Metabolism of lysophospholipids in intact rat islets

The insulin secretagogue *p*-hydroxymercuribenzoic acid impairs lysophosphatidylcholine catabolism and permits its accumulation

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Although recent studies implicate lysophospholipids (lyso-PLs) in stimulus–secretion coupling in the pancreatic islet, almost no data on lyso-PL metabolism therein exist. Therefore, intact rat islets were loaded with insulinotropic and non-toxic concentrations of 1-[¹⁴C]palmitoyl-lysophosphatidylcholine (lyso-PC) via transbilayer movement, and its metabolic fate was studied. The time-dependent hydrolysis of lyso-PC to fatty acid (lysophospholipase activity), its conversion to phosphatidylcholine (putative acyltransferase activity) and, to a lesser degree, the appearance of label in phosphatidylethanolamine (putative transacylase or base exchange activity) were observed. *p*-Hydroxymercuribenzoic acid (PHMB) at 100 μ M (a concentration previously demonstrated to elicit potent exocytotic insulin release) inhibited all three activities (by 56, 46 and 75%, respectively) and led to the intracellular accumulation of lyso-PC. Antimycin A inhibited phosphatidylcholine formation but not lysophospholipase activity; lyso-PC did not accumulate, implying that blockade of both of the major metabolic pathways is required to induce a detectable increment in lyso-PC levels. Calculations derived from data using the lowest effective insulinotropic concentration of lyso-PC suggested that increments in lyso-PC accumulation at critical membrane sites of less than 10–15% above basal values are sufficient to trigger insulin release. Since PHMB elicited increments of 50–100% in lyso-PC after its translocation into islets, support is provided for the earlier contention that lyso-PLs mediate the insulinotropic effect of PHMB. In addition, these studies may provide a more precise experimental paradigm for future studies of islet lyso-PL metabolism.

INTRODUCTION

My recent studies suggested that lysophospholipids (lyso-PLs), especially lysophosphatidylcholine (lyso-PC), might be previously unrecognized coupling signals involved in exocytotic insulin release [1–7]. However, virtually no data exist regarding lyso-PL metabolism in the pancreatic islet. In my initial attempts to study lyso-PL formation and catabolism in intact islets, I labelled the *sn*-1 position, the *sn*-2 position or the C₃ backbone of islet phospholipids (PLs), using [¹⁴C]stearic acid, [³H]arachidonic acid or [³H]glycerol, respectively. However, I was discouraged from continuing to use such conventional approaches since a small but variable degree of non-enzymic hydrolysis of PLs to lyso-PLs occurred during sample preparation (even when acidification during extraction was avoided), rendering assessment of basal and stimulated lyso-PL levels difficult (S. Metz, unpublished work). Therefore, a more novel approach was taken: by modification of methods first used in red blood cells [8–10] and platelets [11], islets were loaded with labelled lyso-PC to desired levels by transbilayer movement. This procedure facilitates the evaluation of the effect of test compounds on the steady-state level of islet lipids and permits studies of lyso-PC metabolism in intact islets.

My earlier studies [2,4,7] also showed that the organic mercurial *p*-hydroxymercuribenzoic acid (PHMB) inhi-

bits the reacylation of islet lyso-PLs by arachidonic acid, and stimulates insulin release. I attributed the latter to the accumulation of lyso-PLs whose clearance mechanisms were impeded; however, that hypothesis was not directly studied. In the current study, I confirm that insulinotropic concentrations of PHMB augment the islet accumulation of lyso-PC.

MATERIALS AND METHODS

1-[1-¹⁴C]Palmitoyl-lyso-PC (40–60 mCi/mmol) was from New England Nuclear. PHMB was from Sigma, and other chemicals were as previously identified [1–3,5].

Intact rat islets were isolated from fed male Sprague–Dawley rats essentially as previously described in detail [1–3,5]. Approx. 120–140 freshly isolated islets were portioned into specially designed siliconized test tubes whose bottom surfaces had been removed and replaced with 62 μ m filters [1–3,5]. These tubes rested in outer, siliconized test tubes containing Krebs–Ringer bicarbonate (KRB) buffer with a composition as described [1–3,5]; the medium was gassed with O₂/CO₂ (19:1). The buffer contained 1.7 mM- or 16.7 mM-D-glucose, as indicated, but no serum albumin. Hepes (10 mM) was also added to prevent minor changes in pH which would be greatly magnified (with resultant non-enzymic PL hydrolysis) when buffers and solvents were rotary-evaporated for sample preparation. Islets were then

Abbreviations used: lyso-PL, lysophospholipid; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PHMB, *p*-hydroxymercuribenzoic acid; KRB, Krebs–Ringer bicarbonate; n.s., not significant.

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incubated with 2 μ Ci of radiolabelled lyso-PC/ml (mass concentration 19.8 μ g/ml) for varying times (after evaporating off the diluent with a stream of argon); in some studies, 100 μ M-PHMB or its diluent was added at the indicated times. This is a concentration of PHMB which reproducibly elicits insulin release at 1.7 mM-glucose in a physiological and non-toxic fashion [2,7,12]. In most studies, after these incubations, islets were rinsed five times (over a period of 15 min) at 2 °C (the low temperature having been chosen to impede any further metabolism or translocation of islet lipids) in 2% fatty-acid-free bovine serum albumin (Sigma) in KRB buffer. This step removes superficially adherent lyso-PC which had not been truly incorporated into the inner or outer membrane leaflet [8–11]. In order to eliminate the possibility that PHMB merely impedes the uptake of label, the incubations in labelled lyso-PC were stopped at 60 min in some studies and the islets were returned to 1 ml of fresh KRB (lacking labelled lyso-PC) in the presence of PHMB or its diluent (3 μ l of 0.1 M-NaOH) for a final 30 min of incubation. Under these conditions, the test agent can alter the metabolism only of that lyso-PC which has already been translocated into the lipid bilayer.

Islets were then extracted and subjected to analysis of lipids by normal-phase h.p.l.c. as described [2,3]. Incubations were rapidly terminated by submersion of the mesh filters containing the islets into 3 ml of ice-cold methanol, in a conical centrifuge tube. Islets were left overnight in the freezer in these tubes, to which had been added internal standards (see below). Total lipid extracts were obtained after warming the tubes to room temperature, adding 3 ml of chloroform, vortex-mixing, and removing the filters. The solvent was removed to a silanized pear-shaped flask. Any residual phospholipid was removed from the original centrifuge tube (or extracted from islet fragments remaining in it) by sequential extractions into 6 ml of methanol/chloroform (1:1, v/v), 6 ml of chloroform/methylene chloride/propan-2-ol (2:1:1, by vol.) and 3 ml of methanol/chloroform (1:2, v/v). Acidification of samples, or complete evaporation to dryness after extraction procedures, was rigorously avoided. All rinses/extracts were pooled, rotary evaporated to near dryness, and injected onto the high performance liquid chromatograph in 10 μ l of chloroform/methanol (1:1, v/v). H.p.l.c. was carried out as previously described [2,3] using a Beckman 5 μ m Ultrasphere-Si silica column (25 cm $\times$ 4.6 mm) purchased from Altex. The mobile phase was acetonitrile/methanol/phosphoric acid (generally at a ratio of 130:1.5:1.5, by vol.). Eluents from the h.p.l.c. were analysed by radionuclide spectrometry as described [2,3]. I had previously used fluorescein as an internal standard [2,3] but rejected it in the current studies due to occasional, unacceptable variations in recovery not appearing adequately to reflect islet PL recovery. In its place, fluorescein was replaced by two new internal standards, *n*-biotinylated phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL, U.S.A), and lyso-PC itself containing unsaturated fatty acid residues (from soybean; Sigma) so that it was visible at 203 nm in the u.v. detector of the h.p.l.c. apparatus. This was possible since islets in the basal state were ascertained to contain insufficient lyso-PC to produce a u.v. signal at 0.05 absorbance units/full scale; in addition, the radiolabelled lyso-PC provided ($[^{14}\text{C}]$ palmitoyl-lyso-PC) has no unsaturated

fatty acid residues and thus is virtually transparent at 203 nm. Both internal standards yielded indistinguishable recovery figures and values were averaged [recoveries $70 \pm 11\%$ (means $\pm$ s.d., $n = 40$)]. In this h.p.l.c. system (Fig. 1 and [2,3]), *n*-biotinylated phosphatidylethanolamine is eluted between fatty acids and phosphatidylserine. All lipids quantified in the current study (fatty acid, phosphatidylethanolamine, phosphatidylcholine, lyso-PC) were baseline separated from each other (Fig. 1). A very small (≈ 150 d.p.m.s/120 islets) peak of phosphatidylserine was present but was sometimes difficult to quantify accurately since it slightly overlapped a much larger peak of phosphatidylethanolamine. Thus in these studies, the 'phosphatidylethanolamine' values reported actually include a small amount (generally less than 5%) of phosphatidylserine.

To evaluate the effects of PHMB, it was important to document that this agent did not alter the non-enzymic catabolism of phospholipids, or affect selectively the recovery of individual lipid moieties. This was accomplished in several ways. First, u.v.-detectable amounts of the relevant lipids [lyso-PC (7.5 μ g) and phosphatidylcholine (0.5 μ g)] and radiolabelled lipids ($[^{14}\text{C}]$ lyso-PC, $[^{14}\text{C}]$ phosphatidylcholine and $[^3\text{H}]$ arachidonic acid, all from New England Nuclear, in amounts comparable with those taken up or produced by islets) were added to control (islet-free) incubations; the medium and the filters used normally to hold the islets were 'extracted' and their lipid content quantified both as u.v.-detectable peak heights and as radioactivity. PHMB did not alter: (1) the total recovery of radioactivity or the radioactivity (or mass) in the individual lipid fractions; (2) the non-enzymic hydrolysis, during extraction, of lyso-PC to fatty acid, or of phosphatidylcholine to fatty acid or lyso-PC; or (3) the relative (percentage) distribution of each radiolabelled lipid among the totals recovered. In addition, the effect of PHMB on lipid recovery in the presence of viable islet tissue was studied in two ways. First, after a 60 min labelling of islets with $[^{14}\text{C}]$ lyso-PC, unincorporated lyso-PL was removed by two 2.5 min washes in 2% (w/v) bovine serum albumin in KRB buffer at 37 °C, followed by a further 30 min incubation period in buffer containing PHMB or its diluent in the absence of label. No acylation or hydrolysis of incorporated lyso-PC is seen during the last 30 min, since complete removal of extracellular lyso-PC by the albumin reverses the concentration gradient, blocks the continued inward translocation ('flip') of lyso-PC and leads to reverse translocation or 'flop' at 37 °C [13] (i.e lyso-PC moves from inner to outer membrane leaflet and some was even ascertained to be released back into the medium). Thus, the effect of PHMB on the recovery of radiolabelled lipids from islets could be assessed under conditions where it could not substantially alter their metabolic interconversions in the inner membrane leaflet. Under these conditions, PHMB did not alter total radioactivity recovered, the percentage distribution between various lipids or the absolute amounts of labelled fatty acids, lyso-PC or phosphatidylcholine recovered. In a second control study, PHMB was added (at the end of a 60 min period of labelling in $[^{14}\text{C}]$ lyso-PC) for an additional 2 minutes only of incubation; this period would be insufficient to alter in a quantitatively important way the metabolic (enzymic) fate of $[^{14}\text{C}]$ lyso-PC, $[^{14}\text{C}]$ phosphatidylcholine or $[^{14}\text{C}]$ fatty acid but would allow PHMB to be carried through the extraction

procedures and h.p.l.c. Again, no differences were observed between experimental and control tubes. Thus PHMB has no direct effects on the binding of intact phospholipid, fatty acid, or lyso-PC to inert materials, nor does it alter their subsequent recovery from islets, filters or media. In addition it does not alter overall recovery, non-enzymic hydrolysis or the chromatography of islet lipids, or increase lyso-PC levels by activating phospholipase A₂.

Values for control tubes containing no islets were subtracted from all values for tubes containing islets in order to correct for adsorption of lipids to the glass tubes or filters. Data are expressed as a percentage of total radioactivity (d.p.m.) recovered, or as d.p.m./120 islets per incubation after correction for the recovery of internal standards. When comparing the absolute radioactivity in various lipid fractions between different experiments, the total radioactivity recovered was adjusted to 200 000 or 400 000 d.p.m./tube (as indicated) in order to normalize for variations of labelling or islet size between studies. In studies involving PHMB or antimycin A, control and experimental tubes were always incubated (and analysed) side by side and comparisons were thus made by paired *t* test.

RESULTS AND DISCUSSION

Uptake and metabolism of lyso-PC (Figs. 1 and 2)

The disposition of labelled lyso-PC inserted by transbilayer translocation into the membranes of intact islets is shown in Figs. 1 and 2. I have previously ascertained that this concentration (19.8 µg/ml) of lyso-PC in the extracellular fluid has no deleterious effects on islet structure or function [1,2,4,7]. Lyso-PC was converted over time to phosphatidylcholine, and to fatty acid ([¹⁴C]palmitate plus, presumably, unlabelled glycerophosphorylcholine). A steady-state proportion of phosphatidylcholine was reached by about 90 min, whereas fatty acid continued to rise over the course of the study. A small amount of phosphatidylethanolamine (1.24 ± 0.16% of d.p.m. recovered) was formed as well (Tables 1 and 2; Fig. 1). The first conversion (lyso-PC to phosphatidylcholine) presumably largely reflects an acyl-CoA:lysophosphatidylcholine acyltransferase which esterifies lyso-PC with fatty acid in a CoA- and ATP-dependent fashion [11,14–18]. The second reaction reflects lysophospholipase activity [16,18,19] in the islet. The third activity probably reflects a transacylase transferring labelled palmitic acid from PC to lyso-PE [20–24] (see section on 'Formation of phosphatidylethanolamine' below) or, possibly, base exchange between phosphatidylethanolamine and phosphatidylcholine. Transacylase activity may be intrinsic to the enzyme effecting lysophospholipase activity [25–27] or that effecting acyltransferase activity, but acting in reverse [21], or it could be due to an entirely separate enzyme.

I [2,3,7] and others [14] have previously presented evidence that the first two enzyme systems are present in islet tissue. Since both enzymes, when present in plasma membranes, are generally felt to reside in the inner membrane leaflet [8–10], the appearance of labelled phosphatidylcholine and palmitate can be taken as an index of translocation of lyso-PC to the cytoplasmic side of the membrane. In preliminary studies,

Fig. 1. Distribution of radioactivity in lipid fractions in 140 intact islets after a 150 min incubation with 1-[¹⁴C]palmitoyl-lysophosphatidylcholine

Abbreviations: FFA, fatty acid (palmitate); PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine. The internal standard *n*-biotinylated-PE is eluted several minutes after FFA but before PS. Data are uncorrected for recovery of internal standard (66.7% in this study) or for background counts (adsorbed to filters or tubes).

Fig. 2. Time course of disappearance of cellular 1-[¹⁴C]palmitoyl-lysophosphatidylcholine and the coincident appearance of label in fatty acid (lysophospholipase activity) or phosphatidylcholine (PC; putative acyltransferase activity).

Numbers of replicate determinations are in parentheses along the abscissa. S.E.M. values were 2–3% for fatty acid, 1–4% for PC and 3–8% for lyso-PC. The inset shows mean values for total radioactivity (d.p.m.) recovered in islets at each time point in the same studies.

Table 1. Effect of antimycin A on lipid levels in intact rat islets

Incubations were for 30 min in the presence of 2 μCi of 1-[^{14}C]palmitoyl-lysophosphatidylcholine/ml $\pm$ 10 μM -antimycin A or its diluent. Lipids were separated and quantified as described in the text. Values are normalized to 200000 d.p.m. recovered. Actual d.p.m. recovered: control, 176984 ± 19399 ; antimycin A, 163207 ± 4380 ($P = \text{n.s.}$). Results are means $\pm$ S.E.M. for four paired samples. The statistical analysis is by paired t test. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine.

	$10^{-3} \times ^{14}\text{C}$ (d.p.m./30 min per 120 islets)				
	Fatty acid (palmitate)	PE	PC	Lyso-PC	Lyso-PC/PC
Control ($n = 4$)	48.43 ± 0.94	2.90 ± 0.12	51.75 ± 2.89	96.14 ± 2.76	1.88 ± 0.16
Antimycin A (10 μM) ($n = 4$)	57.77 ± 2.86	2.01 ± 0.24	39.75 ± 5.24	100.7 ± 7.29	2.74 ± 0.54
P	< 0.10	< 0.05	< 0.05	n.s. (> 0.10)	n.s. (> 0.10)

16.7 mM-glucose had no obvious effect on the rate of lyso-PC uptake or esterification compared with 1.7 mM-glucose (results not shown). These findings were not surprising since the translocation of lyso-PC across the plasma membrane is energy-independent in other cells [10]; in addition, after isolation in the Ficoll gradient, the islets were distributed into individual test tubes from a single dish which contained the higher glucose concentration in the KRB buffer. Thus, the islets probably had more than adequate ATP stores (see below).

Effects of ATP depletion on the metabolism of lyso-PC (Table 1)

Under physiological conditions, the formation of phosphatidylcholine from lyso-PC is believed to proceed via an acyltransferase (the Lands pathway) after the ATP- and CoA-dependent activation of fatty acids to acyl-CoA. However an alternative, usually minor, pathway (the Erbland–Marinetti pathway) exists in some cells; this enzyme catalyses the disproportionation of two molecules of lyso-PC (to yield one molecule of phosphatidylcholine and one of glycerophosphorylcholine) via an intermolecular transesterification reaction [16–19,28]. This reaction is ATP- and CoA-independent, and it proceeds rapidly and not progressively as does the ATP-dependent esterification of lyso-PC by acyl-CoA; it usually predominates only in conditions of very high lyso-PC concentrations, low intracellular pH and/or in the absence of cell particles [17,18,28–30].

Therefore it seemed of interest to examine the effect of reductions in cellular ATP content, using antimycin A, on phosphatidylcholine formation. When islets were preincubated with antimycin A (10 μM) or its diluent for 15 min and then incubated with [^{14}C]lyso-PC in the presence or absence of antimycin A for a further 30 min, phosphatidylcholine formation fell by $24 \pm 7\%$ (mean $\pm$ S.E.M., $n = 4$, $P < 0.05$; Table 1), suggesting, as concluded by Laychock in studies of islet homogenates [14], that a major pathway in the islet converting lyso-PC to phosphatidylcholine is ATP- (and CoA-) dependent. Since 10 μM -antimycin A leaves about 20–25% of resting ATP levels in islets [31], the residual, antimycin-resistant formation of phosphatidylcholine probably reflects a submaximal reduction of ATP availability. Indeed, islet ATP concentrations are in the millimolar range, and at least some acylation pathways have a K_m for ATP in the micromolar range [32]. These studies, however, clearly

cannot exclude the concomitant presence of an ATP-independent mechanism converting lyso-PC to phosphatidylcholine in islets, perhaps an Erbland–Marinetti pathway or a related transacylase. [Indeed, this possibility would perhaps be expected, since in some cells transacylase-like reactions may be carried out by the same enzyme having lysophospholipase activity [25,26,33], with the fatty acid moiety being transferred from lyso-PC to another molecule of lyso-PC, instead of to water. In fact, I have previously provided evidence [3] that the release and re-esterification of [^3H]arachidonic acid from and into membrane lysophosphoacylglycerols of intact rat islets involves two ‘compartments’, one a basal, energy-independent ‘pool’ (resembling a transesterification mechanism in its characteristics) and one a glucose-stimulated, energy-dependent ‘pool’ (having the characteristics expected of a deacylation–reacylation, Lands’, cycle).] Possibly a transacylation pathway supervenes when ATP (or endogenous free fatty acids) needed for the acyltransferase cascade are exhausted by antimycin A or the initial metabolism of an exogenous load of lyso-PC.

During the reduction in conversion of lyso-PC to phosphatidylcholine induced by antimycin A, lyso-PC levels did not rise in absolute amounts ($+5 \pm 6\%$ of control; $P = \text{n.s.}$; Table 1) or as a percentage of total radioactivity counts recovered (control, $48 \pm 1\%$, antimycin, $51 \pm 5\%$; $n = 4$, $P = \text{n.s.}$). Instead, lyso-PC seemed to be effectively cleared by lysophospholipase activity, since fatty acids rose from $24 \pm 0.4\%$ to $29 \pm 1\%$ ($P < 0.10$; Table 1). Thus (partial) inhibition of only one pathway of lyso-PL clearance alone will not lead to lyso-PL accumulation, due to the presence of duplicative pathways for its removal. The lack of effect of antimycin A on the net uptake of [^{14}C]lyso-PC (Table 1) or on lysophospholipase activity (Table 1) is in accord with observations of others [10,26], that the uptake and transbilayer movement of lyso-PC, and its metabolism via lysophospholipases, do not require metabolic energy.

Effects of PHMB on the metabolic fate of lyso-PC (Table 2)

The effects of 100 μM -PHMB during a 30 min incubation period were then studied at 1.7 mM-glucose. These conditions were chosen since: (1) this concentration of PHMB consistently elicits significant, but submaximal, insulin release at 1.7 mM-glucose [2,4,7]; (2)

Table 2. Effect of 100 μ M-PHMB on lipid levels in intact rat islets

PHMB (or its diluent) was added at the times indicated for 30 min. Lipids were separated and quantified as described in the text. Results are mean $\pm$ S.E.M. for the number of paired (replicate) samples indicated in parentheses. The statistical analysis is by paired *t* test. Abbreviations are the same as used in Table 1. Condition 1: islets were incubated with [14 C]lyso-PC for 0–30 min in the presence or absence of PHMB (100 μ M). Values are normalized to 200 000 d.p.m. recovered. Actual d.p.m. recovered: control, 135 497 $\pm$ 16 069; PHMB, 128 396 $\pm$ 15 810 ($n = 5$ each, $P = \text{n.s.}$). Condition 2: islets were incubated with [14 C]lyso-PC for 60 min, followed by transfer to new medium in the presence or absence of PHMB (but without [14 C]lyso-PC) for a final 30 min. Values are normalized to 400 000 d.p.m. recovered. Actual d.p.m. recovered: control, 320 404 $\pm$ 27 692 ($n = 7$); PHMB, 317 260 $\pm$ 56 934 ($n = 8$); $P = \text{n.s.}$

		$10^{-3} \times ^{14}\text{C}$ [d.p.m./30 min (condition 1) or 90 min (condition 2) per 120 islets]				
Condition		Fatty acid (palmitate)	PE	PC	Lyso-PC	Lyso-PC/ PC
1	Control ($n = 5$)	43.6 $\pm$ 6.2	2.27 $\pm$ 0.59	41.1 $\pm$ 8.3	112.7 $\pm$ 14.8	3.69 $\pm$ 1
	PHMB (100 μ M) ($n = 5$)	19.9 $\pm$ 3.9	0.51 $\pm$ 0.16	19.6 $\pm$ 8.3	160.0 $\pm$ 5.8	9.81 $\pm$ 2
	<i>P</i>	< 0.01	< 0.05	< 0.05	< 0.02	< 0.10
2	Control ($n = 7$)	185.3 $\pm$ 23.3	1.85 $\pm$ 0.07	108.2 $\pm$ 12.0	105.1 $\pm$ 20.1	1.19 $\pm$ 0.34
	PHMB (100 μ M) ($n = 8$)	155.1 $\pm$ 17.0	1.39 $\pm$ 0.08	89.2 $\pm$ 8.2	155.0 $\pm$ 10.3	1.93 $\pm$ 0.32
	<i>P</i>	< 0.05	< 0.01	< 0.05	< 0.02	< 0.01

30 min incubations had been used in those studies; (3) this concentration of PHMB is virtually free from functional or morphological islet toxicity [2,4,12].

When present throughout a labelling period from 0 to 30 min, PHMB inhibited the accumulation of phosphatidylcholine from 21 $\pm$ 4 to 10 $\pm$ 2% of total radioactivity recovered ($n = 5$, $P < 0.05$) and fatty acid from 22 $\pm$ 3 to 10 $\pm$ 2% ($P < 0.01$); consequently, lyso-PC increased from 57 $\pm$ 7 to 80 $\pm$ 3% ($P < 0.02$). The lyso-PC/phosphatidylcholine ratio also rose (Table 2). PHMB did not alter the total radioactivity recovered (Table 2). In paired comparisons of the absolute values, lyso-PC levels also increased to 150 $\pm$ 17% of control values (Table 2) whereas fatty acid fell to 44 $\pm$ 11%, and phosphatidylcholine to 54 $\pm$ 11% of control values. In preliminary studies ($n = 2$), PHMB, 200 μ M, reduced phosphatidylcholine further to 6 $\pm$ 1% of total radioactivity recovered and fatty acid to 6 $\pm$ 5%; lyso-PC increased to 89 $\pm$ 6% of total radioactivity. In absolute terms, the formation of phosphatidylcholine over 30 min was inhibited 51% by 200 μ M-PHMB. Thus PHMB appeared to paralyse both major enzymes clearing lyso-PC, leading to its accumulation (in relative and absolute terms), in contrast with antimycin A, which inhibited only its conversion to phosphatidylcholine (see above).

In an additional series of studies, 100 μ M-PHMB was added between 60 and 90 min of the labelling period when the percentage conversion of lyso-PC had reached an apparent steady state (Fig. 2). Phosphatidylcholine again was decreased (27 $\pm$ 1 to 20 $\pm$ 2% of total radioactivity; $n = 5$, $P < 0.01$), the fatty acid level fell, although non-significantly (47 $\pm$ 3 to 43 $\pm$ 3% of total; $n = 5$, $P = \text{n.s.}$) and lyso-PC again rose (from 26 $\pm$ 3 to 36 $\pm$ 4%; $n = 5$, $P < 0.05$).

However (in contrast with the first series of studies), the total radioactivity recovered in PHMB-treated islets seemed to be somewhat (21 $\pm$ 1%) less than that in control islets (control, 370 955 $\pm$ 51 489; PHMB,

302 941 $\pm$ 31 719 d.p.m.; $n = 5$, $P < 0.01$). Therefore, we felt it necessary to exclude completely the trivial possibility that PHMB merely altered the uptake of labelled lyso-PC into the cell, thereby altering lyso-PC metabolism only secondarily. Thus, the latter set of studies was repeated but with the following modification: during the final 30 min (during which PHMB was present), exogenous lyso-PC was removed from the medium. Thus, only the lyso-PC already incorporated into the lipid bilayer was subject to metabolism and its perturbation by PHMB. PHMB still inhibited the accumulation of phosphatidylcholine (control, 27 $\pm$ 3%; PHMB, 22 $\pm$ 2% of total radioactivity recovered; $n = 9$, $P < 0.05$) and of fatty acid (control, 46 $\pm$ 2%; PHMB, 39 $\pm$ 2%; $P < 0.05$) and augmented that of lyso-PC (control, 26 $\pm$ 5%; PHMB, 39 $\pm$ 2%; $n = 5$, $P < 0.02$). The absolute values for lyso-PC in the presence of PHMB were 201 $\pm$ 64% of control values, in paired comparisons (Table 1). PHMB again increased the lyso-PC/phosphatidylcholine ratio (Table 2). Under these circumstances, PHMB had no effect on total radioactivity recovered (Table 2).

During this last protocol, PHMB could have only a moderate effect on final lipid levels since it was present only during the last 30 min of a 90 min incubation period. However, the percentage inhibition of the acyltransferase during the final 30 min experimental part of this study could be calculated by determining lipid levels at both 60 and 90 min and ascertaining the percentage of this increment in phosphatidylcholine between 60 and 90 min which was blocked by the presence of PHMB. Using this calculation, 100 μ M-PHMB inhibited acyltransferase activity between 60 and 90 min by 48 $\pm$ 15% in paired comparisons ($n = 8$), reducing the increment in phosphatidylcholine from 37 294 $\pm$ 7653 to 21 027 $\pm$ 7386 d.p.m./30 min. This 48% reduction is comparable with the 46% reduction determined by the first study. It is also reasonably consistent with my earlier observations that this

concentration of PHMB inhibits the esterification of [^3H]arachidonic acid into islet phospholipids by 55–65% during 90 min incubations [3].

Formation of phosphatidylethanolamine

Antimycin A reduced phosphatidylethanolamine from $1.46 \pm 0.06\%$ of total radioactivity recovered to $1.01 \pm 0.12\%$ ($P < 0.05$, $n = 5$) at 0–30 min. PHMB also inhibited phosphatidylethanolamine appearance from 1.13 ± 0.29 to $0.25 \pm 0.08\%$ of total ($P < 0.05$, $n = 5$) when added at 0–30 min and from $0.33 \pm 0.04\%$ to $0.26 \pm 0.04\%$ ($P < 0.02$, $n = 9$) when added between 60 and 90 min. The absolute values for phosphatidylethanolamine were also significantly inhibited by either test agent (Tables 1 and 2): by PHMB ($-75 \pm 9\%$) or antimycin A ($-30 \pm 8\%$) between 0 and 30 min. This inhibition could reflect the direct inhibition of a putative ATP-dependent transacylase by these agents. Formation of phosphatidylethanolamine from phosphatidylcholine via base exchange seems less likely since such reactions are generally resistant to inhibition by organic mercurials such as PHMB [34]. However, we cannot exclude the possibility that it merely reflects the PHMB-induced decline in the donor phospholipid (phosphatidylcholine) involved in fatty acid donation or base exchange.

Relevance of lyso-PC metabolism in islets to insulin release

The appearance of the metabolic products of lyso-PC occurred at least as early as 5 min of incubation (Fig. 2), by which time 6% of total radioactivity had been converted to phosphatidylcholine and 8% to fatty acid ($n = 2$). Since lyso-PC-induced insulin release occurs within 3–5 min of application [1,2,7], it is evident that, on temporal grounds, the insulin release induced by exogenous lyso-PLs is consistent with a rapid, albeit limited, diffusion of lyso-PC to the inner leaflet where it is normally formed by the action of phospholipase A_2 [35]. Both in intact adult rat islets [1] and in monolayer-cultured neonatal rat islet cells (W. Fujimoto & S. Metz, unpublished work), lyso-PC-induced insulin release is biphasic, with an initial peak followed by a trough at 5–10 min, followed by a secondary rise. In the latter system, the second phase of insulin release reaches a plateau after about 50–60 min. The dynamics of lyso-PC metabolism at the time of the trough in insulin secretion was not investigated in detail in the current study. However, the later plateau seems to correlate temporally with an apparent steady-state in the absolute amounts of lyso-PC persisting unmetabolized in islets after about 60 min in the current study (cf. Fig. 2 and inset, where it can be seen that the total radioactivity in islets continues to rise as the percentage in the lyso-PC fraction declines: absolute radioactivity of unmetabolized lyso-PC in the islets appears to reach a peak by 60 min and then stabilize at a somewhat lower level).

The data also permit approximate calculations of the increment in islet lyso-PC required to initiate insulin release. Using the protocol described above, it was observed that a mean of 151000 ± 15000 d.p.m. ($n = 9$), or 2.88% of the total lyso-PC added, was taken up into islets during a 30 min incubation. Since the medium contained $19.8 \mu\text{g}$ of lyso-PC/ml, approx. 570 ng was taken up by 120 islets, or 4.75 ng/islet. Using Hallberg's data [36] it can be calculated that resting rat islets contain approximately 4.4 ng of lyso-PC/islet. Since the

minimal effective concentration for exogenous lyso-PC to induce insulin release at 1.7 mM-glucose during similar 30 min incubations is only $10 \mu\text{g/ml}$ [1,2,7], it can be estimated that an increment in islet lyso-PC content of about 2.4 ng/islet, or 55% over basal levels, may be sufficient to induce insulin release. However, even this value is likely to be an overestimation, since during such a 30 min incubation, an average of only 22–23% of lyso-PC (0% at the outset, 45% at the end of 30 min; Fig. 2) has reached the inner membrane leaflet (as assessed by conversion to phosphatidylcholine and/or fatty acid) where endogenous lyso-PC is normally formed by the action of phospholipase A_2 [35]. Thus, an increment in inner leaflet lyso-PC perhaps as small as 10–15% above basal values might be sufficient to initiate insulin release. It seems likely therefore, as I previously postulated [5,7], that the apparently 'high' concentrations of lysophospholipids in the extracellular medium required to trigger insulin release are merely those needed to achieve small but effective fractional changes in lysophospholipid content of the inner membrane leaflet (one putative site of physiological formation and action). In fact, a slower rate of uptake may explain the ineffectiveness of lyso-PS or lyso-PE in inducing insulin release [1,2,4,7]; although these amino-lysophospholipids are translocated to the inner membrane leaflet after uptake at least as fast as lyso-PC [13], they may be taken up into islets to a lesser degree than is lyso-PC [30]. Earlier data (especially those demonstrating the inhibitability of lyso-PL-induced insulin release by La^{3+} [1,2,5,7]) suggested that lyso-PLs may release Ca^{2+} stores located at the inner plasma membrane leaflet of the β -cell [37,38]. The current data are compatible with the formulation that a small accumulation of lyso-PLs at this critical location may trigger Ca^{2+} -dependent insulin release.

It should be noted these conclusions are based on the use solely of lyso-PC with a C_{16} (palmitoyl) residue in the *sn*-1 position. Islets may contain more C_{18} than C_{16} acyl chains [39]. However, it is unlikely that the metabolism of such forms of lyso-PC by the islet differs greatly from that of the lyso-PC used in the current study. In erythrocytes, at least, increasing acyl chain length does slow the rate of the translocation (and of the metabolism) of lyso-PC [40]; however, this effect would probably be more than offset by the more rapid translocation induced by a greater degree of unsaturation [41] seen in some longer chain fatty acids. Furthermore, I have observed generally similar insulinotropic effects of lyso-PC whether the fatty acid moiety is: (1) saturated or unsaturated; (2) in an ether or ester linkage; (3) of different complex compositions, such as in soybean or egg lyso-PC; or (4) of C_{16} or predominantly of C_{18} chain length [1,2].

To achieve increments in lyso-PC accumulation, PHMB had to paralyze at least two enzymes of lyso-PC clearance (acyltransferase, lysophospholipase and possibly transacylase). Indeed, in most studies [15,42,43] of other cells, PHMB has been observed to inhibit both acyltransferase and lysophospholipase reactions, although the latter finding has been more variable [42,44]. The calculations above suggest that the increment in lyso-PC accumulation induced by PHMB (50–100%) would be sufficient to explain its insulinotropic ability, as I previously suggested [2,4,7]. In red blood cells, a similar increment of membrane lyso-PC content (a doubling or less to 2.1% of total phospholipid content) is sufficient to

facilitate Ca^{2+} fluxes across the membrane, whereas at least four times as much is required to initiate some cell lysis [45]. In fact, I have demonstrated that it is possible to achieve equivalent or greater changes in islet membrane lyso-PC content (with a commensurate, large release of insulin) by using melittin, an activator of endogenous phospholipase A_2 [3]. However, it is important to emphasize that it has not yet been demonstrated that physiological β -cell stimuli promote the generation or accumulation of lyso-PC. However, the current approach may provide an improved method for the study of the effect of physiological agonists on the metabolism of lysophospholipids and phospholipids in intact islets.

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