b) 2-acyl-1-[^{14}C]stearoylglycerophosphoinositol to lysophosphatidylinositol (■), non-esterified fatty acid (▲) and 1,2-diacylglycerol (●). Incubations were terminated by boiling for 2 min and lipids extracted in *n*-butanol. Hydrolysis is expressed as the amount of product formed as a percent of the total radioactivity present. Each point is the mean of duplicate incubations from a single representative experiment. Similar data were obtained in three separate experiments.

products formed at pH 5, namely 2-[^{14}C]arachidonoylglycerophosphoinositol and [^{14}C]stearic acid, are indicative of phospholipase A_1 activity. The appearance of [^{14}C]arachidonic acid at neutral pH is probably not a consequence of phospholipase A_2 activity since no 1-[^{14}C]stearoylglycerophosphoinositol accumulated at this pH. Furthermore, the maintained high levels of [^{14}C]stearic acid at pH 7 cannot be wholly accounted for by phospholipase A_1 activity at this pH. The major product at pH 8 and 9 is 1,2-diacylglycerol and is indicative of phospholipase C activity. This is confirmed when phosphatidyl-*myo*-[^3H]inositol is employed as a substrate as indicated by the production of [^3H]phosphoinositol (Fig. 2). Optimal activity occurs around pH 8.0. However, considerable activity is also present at pH 6 and 7 in the apparent absence of any significant 1,2-diacylglycerol accumulation (Fig. 1). As reported previously in other tissues (Shukla, 1982), the phospholipase C requires Ca^{2+} for full activity (Fig. 2). The less effective suppression of activity in the absence of Ca^{2+} at pH 5 could be due to some contribution by a lysosomal phospholipase C which is inhibited by Ca^{2+} in a number of tissues and optimally active around this pH (Shukla, 1982).

Thus, in two separate experiments phospholipase C activity was reduced by a mean 20% at pH 5 by removal of Ca^{2+} and by 90% at pH 7.

The temporal profile of 1,2-diacylglycerol and arachidonic acid accumulation due to 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol hydrolysis by islet sonicated material is shown in Fig. 3. After 30 min incubation at pH 6 arachidonic acid was the major product. However, after 2 min a transient increase in 1,2-diacylglycerol production was apparent before significant accumulation of non-esterified arachidonic acid (Fig. 3). This precursor-product relationship is suggestive of a sequential action of phospholipase C followed by diacylglycerol lipase. Monoacylglycerol production also increased up to 5 min, but did not further accumulate after this time.

Phospholipase activity in islet and acinar tissue

Since previous studies have reported the presence of a secreted phospholipase A_1 from guinea-pig pancreas (White *et al.*, 1971) it was desirable to compare the activity of this enzyme in isolated islets with that in acinar tissue. The hydrolysis of 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol at pH 5, 6 and 9 by islet and acinar tissue was measured. The production rate of 1,2-diacylglycerol

Fig. 2. The effect of pH and Ca^{2+} on phospholipase C activity in islet sonicated material

Reactions were initiated by addition of islet protein (80 μg) to buffer (50 mM) containing phosphatidyl- myo - $[\text{H}]$ inositol (5 μM) and terminated after 20 min by boiling. Lipid was extracted into *n*-butanol and radioactivity in the aqueous phase was analysed as described in the Experimental section. Incubations were performed in the presence of Ca^{2+} (1 mM; ●) or EGTA (1 mM; ○). Phospholipase C activity is expressed as the percentage of the total radioactivity released as $[\text{H}]$ phosphoinositol. Values represent the mean of duplicate incubations. Similar profiles were obtained in separate experiments.

and arachidonic acid at pH 6 and 9 was essentially the same in both tissues (results not shown). However, phospholipase A_1 activity at pH 5 was about 20-fold higher in acinar tissue. Thus, during a 5 min incubation at pH 5 labelled lysophosphatidylinositol production was 0.7 ± 0.1 and 14.0 ± 2.0 nmol/mg of protein in islet and acinar tissue homogenates respectively (means $\pm$ S.E.M., $n=4$). This large difference probably reflects the presence of a highly active secretory phospholipase A_1 in acinar tissue. Whether phospholipase A_1 activity detected in isolated islets is due to small amounts of contaminating acinar tissue or is indeed due to an endogenous islet enzyme remains to be resolved.

Fig. 3. Time course of 1-acyl-2- $[\text{H}]$ arachidonoyl-glycerophosphoinositol hydrolysis by islet sonicated material

Incubations contained islet protein (60 μg) in Tris/HCl buffer (50 mM), pH 6, Ca^{2+} (1 mM) and 1-acyl-2- $[\text{H}]$ arachidonoyl-glycerophosphoinositol (5 μM). Reactions were terminated at specific times by addition of chloroform/methanol (1:2, v/v). Activity is expressed as the amount of product formed per mg of protein: ○, 1,2-diacylglycerol; ■, monoacylglycerol; ▲, arachidonic acid. Each point represents the mean value from two separate experiments each performed in duplicate.

Substrate specificity of islet phospholipase activity

The hydrolysis of $[\text{H}]$ arachidonic acid-labelled phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol by islet sonicated material is shown in Table 1. With regard to the production of 1,2-diacylglycerol and arachidonic acid there appears to be a preference for phosphatidylinositol as substrate. This probably reflects the action of a phosphatidylinositol-specific phospholipase C acting in concert with a diacylglycerol lipase. The phospholipase A_1 activity appears equally directed towards phosphatidylinositol and phosphatidylethanolamine (Table 1). However, non-esterified arachidonic acid production from phosphatidylinositol is more than 10 times higher than from phosphatidylethanolamine. This would be inconsistent with an interpretation that proposes a combination of phospholipase A_1 and a lysophospholipase as a mechanism for releasing non-esterified arachidonic acid. Furthermore, the hydrolysis of phosphatidylinositol by phospholipase A_1 is inhibited by Ca^{2+} , whereas arachidonic acid production is stimulated by Ca^{2+} . Thus, in two experiments employing 1-acyl-2- $[\text{H}]$ arachidonoyl-glycerophosphoinositol as substrate the

Table 1. *Substrate specificity of phospholipase activity in islet sonicate*

Islet sonicated material (about 50 µg) was incubated in the presence of [14 C]arachidonic acid-labelled phospholipid (20 µM) in Tris/HCl (50 mM), pH 6.0, containing Ca^{2+} (1 mM) for 20 min. After n-butanol extraction the amount of product formed was determined. Values are means $\pm$ S.E.M. from three separate experiments. Abbreviation used: ND, non-detectable.

[14 C]Arachidonate-labelled substrate	Product (nmol/mg of protein)		
	Diacylglycerol	Non-esterified fatty acid	Lysophospholipid
Phosphatidylinositol	4.3 $\pm$ 0.8	28.3 $\pm$ 4.1	19.8 $\pm$ 1.9
Phosphatidylethanolamine	0.9 $\pm$ 0.4	2.4 $\pm$ 0.5	21.2 $\pm$ 6.2
Phosphatidylcholine	ND	0.7 $\pm$ 0.1	4.8 $\pm$ 2.1

production of 2-[14 C]arachidonoylglycerophosphoinositol by islet sonicated material was increased by a mean 95% when 1.0 mM- Ca^{2+} was replaced with 1.0 mM-EGTA, whereas [14 C]arachidonic acid production was reduced by a mean 58%.

Subcellular distribution of phospholipase C activity in islets

The distribution of phospholipase C activity between a high-speed (100 000 g) pellet and corresponding supernatant fraction is shown in Table 2 and appears to be predominantly cytoplasmic in origin. Activity in the 100 000 g supernatant is approx. 10-fold higher than in the pellet. This is consistent with previous studies in other tissues where the enzyme was reported to be mainly soluble (Shukla, 1982).

Ca^{2+} requirement of a soluble fraction of islet phospholipase

The simultaneous hydrolysis of 1-acyl-2-[14 C]-arachidonoylglycerophosphoinositol and phosphatidyl-*myo*-[^3H]inositol by the 100 000 g supernatant from islet homogenate at different Ca^{2+} concentrations is shown in Fig. 4. Addition of 1 mM- Ca^{2+} was necessary to elevate activity above that present in the absence of added Ca^{2+} . However, chelation of endogenous Ca^{2+} present in the supernatant with EGTA (1 mM) was sufficient to further reduce activity 3-fold. The changes in production of phosphoinositol and the sum of arachidonic acid and 1,2-diacylglycerol with changes in Ca^{2+} concentration clearly parallel one another. This would be consistent with the proposal that arachidonic acid production is dependent on phospholipase C activity.

Diacylglycerol lipase activity in islets of Langerhans

A role for diacylglycerol lipase has already been inferred in the generation of non-esterified

Table 2. *Subcellular distribution of phospholipase C activity in islets of Langerhans*

Phospholipase C activity was measured by incubating portions of 1000 g supernatant, 100 000 g pellet or 100 000 g supernatant obtained from islet homogenates (see the Experimental section) with phosphatidyl-*myo*-[^3H]inositol (5 µM) in Tris/HCl buffer (50 mM), pH 7.5, for 15 min. Incubations were terminated by boiling for 2 min and phospholipid was extracted in n-butanol. The fraction of radioactivity present in the aqueous phase as phosphoinositol was determined as a measure of phospholipase C activity (see the Experimental section). Values represent means $\pm$ S.E.M., from three separate experiments.

Subcellular fraction	Protein (µg)	Phosphoinositol production	
		Activity (pmol/min)	Specific activity (pmol/mg of protein per min)
1000 g supernatant	59	29 $\pm$ 3	473 $\pm$ 25
100 000 g pellet	21	4 $\pm$ 1	181 $\pm$ 27
100 000 g supernatant	36	46 $\pm$ 4	1318 $\pm$ 124

arachidonic acid from the diacylglycerol product of phosphatidylinositol-specific phospholipase C action. Diacylglycerol lipase was measured by monitoring the hydrolysis of 1-acyl-2-[14 C]arachidonoylglycerol. Activity was readily detectable at pH 6.0 as measured by the release of [14 C]arachidonic acid and was higher in particulate fractions compared with a high-speed supernatant. Thus, 64 $\pm$ 9% (mean $\pm$ S.E.M., $n = 5$) of the total homogenate activity was present in the 100 000 g pellet. The activity of this enzyme was also increased in the presence of Ca^{2+} . Thus, diacylglycerol lipase activity in islet sonicated material was increased by 55 $\pm$ 5% (mean $\pm$ S.E.M., $n = 3$) when measured in the presence of 1 mM- Ca^{2+} relative to Ca^{2+} -free conditions (i.e. no added Ca^{2+} plus 0.1 mM-EGTA).

The specific activity of diacylglycerol lipase in islet homogenate was 192 $\pm$ 36 pmol/mg of protein per min (mean $\pm$ S.E.M., $n = 5$) and is probably sufficient to account for the observed breakdown of diacylglycerol derived from phosphatidylinositol. In two separate experiments comparing diacylglycerol hydrolysis in acinar tissue with that in islet tissue, the mean specific activity in acinar tissue was 5.4 times higher than that present in islet tissue. In the same experiments the mean specific activity of phospholipase A_1 was 18.5 times higher in acinar tissue compared with that in islet tissue. Thus, on the basis of the relative activities of these two enzymes in both tissues it seems improbable that the diacylglycerol lipase detectable in islet tissue is entirely attributable to contaminating acinar tissue.

The presence of diacylglycerol lipase activity is consistent with two former observations, namely the

Fig. 4. The effect of Ca^{2+} on phosphatidylinositol hydrolysis by 100 000 g supernatant of islet homogenate. Portions of a soluble fraction of islet homogenate (25 μg of protein) were incubated for 15 min in Tris/HCl buffer (50 mM, pH 7.5, containing 1-acyl-2-[^{14}C]arachidonoylphosphoinositol (5 μM) and phosphatidyl-*myo*-[^3H]inositol (5 μM) either in the presence of EGTA (1 mM) or with increasing Ca^{2+} concentrations (0–1 mM). Reactions were terminated and lipids extracted in *n*-butanol. Hydrolysis products were measured after t.l.c. of portions of both organic and aqueous fractions. Phospholipase activity is expressed as the percent of total radioactivity (^3H or ^{14}C) released as [^3H]phosphoinositol (○), 1,2-[^{14}C]diacylglycerol (▲), [^{14}C]arachidonic acid (■) or the sum of 1,2-[^{14}C]diacylglycerol and [^{14}C]arachidonic acid (●). Each point is the mean value of duplicate incubations from a representative experiment.

production of monoacylglycerol during phosphatidylinositol hydrolysis and the lack of a 1:1 stoichiometry between phosphoinositol and diacylglycerol production. The positional specificity of this enzyme has yet to be resolved, thus a diacylglycerol lipase acting in conjunction with a monoacylglycerol lipase could be involved in the release of non-esterified arachidonic acid.

Discussion

The present study has employed a variety of radiolabelled exogenous phospholipid substrates to measure phospholipase activity in isolated guinea-pig islets of Langerhans. Depending on the incubation conditions a variety of enzyme activities have been detected. Optimal hydrolysis by islet sonicated material of 1-acyl-2-[^{14}C]arachidonoylglycerophosphoinositol to release lysophosphatidylinositol, non-esterified arachidonic acid and 1,2-diacylglycerol occurred at pH 5, 7 and 9 respectively (Fig. 1a). This suggests the involvement of phospholipase A_1 and phospholipase C but the lack of any significant lysophosphatidylinositol production when 2-acyl-1-[^{14}C]stearoylglycerophosphoinositol was hydrolysed (Fig. 1b) argues against a role of phospholipase A_2 in the release of arachidonic acid (Fig. 1a). The apparent lack of 1,2-diacylglycerol accumulation at pH 6 and 7 (Fig. 1) in the presence of phospholipase C activity (Fig. 2) can be reconciled by proposing a further degradation of the 1,2-diacylglycerol product of phospholipase C action by a diacylglycerol lipase. Further evidence for this interpretation is provided by the temporal precursor-product relationship between 1,2-diacylglycerol and arachidonic acid at pH 6 (Fig. 3) and the fact that diacylglycerol lipase activity was detectable in islets. The involvement of phospholipase C and a diacylglycerol lipase in the liberation of arachidonic acid from phosphatidylinositol has been observed in a number of other tissues including platelets (Rittenhouse-Simmons, 1979) and human foetal membranes and uterine decidua (Di Renzo *et al.*, 1981).

The presence of a soluble phospholipase C specific for phosphatidylinositol hydrolysis has been reported in several tissues (Shukla, 1982) and the present study extends this to guinea-pig islets. Thus phospholipase C activity was ten times higher in a 100 000 g supernatant compared with activity in the pellet (Table 2). Furthermore, the high specificity of arachidonic acid release from phosphatidylinositol compared with that from phosphatidylethanolamine and phosphatidylcholine (Table 1) in the apparent absence of any phospholipase A_2 activity (Fig. 1b) is also supportive of a role for a phosphatidylinositol-specific phospholipase C in arachidonic acid release. Although phospholipase C activity was present at pH 6 the enzyme was optimally active around pH 8, which is higher than that reported in some studies but consistent with that measured in brain (Hirasawa *et al.*, 1981).

The presence of a secretory phospholipase A_1 in guinea-pig pancreas has been reported previously (White *et al.*, 1971). The relatively high activity of this enzyme in acinar tissue compared with that in islet tissue would be expected if this enzyme assumes

the role of a secretory phospholipase A_1 . In the present study a 5% contamination of the isolated islets with acinar tissue would account for the phospholipase A_1 activity detectable in the former. However, the possibility of an endogenous islet phospholipase A_1 cannot be ruled out. Indeed, such an enzyme has been reported in other tissues such as brain (Hirasawa *et al.*, 1981).

The dependence of phosphatidylinositol-specific phospholipase C of mammalian origin on Ca^{2+} for optimal activity has been previously reviewed (Shukla, 1982). In this respect the islet enzyme is no exception and activity around neutral pH also requires the presence of Ca^{2+} for full activity (Figs. 2 and 4). The Ca^{2+} -dependent nature of this enzyme does not necessarily mean its activity is directly regulated by changes in the intracellular levels of Ca^{2+} (Irvine, 1982). Indeed, a sensitivity to changes in Ca^{2+} concentration in the micromolar range would be a prerequisite for such a mechanism. In the present study appreciable increases in islet phospholipase C activity above levels observed in the absence of added Ca^{2+} were only seen at 1 mM- Ca^{2+} (Fig. 4). However, removal of endogenous Ca^{2+} by chelation with EGTA (1 mM) considerably reduced activity by about 75%. It is of interest that recent studies on the phosphatidylinositol-specific phospholipase C in rat brain showed that proteolytic activation results in an enzyme whose activity is highly sensitive to changes in Ca^{2+} from 0.1–1.0 μ M (Hirasawa *et al.*, 1982).

A functional role has yet to be ascribed to phosphatidylinositol-specific phospholipase C in islet tissue. Previous studies have shown that insulin secretagogues such as high glucose levels stimulate the turnover of the polar head group of endogenous phosphatidylinositol in intact rat islets (Freinkel *et al.*, 1975; Clements & Rhoten, 1976) presumably by activation of phospholipase C. More recently we have shown that high glucose stimulated a Ca^{2+} -dependent loss of radiolabel from [3H]glycerol labelled guinea-pig islet phospholipid accompanied by an increase in 1,2-diacylglycerol (Montague & Parkin, 1980). A role for phospholipase A_2 has also been implicated in glucose-stimulated rat islets (Laychock, 1982). Furthermore, the turnover of arachidonic acid within phosphatidylethanolamine and phosphatidylinositol was also increased on exposure to high glucose concentrations and was accompanied by increased prostaglandin production (Evans & Clements, 1981). The significance of the phospholipase C-mediated turnover of phosphatidylinositol during insulin secretion remains a subject for speculation. The fate of the diacylglycerol product of this hydrolysis could play an important role in this respect. An increase in intracellular diacylglycerol generated during β -cell stimulation could be important in the activation of

the Ca^{2+} and phospholipid-dependent protein kinase C reported in various tissues including islets (Tanigawa *et al.*, 1982). The enzyme diacylglycerol kinase permits a recycling of the diacylglycerol product back into phosphatidylinositol via phosphatidic acid. Alternatively, the diacylglycerol could be further hydrolysed by a lipase to release non-esterified fatty acids such as arachidonic acid. In human amniotic tissues it has been shown recently that increases in Ca^{2+} concentration increase the ratio of activity of diacylglycerol lipase to diacylglycerol kinase (Sagawa *et al.*, 1982), possibly regulating the response to phosphatidylinositol hydrolysis and favouring the release of arachidonic acid. In the present study arachidonic acid is certainly the major product of Ca^{2+} -dependent phosphatidylinositol hydrolysis around neutral pH and diacylglycerol lipase activity was also increased considerably in the presence of Ca^{2+} , which is also the case for this enzyme in platelets (Bell *et al.*, 1979). It is thus possible that β -cell stimulation causes an increase in phosphatidylinositol turnover, which is accompanied by elevated cytosolic Ca^{2+} levels and an increased release of arachidonic acid from the diacylglycerol generated. The further metabolism of this arachidonic acid in the β -cell by either the lipoxygenase pathway (Metz *et al.*, 1982) or the cyclo-oxygenase pathway (Metz, 1981) may play an important role in the regulation of insulin.

We thank the Wellcome Trust for their continued support of these studies.

References

- Bell, R. L., Kennerly, D. A., Stanford, N. & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3241
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Chen, Jr., P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Clements, R. S. & Rhoten, W. B. (1976) *J. Clin. Invest.* **57**, 684–691
- Di Renzo, G. C., Johnston, J. M., Okazaki, T., Ojita, J. R., MacDonald, P. C. & Bleasdale, J. E. (1981) *J. Clin. Invest.* **67**, 847–856
- Evans, M. H. & Clements, R. S. (1981) *Clin. Res.* **29**, 404A
- Freinkel, N., El Younsi, C. & Dawson, R. M. C. (1975) *Eur. J. Biochem.* **59**, 245–252
- Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1981) *Eur. J. Biochem.* **120**, 53–58
- Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982) *Biochem. J.* **206**, 675–678
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Lacy, P. E. & Kostianovsky, M. (1967) *Diabetes* **16**, 35–39
- Laychock, S. G. (1982) *Cell Calcium* **3**, 43–54

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Metz, S. A. (1981) *Prostaglandins Med.* **7**, 581–589
- Metz, S. A., Fujimoto, W. Y. & Robertson, R. P. (1982) *Endocrinology* **111**, 2141–2143
- Michell, R. & Kirk, C. J. (1981) *Trends Pharmacol. Sci.* **2**, 86–89
- Montague, W. & Parkin, E. N. (1980) *Horm. Metab. Res. Suppl.* **10**, 153–157
- Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 580–587
- Rubin, R. P. (1982) in *Calcium and Cellular Secretion*, pp. 126–133, Plenum Press, New York and London
- Sagawa, N., Okazaki, T., MacDonald, P. C. & Johnston, J. M. (1982) *J. Biol. Chem.* **257**, 8158–8162
- Schrey, M. P. & Rubin, R. P. (1979) *J. Biol. Chem.* **254**, 11234–11241
- Shukla, S. D. (1982) *Life Sci.* **30**, 1323–1335
- Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, T. & Nishizuka, Y. (1982) *FEBS Lett.* **138**, 183–186
- Wassef, M. K. & Horowitz, M. I. (1981) *Biochim. Biophys. Acta* **665**, 234–243
- White, D. A. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 441–482, Elsevier, Amsterdam
- White, D. A., Pounder, D. J. & Hawthorne, J. N. (1971) *Biochim. Biophys. Acta* **242**, 99–107
- Wightman, P. D., Dahlgren, M. E., Hall, J. C., Davies, P. & Bonney, R. J. (1981) *Biochem. J.* **197**, 523–526