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Stimulation of insulin release by phospholipase D

A potential role for endogenous phosphatidic acid in pancreatic islet function

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Although exogenous phosphatidic acid (PA) has been shown to promote insulin release, the effects of endogenous PA on endocrine function are largely unexplored. In order to generate PA *in situ*, intact adult-rat islets were treated with exogenous phospholipases of the D type (PLD), and their effects on phospholipid metabolism and on insulin release were studied in parallel. Chromatographically purified PLD from *Streptomyces chromofuscus* stimulated the accumulation of PA in [^{14}C]arachidonate- or [^{14}C]myristate-prelabelled islets, and also promoted insulin secretion over an identical concentration range. During 30 min incubations, insulin release correlated closely with the accumulation of [^{14}C]arachidonate-labelled PA ($r^2 = 0.98$; $P < 0.01$) or [^{14}C]myristate-labelled PA ($r^2 = 0.97$; $P < 0.01$). Similar effects were seen both in freshly isolated and in overnight-cultured intact islets. In contrast, PLDs (from cabbage or peanut) which do not support phospholipid hydrolysis at the pH of the extracellular medium also did not promote insulin release. The effects on secretion of the active PLD preparation were inhibited by modest cooling (to 30 °C); dantrolene or Co^{2+} also inhibited PLD-induced secretion without decreasing PLD-induced PA formation. Additionally, the removal of PLD left the subsequent islet responsiveness to glucose intact, further supporting an exocytotic non-toxic mechanism. PLD-induced insulin release did not appear to require influx of extracellular Ca^{2+}, nor could the activation of protein kinase C clearly be implicated. During incubations of 30 min, PLD selectively generated PA; however, more prolonged incubations (60 min) also led to production of some diacylglycerol and free arachidonic acid concomitant with progressive insulin release. These data suggest that PLD activation has both rapid and direct effects (via PA) and more delayed, secondary, effects (via other effects of PA or the generation of other lipid signals). Taken in conjunction with our demonstration that pancreatic islets contain an endogenous PLD which generates PA [Dunlop & Metz (1989) Biochem. Biophys. Res. Commun. **163**, 922–928], these studies provide evidence suggesting that PLD activation (and possibly other pathways leading to PA formation) could play a role in stimulus–secretion coupling in pancreatic islets.

INTRODUCTION

In many cells, exogenous phosphatidic acid (PA) has potent effects on cyclic nucleotide formation [1,2], inositol lipid hydrolysis [1,3], protein kinase C (PKC) activity [3,4], Ca^{2+} influx or intracellular Ca^{2+} mobilization [1–3,5,6], and growth [6,7]. Recent studies demonstrate that exogenous PA can mobilize Ca^{2+} stores and stimulate insulin release in neonatal-rat islet cells [1]. Thus PA has been considered to have a potential role in signal transduction; however, the role of endogenously generated PA has rarely been assessed. It is known that PA can be generated in islets by several possible mechanisms. For example, the synthesis of PA *de novo* can be stimulated by the metabolism of glucose, the major physiological insulin secretagogue [8,9]. Furthermore, diacylglycerol (DG), which can be increased in islets via several mechanisms [10], can potentially be metabolized to PA via a DG kinase; indirect studies suggest the presence of this enzyme in islet tissue [10,11]. Recently, we described [12] in islet cells a phospholipase of the D type (PLD), which hydrolyses phosphatidylcholine (PC) to produce PA (and free choline). Unfortunately, specific activators or inhibitors of islet PLD have not yet been identified. Therefore, in order to examine further the idea that the generation of PA endogenously promotes insulin release, intact adult rat islets were treated with exogenous bacterial PLD, and its effects on phospholipid metabolism and insulin secretion were studied in parallel. These studies together are compatible with a role for endogenously formed PA in stimulus–secretion coupling in pancreatic islet cells.

MATERIALS AND METHODS

Materials

[1-^{14}C]Arachidonic acid (53 mCi/mmol) and [1-^{14}C]myristic acid (40–60 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Silica-Gel 60a t.l.c. plates (LK6D) were from Whatman (Clifton, NJ, U.S.A.). Solvents for t.l.c. were from Fisher (Fair Lawn, NJ, U.S.A.). Chemicals were generally from Sigma (St. Louis, MO, U.S.A.), except where indicated below. Phospholipases were from Sigma: PLD from *Streptomyces chromofuscus* (type VI), from peanut (type III) and from cabbage (type I). The bacterial PLD had been purified by the method of Imamura & Horiuti [13], which included, in succession, acetone precipitation, column chromatography on palmitoylated gauze and DEAE-cellulose, chromatography on Sephadex G-150 and finally identification of a single band on SDS/polyacrylamide-disc-gel electrophoresis [13]. Unlabelled arachidonic acid was from Nu-Chek Prep (Elysian, MN, U.S.A.).

Abbreviations used: PLD, phospholipase D; PA, phosphatidic acid; DG, diacylglycerol; MG, monoacylglycerol; PKC, protein kinase C; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; PMA, phorbol 12-myristate 13-acetate (TPA, 12-*O*-tetradecanoylphorbol 13-acetate); PC, phosphatidylcholine; df, degrees of freedom.

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Dantrolene was a gift from Norwich–Eaton Pharmaceuticals (Norwich, NY, U.S.A.). The diluent for stock solutions of the phorbol esters and dantrolene used in insulin secretion studies was dimethyl sulphoxide; all control tubes were given exactly the same nature and quantity of diluent as experimental tubes.

For the production of ^{14}C-labelled PA and DG standard, 2 μCi of 1,2-di[1-^{14}C]palmitoyl PC (114 mCi/mmol; New England Nuclear) and 2 μmol of unlabelled PC were solubilized in 2 ml of diethyl ether. Enzyme [PLD, from cabbage (Sigma, 500 units/tube) or phospholipase C, from *Bacillus cereus* (Sigma, 20 units/tube)] was then added in 500 μl of medium (100 mM-Tris/HCl, pH 6.0 or 7.2 respectively, and containing 5 mM-$CaCl_2$) in a screw-top glass vial. Incubations were carried out for 2–4 h at 37 °C, with frequent vigorous vortex-mixing or sonication. The ether layer was then removed, and the aqueous layer was back-extracted with 2 × 1 ml of ether and then 2 × 1 ml of chloroform, and the organic phases were combined.

Radiolabelling and incubation of intact adult islets and dispersed neonatal islet cells

Approx. 1500–4000 intact adult islets were obtained from 8–16 male Sprague–Dawley rats (280–380 g body wt.) by using collagenase digestion and discontinuous Ficoll gradients as previously described [14,15]. Islets were cultured overnight (18 h) in polystyrene Petri dishes (60 mm × 15 mm; Falcon 1007; Becton Dickinson, Lincoln Park, NJ, U.S.A.) containing 2.5 ml of RPMI 1640 medium ([glucose] = 11.1 mM), 5% (v/v) fetal-calf serum, 20 mM-Hepes, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and either 3–6 μCi of arachidonic acid/ml or 4 μCi of myristic acid/ml. Plates were incubated overnight at 37 °C in O_2/CO_2 (19:1). The next morning, 200 islets were picked into each inner borosilicate tube (12 mm × 75 mm) containing a 62 μm (pore size) polyethylene filter attached at the bottom [14]. Diligent care was taken to select only islets free of attached acinar tissue, by using stereomicroscopic control to pick islets. Islets were then placed into outer borosilicate tubes (16 mm × 100 mm) containing 0.5 ml of Krebs–Ringer bicarbonate buffer with 20 mM-Hepes, of composition as described in [14], and were washed three times by successive transfer into fresh medium of the same composition (glucose = 5.5 mM, radionuclide absent, 0.5% fatty-acid-free BSA present in order to remove any unincorporated label). In general (and except where indicated below), islets were then preincubated for 20 min in a gently shaking water bath, followed by an incubation period of variable duration, both at 3.3–5.5 mM glucose, all in the same medium, except that BSA was excluded. Test compounds were generally present only in the incubation period except where indicated otherwise. In studies of [^{14}C]arachidonate-prelabelled islets, a 'chase' with 10 μM unlabelled arachidonate was provided during both preincubation and incubation periods. Incubations were terminated by removing the inner tubes containing the islets and placing them in ice-cold methanol, followed shortly thereafter (30–60 min) by the addition of chloroform to initiate extractions.

Lipid and metabolite extractions and analyses

For quantification of the cellular content of PA and other lipids, islets were extracted overnight in a conical centrifuge tube gassed with argon at 4 °C in 3 ml of chloroform/methanol/conc. HCl (200:100:1, by vol). The next morning, 0.75 ml of 500 mM-KCl/50 mM-EDTA was added. The mixture was vortex-mixed, and phases were separated by centrifugation at 1200 rev./min for 6 min. The organic phase was removed with a glass Pasteur pipette, and the organic solvent was removed under a stream of argon. For application to pre-activated t.l.c. plates, samples were reconstituted in 50 μl of chloroform/methanol (2:1, v/v) containing 250 ng of PA as carrier, vigorously vortex-mixed, and applied to plates with capillary pipettes with the aid of a Captrol Controller (Drummon Scientific Co., Broomall, PA, U.S.A.). Generally, 20 μl samples were applied in duplicate to plates for use in t.l.c. system 1; the remaining 10 μl was washed to the bottom of the tube with acidified chloroform/methanol (2:1, v/v) and then was applied to plates which were developed with t.l.c. solvent system 2 for confirmation of the results obtained with system 1. Extraction of fatty acid-labelled metabolites from medium was accomplished by a similar technique, except that samples were first centrifuged and the cell-free media (500 μl) were transferred to new tubes in which they were extracted overnight in 2 ml of chloroform, 2 ml of methanol and 25 μl of 200 mM-EDTA. The next morning, 2 ml of chloroform, 1 ml of KCl/EDTA (in the proportions indicated above), 10 μl of conc. HCl and 250 ng of PA as carrier were added. After vortex-mixing and centrifugation (1200 rev./min for 6 min), the organic phases were collected.

Two t.l.c. systems were routinely used for the analysis of lipid extracts: system 1 employs LK6D silica-coated plates, developed in paper-lined tanks with the upper (organic) phase of iso-octane (2,2,4-trimethylpentane)/ethyl acetate/acetic acid/water (9:5:2:10, by vol.); system 2 uses the solvent system pyridine/chloroform/88% (v/v) formic acid (30:50:7, by vol.), otherwise as described for system 1.

Spots were scraped according to the superimposition of the individual corresponding autoradiograph, placed into scintillant (6 ml of Biocount; Research Products International, Mt. Prospect, IL, U.S.A.), shaken vigorously and counted for radioactivity.

Insulin secretion

Intact adult islets (10 per tube) were studied in static batch-type incubations of 10, 30 or 60 min as previously described [14,15]. Islets were studied either freshly isolated or after having been cultured overnight. In the latter case, they were treated similarly to the islets used in lipid studies, except that: (1) radiolabel was excluded from the overnight culture period; (2) 10% instead of 5% fetal-calf serum was present during the overnight culture period, and Hepes was excluded; and (3) during the acute experimental (10–60 min) incubation periods, the 'chase' with unlabelled arachidonic acid was omitted and 0.5% BSA was generally present. Insulin was measured by radioimmunoassay by using rat insulin standard from Novo Research Institute (Bagsvaerd, Denmark).

Data presentation and analysis

Each data point (determination) in lipid studies was obtained by averaging two replicate determinations, each of which comprised 40% of the total organic residue. Data for PA, DG, monoacylglycerols (MG) or non-esterified fatty acid are generally expressed as percentages of total phospholipids (in order to normalize minor variations either in islet mass in each tube, or in the exact amount applied to each t.l.c. loading zone). Data from multiple such determinations were then averaged and data are presented as means (±S.E.M.) for (n) determinations, with data analysis for statistical significance generally via a non-paired t test; $P < 0.05$ was accepted as significant. The findings by these methods were generally confirmed by using the second t.l.c. system to analyse the remaining 20% of the organic sample. Data from insulin-secretion studies (expressed as μ-units secreted/10 islets during an incubation of a given length) are analysed similarly, except in Fig. 1, where (n) = number of separate experiments.

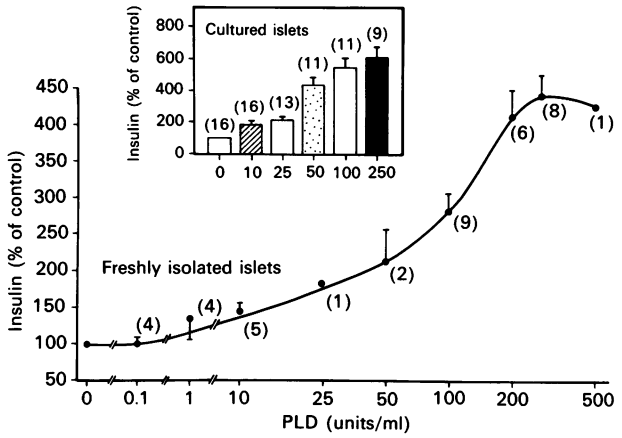

Fig. 1. Effects of exogenous PLD (*S. chromofuscus*) on insulin release from intact adult rat islets during 30 min incubations

Main panel depicts effects in freshly isolated adult islets at 1.7–6.6 mM-glucose (results were indistinguishable at different glucose concentrations in this range, and therefore the data are pooled). Values are means (±S.E.M.) for the numbers of separate experiments indicated in parentheses, each data point comprising the mean of 4–9 separate tubes (determinations). Inset shows results from two experiments using overnight-cultured islets, where (*n*) = number of individual tubes (determinations), each containing 10 islets. All values are expressed as % of control values (i.e. no PLD present).

RESULTS

Insulin release; concentration- and time-dependence, and specificity

In freshly isolated intact adult islets, exogenous PLD (from *S. chromofuscus* and purified via chromatography and electrophoresis) reliably stimulated insulin release over a large number of experiments, at any glucose concentration between 1.7 mM and 6.7 mM (Fig. 1; main panel). The threshold was approx. 10 units/ml (Fig. 1); 25 units/ml led to a substantial increase in secretion (control, 225±19 μ-units/10 islets; with 25 units of PLD/ml, 409±33; df 13; $P < 0.001$). A similar concentration-dependence for PLD was also observed with overnight-cultured islets (Fig. 1, inset). In contrast, the effect of PLD was not fully additive to that of a maximally stimulatory glucose concentration. For example, 100 units of PLD/ml had no additional effect above that of 16.7 mM-glucose alone (Table 1, Expt. 1), whereas the effect of 200 units of PLD/ml was only partially additive (incremental response of PLD above 5.5 mM-glucose = 785±75 μ-units/10 islets; incremental response above 16.7 mM-glucose = 281±55; df 8; $P < 0.001$). PLD-stimulated insulin release could be seen at least as soon as 10 min after application (control, 152±11 μ-units/10 islets; with 200 units of PLD/ml, 288±6; df 13; $P < 0.001$); such values rose progressively to 786±87 by 30 min (df 10; $P < 0.001$ versus 10 min value) and to 1002±43 (df 11; $P < 0.05$) by 60 min.

In contrast, PLD from cabbage (0.1–300 units/ml) had no effect at all on insulin release during 10 or 30 min incubations at 1.7 mM-, 3.3 mM-, 5.5 mM-, 6.7 mM- or 16.7 mM-glucose (cf. Table 2; and S. A. Metz, unpublished work). {PLD from peanut (10–300 units/ml) actually appeared to inhibit insulin secretion by 37–79% (at 1.7–16.7 mM-glucose). This effect was traced to the fact that peanut PLD is supplied by the manufacturer in the presence of dithioerythritol as preservative. The latter, by reducing disulphide bonds in the insulin molecule, impedes its quantitative recovery, as described by Landgraf-Leurs *et al.* [16]. Indeed, peanut PLD, at a concentration of 50 units/ml, decreased insulin recovery in the radioimmunoassay by 27–52%. Therefore, its further use was abandoned. PLD from *S. chromofuscus*, in contrast, did not alter the radioimmunoassay for insulin.}

In an attempt to study the reversibility of the effects of PLD (*S. chromofuscus*), islets were treated for 30 min with 200 units of PLD/ml during a preincubation period, and then were incubated in PLD-free medium for two additional periods of 15 and 30 min each (Table 3). Unexpectedly, after the removal of PLD, insulin-secretion rates seemed to rise slightly for the next 15 min and then declined somewhat, but over the next 30 min still remained above control rates for islets which had never been exposed to PLD. These findings, however, were readily explained by the phospholipid studies (see below). Further evidence that this delayed secretion was not merely due to cell leakiness or lysis (induced by the prior exposure to PLD) came from examining the responses to 16.7 mM-glucose during the last incubation period; that is, whereas basal insulin levels remained elevated in islets previously exposed to PLD, the incremental response to glucose nonetheless was completely intact (Table 3).

Effect of potential inhibitors on PLD-induced insulin release

The effect of PLD on secretion was markedly inhibited by decreasing ambient temperature to 30 °C (−66%) or 21 °C (90%) (Table 1, Expt. 2). This is not likely to be due to blocking the action of PLD on phospholipid hydrolysis, since exogenous phospholipases of the D type actually are maximally active at 30 °C [17]. The insulinotropic effect of PLD was not due to a physiological (or a toxic) stimulation of the entry of Ca^{2+} from the extracellular space, since removal of extracellular Ca^{2+} failed to decrease PLD-induced insulin release (results not shown). PLD-induced insulin release was, however, inhibited by 75% by 2 mM-Co^{2+} (Table 1, Expt. 3), possibly reflecting a direct inhibition of the ionophoretic effect of PA [18] or an intracellular effect to decrease exocytotic secretion [19]. Dantrolene (100 μM) also markedly decreased PLD-induced insulin release at 30 min of incubation (Table 1, Expt. 4) or at 60 min (−76±2%; df 10, $P < 0.001$). The effects of 200 units of PLD/ml were not significantly decreased by inhibitors of other phospholipases, such as *p*-bromophenacyl bromide (25 μM) or mepacrine (150 μM) (results not shown). A number of protease inhibitors provided together (250 μM-phenylmethanesulphonyl fluoride plus 0.1 μM-aprotinin plus 2 μM-leupeptin) also failed to decrease PLD-induced release (97±16% of control; df 11; not significant).

Effects of PKC inhibition or depletion on insulin release

In order to examine a possible role for the activation of PKC in the insulinotropic effects of PLD, the PKC inhibitors staurosporine (500 nM) or H-7 (150 μM) were provided. These are concentrations of the drugs which abrogate phorbol-ester-induced insulin release (refs. [20,21]; S. A. Metz, unpublished work). In 30 min incubations of freshly isolated islets, neither agent decreased PLD-induced secretion. For example, insulin release rose from 223±32 μ-units/10 islets ($n = 5$) to 687±41 ($n = 6$) in the presence of PLD (300 units/ml) alone; in the presence of H-7 or staurosporine, the respective values were 702±57 ($n = 7$) and 658±18 ($n = 7$), respectively (not significant). Furthermore, in overnight-cultured islets, 'down-regulation' of PKC by prolonged incubation of islets in the presence of 1 μM-TPA [20,21] failed to decrease the effect of PLD on insulin release during 30 or 60 min incubations (Table 1, Expt. 5). Lastly, the effects of PLD (200 units/ml) remained additive to the insulinotropic actions of high concentrations (1–2 μM) of PMA, or of mezerein, a dissimilar activator of PKC (results not shown).

Since it was possible that the later effects of PLD are mediated

Table 1. Effect of exogenous PLD (*S. chromofuscus*) on insulin secretion

Intact adult rat islets (10/tube) were incubated for 30 min (except where indicated otherwise) as described in the Materials and methods section. Freshly isolated islets were studied, except where indicated otherwise (Expt. 5.) Data are expressed as means (±S.E.M.) for (*n*) determinations: *incremental response versus control = $P < 0.05$ (or greater); n.s., not significant. Abbreviation: DMSO, dimethyl sulphoxide.

	Insulin (μ-units/ml)	
Expt. 1		
(a) Glucose (5.5 mM)	271±16 (5)	
(b) Glucose (5.5 mM)+PLD (100 units/ml)	514±35* (5)	
(c) Glucose (16.7 mM)	1584±105 (7)	
(d) Glucose (16.7 mM)+PLD (100 units/ml)	1496±77 (6)	[n.s. versus (c)]
Expt. 2		
(a) Glucose (5.5 mM) (37 °C)	236±33 (4)	Δ = 797*
(b) Glucose (5.5 mM)+PLD (200 units/ml) (37 °C)	1033±58 (7)	
(c) Glucose (5.5 mM) (30 °C)	231±13 (5)	Δ = 274* [−66% versus (b−a)]
(d) Glucose (5.5 mM)+PLD (200 units/ml) (30 °C)	505±49 (7)	
(e) Glucose (5.5 mM) (21 °C)	179±45 (5)	Δ = 77 (n.s.) [−90% versus (b−a)]
(f) Glucose (5.5 mM)+PLD (200 unis/ml) (21 °C)	256±58 (7)	
Expt. 3		
(a) Glucose (3.3 mM)	240±25 (6)	Δ = 355
(b) Glucose (3.3 Mm)+PLD (100 units/ml)	595±23* (16)	
(c) Glucose (3.3 Mm)+$CoCl_2$ (2 mM)	69±9 (7)	Δ = 90 [$P < 0.01$ versus (b−a)]
(d) Glucose (3.3 mM)+PLD+$CoCl_2$	157±17 (8)	
Expt. 4		
(a) Glucose (3.3 mM)	245±6 (3)	Δ = 1345
(b) Glucose (3.3 mM)+PLD (200 units/ml)	1590±206* (7)	
(c) Glucose (3.3 mM)+dantrolene (100 μM)‡	272±46 (3)	Δ = 163 [$P < 0.001$ versus (b−a)]
(d) Glucose (3.3 mM)+PLD+dantrolene‡	435±40* (7)	
Expt. 5 (overnight-cultured islets)		
Overnight incubation in diluent (DMSO)		
(a) Glucose (3.3 mM) for 30 min	27±2 (4)	Δ = 142
(b) Glucose (3.3 mM)+PLD (200 units/ml) for 30 min	169±10* (5)	
(c) Glucose (3.3 mM) for 60 min	39±5 (4)	Δ = 314
(d) Glucose (3.3 mM)+PLD for 60 min	353±37* (6)	
Overnight incubation in PMA (1 μM)		
(e) Glucose (3.3 mM) for 30 min	55±5 (4)	Δ = 150 [n.s. versus (b−a)]
(f) Glucose (3.3 mM)+PLD (200 units/ml) for 30 min	205±39* (5)	
(g) Glucose (3.3 mM) for 60 min	88±10 (4)	Δ = 391 [n.s. versus (d−c)]
(h) Glucose (3.3 mM)+PLD for 60 min	479±63* (6)	
Expt. 6†		
(a) Glucose (3.3 mM)	206±26 (5)	Δ = 629
(b) Glucose (3.3 mM) (after removal of PLD)	835±57* (8)	
(c) Glucose (3.3 mM)+staurosporine (500 nM)‡	199±19 (4)	Δ = 808 [n.s. versus (b−a)]
(d) Glucose (3.3 mM)+staurosporine‡ (after removal of PLD)	1007±94* (8)	
(e) Glucose (3.3 mM)+dantrolene (100 μM)‡	290±20 (5)	Δ = 298 [$P < 0.01$ versus (b−a)]
(f) Glucose (3.3 mM)+dantrolene‡ (after removal of PLD)	588±60* (8)	

† PLD (200 units/ml) was present during a 30 min preincubation period only and was absent from the incubation period; staurosporine and dantrolene were present in both periods. Data shown are from the incubation period.

‡ Dantrolene or staurosporine were present in the preincubation and incubations periods in these studies.

differently from its immediate effects (see above), another type of experiment was carried out. Islets were incubated in the presence of PLD (200 units/ml) for 30 min, after which PLD was removed and a subsequent 30 min incubation period was carried out. The delayed insulinotropic effects of PLD, described above, remained resistant to blockade by staurosporine (Table 1, Expt. 6). Interestingly, however, dantrolene did decrease by 53% the delayed insulinotropic effect of PLD (Table 1, Expt. 6) as well as its more immediate effects (Table 1, Expt. 4). Since dantrolene appears to inhibit the PKC-dependent insulin release which is induced by phorbol esters or by fatty acids such as arachidonate (ref. [22]; see the Discussion section), these findings may indicate a subtle role of PKC in PA-induced insulin release.

Phospholipid metabolism: general characteristics, and effects of potential inhibitors

In islets prelabelled with ether [^{14}C]arachidonate or [^{14}C]myristate, treatment with PLD for 30 min led to dose-dependent increases in PA accumulation; this was already evident by a PLD concentration of 25 units/ml and rose up to 250 units/ml (Fig. 2; Tables 2 and 4). These concentrations are similar to those stimulating insulin release. In fact, cellular PA correlated closely with insulin release ($r^2 = 0.99$ or 0.97 for arachidonate- or myristate-labelled PA respectively; Fig. 2; $P < 0.01$ each); the lines for the two labels relating PA to insulin release were quite similar and seemed to be parallel (Fig. 2).

Exogenous PLD also stimulated lesser increases in a second band which was labelled with either [^{14}C]arachidonate or [^{14}C]myristate and which migrated on t.l.c. with an R_F slightly greater than that of the PA standard (R_F = 0.076 versus 0.059 on t.l.c. System 1 and R_F = 0.72 versus 0.60 on System 2) (Table 4 and Fig. 3). This band had not been conclusively identified, but is presumed to be a second PA species (see the Discussion section). Changes in this presumptive PA species correlated less well with insulin release than did changes in the major PA species (cf. Table 4 and Fig. 2, legend). Much smaller amounts of PA were found in media extracts, and in general paralleled the findings in the cell extracts. During these 30 min incubations, PLD did not consistently increase cellular levels of MG, DG or non-esterified arachidonic acid.

In contrast with results derived by using bacterial PLD, neither PLD from cabbage (cf. Table 2) nor that from peanut (results not shown) increased the amounts of PA in islets, even at concentrations as high as 500 units/ml; likewise, they failed to stimulate insulin release (see above and Table 2).

The inhibition of PLD-induced insulin release induced by Co^{2+} or dantrolene (see above) was not due to inhibition of PLD-induced phospholipid hydrolysis. For example, the accumulation of [^{14}C]myristoyl-PA induced by 100 units of PLD/ml was not altered by dantrolene, and was actually increased by Co^{2+} (con-

Table 2. Comparison of the effects of PLD from cabbage (type I) and from *S. chromofuscus* (type VI) on PA generation and insulin release

Intact adult rat islets were studied during 30 min static incubations, as described in the Materials and methods section. For PA production, islets had been prelabelled with [^{14}C]myristic acid. Values for PA are expressed as % of total phospholipids, and values for insulin are expressed as μ-units/10 islets; all means (±S.E.M.) for (n) determinations: *$P < 0.05$ (or greater) versus control.

	PA (%)
(I) PA production ([glucose] = 3.3 mM)	
(a) Control (3)	0.52±0.01
(b) Bacterial PLD (200 units/ml) (4)	2.26±0.03*
(c) Cabbage PLD (200 units/ml) (4)	0.58±0.03
	Insulin (μ-units/ml)
(IIA) Insulin release ([glucose] = 3.3 mM)	
(a) Control (7)	245±30
(b) Bacterial PLD (200 units/ml) (7)	1073±118*
(c) Cabbage PLD (200 units/ml) (5)	207±32
(IIB) Insulin release ([glucose] = 5.5 mM)	
(a) Control (10)	189±22
(b) Bacterial PLD (300 units/ml) (10)	745±66*
(c) Cabbage PLD (300 units/ml) (10)	217±15

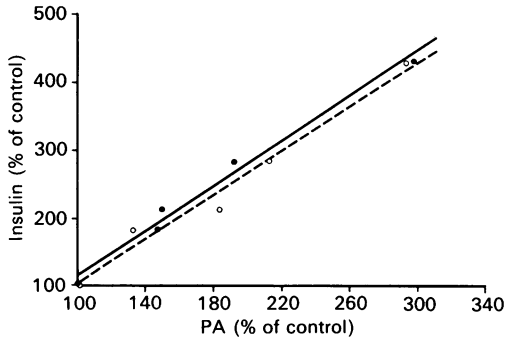

Fig. 2. Correlation between total cellular PA accumulation (abscissa; % of control) and insulin release (ordinate; % of control) in intact adult rat islets stimulated with various concentrations of PLD (*S. chromofuscus*) (0, 25, 50, 100 and 250 units/ml)

PA data are from Table 4, plus additional data for myristate-labelled islets (n = 3–4 determinations at each concentration of PLD). Insulin data are from the main panel of Fig. 1. Cellular PA data represent the sum of putative acyl-linked PA species plus putative alkyl-linked PA species (see the Discussion section); a similar linear correlation is seen if acyl-linked PA alone is analysed, whereas the putative alkyl-linked PA alone correlated poorly with insulin release. The correlative equation derived for arachidonate-labelled PA (●,——) is: $y = 1.66x - 53.04$ ($r^2 = 0.98$; df 3; $P < 0.01$); that for myristate-labelled PA (○,----) is remarkably similar: $y = 1.64x - 61.81$ ($r^2 = 0.97$; df 3; $P < 0.01$).

Table 3. Time course of the effect of PLD (*S. chromofuscus*) on insulin release

Intact adult rat islets (10/tube) were studied during three static incubation periods. In Incubation 1, islets were incubated for 30 min in the absence or presence of PLD (*S. chromofuscus*, 200 units/ml). Islets were then briefly washed and further incubated (in the absence of PLD) for a 15 min period. They were then incubated for a final 30 min period without any further preceding wash. Glucose was 4.5 mM throughout, except that, in Incubation 3, half of the batches of islets were exposed to a maximally stimulatory glucose concentration (16.7 mM). Data (μ-units/10 islets) are expressed as means (±S.E.M.) for (n) determinations; incremental data were calculated by subtracting control data from experimental data (PLD-treated islets) and then were converted into secretion rates (per h) by multiplying values from Incubations 1 and 3 (30 min) by 2 and from Incubation 2 (15 min) by 4.

	Incubation 1 (30 min) (glucose 4.5 mM; ±PLD)	Incubation 2 (15 min) (glucose 4.5 mM; PLD removed)	Incubation 3 (30 min) (glucose 4.5 or 16.7 mM; PLD absent)
Glucose (4.5 mM)	180±13 (10)	→ 72±7 (10)	→ glucose 4.5 mM: 68±9 (5) → glucose 16.7 mM: 1104±44 (5)
Glucose (4.5 mM)+PLD (200 units/ml)*	625±35 (12)	→ 423±17 (12)	→ glucose 4.5 mM: 544±19 (6) → glucose 16.7 mM: 1734±49 (5)
Calculated incremental secretion rate induced by PLD (at 4.5 mM-glucose) (μ-units/h per 10 islets)	990	→ 1404	→ 952

* PLD was present in Incubation 1 only.

Table 4. Effects of increasing concentrations of PLD on the accumulation of PA

Intact adult rat islets were prelabelled with [^{14}C]arachidonic acid and then were stimulated for 30 min with PLD (*S. chromofuscus*). Lipid values are expressed as % of total phospholipids, means (±S.E.M.) for (*n*) determinations: **P* < 0.05 (or greater) versus control. The identity of 'alkyl-linked' species of PA is tentative (see the Discussion section and Fig. 3). Abbreviation: AA, arachidonic acid.

	Content (%)				
	PA	'Alkyl-linked' PA	MG	DG	AA
Control (4)	0.49±0.04	0.15±0.01	0.30±0.03	8.19±0.34	2.22±0.22
PLD (25 units/ml) (4)	0.74±0.08*	0.21±0.025	0.33±0.02	8.37±0.39	2.47±0.18
PLD (50 units/ml) (4)	0.76±0.05*	0.20±0.06	0.31±0.02	8.35±0.35	2.31±0.20
PLD (100 units/ml) (3)	1.00±0.11*	0.22±0.06	0.33±0.03	8.86±0.25	2.29±0.32
PLD (250 units/ml) (2)	1.59±0.28*	0.31±0.02*	0.32±0.04	8.58±0.16	2.21±0.09

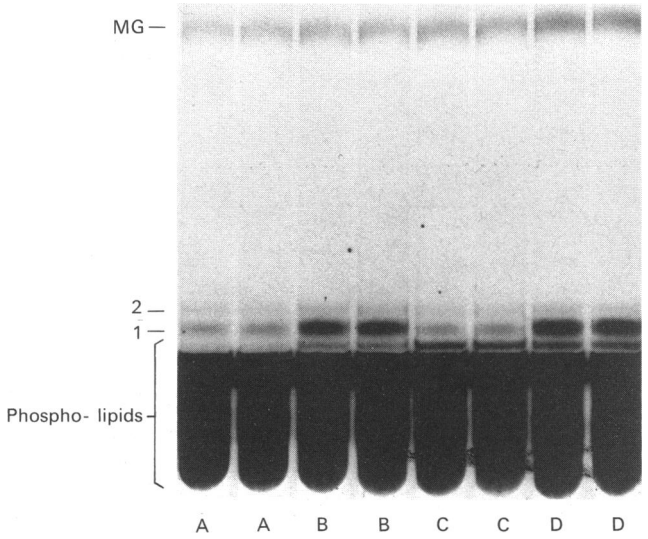

Fig. 3. Autoradiographs of putative acyl-linked PA (Band 1) and putative alkyl-linked PA (Band 2) from intact adult rat islets, which accumulated during either a 60 min preincubation period alone (lanes A and B) or during two successive 60 min incubation periods (lanes C and D)

In B, 200 units of PLD (*S. chromofuscus*)/ml was present during the first incubation; in D, PLD was present during the first incubation period, but was removed from the medium for the second incubation period. Each lane was run in duplicate.

trol = 0.57±0.06% of phospholipids; PLD = 1.40±0.06%, df 6; $P < 0.001$; PLD and 100 μM-dantrolene = 1.42±0.04%, df 8; not significant versus PLD alone; PLD and 2 mM-Co^{2+} = 2.01 ±0.11%, df 8, $P < 0.01$ versus PLD alone).

Effects of exogenous PLD on other lipids

During 30 min incubations, PLD did not consistently augment the accumulation of MG, DG or non-esterified cellular arachidonic acid (Table 4). In contrast, during 60 min incubations, in addition to increments in PA, there were significant increments in cellular arachidonic acid and DG, and a (non-significant) rise in MG (Table 5). These increments could be derived either directly from the degradation of PA (via PA phosphohydrolase present in islet cells; ref. [23]), or indirectly via a PA-induced activation of a phospholipase C directed towards inositol phospholipids [1,3]. In preliminary data (Table 5), 2.5 mM-neomycin (which inhibits phospholipase C [24], probably by binding inositol phospholipids) had no, or only minimal, inhibitory effects on the accumulation of PA (+8%), MG (+7%), arachidonic acid (−14%) or DG (−29%), suggesting that the second possibility was not valid.

Similar results were seen in islets prelabelled with [^{14}C]myristate. After 30 min of stimulation with PLD (200 units/ml), PA rose to 435% of control (Table 2) in the absence of consistent increments in other lipids. When islets were stimulated for 60 min, PA rose similarly to responses in 30 min incubations (450%), but now DG plus non-esterified fatty acid also increased (Table 5). These findings suggested that at early points (10–30 min) PLD-stimulated insulin release was entirely due to PA accumulation directly, whereas after 30 min other lipid mediators accumulate secondarily and could contribute to the greater insulin release after 60 min compared with that after a 30 min exposure to PLD. To determine if this formulation could also help to explain the slow reversibility of the effects of PLD on secretion (see above, [^{14}C]arachidonate-prelabelled islets were preincubated for 30 min with or without 200 units of PLD/ml and then were incubated for a further 30 min after removal of PLD (Table 6; Fig. 3). During the first 30 min period, only PA significantly increased. After the removal of PLD, PA, which moves only slowly through membranes [25], remained elevated; however, in addition, DG and cellular arachidonic acid now accumulated (Table 6) and could have contributed to the delayed insulinotropic effects of PLD after its removal from the islets.

DISCUSSION

Exogenous PLD promoted a dose- and time-dependent insulin release from freshly isolated or cultured intact rat islets. Several lines of evidence suggest that this effect specifically reflects phospholipid hydrolysis, and the generation of a specific lipid mediator(s), rather than the induction of toxic perturbations in the plasma membrane (causing, for example, leakiness to cations such as Ca^{2+} or non-specific cell lysis with leakage of insulin). Firstly, the insulin secretion induced by PLD was closely correlated quantitatively and temporally with a selective accumulation of PA. Specifically, an increment in PA of ⩽33% above basal was sufficient to augment secretion. Second, other PLD preparations (from cabbage or peanut), which are most active at a pH (approx. 5.6) well below that of the extracellular medium [17,26,27], were unable to support either phospholipid hydrolysis or insulin release, whereas PLD from *S. chromofuscus*, which has a pH optimum at 7.5–8.0 [13,27], was effective in both regards. Thus the negative findings with other PLD preparations provide strong control data to compare with the findings associated with the use of bacterial PLD. Thirdly, insulinotropic concentrations of PLD (up to 300 units/ml) do not induce a

Table 5. Effects of exogenous PLD on lipid metabolism during 60 min incubations

Intact adult rat islets, prelabelled with [^{14}C]arachidonic acid or [^{14}C]myristic acid, were stimulated with PLD (*S. chromofuscus*, 200 units/ml) in the presence or absence of neomycin. Glucose was 3.3 mM. In I(c), neomycin (2.5 mM) was present in the entire incubation period. Values are expressed as % of total phospholipids, means ± S.E.M. for (*n*) determinations each: $*P < 0.05$ (or greater) versus control. Absolute total d.p.m./200 islets in [^{14}C]arachidonate-labelled phospholipids were 183383 ± 8826 for controls and 182409 ± 7764 for PLD-treated islets. Equivalent phospholipid totals for [^{14}C]myristic acid label were 39466 ± 1355 (control) and 41444 ± 1415 (PLC-treated). Abbreviation: NEFA, non-esterified fatty acid.

	Content (%)			
	PA	MG	DG	NEFA (arachidonate)
(I) Arachidonic acid label				
(a) Control (5)	0.42 ± 0.01	0.24 ± 0.01	8.42 ± 0.34	1.98 ± 0.05
(b) PLD (200 units/ml) (5)	0.90 ± 0.09*	0.31 ± 0.03	9.47 ± 0.28*	2.70 ± 0.28*
(c) PLD (200 units/ml) plus neomycin (2)	0.82, 1.06	0.30, 0.33	9.31, 9.02	2.53, 2.67

	Content (%)		
	PA	MG	DG + NEFA (myristate)†
(II) Myristic acid label			
(a) Control (5)	0.42 ± 0.03	0.60 ± 0.01	7.35 ± 0.23
(b) PLD (200 units/ml) (6)	1.89 ± 0.06*	0.63 ± 0.02	8.58 ± 0.30*

† The bands for DG and myristic acid did not have an adequate clear zone between them to scrape them accurately individually, and therefore the two fractions are combined.

Table 6. Immediate and delayed effects of exogenous PLD on lipid metabolism

Intact adult rat islets prelabelled with [^{14}C]arachidonic acid were incubated for 30 min in the presence or absence of PLD (*S. chromofuscus*). At the end of the first incubation, the incubation of one group of islets was terminated (A, B). The rest of the islets were incubated for an additional 30 min period in the absence of PLD. Glucose was 4.5 mM throughout. Values (except for total phospholipids) are expressed as % of total phospholipids, means ± S.E.M. for (*n*) determinations: $*P < 0.05$ (or greater) versus relevant control. Abbreviation: AA, arachidonic acid.

Conditions			Content (%)				
First 30 min	Second 30 min	(*n*)	PA	MG	DG	AA	Total phospholipids (d.p.m./tube)
(A) Control	–	3	0.51 ± 0.08	0.37 ± 0.03	9.82 ± 0.77	2.90 ± 0.39	(159085 ± 4485)
(B) PLD (200 units/ml)	–	6	1.44 ± 0.06*	0.44 ± 0.02	10.82 ± 0.35	3.72 ± 0.22	(156654 ± 3432)
(C) Control	Control (PLD absent)	3	0.56 ± 0.05	0.37 ± 0.01	9.85 ± 0.40	3.86 ± 0.24	(166537 ± 7609)
(D) PLD (200 units/ml)	Control (PLD absent)	5	1.59 ± 0.09*	0.50 ± 0.01*	11.74 ± 0.45*	4.93 ± 0.27*	(155168 ± 2950)

generalized cell leakiness, as assessed by the lack of permeation from islet cells of small-molecular-mass, water-soluble, phosphorylated metabolites of choline such as phosphocholine or glycerophosphocholine ([12]; S. A. Metz & M. Dunlop, unpublished work). Fourth, after removal of the PLD, the insulinogenic response to glucose (which is sensitive to inhibition by permeabilization of the plasma membrane; ref. [28]) remained intact. Fifth, PLD only increased insulin levels in the medium in the presence of sub-maximal or non-stimulatory glucose levels, a finding which would not be in accord with mere cell toxicity. Furthermore, PLD-induced insulin release appeared to be saturable. Sixth, the insulin responses were inhibitable by modest decreases in ambient temperature. Since the latter occurred even at the optimum temperature for the PLD effect on phospholipid hydrolysis [17], it seems that the effect of cooling was to decrease the resulting insulin secretion; exocytotic insulin secretion is quite sensitive to small decrements in temperature [29]. The secretory effects of PLD were also inhibitable pharmacologically (e.g. by Co^{2+} or dantrolene) in the absence of decreases in PLD-induced phospholipid hydrolysis. These findings suggest that these pharmacological agents either inhibit specifically the cellular responses to PA or act at an intracellular site to impede exocytotic secretion; both actions have previously been ascribed to Co^{2+} [18,19] in other cell types. Lastly, the lack of inhibition of the PLD effect by the removal of extracellular Ca^{2+} suggests that simple permeabilization of the membrane to Ca^{2+} (or an ionophoretic effect at the plasma membrane) cannot alone explain the effects of PLD.

The mechanism whereby insulin release is stimulated by PA (presumably formed in the outer leaflet of the plasma membrane by extracellular PLD) is not elucidated by these studies. Exogenously provided PA (which can be rapidly incorporated into the outer membrane leaflet; ref. [25]) also promotes insulin release from dispersed neonatal islet cells, in association with a mobilization of EGTA-insensitive intracellular Ca^{2+} stores, a rise in cyclic AMP, and hydrolysis of inositol-containing phospholipids [1]. Likewise, in human A431 carcinoma cells, either exogenous PA or exogenous bacterial PLD promotes

increments in cytosolic free Ca^{2+} concentration which are not blocked by EGTA [6]. In islet cells, the effect of a rise in cytosolic free [Ca^{2+}] on insulin secretion is potentiated by cyclic AMP [30,31], and these two events together could explain the observed insulin release. Although we did not find a rise in DG (after 30 min of stimulation by PLD) to support the formulation that inositol lipids were degraded owing to interaction of PA with a surface receptor, as previously reported [1], it is possible that such a rise occurred at earlier time points than were sampled. Alternatively, PA formed in the exterior plasmalemmal leaflet could have undergone transbilayer movement to reach intracellular sites. However, using a fluorescent derivative of PA and Chinese-hamster fibroblasts, Pagano & Longmuir [25] reported that exogenous PA enters cells only by its dephosphorylation to DG, which crosses to the inner leaflet and is rephosphorylated to PA. Our failure to find increments in DG argues more strongly against this formulation. Clearly, further studies will be needed to elucidate the exact site and mechanism(s) of action of PA. The fact that increments in PA and insulin release both persisted after removal of PLD from the medium presumably reflects the continued association of some PLD with membrane phospholipids and/or, a slow rate of translocation [25] and degradation of PA in the membrane.

Interestingly, however, two types of secretion induced by exogenous PLD could be functionally distinguished. During the first 30 min of PLD stimulation, there was a selective accumulation of PA unaccompanied by increases in DG or arachidonic acid. This pattern of insulin release was not additive to that induced by maximum glucose stimulation. This latter observation allows speculation that glucose itself promotes PA accumulation from synthesis *de novo* [8,9] and possibly via other biochemical pathways such as, possibly, the activation of an endogenous PLD; however, further studies will be needed to examine this hypothesis directly. After 30 min, insulin release induced by PLD increased further; this was accompanied by a continued, quantitatively unchanged, accumulation of PA plus, now, the appearance of increases in DG and arachidonic acid, presumably reflecting the catabolism of PA. Insulin secretion at these time points was additive to that induced by glucose. One interpretation of these findings may be that PLD also stimulates a delayed and qualitatively different type of insulin release. This might be due either to a second, distinct, time-dependent effect of PA directly (possibly reflecting its translocation from the outer leaflet of the plasma membrane to the cell interior; see above), or to the secondary rise in the other lipid mediators. For example, both DG [32] and arachidonic acid [33] can stimulate insulin release. However, secretion induced by DG or arachidonic acid might be expected to be at least partly dependent on PKC activation. For example, the 'down-regulation' of PKC eliminates or blunts the insulinotropic effects of exogenous arachidonate or of PMA (a DG mimic) [20]; the effects of the latter are also blocked by the PKC inhibitors H-7 [20] or staurosporine [21]. However, both the early and the later effects of PA were resistant to such manoeuvres.

It is also reasonable to question whether the immediate effects of PA were mediated by PKC, since, in certain cells, PA has been suggested to play a role in the activation of PKC [3,4]. This could occur since PA is an anionic phospholipid, which might substitute for phosphatidylserine [34,35] as a necessary cofactor for PKC. Alternatively, PA could be directly converted into DG, an accepted endogenous activator of PKC, through the action of a PA phosphohydrolase present in the islet [23]. A third means for PA to augment PKC activity might be to stimulate DG accumulation secondarily via the activation of inositol lipid hydrolysis [1,3]. However, the insulin secretion induced by PA at early time points is also likely to be PKC-independent, since it too was resistant to blockade by staurosporine, H-7, or the prior down-regulation of PKC. However, a mediation via isoforms of PKC which are resistant to such inhibitory manoeuvres [36] cannot be completely excluded; conversely, the potency of PKC blockers may vary, depending upon the agonist [37]. For example, the effects of exogenous arachidonic acid in islets are resistant to inhibition by H-7 or staurosporine [33], but are blunted by spermine, a third PKC inhibitor [33]. Thus it is theoretically possible that PA activates a form of PKC which is resistant to blockade by such inhibitory manoeuvres; however, the current studies provide no direct support for such a possibility.

Along this line of thinking, it is interesting that both the immediate and the delayed insulinotropic effects of PA were decreased by dantrolene. Although dantrolene is frequently used as a purported inhibitor of intracellular Ca^{2+} mobilization [38], we have observed that this agent blocks the insulin release induced by exogenous fatty acids or by phorbol esters, in the absence of demonstrable effects on Ca^{2+} mobilization [22] and even when cytosolic Ca^{2+} concentrations are 'clamped' in permeabilized islets [33]. This might be interpreted to indicate that a major mechanism whereby dantrolene decreases insulin release is an inhibition of PKC. This formulation may explain why dantrolene inhibits insulin release induced by exogenous phospholipase A_2 (or by its lipid byproduct, arachidonic acid [22,33]), by exogenous phospholipase C (or by its lipid by-product, DG [22]) or by PLD (or by its lipid by-product, PA; the present work) in the absence of evidence of direct phospholipase inhibition ([22]; the present work). Such observations may make dantrolene a useful probe of phospholipase-dependent insulin release. However, further studies are clearly needed to determine definitively whether these effects of dantrolene do in fact reflect subtle inhibitory actions on PKC or some other effect. There is evidence that PA, DG or arachidonic acid may all have direct effects on insulin release which are independent of PKC activation. Such effects may, for example, involve Ca^{2+} fluxes (see refs. [1,22,33,38,39]), leading to elevations in cytosolic free Ca^{2+} concentrations. PA could also have indirect effects attributable to the generation of lyso-PA, produced either via the action of phospholipase A_2 on PA, or by the concomitant hydrolysis of lysophospholipids by exogenous PLD [13]. However, in our previous studies of intact adult islets, exogenous lyso-PA, at least, was not insulinotropic [40].

The phospholipid source and chemical composition of individual lipid species might also modify their effects in islets. PLD from *S. chromofuscus* is known to attack primarily choline-containing phosphoacylglycerols [13]; furthermore, this PLD releases free choline from PC ([12]; S. A. Metz, unpublished work). The current data derived by using [^{14}C]myristate-labelled islets also support the conclusion that the PA generated was derived from PC, since, unlike arachidonic acid, myristate is incorporated in a highly selective fashion into PC (S. A. Metz. & M. Dunlop, unpublished work; cf. also [41]). Presumably, PC-derived PA, in turn, is directly metabolized over time to yield DG and arachidonic acid. These conclusions are supported by the data obtained with neomycin, which failed to inhibit the delayed rise in DG or arachidonic acid, suggesting that a secondary activation of a phospholipase C directed against inositol phosphoacylglycerols was not involved. In preliminary studies, we recently observed that dispersed cultured neonatal-rat islet cells also generate PA and release insulin in response to exogenous PLD (M. Dunlop & S. A. Metz, unpublished work). However, although these cells produced at least as much total PA in response to PLD as did intact islets, the insulin release was less. Although the cause of this difference has not been conclusively identified, it is of interest that we observed, in preliminary studies, that the PA generated by PLD in neonatal-rat cells

predominantly (63 %) comprised the putative second PA species having the higher R_F value, in contradistinction to the predominant generation of the PA species having the lower R_F value in adult islets. The exact identity of the former compound was not established, owing to its low abundance, although such an unidentified band has been observed by other investigators [7,42]. The slightly greater mobility of this band (in both t.l.c. systems) suggests that it may be a 1-alkyl 2-long-chain-acyl species of PA, since the R_F of PA in t.l.c. System 2 is increased slightly by alkyl linkages and, to a greater extent, by increasing the chain length and/or the degree of unsaturation of the fatty acids [43]. Some support for this identification also came from studies in which neonatal islet cells were prelabelled with [^{14}C]hexadecanol, a fatty alcohol which is incorporated preferentially into alkyl linkages of phospholipids. During such studies, a second putative PA band was seen on t.l.c. compatible with Band 2 in Fig. 3 (M. Dunlop, unpublished work). Thus it is possible that some PA species are more insulinotropic than others; further studies should be directed to this possibility.

In conclusion, these data suggest for the first time that PLD activation (working via the generation of endogenous PA and possibly other lipid mediators) promotes insulin release. Since a PLD exists in pancreatic islet cells ([12]; S. A. Metz & M. Dunlop, unpublished work), it is possible that this pathway is relevant to physiological insulin release. By similar logic, PA generated from glucose metabolism via synthesis *de novo* or via the phosphorylation of DG may be speculated to promote secretion. Further studies will be needed to examine each of these hypotheses.

Note added in proof (received 11 July 1990)

Recently, we have confirmed the presence of a PLD in pancreatic islets through studies of the production of phosphatidylethanol, a specific marker for PLD [7,27,42] (S. Metz & M. Dunlop, unpublished work).

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