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# Secretagogue-induced formation of inositol phosphates in rat exocrine pancreas

## Implications for a messenger role for inositol trisphosphate

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The formation of inositol phosphates in response to secretagogues was studied in rat pancreatic acini preincubated with [$^{3}$H]inositol. Carbachol caused rapid increases in radioactive inositol phosphate, inositol bisphosphate and inositol trisphosphate. This effect was blocked by atropine, and also elicited by caerulein, but not by ionomycin or phorbol dibutyrate. Thus phospholipase C-mediated breakdown of polyphosphoinositides, with the resulting formation of inositol phosphates, may be an early step in the stimulus–secretion coupling pathway in exocrine pancreas. Inositol trisphosphate may function as a second messenger in the exocrine pancreas, coupling receptor activation to internal $Ca^{2+}$ release.

Encompassing the cellular events associated with $Ca^{2+}$-dependent exocytotic secretion is the turnover of membrane phospholipids. Attempts to correlate changes in specific phospholipids with functional activity in secretory glands have dealt mainly with PtdIns (Laychock & Putney, 1982). In the exocrine pancreas, two separate pathways for stimulated PtdIns degradation appear to exist, in that secretagogues induce the release of arachidonic acid from PtdIns (Marshall *et al.*, 1980; Halenda & Rubin, 1982), as well as the breakdown and resynthesis of PtdIns by way of diacylglycerol formation and subsequent conversion of diacylglycerol into phosphatidic acid (PtdIns cycle) (Hokin, 1968; Hokin-Neaverson, 1977; Halenda & Rubin, 1982).

The phosphorylated derivatives of PtdIns [PtdIns(4,5)$P_2$ and PtdIns4$P$], generally termed the polyphosphoinositides, are also degraded during stimulation of the exocrine pancreas, with a more rapid time course than for PtdIns breakdown (Putney *et al.*, 1983). The pathway involved in this hydrolysis in fly salivary glands appears to be via a phosphodiesterase that acts directly on PtdIns(4,5)$P_2$ to form diacylglycerol and Ins$P_3$. The latter is then degraded sequentially to Ins$P_2$, Ins1$P$ and *myo*-inositol (Berridge, 1983). Berridge (1983) has suggested that Ins$P_2$ or Ins$P_3$ may function as messenger(s) to transmit the signal expressed by surface receptors to the interior of the cell. Consistent with this concept is the finding that Ins(1,4,5)$P_3$ in micromolar concentrations releases $Ca^{2+}$ from a non-mitochondrial store in permeabilized pancreatic acinar cells (Streb *et al.*, 1983). However, the formation of Ins$P_3$ or other inositol phosphates in exocrine pancreas has not been demonstrated. The present paper reveals that inositol phospholipid breakdown produced by stimulation of pancreatic acini brings about the formation of inositol phosphates, which coincides temporally with the secretory response and could thus function to signal internal $Ca^{2+}$ release in this tissue.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4$P$, phosphatidylinositol 4-phosphate; PtdIns(4,5)$P_2$, phosphatidylinositol 4,5-bisphosphate; Ins1$P$, inositol 1-phosphate; Ins(1,4)$P_2$, inositol 1,4-bisphosphate; Ins(1,4,5)$P_3$, inositol 1,4,5-trisphosphate; Ins$P_2$, inositol bisphosphate; Ins$P_3$, inositol trisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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## Materials and methods

*myo*-[2-$^{3}$H($n$)]Inositol (sp. radioactivity 16 Ci/mmol) was purchased from New England Nuclear, Boston, MA, 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 (fatty acid free), carbamoylcholine chloride (carbachol). Collagenase (chro-

matographically purified; 402units/mg dry wt.) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Caerulein was acquired from Peninsula Laboratories, San Carlos, CA, U.S.A. The calcium ionophore ionomycin was a gift from the Squibb Institute for Medical Research, Princeton, NJ, U.S.A.

Dispersed pancreatic acini were prepared from a single rat as described previously for preparing acini from guinea-pig pancreas (Peikin *et al.*, 1978). Acini were then washed and preincubated for 30min in bicarbonate-free Krebs–Hensleit medium containing 0.1mg of trypsin inhibitor, 10mM-glucose, 4% (w/v) bovine serum albumin, 6mM-$\beta$-hydroxybutyrate and 5mM-Hepes. All solutions were gassed with 100% $O_2$, and all experiments were performed at 37°C and pH7.4. To measure formation of radiolabelled inositol phosphates, acini were incubated in 3ml of medium containing 1% bovine serum albumin plus 18$\mu$Ci of [$^3$H]inositol. Tolbert *et al.* (1980) reported that $Mn^{2+}$ markedly increases [$^3$H]inositol incorporation into PtdIns of rat hepatocytes. In preliminary experiments with pancreatic acini, 1mM-$Mn^{2+}$ enhanced label incorporation into Ins$P_2$ and Ins$P_3$ by 2.4 ($\pm$0.3)- and 2.0 ($\pm$0.3)-fold respectively ($n = 5$). Therefore 1mM-$Mn^{2+}$ was included in the incubation medium during the labelling procedure.

The acini were centrifuged (600*g* for 10s), and the supernatant containing the unincorporated inositol was removed. The acini were then resuspended in 3ml of medium without bovine serum albumin plus 10mM-LiCl. $Li^+$ inhibits the enzyme that converts *myo*-inositol 1-phosphate into *myo*-inositol, and therefore would lead to an accumulation of [$^3$H]Ins1*P* (see Berridge *et al.*, 1982). Indeed, in preliminary experiments, $Li^+$ not only enhanced the amount of [$^3$H]Ins*P* by an average of 2.7-fold, but also increased [$^3$H]Ins$P_2$ and [$^3$H]-Ins$P_3$ by 1.7- and 3.0-fold respectively ($n = 3$). Therefore, to maximize responses, all experiments were performed in the presence of $Li^+$. Samples (300$\mu$l) were taken at various time intervals, and the reaction was stopped by adding 1.2ml of ice-cold 4.5% (v/v) $HClO_4$. The samples were cooled for 10–20min on ice, and then an additional 300$\mu$l of medium was added and the samples were centrifuged at 2000*g* for 5min. A 1.6ml sample of the supernatant was then neutralized with a sufficient volume of 0.5M-KOH/9mM-$Na_2B_4O_7$/1.9mM-EDTA/3.8mM-NaOH to give pH8–9. [$^3$H]-Inositol-labelled water-soluble compounds were analysed by anion-exchange chromatography on Dowex-1 (X8; formate form) columns as described previously (Berridge *et al.*, 1983). Since this technique does not distinguish between various possible isomers of the inositol phosphates, the fractions are designated as Ins1*P*, Ins$P_2$ and Ins$P_3$ without reference to the position of the phosphate groups on the inositol ring. Radioactivity was determined by scintillation counting, by using 8ml of Beckman Ready Solv EP plus 50$\mu$l of concentrated acetic acid.

To measure [$^3$H]inositol radioactivity in the phospholipids, 200$\mu$l of acini was mixed with 400$\mu$l of medium before the addition of drugs; 2.25ml of chloroform/methanol/6M-HCl (40:80:1, by vol.) was then added, and the phospholipids were extracted for 30min at room temperature. Chloroform (750$\mu$l) and 2M-KCl (750$\mu$l) were then added, and two phases were obtained by centrifugation for 5min at 2000*g*. The lower phase was removed, dried under a stream of $N_2$, and counted for radioactivity by liquid-scintillation spectrometry. Amylase content of medium and acinar lysate was determined as previously described (Halenda & Rubin, 1982). All values are presented as means$\pm$S.E.M. Statistical evaluation of the data was by paired Student's *t* test.

## Results

In a previous study, carbachol induced a rapid decrease in $^{32}$P label in PtdIns(4,5)$P_2$ in pancreatic acini (Putney *et al.*, 1983). The present study supports the previous contention (Putney *et al.*, 1983) that a phosphodiesterase is the primary enzymic mechanism for this receptor-mediated effect by demonstrating a rapid increase in [$^3$H]inositol phosphates, specifically Ins$P_3$, in carbachol-treated acini. After 1min, 8.2 ($\pm$1.8)- and 6.8 ($\pm$0.3)-fold increases were observed in the [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ fractions respectively ($P<0.05$). Fig. 1(*a*) shows the nearly linear time course of [$^3$H]Ins1*P* formation after the addition of carbachol. There were also substantial increases in [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ with time, and after 30min [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ were increased by 9.2 ($\pm$1.2)- and 17 ($\pm$5)-fold respectively (Figs. 1*b* and 1*c*). At 1 and 2min, carbachol elicited relatively small changes in the contents of [$^3$H]Ins1*P* [1.4 ($\pm$0.3)- and 1.6 ($\pm$0.3)-fold respectively] ($P>0.05$). After 30min, carbachol induced a 5.2 ($\pm$0.8)-fold increase in [$^3$H]Ins1*P* (Fig. 1*a*). All of the changes induced by carbachol were inhibited by atropine (Table 1). In unstimulated acini, the contents of [$^3$H]inositol phosphates remained very low over the 30min incubation period (Fig. 1).

The effects of other pancreatic secretagogues on the formation of water-soluble inositol phosphates were also determined for comparison with the actions of carbachol. Peptidergic stimuli, like carbachol, are $Ca^{2+}$-mobilizing secretagogues that also cause breakdown of PtdIns and polyphosphoinositides (Hokin-Neaverson, 1977; Putney *et al.*, 1983). After a 30min exposure to caerulein,

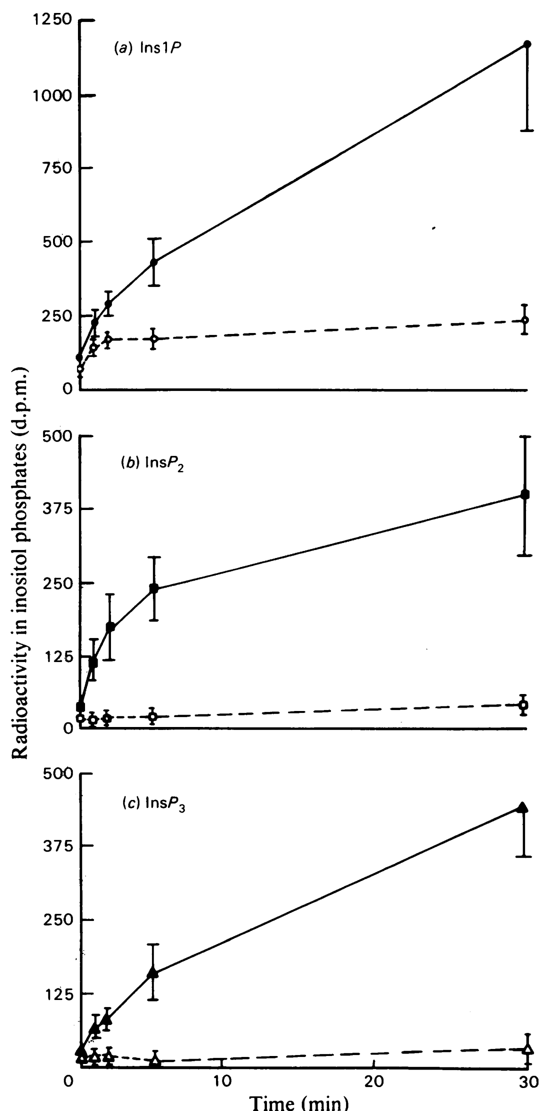


Fig. 1. *Time course of carbachol-stimulated* [$^3$H]*inositol phosphate formation*

[$^3$H]Inositol-labelled pancreatic acini were incubated with (——) or without (----) 10$\mu$M-carbachol. The incubations were stopped at various time intervals and analysed for radiolabelled (*a*) Ins1*P*, (*b*) Ins$P_2$ and (*c*) Ins$P_3$ as described in the Materials and methods section. Results are means ± S.E.M. for four or five different preparations.

there were large increases in Ins1*P*, Ins$P_2$ and Ins$P_3$. The biggest change was found in the Ins$P_3$ fraction, which increased 17-fold (Table 1).

In previous studies, ionomycin was a potent stimulus for amylase secretion, although its effects on inducing the breakdown of PtdIns or polyphosphoinositides did not mimic those of carbachol (Halenda & Rubin, 1982; Putney *et al.*, 1983). In the present study, ionomycin elicited a modest but significant increase in Ins1*P* (2.5-fold) and Ins$P_2$ (5-fold), but failed to alter significantly the content of Ins$P_3$ (Table 1). This finding lends support to our previous contention (Putney *et al.*, 1983) that ionomycin-induced polyphosphoinositide breakdown occurs by a mechanism other than that mediated by phospholipase C. Phorbol dibutyrate failed to elicit any significant alteration in the amount of Ins1*P*, Ins$P_2$ or Ins$P_3$ (Table 1), although amylase secretion increased from 2.7 (±1.6)% at 5 min to 18 (±4)% of total at 30 min (stimulated minus basal) ($n = 6$). Ionomycin and phorbol esters are both secretagogues that bypass surface receptor mechanisms: ionomycin by direct ionophoric transport of $Ca^{2+}$ (Kauffman *et al.*, 1980) and phorbol esters by direct activation of $Ca^{2+}$–phospholipid-dependent protein kinase (protein kinase C) (Castagna *et al.*, 1982; Kraft & Anderson, 1983). The inability of either agent to mimic the effects of carbachol on inositol phosphate formation supports the hypothesis that the latter is a direct consequence of receptor activation and represents an early step in the stimulus–response coupling pathway.

## Discussion

In exocrine pancreas two separate pathways coexist for PtdIns degradation, involving phospholipases $A_2$ and C (Halenda & Rubin, 1982). Although the receptor mechanisms regulating arachidonic acid turnover and the PtdIns cycle are closely linked (Halenda & Rubin, 1982), the breakdown of PtdIns and the polyphosphoinositides is an early receptor-coupled event in the exocrine pancreas which, in contrast with the release of arachidonic acid, is not dependent on agonist-induced $Ca^{2+}$ release from cellular stores (Putney *et al.*, 1983).

The present study provides deeper insight into the role of phosphoinositide turnover in the secretory process by demonstrating that the pancreatic secretagogue carbachol causes a rapid and striking increase in the formation of inositol phosphates. Since the breakdown of inositol phospholipids and (presumably) the resulting formation of inositol phosphates is not dependent on $Ca^{2+}$ mobilization, this biochemical sequence, expressed through muscarinic receptors, may be a progenitor of the common $Ca^{2+}$ signal culminating in enhanced secretion. Indeed, the time course of Ins$P_3$ formation coincided with the time course of secretagogue-induced amylase secretion (Halenda & Rubin, 1982). Moreover, Streb *et al.* (1983) have shown that Ins(1,4,5)$P_3$, but not Ins(1,4)$P_2$, promotes $Ca^{2+}$ release (as measured with a $Ca^{2+}$-

Table 1. *Effect of secretagogues on the contents of inositol phosphates in isolated rat pancreatic acini*
Equal samples of acini prelabelled with [$^3$H]inositol for 60min were incubated for 30min after various drug treatments. The incubations were stopped by adding $HClO_4$, and [$^3$H]inositol-labelled metabolites in the soluble fraction were analysed by anion-exchange chromatography as described in the Materials and methods section. Results are means ± S.E.M. obtained from three to five different preparations. *$P<0.05$ compared with control, and **$P<0.05$ compared with carbachol alone, by paired Student's *t* test.

| | Radioactivity in inositol metabolites (d.p.m.) | | |
|---|---|---|---|
| Drug additions | Ins1$P$ | Ins$P_2$ | Ins$P_3$ |
| None (control) | 70±8 | 10±3 | 8±3 |
| Carbachol (10μM) | 625±10* | 235±50* | 315±68* |
| Carbachol + atropine (10μM) | 105±10** | 23±3** | 10±3** |
| Caerulein (0.1μM) | 258±45* | 68±13* | 110±28* |
| Ionomycin (1μM) | 173±15* | 50±13* | 13±3 |
| Phorbol dibutyrate (1μM) | 88±18 | 18±5 | 10±5 |

sensitive macroelectrode) from endoplasmic reticulum of saponin-permeabilized pancreatic acini. In this context, Berridge (1983) observed a large increase in Ins$P_3$ 5s after exposing the blowfly salivary gland to 5-hydroxytryptamine, with no change in the amounts of Ins1$P$ or free inositol. These results, taken together with our own findings and those of Streb *et al.* (1983), strongly implicate Ins(1,4,5)$P_3$ as a potential second messenger linking receptor activation to $Ca^{2+}$ release from cellular stores.

The large increase in Ins$P_3$ formation observed during secretagogue-induced cellular activation supports the belief that a primary action of pancreatic secretagogues is to activate a phosphodiesterase (phospholipase C) which acts directly on PtdIns(4,5)$P_2$ to form diacylglycerol and Ins$P_3$. The comparable rapid time course of Ins$P_2$ formation supports the contention that it, too, is formed (at least in part) from its respective phospholipid precursor PtdIns4$P$, although it is likely that a portion of the Ins$P_2$ is derived from hydrolysis of Ins$P_3$. The greater latency in Ins1$P$ production suggests that it is formed from the breakdown of Ins$P_3$ and Ins$P_2$. As first suggested by Michell *et al.* (1981), PtdIns breakdown may then merely reflect a compensatory repletion of the polyphosphoinositide pool by phosphorylation of PtdIns by specific kinases. The proposal that receptor activation causes phosphodiesteratic breakdown of both polyphosphoinositides, but not of phosphatidylinositol, is similar to the conclusion drawn by Aub & Putney (1984) for the parotid gland. By contrast, the low cellular contents of inositol phosphates observed in the absence of stimuli, indicative of low basal phosphodiesterase activity, would be compatible with the low concentration of free $Ca^{2+}$ in the resting pancreatic acinar cell.

A question that necessarily arises from these findings is whether intracellular Ins$P_3$ is likely to increase on receptor activation to concentrations that Streb *et al.* (1983) found adequate to induce $Ca^{2+}$ release. An approximation to the cellular concentrations of Ins$P_3$ can be made on the basis of the radioactivity determinations, if certain assumptions are made. Since the monoester phosphates of the polyphosphoinositides turn over much more rapidly than other parts of the molecule (Michell, 1975), the specific radioactivity of polyphosphoinositides and their breakdown products, the inositol phosphates, may be similar to that of total cellular inositol lipids (largely PtdIns). Thus, from concurrent measurements of lipid $^3$H, the net increases in Ins$P_3$ during the first 1min after carbachol stimulation, during which time the maximum rate of $Ca^{2+}$ release occurs (Stolze & Schulz, 1980), is estimated to be about 0.3% of cellular PtdIns radioactivity. From the knowledge that the pancreatic PtdIns content is 11.8nmol/mg of protein (Farese *et al.*, 1982) and that the pancreatic acini contain about 4μl of intracellular water/mg of protein (Preissler & Williams, 1981), the rise in the intracellular Ins$P_3$ concentration will be about 8.8μM. $Li^+$ increased Ins$P_3$ 3-fold in stimulated cells; thus the corrected value would be about 3μM, which is sufficient to cause maximum release of intracellular $Ca^{2+}$ (Streb *et al.*, 1983). However, it must be emphasized that, if specific pools of PtdIns or polyphosphoinositides are utilized in the pancreas, this calculation could be in error. We hope that the development of more refined biochemical techniques to quantify cellular inositol phosphates will provide deeper insight into the relationships between inositol-lipid breakdown and $Ca^{2+}$ mobilization in pancreatic acinar cells.

In summary, the present study has demonstrated that the phospholipase C-mediated breakdown of the polyphosphoinositides with the resulting formation of inositol phosphates is an early step in the stimulus–secretion coupling pathway in rat exocrine pancreas. Although the existing evidence

suggests that the same receptors may be responsible for stimulating both inositol phospholipid degradation and $Ca^{2+}$ mobilization, further studies may be required to prove whether inositol phosphate production is a necessary prelude for the $Ca^{2+}$ mobilization that triggers amylase secretion.

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