Effects of secretagogues on [32 P]phosphatidylinositol 4,5-bisphosphate metabolism in the exocrine pancreas

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(Received 15 December 1982/Accepted 10 February 1983)

Experiments were carried out to assess the effects of secretagogues on the polyphosphoinositides phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] on preparations of exocrine pancreas *in vitro*. Carbachol and caerulein provoked a rapid (<1 min) breakdown of 15–20% of [32 P]PtdIns(4,5) P_2 in isolated pancreatic acini, but did not affect [32 P]PtdIns4P. In contrast, the Ca^{2+} ionophore ionomycin had no immediate effect on the levels of either inositide but caused a parallel fall in both lipids after 5–10 min. A similar decrease in [32 P]PtdIns(4,5) P_2 due to carbachol was obtained with isolated acini and isolated cells, despite the fact that the secretory response of isolated cells was considerably less than that of isolated acini. Loss of [32 P]PtdIns(4,5) P_2 elicited by carbachol or caerulein was unaffected either by the addition of EGTA in excess of extracellular Ca^{2+} or when a protocol was employed that eliminated caerulein-induced intracellular Ca^{2+} -release. These results suggest that agonist-induced PtdIns(4,5) P_2 breakdown in the exocrine pancreas may be an early step in the stimulus–response coupling pathway and also suggest that this breakdown is not dependent on Ca^{2+} -mobilization.

A number of different cell types show changes in phospholipid metabolism in response to activation of surface receptors (Michell, 1975). One specific phospholipid response involves the net loss of PtdIns, perhaps due to phospholipase C, and subsequent synthesis of phosphatidic acid. This response, measured initially as altered labelling with [32 P]P $_1$, was first described by Hokin & Hokin (1953) for the exocrine pancreas. As this reaction appears to be associated exclusively with Ca^{2+} -mobilizing receptors, but does not in general appear to be a consequence of Ca^{2+} mobilization, Michell (1975) has suggested that PtdIns breakdown may be directly coupled to receptor activation and is in some manner involved in the mechanism by which cellular Ca^{2+} is mobilized.

Recently, a number of investigators have reported effects of receptor activation on a phosphorylated derivative of PtdIns, PtdIns(4,5) P_2 (Abdel-Latif *et*

al., 1977; Kirk *et al.*, 1981; Weiss *et al.*, 1982; Billah & Lapetina, 1982). These findings have raised the question as to whether PtdIns or PtdIns(4,5) P_2 may be the lipid involved in the primary receptor-mediated effect (Kirk *et al.*, 1981; Weiss *et al.*, 1982). The purpose of the present study was to determine the nature of the effects of Ca^{2+} -mobilizing secretagogues on polyphosphoinositides in the exocrine pancreas, the tissue for which altered phosphoinositide metabolism was first described. In addition, the role of Ca^{2+} in these biochemical events was investigated. The results reveal that cholinergic or peptidergic agonists (caerulein) cause a rapid (<1 min) breakdown of PtdIns(4,5) P_2 , but not of PtdIns4P, and suggest that this effect occurs independently of Ca^{2+} mobilization.

Experimental

Rat pancreatic acini or cells were prepared and incubated by procedures described previously in detail. The method for acini was essentially that described by Williams *et al.* (1978) as modified by Halenda & Rubin (1982). The acini were incubated in a Krebs–Henseleit medium with 0.1 mg of trypsin

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate.

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inhibitor/ml and 1.2 mM-CaCl₂. Cells were prepared as described by Putney *et al.* (1980) and incubated in a medium of the following composition (mM): NaCl, 120; KCl, 5.0; MgCl₂, 1.2; CaCl₂, 1.0; sodium β -hydroxybutyrate, 5.0; Tris, 10. All experiments were carried out at 37°C, and at a pH of 7.4. For acini the gas phase was CO₂/O₂ (1:19) and for cells 100% O₂ was used. Cell preparations were >90% viable and acini were 80–90% viable in isolation [if a highly purified collagenase (Sigma type VII) was used], as assessed by Trypan Blue exclusion. On prolonged incubation, viability of the acini (but not the cells) tended to fall somewhat, but this was difficult to quantify because of the strong tendency of the acini to clump and to stick to the surface of incubation vessels (polycarbonate). This made sampling of the acini more difficult, but reasonably consistent data could be obtained if individual samples were evaluated for protein content along with other experimental parameters. The acini apparently did not lose the ability to respond to stimuli by release of amylase or ⁴⁵Ca²⁺ or breakdown of [³²P]PtdIns[4,5]P₂. By comparison, isolated cells did not clump or stick to the sides of vessels and maintained >90% viability throughout the experimental procedures. The reason for this difference in behaviour of the two preparations is not known.

In phospholipid labelling experiments, the concentration of P_i in the medium was 10 μ M. Cells or acini were incubated in medium containing 5 μ Ci of [³²P]P_i/ml for at least 60 min, after which time radioactivity associated with PtdIns4P or PtdIns(4,5)P₂ did not increase further. Agonists were added to some of the suspensions, 0.6 ml portions were removed and extracted in 2.25 ml of chloroform/methanol (1:2, v/v) plus 20 μ l of 6M-HCl. PtdIns4P and PtdIns(4,5)P₂ were purified as previously described (Weiss *et al.*, 1982). Cellular protein, precipitated during the extraction, was dissolved in 0.1M-NaOH and assayed by the method of Lowry *et al.* (1951). As there was insufficient PtdIns4P or PtdIns(4,5)P₂ for chemical determination, the extent of radioactive labelling is generally expressed as a percentage of a control value, usually just before drug additions.

Movements of ⁴⁵Ca²⁺ were measured as described by Poggioli & Putney (1982). Briefly, cells or acini were incubated in 4 μ Ci of ⁴⁵Ca²⁺/ml for 55–65 min, after which time cellular ⁴⁵Ca²⁺ appears to be in a quasi-steady state (Stolze & Schulz, 1980). Portions of the suspensions were taken at various times before and after the addition of agonists and antagonists, and diluted 10-fold in ice-cold iso-osmotic sucrose (310 mM) containing 4 mM-EGTA and tracer quantities of [³H]sucrose. The cells or acini were rapidly

Fig. 1. Effect of carbachol (10 μ M), caerulein (0.1 μ M) and ionomycin (2 μ M) on ³²P-labelled polyphosphoinositides in rat pancreatic acini

Cells were pre-incubated for 60 min in [³²P]P_i and samples taken just before ($t = 0$) and 1, 2, 5 and 10 min after the addition of agonists. (a), [³²P]PtdIns(4,5)P₂; (b), [³²P]PtdIns4P; (c), [³²P]PtdIns(4,5)P₂/[³²P]PtdIns4P radioactivity ratio. ●, Control; ○, carbachol; ▲, ionomycin; △, caerulein. Results are means for five experiments. S.E.M. values are included where practicable. The average S.E.M. values were: (a), 4.08; (b), 4.03; (c), 4.12.

centrifuged, washed with 1 ml of ice-cold unlabelled sucrose, resuspended and lysed, and the associated $^{45}\text{Ca}^{2+}$, [^3H]sucrose and protein was determined. The data are expressed either as nmol of $^{45}\text{Ca}^{2+}$ /mg of protein, calculated by using the specific radioactivity of the radioactive medium, or as a percentage of a reference value obtained just before drug additions.

The sources of radiochemicals, biochemicals and materials for preparing cells or acini were as previously given (Putney *et al.*, 1980; Halenda & Rubin, 1982), except that for preparation of acini, Sigma type VII collagenase (Sigma Chemical Co., St. Louis, MO, U.S.A.) was generally used.

Results

The effects of carbachol (10 μM), caerulein (0.1 μM) and the Ca^{2+} ionophore, ionomycin (2 μM) on [^{32}P]PtdIns(4,5) P_2 and [^{32}P]PtdIns4P in pancreatic acini are shown in Fig. 1. The PtdIns(4,5) P_2 /PtdIns4P radioactivity ratio after 60 min in [^{32}P]P_i was 1.92 ± 0.06 ($n = 20$). The effects of the secretagogues on this parameter were determined as well and are also shown in Fig. 1. Carbachol and caerulein caused a rapid and significant decrease in [^{32}P]PtdIns(4,5) P_2 , which was maximal between 1 and 2 min, but these secretagogues had no statistically significant effect on [^{32}P]PtdIns4P. Ionomycin, on the other hand, did not affect [^{32}P]PtdIns(4,5) P_2 or [^{32}P]PtdIns4P for the first 2 min after stimulation but significantly decreased both radiolabelled inositides at 5 and 10 min. When the ratio [^{32}P]PtdIns(4,5) P_2 /[^{32}P]PtdIns4P was examined, carbachol and caerulein caused a substantial decrease, whereas ionomycin had no effect.

The possible involvement of Ca^{2+} -mobilization in the agonist-induced decrease in [^{32}P]PtdIns(4,5) P_2 was examined by comparing the effects of agonists on $^{45}\text{Ca}^{2+}$ and [^{32}P]PtdIns(4,5) P_2 under different experimental conditions. Fig. 2 duplicates an experiment first described by Stolze & Schulz (1980) wherein the sequential effects of a cholinergic stimulus, cholinergic blocker and peptide stimulus on cellular $^{45}\text{Ca}^{2+}$ content are examined. Carbachol stimulated $^{45}\text{Ca}^{2+}$ efflux from $^{45}\text{Ca}^{2+}$ -labelled acini; subsequent addition of atropine caused re-uptake of $^{45}\text{Ca}^{2+}$, and caerulein was then able to evoke additional $^{45}\text{Ca}^{2+}$ loss. If, however, EGTA in excess of Ca^{2+} was added to the medium before carbachol, only the first stimulus caused an accelerated $^{45}\text{Ca}^{2+}$ efflux. The interpretation of this result is that the addition of atropine permits reloading of the receptor-regulated Ca^{2+} pool from the extracellular space (Stolze & Schulz, 1980) and that this reloading is prevented by chelation of external Ca^{2+} .

Acini whose lipids were prelabelled with [^{32}P]P_i for 1 h were subjected to the same sequence of

Fig. 2. Effect of carbachol (10 μM), atropine (10 μM) and caerulein (0.1 μM) on $^{45}\text{Ca}^{2+}$ content of pancreatic acini

Two batches of acini were incubated in Krebs-Henseleit medium containing $^{45}\text{Ca}^{2+}$ for 60 min, at which time 3 mM-EGTA (in excess of 1.2 mM- Ca^{2+}) was added to one batch (O). To both batches, carbachol (carb) was added at 65 min, atropine (atrop) at 70 min and caerulein (caer) at 75 min. Portions were taken at various times and cell-associated $^{45}\text{Ca}^{2+}$ was determined. Results are means $\pm$ S.E.M. for six experiments.

agents, and the relative effects of carbachol and caerulein on [^{32}P]PtdIns(4,5) P_2 were determined. Parallel controls were incubated for the same periods but no drugs were added. The data were subjected to two-way analysis of variance, which generates *F*-statistics (variance ratios) for the effect of Ca^{2+} , the effect of carbachol or caerulein and the interaction of the two parameters. The values obtained (Table 1) revealed no significant effect of Ca^{2+} , whereas both carbachol and caerulein caused significant changes. Further, the insignificant *F*-statistics for interaction indicate that the effects of agonists were not affected by the absence of Ca^{2+} . In other words, the results of these experiments show that carbachol (first stimulus) caused an equivalent loss of [^{32}P]PtdIns(4,5) P_2 with or without chelation of external Ca^{2+} . Likewise, the ability of caerulein (second stimulus) to cause [^{32}P]PtdIns(4,5) P_2 loss was not affected by excess EGTA, despite the demonstration in Fig. 2 that intracellular Ca^{2+} -mobilization was ablated by this procedure.

The effects of carbachol on [^{32}P]PtdIns(4,5) P_2 and $^{45}\text{Ca}^{2+}$ in dispersed cells were also determined for comparison with the results obtained with acini. These data are summarized in Table 2. In cells, carbachol caused a significant loss of [^{32}P]PtdIns(4,5) P_2 and $^{45}\text{Ca}^{2+}$. The magnitudes of these effects were similar to those obtained with acini.

Table 1. *Effect of carbachol (10 μ M) and caerulein (0.1 μ M) on 32 P-labelled PtdIns(4,5) P_2 in pancreatic acini*

The protocol for these experiments was identical with that described in the legend to Fig. 2. Cells were incubated in [32 P] P_i -containing medium for 65 min, at which time a sample was taken, and 10 μ M-carbachol was added. At 67 min, a second sample was taken. Atropine (10 μ M) was added at 70 min, and at 75 min a sample was taken and caerulein (0.1 μ M) was added. At 77 min a sample was taken. The radioactivity associated with PtdIns(4,5) P_2 was determined and the value obtained 2 min after carbachol or caerulein addition was expressed as a percentage of the value just before each addition. For some of the experiments 3 mM-EGTA was added 5 min before carbachol. Values are means $\pm$ S.E.M. for the numbers of replications shown in parentheses. Abbreviation used: N.S., not significant. For *F*-statistics, the 'calcium' value is for the effect of removal of Ca^{2+} (i.e. +EGTA) and the 'carbachol' and 'caerulein' values are for the effects of the respective secretagogue; the 'Interaction' value is for the interaction of the two effects.

		Radioactivity %	
		Normal Ca^{2+}	EGTA
First stimulus			
Control		96.1 $\pm$ 3.8 (6)	93.6 $\pm$ 2.6 (7)
Carbachol		87.3 $\pm$ 2.7 (10)	86.1 $\pm$ 4.2 (7)
<i>F</i> -Statistics			
Calcium	0.05 (N.S.)		
Carbachol	5.59 ($P < 0.05$)		
Interaction	0.27 (N.S.)		
Second stimulus			
Control		99.9 $\pm$ 2.2 (6)	102.5 $\pm$ 2.3 (7)
Caerulein		88.1 $\pm$ 2.5 (8)	89.8 $\pm$ 4.4 (6)
<i>F</i> -Statistics			
Calcium	1.43 (N.S.)		
Caerulein	18.36 ($P < 0.01$)		
Interaction	0.87 (N.S.)		

Table 2. *Effect of carbachol (10 μ M) on [32 P]PtdIns(4,5) P_2 , $^{45}Ca^{2+}$ and amylase in pancreatic acini and cells*

For [32 P]PtdIns(4,5) P_2 and $^{45}Ca^{2+}$, cells were pre-incubated in $^{45}Ca^{2+}$ or [32 P] P_i for 55–65 min, after which time no significant further labelling of PtdIns(4,5) P_2 or cellular Ca^{2+} occurred. Samples were taken just before ($t = 0$) and 5 min after the addition of 10 μ M-carbachol or vehicle (control). Values are expressed as the radioactivity after 5 min as a percentage of the radioactivity at $t = 0$. For amylase, the percentage of total cellular enzyme secreted in 30 min in the presence or absence of 10 μ M-carbachol is given. Results are means $\pm$ S.E.M. for the numbers of replications shown in parentheses.

	Radioactivity (%)			
	Cells		Acini	
	Control	Carbachol*	Control	Carbachol*
[32 P]PtdIns(4,5) P_2	104.0 $\pm$ 7.7 (4)	84.8 $\pm$ 6.1 (4)	99.0 $\pm$ 3.2 (5)	82.8 $\pm$ 7.5 (5)
$^{45}Ca^{2+}$ †	98.8 $\pm$ 2.5 (4)	69.5 $\pm$ 1.3 (4)	92.4 $\pm$ 2.9 (6)	66.3 $\pm$ 8.8 (6)
Amylase‡	1.0 $\pm$ 0.8 (3)	3.1 $\pm$ 0.3 (3)	9.0 $\pm$ 3.0 (4)	34.0 $\pm$ 4.0 (4)

* For all three parameters, data for carbachol stimulation are significantly different from control by analysis of variance ($P < 0.05$).

† Values for $^{45}Ca^{2+}$ content after 60 min incubation in $^{45}Ca^{2+}$ were (nmol/mg of protein): cells, 1.85 ± 0.12 ($n = 4$); acini, 1.80 ± 0.24 ($n = 6$).

‡ Amylase data from Putney *et al.* (1980) (cells) and Halenda & Rubin (1982) (acini).

Also included in Table 2 are previously determined rates of amylase secretion for the two preparations. The rate of amylase secretion induced by carbachol from acini was substantially greater than that obtained from cells, in agreement with previous findings (Gardner & Jensen, 1981).

Discussion

These results show that in pancreatic acini and isolated cells, Ca^{2+} -mobilizing secretagogues cause a rapid net breakdown of radiolabelled PtdIns(4,5) P_2 , but not of PtdIns4 P . Another laboratory investigated drug effects on polyphosphoinositides in

pancreatic fragments and reported no changes in cellular content (Farese *et al.*, 1982). The reason for this discrepancy is not clear, although in the cited study the precise details of the polyphosphoinositide experiments were not given.

The role of Ca^{2+} in agonist effects on $\text{PtdIns}(4,5)\text{P}_2$ is of interest in the light of hypotheses implicating alterations in inositide metabolism in the mechanisms of receptor-mediated Ca^{2+} mobilization. Michell (1975) has noted that agonist-induced breakdown of PtdIns is always associated with receptors that use Ca^{2+} as second messenger; but in most instances, it has been found that the effects on phospholipid metabolism are not secondary to the elevation in intracellular Ca^{2+} . Rather, alterations in PtdIns metabolism appear to result more directly from receptor activation and so it has been proposed that they may precede Ca^{2+} -mobilization in the stimulus-response coupling pathway (Michell, 1975; Michell *et al.*, 1981; but see Cockcroft, 1981; Hawthorne, 1982). A number of laboratories have demonstrated that Ca^{2+} -mobilizing agonists cause $\text{PtdIns}(4,5)\text{P}_2$ breakdown (Abdel-Latif *et al.*, 1977; Kirk *et al.*, 1981; Weiss *et al.*, 1982; Billah & Lapetina, 1982). In iris smooth muscle, this reaction appears to be Ca^{2+} -dependent (Akhtar & Abdel-Latif, 1978) but for the liver, parotid gland and platelets, agonist-induced $\text{PtdIns}(4,5)\text{P}_2$ breakdown seems to be Ca^{2+} -independent (Kirk *et al.*, 1981; Weiss *et al.*, 1982; Billah & Lapetina, 1982).

The results of the present study suggest that $\text{PtdIns}(4,5)\text{P}_2$ breakdown may also be an early receptor-coupled event in the exocrine pancreas. The comparison of responses in cells and acini is important in this regard, since isolated cells generally secrete poorly in response to secretagogues in comparison with acini. Although the precise mechanism for this disparity is unknown, the receptor populations and receptor-induced Ca^{2+} release mechanisms do not apparently differ in these two preparations (Gardner & Jensen, 1981). Accordingly, the finding that the relative degree of carbachol-induced $\text{PtdIns}(4,5)\text{P}_2$ breakdown is similar in cells and acini suggests that this reaction occurs early in the stimulus-response pathway and is not related to the rate of exocytotic amylase release.

The data in Figs. 1 and 2 and Table 1 collectively suggest that agonist-induced Ca^{2+} mobilization does not trigger breakdown of $\text{PtdIns}(4,5)\text{P}_2$. In the exocrine pancreas, Ca^{2+} mobilization occurs primarily by release of Ca^{2+} from specific cellular stores (Williams, 1980; Gardner & Jensen, 1981). As demonstrated by Stolze & Schulz (1980), this Ca^{2+} release can be readily measured as net $^{45}\text{Ca}^{2+}$ loss under the experimental conditions for Fig. 2. The finding that excess EGTA does not prevent

carbachol-induced $\text{PtdIns}(4,5)\text{P}_2$ breakdown is not surprising since the data in Fig. 2 show that Ca^{2+} release still occurs under these conditions. However, with the second stimulus, caerulein, the continued presence of EGTA apparently prevents refilling of the receptor-regulated pool so that no agonist-induced Ca^{2+} release can occur. As shown in Table 1, however, caerulein is still capable of inducing a $\text{PtdIns}(4,5)\text{P}_2$ breakdown response. This finding suggests that neither the presence of extracellular Ca^{2+} , nor the release of Ca^{2+} internally is necessary for caerulein to cause $\text{PtdIns}(4,5)\text{P}_2$ breakdown.

The results obtained with the Ca^{2+} -ionophore are consistent with this interpretation. Ionomycin, for the first 2 min after addition, did not mimic the effects of carbachol or caerulein in inducing $\text{PtdIns}(4,5)\text{P}_2$ breakdown. At this concentration, ionomycin is capable of stimulating amylase release at a rate greater than or equal to $10\text{ }\mu\text{M}$ -carbachol, and the response is maximal within 2 min (Halenda & Rubin, 1982). The reason for the delayed (5–10 min) effect of ionomycin on both polyphosphoinositides is not clear, although a greater latency is also observed with ionomycin-induced stimulation of phosphatidic acid and PtdIns turnover (Halenda & Rubin, 1982). A toxic mechanism could explain the parallel depletion of $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}4\text{P}$, since the pattern is similar to the effects of antimycin in the parotid gland (Poggioli *et al.*, 1983). Furthermore, the concentration of ionomycin ($2\text{ }\mu\text{M}$) is just below the threshold for increased cell death as measured by Trypan Blue uptake (Halenda & Rubin, 1982).

In summary, these data indicate that in pancreatic acini and cells carbachol and caerulein cause a rapid breakdown of $\text{PtdIns}(4,5)\text{P}_2$ but not of $\text{PtdIns}4\text{P}$. This differential effect is not mimicked by the Ca^{2+} -ionophore, ionomycin, and occurs independently of agonist-induced Ca^{2+} -mobilization, thus suggesting that $\text{PtdIns}(4,5)\text{P}_2$ changes may occur early in the stimulus-response coupling pathway. The possible relationship of this effect to cellular Ca^{2+} -mobilization and to other previously described changes in acinar phospholipids (Hokin-Neaverson, 1977; Marshall *et al.*, 1981; Halenda & Rubin, 1982) is not presently known and should be the focus of future investigations.

This research was supported by grants from the N.I.H. (nos. AM-28029 and DE-05764). G. M. B. was supported in part by the Wellcome Trust.

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