Phospholipid turnover in isolated rat pancreatic acini

Consideration of the relative roles of phospholipase A₂ and phospholipase C

Stephen P. HALENDA and Ronald P. RUBIN*

Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond,
VA 23298, U.S.A.

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The purpose of the present study was to explore the interaction of phosphatidylinositol breakdown and the turnover of arachidonic acid in isolated rat pancreatic acini by using receptor agonists and the calcium ionophore ionomycin. Acini prelabelled with *myo*-[³H]inositol *in vivo* responded to carbachol with a rapid breakdown of phosphatidylinositol. In the presence of [³²P]P_i, carbachol increased labelling of phosphatidic acid and phosphatidylinositol within 1 and 5 min respectively. Carbachol also rapidly stimulated the incorporation of [¹⁴C]arachidonic acid into phosphatidylinositol within 2 min, and the peptidergic secretagogue caerulein caused the loss of radioactivity from phospholipids prelabelled with arachidonic acid. Ca²⁺ deprivation partially impaired the stimulatory action of carbachol on arachidonic acid turnover. In contrast with its stimulatory effects on [³²P]P_i and [¹⁴C]arachidonate incorporation, carbachol inhibited the incorporation of the saturated fatty acid stearic acid into phosphatidylinositol. Whereas ionomycin stimulation of phosphatidylinositol breakdown and [³²P]P_i labelling of phospholipids was slower in onset and less effective than carbachol stimulation, the ionophore effectively promoted (arachidonyl) phosphatidylinositol turnover within 2 min. These results implicate two separate pathways for stimulated phosphatidylinositol degradation in the exocrine pancreas, involving phospholipases A₂ and C. Whereas mobilization of cellular Ca²⁺ appears sufficient to cause activation of phospholipase A₂ and amylase secretion, additional events triggered by receptor activation may be required to act in concert with Ca²⁺ to optimally stimulate phospholipase C. The nature of the interaction between phospholipases A₂ and C and their specific physiological roles in pancreatic secretion remain to be elucidated.

One approach to elucidating the nature of the cellular events associated with Ca²⁺-dependent exocytotic secretion concerns the turnover of membrane phospholipids. Changes in the composition of these phospholipids may mediate alterations in membrane permeability, activation of membrane-bound enzymes or fusion of cellular membranes. Attempts to correlate changes in specific phospholipids with functional activity in secretory glands have dealt mainly with phosphatidylinositol (Berridge, 1981). The turnover of the polar head group and of arachidonic acid in position 2 of phosphatidylinositol is catalysed by phospholipases C

and A₂. Both of these reactions have been implicated in the events associated with activation of secretion. More particularly, the blood platelet release reaction stimulated by thrombin involves an initial activation of the phospholipase C reaction resulting in the degradation and resynthesis of phosphatidylinositol (phosphatidylinositol cycle); and stimulation of arachidonic acid turnover in phospholipids is an accompanying event (Lapetina, 1982). We have also previously reported that a Ca²⁺-dependent activation of phospholipase A₂ degrades phosphatidylinositol in certain other secretory organs (Rubin & Laychock, 1978; Schrey & Rubin, 1979; Rubin *et al.*, 1981*a,b*). This deacylation reaction together with a subsequent reacylation of lysophosphatidylinositol appears to be

* To whom correspondence and reprint requests should be sent.

involved in the mechanism regulating secretion (Rubin, 1982).

In the exocrine pancreas, secretagogues promote the turnover of [^{32}P]P_i and radiolabelled inositol, depress phosphatidylinositol levels and raise phosphatidic acid levels (Hokin, 1968; Hokin-Neaverson, 1977; Farese *et al.*, 1980). The hypothesis has been offered that phosphatidylinositol breakdown might play a key role in the reactions that couple the receptor responses to Ca²⁺ gating; however, it is still uncertain as to whether phosphatidylinositol degradation is a consequence, rather than a cause, of Ca²⁺ flux (Berridge, 1981; Michell *et al.*, 1981). More recent evidence supports the concept that arachidonic acid release from phosphatidylinositol induced by phospholipase A₂ and the resultant production of arachidonic acid metabolites, e.g., prostaglandins and hydroxy-acids, may also be a key event in the activation of pancreatic secretion, although evidence in this regard is conflicting (Banschbach & Hokin-Neaverson, 1980; Marshall *et al.*, 1980, 1981; Bauduin *et al.*, 1981; Rubin *et al.*, 1982).

In the present paper we explore the interaction of the phosphatidylinositol cycle and the turnover of arachidonic acid in rat pancreatic acini by using receptor agonists and the calcium ionophore ionomycin. Analysis of the time course of events reveals that mobilization of Ca²⁺ produced by the ionophore predominantly stimulates the deacylation-reacylation of phosphatidylinositol. On the other hand, the action of carbachol is expressed by the turnover of both arachidonyl and inositol phosphate moieties of phosphatidylinositol.

Materials and methods

Materials

The following radiolabelled materials were purchased from New England Nuclear, Boston, MA, USA: [^{14}C]stearic acid (sp. radioactivity 52 Ci/mol); *myo*-[^3H]inositol (sp. radioactivity 12 Ci/mol) and [^{32}P]orthophosphoric acid (carrier-free). [^{14}C]Arachidonic acid (sp. radioactivity 58 Ci/mol) was obtained from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. The following biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: soya-bean trypsin inhibitor, bovine serum albumin (fraction V), carbamoylcholine chloride (carbachol) and all phospholipid standards. Collagenase (class IV) was acquired from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Caerulein was obtained from Peninsula Laboratories, San Carlos, CA, U.S.A. Radiolabelled fatty acids were stored in toluene under argon at -40°C. Before use, toluene was evaporated with dry N₂ and fatty acids were

redissolved in a small volume of dimethyl sulphoxide, yielding a final concentration of 0.1% (v/v) per incubation. The ionophore ionomycin (compound SQ 23,377; calcium salt), a gift from the Squibb Institute for Medical Research, Princeton, NJ, U.S.A., was stored frozen as a stock solution in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide in the medium, which was not more than 0.2% (v/v), had no effect on amylase secretion or incorporation of radiolabel into phospholipids. All solvents (reagent grade) were purchased from Fisher Chemical Co., Pittsburgh, PA, U.S.A.

Methods

Pancreatic acini were prepared from starved male Sprague-Dawley rats (250–300 g) by a modification of the method of Williams *et al.* (1978), utilizing the following procedure. The rat was guillotined and exsanguinated. The common bile duct was ligated at its juncture with the duodenum and 5 ml of a Krebs-Henseleit medium (pH 7.4) containing 70 units of collagenase/ml, 0.1 mg of trypsin inhibitor/ml, 0.25% (w/v) bovine serum albumin and 0.1 mM-CaCl₂ was injected into the duct to distend the pancreas. The pancreas was rapidly dissected free of extraneous tissues and then incubated for 10 min in 5 ml of the same medium. Excess medium was aspirated and replaced with fresh medium, and incubation was continued for an additional 35 min. Acini were dispersed by gently drawing the digested tissue several times through a 5 ml Finn timer, and the suspension was filtered through 6-ply gauze. The filtrate was layered over 8 ml cushions of medium containing 4% bovine serum albumin, 0.1 mg of trypsin inhibitor/ml and 1.2 mM-CaCl₂ and then centrifuged for 5 min at 50 g to remove blood cells and other extraneous tissue. Acini were then washed and pre-incubated for 30 min in the same medium, followed by resuspension in 5 ml of Krebs-Henseleit medium containing 0.1 mg of trypsin inhibitor/ml and 1.2 mM-CaCl₂. This medium was used for all subsequent incubations, except when prelabelling acini with [^{32}P]orthophosphate, in which case the KH₂PO₄ concentration of the medium was reduced from 1.2 mM to 10 μM . In experiments involving Ca²⁺ deprivation, the medium was constituted from stock solutions in concentrations identical with those of normal medium, except for the absence of Ca²⁺ and the presence of 1 mM-EGTA.

For amylase determinations, acini were incubated at 37°C in 500 μl volumes for various times with or without drugs, then placed on ice and quickly centrifuged for 20 s at 15 900 g. A portion of the supernatant was saved, and the pellet was resuspended in water. Acini were lysed by three freeze-thaw cycles, centrifuged for 2 min at 15 900 g

and a portion of supernatant was taken. Amylase content of medium and acinar lysate was determined by the method of Bernfeld (1955). Enzyme release was calculated as a percentage of total (acinar plus medium) amylase activity present in the medium at various time periods after subtraction of the amylase activity of medium at time zero. To measure radiolabel incorporation into phospholipids, incubations were carried out in a volume of 500 μ l at 37°C, shaken at 200 cycles/min. Incubations were begun by pipetting 100 μ l of acinar suspension into 400 μ l of medium containing either 0.2 μ Ci of [14 C]arachidonic acid or 0.2 μ Ci of [14 C]stearic acid. When 32 P-turnover studies were conducted, acini were pre-incubated with 1 μ Ci of [32 P]orthophosphate for 30 min before the addition of drugs. For prelabelling studies, the acini were pre-incubated with 0.2 μ Ci of [14 C]arachidonic acid plus 1 mM-carbachol for 30 min. The acini were then resuspended for 30 min in fresh medium containing 100 μ M-atropine plus 1% bovine serum albumin to remove the excess arachidonic acid, and then resuspended in medium devoid of bovine serum albumin and continued presence of atropine plus 700 nM-caerulein. Incubations were terminated after various time intervals by adding 3 ml of chloroform/methanol (1:2, v/v) containing 0.1 mg of butylated hydroxytoluene/ml.

Phospholipids were extracted as previously described (Schrey & Rubin, 1979) and purified by the one-dimensional two-solvent-system method of Weiss *et al.* (1982). Briefly, lipid extracts were applied to heat-activated t.l.c. plates (Silica gel 60 from Merck) with a multispotter and developed in chloroform/methanol/acetic acid/water (40:10:10:1, by vol.). The plates were dried, then developed to a level approx. 1.2 cm below the first solvent front in a system containing the same solvents in the proportions 120:46:19:3 (by vol.). Phospholipids were visually detected by exposure to I_2 vapour. R_F values for phospholipids of interest were: phosphatidylcholine, 0.2; phosphatidylinositol, 0.47; phosphatidylserine, 0.55; phosphatidylethanolamine, 0.70; phosphatidic acid, 0.81. This system afforded reliable separation of phosphatidylinositol from phosphatidylserine, and of phosphatidic acid from neutral lipids and non-esterified fatty acids, which migrate at the solvent front. Spots corresponding in R_F values to phospholipid standards were scraped and counted for radioactivity for 10 min in 5 ml of Beckman Ready-Solv EP by using an LKB 1211 liquid-scintillation spectrometer with a counting efficiency for 14 C and 3 H of 91 and 65% respectively. There was no decrease in efficiency when 14 C or 3 H from silica-gel scrapings was counted for radioactivity. Each experiment was carried out in duplicate and was generally replicated on at least three different preparations. The

statistical significance of differences between control and drug treatments was obtained by Student's *t* test for either paired or unpaired comparisons.

Results

Amylase release

The muscarinic receptor agonist carbachol and the Ca^{2+} ionophore ionomycin stimulate amylase release from pancreatic acini. Fig. 1 shows that the onset of release is equally rapid with these two secretagogues and secretion is virtually complete within 15 min. During a 30 min incubation secretion was raised from a basal level of 9 (± 3)% of total tissue amylase activity to 34 (± 4) and 41 (± 1)% by 10 μ M-carbachol and 1 μ M-ionomycin respectively. Ionomycin (2 μ M) elevated output to 52 (± 5)% ($n = 11$). Concentrations of ionomycin exceeding 2 μ M elicited even greater secretory responses, presumably owing to a toxic effect, since acini were unable to exclude Trypan Blue at these higher concentrations.

Stimulated breakdown of phosphatidylinositol and [32 P] P_i turnover

Two types of experiments were performed to confirm the presence of a phosphatidylinositol-

Fig. 1. Time course of ionomycin- and carbachol-stimulated amylase release from rat pancreas

Isolated pancreatic acini were incubated with secretagogue for the indicated time periods. Values are means derived from at least four determinations. Spontaneous release of enzyme was subtracted from stimulus-induced secretion at each time point. Δ , 1 μ M-ionomycin; $\bullet$, 10 μ M-carbachol.

hydrolysing phospholipase C in the exocrine pancreas. Acini were prepared from rats injected 18h previously with *myo*-[^3H]inositol to label endogenous phosphatidylinositol. Carbachol induced a rapid breakdown of phosphatidylinositol with no apparent latency; this breakdown proceeded in a linear manner for 15min and then approached completion by 30min (Fig. 2). A concentration of ionomycin ($2\mu\text{M}$) that stimulated amylase secretion more effectively than $10\mu\text{M}$ -carbachol also stimulated the breakdown of phosphatidylinositol. Ionomycin induced degradation developed more slowly, but by 30min phosphatidylinositol was decreased to a level comparable with that obtained with carbachol (Fig. 2).

The activation of the phosphatidylinositol cycle as analysed by [^{32}P]P_i turnover is a reflection of a sequential increase in phosphatidic acid followed by the resynthesis of phosphatidylinositol (Michell *et al.*, 1977). The time course of phosphatidylinositol and phosphatidic acid labelling with [^{32}P]orthophosphate over a 10min period is illustrated in Fig. 3. Carbachol induced a time-dependent stimulation of [^{32}P]P_i incorporation into phosphatidylinositol, although a significant stimulation of label incorporation was not observed until 5min (Fig. 3a). Incorporation of [^{32}P]P_i into phosphatidic acid preceded that into phosphatidylinositol. Thus, phosphatidic acid labelling was significantly enhanced by 1min, reaching maximum levels of stimulation by 5min (Fig. 3a).

Fig. 2. Effect of carbachol and ionomycin on phosphatidylinositol breakdown

Rats were injected with *myo*-[^3H]inositol and acini were prepared as described in the Materials and methods section. Acini were incubated for indicated time periods in the presence or absence of $10\mu\text{M}$ -carbachol (O) or $2\mu\text{M}$ -ionomycin (●). Lipids were extracted and radioactivity was determined. Data are means ($\pm$ S.E.M., indicated by the bars) derived from four different preparations. Radioactivity of control samples remained constant over the time course of the experiment.

Ionomycin ($2\mu\text{M}$) was less effective than carbachol in promoting the labelling of phosphatidylinositol and phosphatidic acid (Fig. 3b). A small time-dependent stimulation of [^{32}P]P_i incorporation into phosphatidylinositol was observed with the ionophore, although a significant effect was not observed until 5min. Phosphatidic acid labelling was also not significantly enhanced by ionomycin until 5min (Fig. 3b), whereas carbachol stimulated [^{32}P]P_i incorporation into phosphatidic acid within 1min (Fig. 3a). The enhancement of [^{32}P]P_i incorporation into phosphatidic acid induced by ionomycin (62%) was also less than that induced by carbachol (162%) after 5min (Figs. 3a and 3b). Similarly, ionomycin and carbachol stimulated

Fig. 3. Effect of carbachol and ionomycin on [^{32}P]P_i incorporation into acinar phosphatidic acid and phosphatidylinositol

Acini were pre-incubated for 30min in the presence of $1\mu\text{Ci}$ of [^{32}P]P_i. In (a) $10\mu\text{M}$ -carbachol and in (b) $2\mu\text{M}$ -ionomycin were added and incubations were terminated at the indicated time intervals. Values are means ($\pm$ S.E.M., indicated by the bars) of four to nine experiments * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, versus control at the same time point (unpaired differences). ●, phosphatidylinositol; ○, phosphatidic acid. Control radioactivity (excluded for clarity) gradually increased from $100 \pm 6\%$ of the zero-time value at 1min to $116 \pm 14\%$ at 10min for phosphatidylinositol and from 111 ± 6 to $123 \pm 8\%$ of the zero-time value for phosphatidic acid.

labelling of [^{32}P]phosphatidylinositol by 83 and 240% respectively after 10 min. Ionomycin ($1\mu\text{M}$), which effectively augments enzyme release, did not significantly increase [^{32}P]P_i incorporation into phosphatidylinositol; a significant enhancement of phosphatidic acid labelling was observed only after 10 min ($74 \pm 28\%$) ($P < 0.05$). In contrast with their stimulant actions on the labelling of phosphatidylinositol and phosphatidic acid, carbachol and ionomycin ($2\mu\text{M}$) did not promote [^{32}P]P_i incorporation into phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine (results not shown).

Fatty acid incorporation

Phosphatidylinositol of the exocrine pancreas consists almost entirely of the 2-arachidonyl-1-stearoyl molecular species (Hokin-Neaverson, 1977), so the turnover of these fatty acyl groups during secretory activity was next examined. The relative distribution of [^{14}C]arachidonic acid in the various phospholipids is given in Table 1. Although acinar phosphatidylcholine accounted for the largest amount of radiolabelled phospholipid, phosphatidylinositol and phosphatidic acid also accumulated considerable amounts of radioactivity (Figs. 4*a* and 4*b*). The rapid accumulation of arachidonic acid by phosphatidic acid was most striking, attaining peak levels within 2 min and then gradually declining over the next 30 min (Fig. 4*b*).

Carbachol stimulated the incorporation of arachidonic acid into phosphatidylinositol and phosphatidic acid within 2 min (Figs. 4*a* and 4*b*), but had no significant effect on label incorporation into phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine (results not shown). Whereas carbachol increased incorporation of arachidonic acid into phosphatidylinositol in a time-dependent manner (Fig. 4*a*), the stimulation of arachidonyl phosphatidic acid labelling decreased after 2 min (Fig. 4*b*). The rapid stimulatory effect on the turnover of the fatty acyl chain of phosphatidylinositol was further demonstrated by pre-incubating

acini with [^{14}C]arachidonic acid for 15 min; a 1 min exposure to carbachol ($10\text{ }\mu\text{M}$) stimulated arachidonate incorporation into phosphatidylinositol by $10\text{ }(\pm 2)\%$ ($P < 0.02$). The extent of the enhancement of radiolabelled arachidonic acid incorpo-

Fig. 4. Effect of carbachol on arachidonic acid incorporation into phosphatidylinositol and phosphatidic acid

Acini were incubated with [^{14}C]arachidonic acid in the presence (○) or absence (●) of $10\text{ }\mu\text{M}$ -carbachol. Incubations were terminated at indicated time intervals and extracts were processed for (a) phosphatidylinositol and (b) phosphatidic acid analysis (see the Materials and methods section). Values are means ($\pm$ S.E.M., indicated by the bars) for four to 12 experiments. * $P < 0.05$; ** $P < 0.001$ (paired differences).

Table 1. *Effect of ionomycin on [¹⁴C]arachidonic acid incorporation into acinar phospholipids*

Acini were incubated for 10 min with 0.2 μ Ci of [14 C]arachidonic acid per 0.5 ml incubation volume in the absence (control) or presence of 1 μ M-ionomycin. Incubations were terminated and phospholipid radioactivity was determined as described in the Materials and methods section. Values are means $\pm$ S.E.M. for 11 preparations. * P < 0.05; ** P < 0.01; *** P < 0.001, for ionomycin-treated versus control acini by paired t -test.

Phospholipid	Radioactivity (c.p.m./mg of protein)	
	Control	Ionomycin
Phosphatidylinositol	1194 ± 107	2054 ± 229***
Phosphatidic acid	776 ± 98	1090 ± 157*
Phosphatidylcholine	18621 ± 995	16631 ± 888***
Phosphatidylethanolamine	2730 ± 199	2523 ± 194**
Phosphatidylserine	403 ± 22	473 ± 34**

ation elicited by carbachol may be blunted by the concomitant fall in endogenous levels of phosphatidylinositol (cf. Fig. 2).

Ionomycin, like carbachol, promoted a rapid and time-dependent stimulation of (arachidonyl) phosphatidylinositol turnover (Fig. 5a). During a 10 min incubation period, ionomycin and carbachol stimulated (arachidonyl) phosphatidylinositol turnover by 72 (± 9) and 110 (± 8)% respectively (Figs. 4a and 5a). However, ionomycin, unlike carbachol, failed to stimulate (arachidonyl) phosphatidate turnover within 2 min (Fig. 5b). Thus the specific enhancement of arachidonate incorporation into phosphatidic acid induced by carbachol at this time interval was probably a reflection of the net increase in phosphatidic acid triggered by phospholipase C activation, rather than by turnover of the fatty acyl chain. Ionomycin also differed from carbachol in modifying arachidonic acid incorporation into other phospholipids. After stimulation with 1 μ M-ionomycin, arachidonic acid incorporation into phosphatidylserine was also signifi-

cantly enhanced whereas incorporation into phosphatidylcholine and phosphatidylethanolamine was depressed (Table 1).

To assess the Ca^{2+} -dependency of carbachol-stimulated arachidonic acid turnover, acini were incubated for 30 min in a medium devoid of added Ca^{2+} and containing EGTA (1 mM). Ca^{2+} deprivation reduced the stimulatory effect of carbachol (5–10 μ M) on arachidonic acid incorporation into phosphatidylinositol from 93 (± 13)% to 49 (± 11)% ($n = 10$); ($P < 0.02$). Under identical incubation conditions, calcium deprivation reduced carbachol-induced amylase secretion by an average of 34% ($n = 2$).

Acinar phospholipids also incorporated the saturated fatty acid [^{14}C]stearic acid. Phosphatidylinositol accounted for the largest amount of radioactivity (2518 ± 265 c.p.m./mg of protein), compared with 186 ± 48 , 926 ± 128 , 529 ± 67 and 160 ± 23 c.p.m./mg of protein for phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine respectively during a 30 min incubation. In contrast with its stimulatory effects on [^{32}P]P_i and [^{14}C]arachidonic acid incorporation, carbachol actually inhibited stearate incorporation into phosphatidylinositol by 26 (± 5)% after 30 min ($n = 4$) and had no significant effect on phosphatidic acid radioactivity (results not shown). The inhibition of stearate incorporation may be a reflection of the decrease in endogenous phosphatidylinositol levels observed during carbachol stimulation (cf. Fig. 2).

Fig. 5. Effect of ionomycin on arachidonic acid incorporation into (a) phosphatidylinositol and (b) phosphatidic acid

The protocol for this experiment was similar to that described in the legend to Fig. 4 except that acini were incubated in the presence (○) or absence (●) of 1 μ M-ionomycin. Values are means ($\pm$ S.E.M., indicated by the bars) for four to 11 experiments.

* $P < 0.05$; *** $P < 0.001$ (paired differences).

Loss of arachidonic acid from prelabelled acini

Incorporation of [^{14}C]arachidonic acid is not a direct measure of deacylation but is a reflection of a reacylation reaction at the *sn*-2 position of glycerophospholipids that presumably follows deacylation. Experiments were therefore conducted to demonstrate the release of [^{14}C]arachidonic acid from prelabelled phospholipids (deacylation). The method of Marshall *et al.* (1981) was employed for labelling phospholipids (see under 'Methods'). Acini prelabelled with arachidonic acid in the presence of carbachol were stimulated with caerulein, a peptide that activates pancreatic secretion by a Ca^{2+} -mobilizing pathway shared by carbachol (Shulz & Stolze, 1980). Caerulein (700 nM) significantly reduced phosphatidylinositol radioactivity by 21% and increased phosphatidic acid radioactivity from prelabelled acini by 59% (Fig. 6). This same concentration of caerulein, when added together with the radiolabel, enhanced arachidonate incorporation into phosphatidylinositol and phosphatidic acid by 150 and 70% respectively during a 30 min incubation. Activation of phosphatidylinositol-specific phospholipase C probably accounts for the

Fig. 6. Effect of caerulein on arachidonic acid release from pancreatic phospholipids

Acini were prelabelled with [^{14}C]arachidonic acid as described in the Materials and methods section and incubated with caerulein for 60 min. Values are means ($\pm$ S.E.M., indicated by the bars) for four experiments. The average control values ($\pm$ S.E.M.) (100%) in phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylinositol plus phosphatidic acid were: 6646 ± 1179 , 354 ± 53 and 7000 ± 1228 c.p.m. respectively. * $P < 0.05$ (paired differences).

increase in phosphatidic acid radioactivity and a portion of the decrease in phosphatidylinositol radioactivity observed with prelabelled acini. However, the sum of the phosphatidylinositol and phosphatidic acid radioactivities decreased with caerulein treatment, indicating that the increase in the radiolabel in phosphatidic acid could not account for the entire loss of label from phosphatidylinositol. Phospholipase A activity likely accounts for the remaining loss, although other intermediates in the phospholipase C pathway (e.g. diacylglycerol) may comprise part of the loss.

Discussion

Previous studies have revealed that phospholipid metabolism is somehow linked to the secretory response of the exocrine pancreas (Hokin, 1968; Hokin-Neaverson, 1977; Marshall *et al.*, 1981). The two reactions stimulated by secretagogues are (a) the release of arachidonic acid from phospholipids (Marshall *et al.*, 1980, 1981) and (b) the hydrolysis

of phosphatidylinositol to diacylglycerol and myo-inositol phosphate by a phospholipase and the resynthesis of phosphatidylinositol by way of the conversion of diacylglycerol into phosphatidic acid (Hokin, 1968; Hokin-Neaverson, 1977). The approach described in the present paper permits an evaluation of these two proposed biochemical signals with regard to the mechanism mediating secretion.

The results of the present study demonstrate that stimulation of muscarinic receptors in isolated rat pancreatic acini induces a prompt decrease in phosphatidylinositol levels and a subsequent increase in phosphatidic acid turnover indicative of a phospholipase C-mediated reaction. Although endogenous phospholipid levels were not directly analysed, the observed decrease in labelled phosphatidylinositol labelled *in vivo* with [^3H]inositol reflects a fall in tissue phosphatidylinositol content. The carbachol-induced increase in [^{32}P]P_i labelling of phosphatidic acid observed within 1 min is deemed a consequence of turnover of the polar head group and not of synthesis *de novo*. Evidence for this derives from the finding that acini pre-incubated with [^{14}C]glycerol for 15 min failed to show significant stimulation of label incorporation during the first 15 min after the addition of carbachol (results not shown). Thus, cholinergic stimulation promotes both the breakdown and reformation of phosphatidylinositol in the exocrine pancreas.

Experiments utilizing [^{32}P]phosphate revealed a 2–5 min lag time for the radiolabel to appear in phosphatidylinositol as the result of its resynthesis from phosphatidic acid. This is of crucial importance in the interpretation of our findings because phosphatidic acid avidly incorporates arachidonic acid in acini, presumably by a phosphatidic acid-specific deacylation–reacylation cycle that has previously been described in equine neutrophils and platelets (Lapetina *et al.*, 1980; Billah *et al.*, 1981). However, the prompt enhancement of arachidonate incorporation into phosphatidylinositol elicited by carbachol with no discernible lag phase when the acini were pre-exposed to [^{14}C]arachidonic acid must be ascribed to a direct activation of the deacylation–reacylation cycle utilizing phosphatidylinositol as the substrate, rather than by way of phosphatidate as an intermediate. Moreover, the fact that the carbachol-induced stimulation of arachidonic acid incorporation into phosphatidic acid did not precede that of phosphatidylinositol rules out the possibility that arachidonic acid release occurred by way of a phospholipase C diacylglycerol lipase pathway (Bell *et al.*, 1979), or a phosphatidic acid-specific phospholipase A₂ (Billah *et al.*, 1981).

Caerulein is a secretagogue that utilizes the same Ca^{2+} -dependent pathway as does carbachol (Schulz & Stolze, 1980). The ability of this peptide to

promote the release of arachidonic acid from prelabelled phosphatidylinositol provides another piece of evidence for the turnover of arachidonic acid during secretory activity and complements similar findings obtained in the mouse pancreas (Marshall *et al.*, 1981). A primary as well as a zymogen-derived form of phospholipase A_2 exists in the rat pancreas (Brockerhoff & Jensen, 1974; Durand *et al.*, 1980; S.P. Halenda, S.G. Laychock & R. P. Rubin, unpublished work), and the activation of pancreatic phospholipase A_2 with the resulting release of non-esterified arachidonate from phosphatidylinositol is in all probability responsible for promoting arachidonic acid metabolism. The ability of receptor agonists to stimulate turnover of the acyl chain in position *sn*-2 is deemed a selective effect; for the finding that carbachol did not enhance the incorporation into phosphatidylinositol of the saturated fatty acid stearic acid, which is incorporated preferentially into position *sn*-1 (Hokin-Neaverson, 1977), is consistent with the proposition that carbachol does not stimulate phospholipase A_1 .

Evidence that carbachol-induced turnover of (arachidonyl) phosphatidylinositol is not completely suppressed by Ca^{2+} deprivation is in harmony with previous results obtained by using rat pancreatic fragments (Calderon *et al.*, 1979). On the other hand, the secretagogue-induced incorporation of arachidonic acid into phosphatidylinositol that is operative in feline adrenocortical cells and rabbit neutrophils is completely abolished by Ca^{2+} deprivation (Rubin, 1982). Non-lysosomal phospholipase A_2 characteristically requires Ca^{2+} as an obligatory cofactor (Brockerhoff & Jensen, 1974; Van den Bosch, 1980). Hence, the demonstration in the present study of only a partial Ca^{2+} requirement may be ascribed to the ability of carbachol to utilize a cellular pool of Ca^{2+} to stimulate phospholipase A_2 , just as this secretagogue is capable of mobilizing a cellular pool of Ca^{2+} as well as increasing Ca^{2+} influx during stimulation of amylase secretion (Schulz, 1980). Similarly, the refractoriness to Ca^{2+} deprivation of stimulus-induced phospholipase A_2 activation in blood platelets has been attributed to a mobilization of cellular Ca^{2+} (Pickett *et al.*, 1977; Van den Bosch, 1980).

The importance of secretagogue-induced mobilization of Ca^{2+} to trigger this deacylation-reacylation reaction is substantiated by the ability of the Ca^{2+} ionophore ionomycin to enhance arachidonate incorporation into phosphatidylinositol. In a similar fashion, ionophore A23187 stimulates (arachidonyl) phosphatidylinositol turnover in adrenocortical cells and neutrophils (Rubin, 1982). So, emerging from our investigations is the realization that phospholipid acylation reactions constitute a characteristic feature associated with altered Ca^{2+} handling in secretory cells.

Additionally, Farese *et al.* (1980) previously demonstrated that relatively high concentrations of ionophore A23187 mimicked the muscarinic-receptor mediated phosphatidylinositol breakdown in rat pancreatic fragments. Our data using isolated acini reveal that concentrations of ionomycin that elicited greater secretory responses than carbachol were less effective in stimulating the turnover of inositol phosphate and elevating phosphatidate levels. Thus, a complex series of co-ordinated events triggered by receptor activation may be required to act in concert with Ca^{2+} to optimally stimulate phospholipase C (Berridge, 1981). This may explain the relative ineffectiveness of ionomycin in stimulating phospholipase C relative to carbachol.

Other investigators have reported that Ca^{2+} -selective ionophores are less effective in stimulating formation of phosphatidic acid and turnover of the polar head group than receptor agonists, but are as effective in stimulating phospholipase A_2 and arachidonic acid metabolite formation (Cockcroft *et al.*, 1981; Rittenhouse-Simmons, 1981; Lapetina, 1982). This suggests that, unlike phospholipase C, phospholipase A_2 is adequately stimulated by the simple mobilization of cellular Ca^{2+} . However, the relative non-selectivity of ionomycin in altering arachidonate incorporation into phospholipids suggests that receptor agonists may mobilize Ca^{2+} from specific compartments rather than as in the case of ionomycin, by causing a generalized increase in intracellular ionic Ca^{2+} .

The function of phospholipase C still remains elusive. It does not appear to be directly linked to the discharge of secretory product, since ionomycin, although only a relatively weak stimulant of the phospholipase C-mediated reaction, is a potent pancreatic secretagogue. By contrast, the time course of ionomycin-induced amylase release paralleled the kinetics of (arachidonyl) phosphatidylinositol turnover. Moreover, ionomycin is a potent activator of the deacylation-reacylation sequence and stimulates both the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism in the exocrine pancreas (Rubin *et al.*, 1982); this suggests a close association between Ca^{2+} metabolism, arachidonic acid metabolism and secretory activity.

In conclusion, this investigation has confirmed that phosphatidylinositol occupies a pivotal position in the events associated with stimulated phospholipid metabolism of the exocrine pancreas by demonstrating that during secretory activity, two separate pathways co-exist for phosphatidylinositol degradation, involving phospholipase A_2 and C. Additionally, the incorporation of arachidonate into phosphatidylinositol, as described herein, provides indirect evidence for the existence in the rat pancreas of an acyltransferase that transfers a CoA-activated

fatty acid to the lysophosphatidylinositol formed by the phospholipase-activated deacylation of phosphatidylinositol. In this regard, Keenan & Hokin (1964) found that the membrane fractions from pigeon pancreas are capable of acylating lysophosphatidylinositol with CoA-activated unsaturated fatty acid. This rapid reacylation may protect the acinar cell from the cytotoxic action of the lysophosphatidylinositol and/or represent a mechanism for determining membrane function by regulating the fatty acid components of membrane phospholipids (Holub & Kuksis, 1978). The observations of this study, coupled with those of previous investigations, indicate that the receptor mechanism regulating arachidonic acid turnover and the phosphatidylinositol cycle in the exocrine pancreas are closely linked. Elucidation of the nature of the interaction between phospholipases A₂ and C and their specific physiological roles should shed light on the molecular events associated with Ca²⁺-dependent secretion.

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