Effects of carbachol and pancreozymin (cholecystokinin-octapeptide) on polyphosphoinositide metabolism in the rat pancreas *in vitro*

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We studied the possibility that hydrolysis of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] may be the initiating event for the increase in [^{32}P] P_i incorporation into phosphatidic acid (PtdA) and phosphatidylinositol (PtdIns) during carbachol and pancreozymin (cholecystokinin-octapeptide) action in the rat pancreas. After prelabelling acini for 2 h, [^{32}P] P_i incorporation into PtdA, $\text{PtdIns}(4,5)\text{P}_2$ and phosphatidylinositol 4-phosphate ($\text{PtdIns}4\text{P}$) had reached equilibrium. Subsequent addition of carbachol or pancreozymin caused ^{32}P in $\text{PtdIns}(4,5)\text{P}_2$ to decrease by 30–50% within 10–15 s, and this was followed by sequential increases in [^{32}P] P_i incorporation into PtdA and PtdIns. Similar changes in ^{32}P -labelling of $\text{PtdIns}4\text{P}$ were not consistently observed. Confirmation that the decrease in ^{32}P in chromatographically-purified $\text{PtdIns}(4,5)\text{P}_2$ reflected an actual decrease in this substance was provided by the fact that similar results were obtained (a) when $\text{PtdIns}(4,5)\text{P}_2$ was prelabelled with [$2\text{--}^3\text{H}$]inositol, and (b) when $\text{PtdIns}(4,5)\text{P}_2$ was measured as its specific product (glycerophosphoinositol bisphosphate) after methanolic alkaline hydrolysis and ion-exchange chromatography. The secretagogue-induced breakdown of $\text{PtdIns}(4,5)\text{P}_2$ was not inhibited by Ca^{2+} deficiency (severe enough to inhibit amylase secretion and Ca^{2+} -dependent hydrolysis of PtdIns), and ionophore A23187 treatment did not provoke $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. The increase in the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ and the increase in [^{32}P] P_i incorporation into PtdA commenced at the same concentration of carbachol in dose-response studies. Our findings suggest that the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ is an early event in the action of pancreatic secretagogues that mobilize Ca^{2+} , and it is possible that this hydrolysis may initiate the Ca^{2+} -independent labelling of PtdA and PtdIns. Ca^{2+} mobilization may follow these responses, and subsequently cause Ca^{2+} -dependent hydrolysis of PtdIns and exocytosis.

Although 30 years have elapsed since Hokin & Hokin (1953) discovered that acetylcholine stimulated [^{32}P] P_i incorporation into pancreatic PtdIns and PtdA, a physiological role for this effect and the mechanism of its induction have not been well defined. Nevertheless, it seems likely that this phospholipid effect is important for mobilization of Ca^{2+} (see Michell, 1975), and, until recently, most data had suggested that hydrolysis of PtdIns, producing diacylglycerol and InsP , was the initiating event, followed by resynthesis of PtdIns from

diacylglycerol through PtdA and CDP-diacylglycerol. However, Farese *et al.* (1982a,b) have shown that the decrease in PtdIns mass (generally taken as evidence for PtdIns hydrolysis) can be dissociated from the increase in ^{32}P -labelling of PtdA and PtdIns in the rat pancreas and submaxillary gland by changing Ca^{2+} availability (the mass change is dependent on Ca^{2+} , whereas labelling is not); these results cast doubt on the assumption that an overt decrease in PtdIns mass, and its presumed hydrolysis, is important as the initiating event for the ^{32}P -labelling effect in these tissues. Moreover, rapid agonist-induced changes in triphosphoinositide [$\text{PtdIns}(4,5)\text{P}_2$] metabolism have now been demonstrated in several tissues (see Kirk

Abbreviations used: PtdA, phosphatidic acid; PtdIns, phosphatidylinositol; InsP , inositol phosphate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-phosphate.

et al., 1981; Downes & Michell, 1982; Weiss *et al.*, 1982; Billah & Lapetina, 1982; Thomas *et al.*, 1983; Putney *et al.*, 1983; Berridge, 1983; Berridge *et al.*, 1983; Creba *et al.*, 1983), and it has been suggested that hydrolysis of PtdIns(4,5) P_2 , rather than PtdIns, may be the initiating event. We have evaluated this possibility in the rat pancreas.

Materials and methods

Non-starved Sprague-Dawley (Holtzman) male rats were killed by decapitation between 09:00h and 10:00h, and pancreata were rapidly excised and placed in ice-cold 0.9% NaCl. Pancreatic acini were prepared by the method of Williams *et al.* (1978), except that the final collagenase concentration in the digestion media was 10mg/ml, as used previously (Farese *et al.*, 1980). The dispersed acini were resuspended in regular (Ca^{2+} , 1.2mM) or Ca^{2+} -free Krebs-Henseleit medium containing 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], glucose (2mg/ml) and 0.5% bovine serum albumin as described by Williams *et al.* (1978). To study PtdIns(4,5) P_2 hydrolysis, [^{32}P]P_i (50 μ Ci/ml) or [2- 3H]inositol (20 μ Ci/ml) was added to the acinar suspension, and 0.5ml portions containing 2–4mg of protein were distributed to siliconized tubes and phospholipids were prelabelled by incubation for 2h under 100% O_2 at 37°C. Appropriate agonists or medium alone (controls) were then added for the indicated times of otherwise uninterrupted incubation at 37°C. Carbachol and pancreozymin (cholecystokinin-octapeptide) were dissolved in 0.1ml of incubation medium and ionophore A23187 in 5 μ l of ethanol (final concentration, 1%), which alone did not influence results. The incubation was terminated by rapidly adding 10ml of chloroform/methanol/HCl (800:400:3, by vol.), followed by 1.5ml of 0.1M-HCl to assure proper acidity for extraction of polyphosphoinositides. After vortex-mixing and centrifugation, the aqueous layer was removed. The organic phase was washed successively with 4ml of chloroform/methanol/0.1M-HCl (3:48:47, by vol.) and then with 4ml of chloroform/methanol/0.01M-HCl (3:48:47, by vol.). The lipid extract was neutralized, dried under N_2 , resuspended in a small volume of chloroform/methanol/water (75:25:2, by vol.), applied to t.l.c. plates coated with 0.5mm silica gel containing 10% magnesium acetate and developed unidimensionally, first with chloroform/methanol/aq. 4.3M- NH_3 (18:13:4, by vol.) and then with propan-1-ol/aq. 4.3M- NH_3 (3:2, v/v) for 4h, as described previously (Farese *et al.*, 1979). The purified phospholipids were identified by comparison with authentic standards after autoradiography and acid-charring. Spots were scraped

and counted for radioactivity in a liquid-scintillation counter. Recovery of PtdIns(4,5) P_2 through extraction and chromatography was determined by adding a known amount of standard to the incubation medium and was found to be approx. 60%. Repeated extraction of the tissue failed to improve the recovery significantly. Results were not corrected for recovery, but the s.e.m. of replicate recovery standards ranged from 2 to 8%, and recoveries of added standard were the same in control and carbachol-treated incubation mixtures. In one experiment, PtdIns(4,5) P_2 was further purified by two-dimensional chromatography: after chromatography in the first dimension using the above two solvent systems, the plates were turned through 90° and developed with butan-1-ol/acetic acid/water (15:2:5, by vol.) for 5h. The PtdIns(4,5) P_2 autoradiographic spots remained intact upon changing from the first to the second dimension, suggesting that purification was adequate after the first dimension. Moreover, the pattern of results obtained in response to carbachol (100 μ M) and pancreozymin (1 μ M) with this two-dimensional chromatography system was the same as that obtained with the unidimensional system cited above.

In some experiments, changes in PtdIns(4,5) P_2 were evaluated by methanolic alkaline hydrolysis of the lipid extract, followed by purification of the specific derivative of PtdIns(4,5) P_2 , i.e. glycerophosphoinositol bisphosphate, by ion-exchange chromatography (Dowex-1, Cl^- form), as described by Downes & Michell (1981).

In all experiments on PtdIns(4,5) P_2 hydrolysis, a control and treatment vessel were subjected to simultaneous manipulations and the data were analysed by the paired *t* test. Statistical significance was accepted if the *P* value was <0.05.

T.l.c. plates were obtained from Supelco. All chemicals and solvents were of ACS grade and were obtained from Sigma or Fisher. Pancreozymin (synthetic cholecystokinin-octapeptide) and A23187 were obtained from Calbiochem. Collagenase (type V) was obtained from Sigma. Carrier-free $H_3^{32}PO_4$ and [2- 3H]inositol (sp. radioactivity 15.8Ci/mmol) were obtained from New England Nuclear.

Results

In preliminary experiments (results not shown), it was found that ^{32}P -labelling and [3H]inositol-labelling of PtdIns(4,5) P_2 , PtdIns4P and PtdA had reached a steady-state plateau (i.e., apparent isotopic equilibrium) after 1–2h of pre-incubation, and these remained nearly constant in control samples during the period of study.

Fig. 1. Carbachol-induced changes in ^{32}P -prelabelled phospholipids of rat pancreatic acini

After prelabelling with ^{32}P for 120 min, a small volume of medium or $100\ \mu\text{M}$ -carbachol in medium were simultaneously added to control and treatment vessels, and the incubation was continued for the indicated times. The mean radioactivity counts (c.p.m./incubation vessel; $\pm$ S.E.M.; $n = 8$) found in PtdA, PtdIns4P and PtdIns(4,5) P_2 in zero-time controls were 5607 ± 645 , 4401 ± 448 and 5374 ± 577 respectively, and did not change significantly over the course of the experiment. Points represent means ($\pm$ S.E.M.) of six comparisons between treatment and control vessels. P values were determined by the paired t test, and asterisks denote a P value of <0.05 .

As shown in Fig. 1, $100\ \mu\text{M}$ -carbachol provoked a 35–50% decrease in the level of ^{32}P found in PtdIns(4,5) P_2 , and this was significant after only 10 s of incubation, the earliest time point examined. On the other hand, no significant change was detected in the level of ^{32}P in PtdIns4P, except for an insignificant decrease after 30 s. The initial decrease in ^{32}P in PtdIns(4,5) P_2 was followed sequentially by increases in labelling of PtdA, which were usually observed after 1 min of incubation. In other experiments (results not shown), it was obvious that enhanced incorporation of ^{32}P into PtdA preceded increases in incorporation of ^{32}P into PtdIns, which were regularly observed after 2 min of incubation.

A similar, but somewhat more pronounced, response pattern was observed in the ^{32}P found in PtdIns(4,5) P_2 , PtdIns4P, PtdA and PtdIns in response to $1\ \mu\text{M}$ -pancreozymin (Fig. 2); decreases in ^{32}P in PtdIns(4,5) P_2 were maximal or near maxi-

Fig. 2. Pancreozymin-induced changes in ^{32}P -prelabelled phospholipids of rat pancreatic acini

Except for employment of $1\ \mu\text{M}$ -pancreozymin (instead of carbachol), experimental conditions were as in Fig. 1. Points represent means ($\pm$ S.E.M.) of four comparisons between treatment and control vessels. Asterisks denote a P value <0.05 .

mal within 15 s, and this was followed by enhanced labelling of PtdA, which preceded the increase in labelling of PtdIns.

The requirement for Ca^{2+} in agonist-induced effects on PtdIns(4,5) P_2 labelling was investigated by examining carbachol-induced changes in Ca^{2+} -free medium containing $1\ \text{mM}$ -EGTA (prelabelling, as well as the treatment, were performed in this medium). As shown in Fig. 3, the effects of carbachol on ^{32}P -labelling of PtdIns(4,5) P_2 and PtdA were unchanged despite severe Ca^{2+} deficiency (note that this degree of Ca^{2+} deficiency completely blocks both amylase secretion and Ca^{2+} -dependent PtdIns hydrolysis; see Farese *et al.*, 1980, 1982a).

The effect of the Ca^{2+} ionophore A23187 ($20\ \mu\text{M}$, a fully effective dose to provoke amylase secretion and Ca^{2+} -dependent PtdIns hydrolysis; see Farese *et al.*, 1980, 1982a) was also examined; as shown in Fig. 4, there was no significant effect on ^{32}P -labelling of PtdIns(4,5) P_2 or PtdA.

Dose-related effects of carbachol on PtdIns(4,5) P_2 labelling were examined to determine whether the changes therein correlated with the more classical 'phospholipid effect', i.e. enhanced labelling of PtdA and PtdIns, and amylase release. As shown in Fig. 5, increases in ^{32}P incorporation into PtdA and PtdIns in acinar tissue commenced at approximately the same carbachol

Fig. 3. Effects of carbachol on PtdIns(4,5)P₂ breakdown and ³²P-labelling of PtdA in Ca²⁺-free media

The experimental conditions were identical with those in Fig. 1, except that the prelabelling and subsequent incubation with treatment were performed in Ca²⁺-free media containing 1 mM-EGTA (these conditions prevent both amylase release and the decrease in phosphatidylinositol mass; see Farese *et al.*, 1980, 1982a). Points represent means $\pm$ S.E.M. for four comparisons between treatment and control vessels. Asterisks denote a *P* value <0.05.

Fig. 4. Failure of A32187 (20 μ M) to influence PtdIns(4,5)P₂ in ³²P-prelabelled rat pancreatic acini. See Fig. 1 and the Materials and methods section for other details. Abbreviation: N.S., not significant.

Fig. 5. Dose-related effects of carbachol on amylase secretion and ³²P-labelling of PtdA and PtdIns in rat pancreatic acini

After a 30 min pre-incubation, pancreatic acini were incubated for 60 min with 25 μ Ci of [³²P]P_i in 0.5 ml of medium. After incubation, media and cells were separated by centrifugation and analysed for amylase release and phospholipids respectively. Points represent means $\pm$ S.E.M. for four determinations. Asterisks denote a *P* value <0.05.

concentration, whereas amylase release was provoked at a slightly lower carbachol concentration. Dose-related effects of carbachol on ³²P-labelling of PtdIns(4,5)P₂ (decrease) and PtdA (increase) were also found to commence at the same approximate dose of carbachol, i.e. over 1 μ M (Fig. 6).

We attempted, on several occasions, to measure the tissue concentration of PtdIns(4,5)P₂, but this was below the level of sensitivity of our assay for inorganic phosphorus, a modified Bartlett procedure (Farese *et al.*, 1979), despite the analysis of relatively large amounts (100–200 mg) of tissue.

In an experiment in which [³H]inositol was used to prelabel inositide phospholipids, we observed that 1 μ M-pancreozymin decreased the level of ³H in PtdIns(4,5)P₂ by approx. 30% [from 970 $\pm$ 69 c.p.m. (mean $\pm$ S.E.M.; *n* = 4) to 680 $\pm$ 60 c.p.m.] over a 30 s-treatment period; ³H in Ptd4P and PtdIns, on the other hand, was not significantly affected by pancreozymin. Similar results were obtained in repeat experiments.

We directly compared secretagogue-induced changes in ³²P-labelled PtdIns(4,5)P₂, as

Fig. 6. Dose-related effects of carbachol on PtdIns(4,5) P_2 breakdown and PtdA labelling with ^{32}P in rat pancreatic acini

After prelabelling for 120 min with [^{32}P] P_i , increasing amounts of carbachol were added and vessels were incubated for 90 s and compared with corresponding control vessels. Points represent means $\pm$ S.E.M. of four comparisons between treatment and control vessels. Asterisks indicate a P value < 0.05 . See Fig. 1 for other details. Abbreviation: N.S., not significant.

measured after purification of PtdIns(4,5) P_2 by unidimensional t.l.c., and after purification of glycerophosphoinositol biphosphate by ion-exchange chromatography after methanolic alkaline hydrolysis of PtdIns(4,5) P_2 in the lipid extracts. As shown in Table 1, results were nearly identical with both methods, and carbachol and pancreozymin apparently induced 40 and 50% decreases in PtdIns(4,5) P_2 , respectively. These results provide strong support for the contention that the ^{32}P -

radioactivity in the PtdIns(4,5) P_2 chromatographic area can be equated with PtdIns(4,5) P_2 itself.

Discussion

Our time-sequence experiments demonstrate a rapid decrease in radioactivity of ^{32}P -prelabelled PtdIns(4,5) P_2 , followed by an increase in ^{32}P -labelling of PtdA, which in turn is followed by an increase in ^{32}P -labelling of PtdIns, during stimulation of rat pancreatic acini by the Ca^{2+} -mobilizing secretagogues, carbachol and pancreozymin. These results are in many respects similar to those observed in rabbit iris smooth muscle (Akhtar & Abdel-Latif, 1978), rat hepatocytes (Kirk *et al.*, 1981; Thomas *et al.*, 1983; Creba *et al.*, 1983), rat parotid gland (Weiss *et al.*, 1982); platelets (Billah & Lapetina, 1982), blowfly salivary gland (Berridge, 1982, 1983; Berridge *et al.*, 1983) and rat pancreas (Putney *et al.*, 1983) during the action of various agents that mobilize Ca^{2+} . Obviously, this appears to be a common response to these agents.

Although our results are in substantial agreement with those reported by Putney *et al.* (1983) for the rat pancreas, the magnitude of the decrease in PtdIns(4,5) P_2 was greater and possibly more rapid in development in our experiments. The reason for this difference is uncertain, but may be due to variations in acinar preparations, conditions of incubation or the use of higher agonists concentrations in our experiments.

The agonist-induced decrease in ^{32}P in PtdIns(4,5) P_2 might occur by several mechanisms. First, conversion of PtdIns4P into PtdIns(4,5) P_2 could be decreased, but this seems unlikely since the ^{32}P in PtdIns4P did not increase. Secondly, conversion of PtdIns(4,5) P_2 into PtdIns4P by phosphomonoesterase could be increased, but this too is unlikely since PtdIns4P labelling did not increase. Thirdly, hydrolysis of PtdIns(4,5) P_2 by phospholipase D (see Kanfer, 1982) could theoretically be increased, but this seems unlikely since the observed increase in ^{32}P -labelling of PtdA lagged behind the decrease in ^{32}P in PtdIns(4,5) P_2 , and this suggests that the increase in PtdA-labelling must be accounted for by another mechanism (i.e., synthesis *de novo* or from diacylglycerol and ATP); furthermore, there is no evidence that inositol phospholipids are degraded by this enzyme. Fourthly, PtdIns hydrolysis might have occurred and diminished the availability of substrate for polyphosphoinositide synthesis; this also seems unlikely as we have not been able to demonstrate Ca^{2+} -independent PtdIns depletion in this tissue, and the decrease in label in PtdIns(4,5) P_2 preceded, and was more prominent than, the decrease in PtdIns4P. Finally, hydrolysis of PtdIns(4,5) P_2

Table 1. *Carbachol- and pancreozymin-induced changes in PtdIns(4,5)P₂ as measured by t.l.c. compared with those by alkaline hydrolysis and purification of glycerophosphoinositol bisphosphate (GroPInsP₂) by Dowex-1 chromatography* After prelabelling pancreatic acini for 120 min with [³²P]P_i, additions were made and incubation was continued for 90 s. After incubation, proteins and lipids were precipitated with ice-cold 5% trichloroacetic acid, and the lipids were extracted as described in the Materials and methods section. Portions of the lipid extracts were subjected to analysis by unidimensional t.l.c., and by alkaline methanolic hydrolysis followed by purification of glycerophosphoinositol bisphosphate by ion-exchange chromatography (Dowex-1, Cl⁻ form). Values are means ± S.E.M. for four determinations. Values in parentheses indicate the percentage changes compared with controls.

Treatment	Radioactivity in PtdIns(4,5)P ₂ /incubation vessel (c.p.m.)	
	T.l.c.	GroPInsP ₂ method
None (control)	5534 ± 229	5739 ± 432
Carbachol (100 μM)	3272 ± 158 (−39%)	3351 ± 124 (−42%)
Pancreozymin (1 μM)	2630 ± 122 (−53%)	2752 ± 60 (−52%)

by phospholipase C to diacylglycerol and inositol triphosphate may have been increased, and this seems most likely in view of the probable increases in the latter substance, as observed during agonist action in other tissues (Berridge, 1982), and, more recently, by Farese and co-workers (R. V. Farese, J. S. Davis & R. E. Larson, unpublished work) in the rat pancreas.

The fact that the decrease in ³²P in PtdIns(4,5)P₂ commenced at approximately the same (relatively high) doses of carbachol as the classical 'phospholipid effect', i.e. enhanced labelling of PtdA and PtdIns, and the fact that all of these changes are independent of Ca²⁺, suggests that these changes in PtdIns(4,5)P₂, PtdA and PtdIns are closely related (in terms of receptor occupancy; see below) events in the metabolic responses to carbachol and pancreozymin. This correlation contrasts with what we have observed in other dose-response experiments (J. L. Orchard, J. S. Davis, R. E. Larson & R. V. Farese, unpublished work) in which we measured changes in PtdIns mass during carbachol action: the carbachol-induced decrease in PtdIns mass was observed to commence at considerably lower doses of carbachol than that required for ³²P-labelling of PtdA and PtdIns. Thus, the Ca²⁺-dependent PtdIns mass effect is readily separable from the PtdIns(4,5)P₂ breakdown and PtdA/PtdIns labelling effects; furthermore, it seems clear that the PtdIns mass effect is provoked by lesser degrees of receptor occupancy than the PtdIns(4,5)P₂ breakdown and PtdA/PtdIns labelling effects.

The presently observed timing of changes in ³²P in PtdIns(4,5)P₂, PtdA and PtdIns is compatible with the following sequence: PtdIns(4,5)P₂ hydrolysis → diacylglycerol generation (presumed) → PtdA synthesis (and labelling) → PtdIns synthesis (and labelling). Since there are progressive increases in ³²P in PtdA and PtdIns, the above sequence, if operative, must be continuous, with

PtdIns(4,5)P₂ being continually replenished via PtdIns4P and ultimately PtdIns (the only known pathway). In this scheme, it may be noted that PtdIns hydrolysis *per se* may not be essential to this initial response, but obviously may occur simultaneously or as a later secondary event; in addition, decreases in PtdIns may be due to its conversion into PtdIns(4,5)P₂ (although this cannot account for the Ca²⁺-dependent decrease in PtdIns observed in the rat pancreas; Farese *et al.*, 1980, 1982a). Other possibilities are that PtdIns(4,5)P₂ hydrolysis may trigger PtdA/PtdIns labelling via a Ca²⁺-independent activation of PtdIns-specific (or other phospholipid-specific) phospholipase C, or that hydrolysis of PtdIns(4,5)P₂ and PtdIns (and subsequent PtdA/PtdIns labelling) are triggered by a common factor in an otherwise unrelated manner. In view of the fact that PtdIns(4,5)P₂ hydrolysis and PtdA/PtdIns labelling are independent of Ca²⁺, and in view of the failure of A23187 to provoke similar effects, it seems unlikely that the 'common factor' is Ca²⁺. Although further studies are needed to be certain of the sequence of metabolic events, our findings are compatible with the possibility that activated receptors directly provoke PtdIns(4,5)P₂ hydrolysis and consequent labelling of PtdA and PtdIns, and this is followed by Ca²⁺ mobilization, and subsequently by Ca²⁺-dependent PtdIns hydrolysis (Farese *et al.*, 1980, 1982a,b) and exocytosis.

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