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Receptor-mediated net breakdown of phosphatidylinositol 4,5-bisphosphate in parotid acinar cells

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The metabolism of phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] in rat parotid acinar cells was investigated, particularly with regard to the effects of receptor-active agonists. Stimulation of cholinergic-muscarinic receptors with methacholine provoked a rapid disappearance of 40–50% of [^{32}P]PtdIns(4,5)P_2, but had no effect on PtdIns4*P*. Adrenaline, acting on α-adrenoceptors, and Substance P also stimulated net loss of PtdIns(4,5)P_2. The β-adrenoceptor agonist, isoprenaline, and the Ca^{2+} ionophore, ionomycin, failed to affect labelled PtdIns(4,5)P_2 or PtdIns4*P*. By chelation of extracellular Ca^{2+} with excess EGTA, and by an experimental protocol that eliminates cellular Ca^{2+} release, it was demonstrated that the agonist-induced decrease in PtdIns(4,5)P_2 is independent of both Ca^{2+} influx and Ca^{2+} release. These results may suggest that net PtdIns(4,5)P_2 breakdown is an early event in the stimulus–response pathway of the parotid acinar cell and could be directly involved in the mechanism of agonist-induced Ca^{2+} release from the plasma membrane.

Much of the current interest in phosphoinositide metabolism centres around a hypothesis offered by Michell (1975) suggesting that phospholipase C-mediated breakdown of PtdIns may be involved in the mechanism by which receptor activation is coupled to Ca^{2+}-mobilization (Michell, 1979; Berridge, 1980; Putney, 1981). The evidence for this idea stems largely from the striking (and apparently exclusive) association of enhanced PtdIns breakdown with receptors that act by mobilizing Ca^{2+}, together with the demonstration that receptor stimulation of PtdIns breakdown is itself apparently not a Ca^{2+}-mediated event. The latter point derives from the experimental findings that phospholipase C-mediated PtdIns breakdown is minimally (or not at all) inhibited by removal of extracellular Ca^{2+}, and is not activated by Ca^{2+}-ionophores (there are exceptions; Cockroft *et al.*, 1980*a*,*b*; Fisher & Agranoff, 1980).

In addition to the breakdown of PtdIns, in some systems it has been demonstrated that phosphorylated derivatives of PtdIns, the polyphosphoinositides, are also broken down in response to receptor activation (Abdel-Latif *et al.*, 1977). This effect, however, has been shown to be Ca^{2+}-dependent and mimicked by Ca^{2+}-ionophores (Akhtar & Abdel-Latif, 1978), leading to the suggestion that polyphosphoinositide breakdown is probably not involved in the mechanism of Ca^{2+}-mobilization but rather may be involved in the mechanism(s) by which the cellular responses to Ca^{2+} are expressed (Akhtar & Abdel-Latif, 1978; Putney, 1978).

In a recent study with hepatocytes, Kirk *et al.* (1981) examined the effects of vasopressin, a Ca^{2+}-mobilizing hormone, on polyphosphoinositide metabolism. These investigators found that vasopressin caused a rapid breakdown of the polyphosphoinositides; the effect was only partially inhibited by Ca^{2+} omission. Additionally, the Ca^{2+}-ionophore A23187 did not activate polyphosphoinositide breakdown. Kirk *et al.* (1981) suggest that polyphosphoinositide breakdown may be an early event in hepatocyte activation, perhaps preceding PtdIns breakdown.

In the present study we have examined the effects of agonists on the ^{32}P-labelled polyphosphoinositides, PtdIns4*P* and PtdIns(4,5)P_2 in the rat parotid acinar cell. The results show that in the

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns-(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

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parotid gland, net PtdIns(4,5)P_2 breakdown occurs, is a Ca^{2+}-independent response to receptor activation and could therefore play some role in the mechanism of Ca^{2+}-mobilization.

Experimental

Parotid glands were removed from anaesthetized (sodium pentobarbitone) male Sprague–Dawley rats (140–200 g) and dispersed acinar cells were prepared as previously described (Putney *et al.*, 1978). The cells were suspended at a concentration of 1–2 mg of protein/ml in a Ringer medium of the following composition (mM); NaCl, 120; KCl, 5.0; $MgCl_2$, 1.2; $CaCl_2$, 1.0; Tris, 20; sodium β-hydroxybutyrate, 5.0; and 0.5% bovine serum albumin. The media were titrated with HCl (about 10 mM final Cl^- concentration) to a pH of 7.4 at 37°C. The gas phase was 100% O_2.

Cells were equilibrated with the above medium for 20 min, at which time 10 μM-[^{32}P]P_i was added as the neutral sodium salt. The specific radioactivity of the [^{32}P]P_i was adjusted so that the 10 μM concentration provided a radioactivity concentration of about 5 μCi/ml. At various times thereafter drugs were added to the media and portions of the cell suspension were taken for analysis. The portions (0.6 ml) were rapidly homogenized in 2.25 ml of chloroform/methanol (1:2, v/v) + 2.0 μl of 6 M-HCl and the phospholipids were extracted by the method of Bligh & Dyer (1959) as modified by Lapetina & Michell (1972). The PtdIns4P and PtdIns(4,5)P_2 were separated from other radioactive phospholipids by t.l.c. in one dimension as described by Schacht (1978) and visually detected by autoradiography. The method employs 10 cm plates coated with 0.25 mm silica gel 60 (EM reagents) and a solvent system of chloroform/methanol/aq. NH_3/water (90:90:7:20, by vol.). The spots described by Schacht (1978) as being PtdIns4P and PtdIns(4,5)P_2 co-migrated with appropriate standards, and remained as a single spot in a few experiments when chromatograms were developed in a second dimension in chloroform/methanol/acetic acid/water (120:46:19:3, by vol.). Additionally, in one experiment (described in the Results section) in which a rat was injected with [^{3}H]inositol, as expected, most of the radioactivity migrated with PtdIns with lesser amounts of radioactivity in the spots attributed to PtdIns4P and PtdIns(4,5)P_2.

The areas corresponding to PtdIns4P and PtdIns(4,5)P_2 were scraped into scintillation vials, 5 ml of water was added and radioactivity was quantified from Čerenkov radiation in a liquid-scintillation counter. As there was insufficient material to measure PtdIns4P and PtdIns(4,5)P_2 chemically, the extent of labelling is generally expressed as a percentage of some control value, usually just before drug additions. Experiments were generally repeated three to four times and means ± S.E.M. are given. All observed effects of drugs etc. are based on demonstrated statistical significance ($P < 0.05$), usually by analysis of variance.

The enzymes used for cell dispersion, acetyl-β-methylcholine, adrenaline, propranolol, isoprenaline, atropin and authentic PtdIns4P and PtdIns(4,5)P_2 were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Substance P was purchased from Peninsula Laboratories, San Carlos, CA, U.S.A. Ionomycin was kindly donated by Mr. S. J. Lucania of the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. [^{32}P]P_i and [^{3}H]-inositol were purchased from New England Nuclear, Boston, MA, U.S.A.

Results

The time course of accumulation of [^{32}P]P_i into PtdIns4P and PtdIns(4,5)P_2 of rat parotid glands is summarized in Fig. 1. Both PtdIns4P and PtdIns(4,5)P_2 are rapidly labelled and with apparently identical kinetics. This may indicate that some step before the phosphorylation of the lipids, such as [^{32}P]P_i uptake or ATP labelling, may be rate-limiting. Both compounds appear to be labelled to near steady-state by 90 min, with a half-time of 19 min.

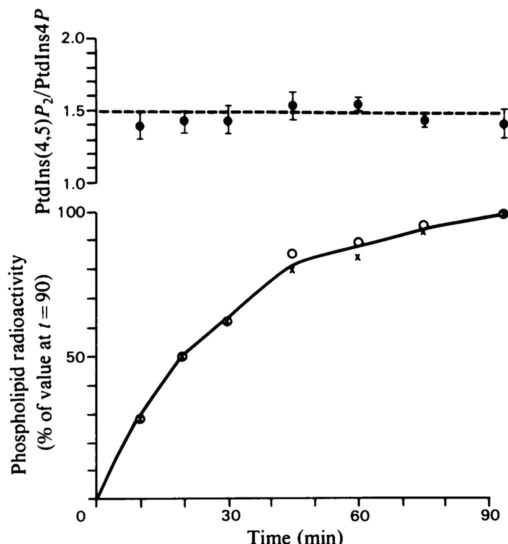

Fig. 1. *Time course of incorporation of* [^{32}P]P_i *in polyphosphoinositides in rat parotid acinar cells*

Data from four experiments were expressed as percentages of the 90 min value. Values are means ± S.E.M. for PtdIns(4,5)P_2/PtdIns4P ratio. ○, PtdIns(4,5)P_2; ×, PtdIns4P.

When the cholinergic-muscarinic agonist, methacholine, was added to the suspension of cells, rapid ($t_{\frac{1}{2}} < 1$ min) disappearance of about 45% of labelled PtdIns(4,5)P_2, but not PtdIns4P, occurred (Fig. 2). The small increase in PtdIns4P at 1 min was also seen in unstimulated controls (not shown) from which these data did not differ significantly. This may result from a small sampling artifact as it was generally seen in the PtdIns(4,5)P_2 controls as well (Fig. 3, for example). The apparent time course for loss of radioactivity from PtdIns(4,5)P_2 was identical whether cells were pre-incubated with [^{32}P]P$_i$ for 30 or 60 min. This suggests that [^{32}P]P$_i$ uniformly labels a homogeneous pool of PtdIns(4,5)P_2 (not necessarily total cellular PtdIns(4,5)P_2), and that the percentage change in radioactive PtdIns(4,5)P_2 probably reflects similar net changes in mass for the specific pool of PtdIns(4,5)P_2 labelled under these conditions. For the remaining experiments *in vitro*, the period of pre-incubation with [^{32}P]P$_i$ was 60 min.

Agents acting on the three known Ca^{2+}-mobilizing receptors in the parotid were compared with respect to their abilities to provoke loss of radioactive PtdIns(4,5)P_2; the results are summarized in Fig. 3. Adrenaline was used together with 10 μM-propranolol to assure activation of only the α-adrenoceptor pathway. Of the three agents (methacoline, Substance P, adrenaline with propranolol), methacholine caused the greatest effect. The temporal patterns of radioactivity loss due to methacholine and adrenaline were similar. The pattern for Substance P differed, however, the effect being equal to that for methacholine for the first minute, but the labelled PtdIns(4,5)P_2 subsequently rose such that the net change was, at 5–10 min, more similar to the less efficacious adrenaline. None of the three agonists had significant effects on PtdIns4P radioactivity (results not shown).

The β-adrenoceptor catecholamine, isoprenaline (10 μM), and the Ca^{2+} ionophore, ionomycin (2.67 μM), were tested for effects on the polyphosphoinositides. Neither compound significantly affected radioactive PtdIns(4,5)P_2 or PtdIns4P (results not shown; less than 5% change), whereas in two concurrent experiments, methacholine caused a decrease similar to the preceding experiments.

The rapidity of the effect of methacholine on PtdIns(4,5)P_2 was examined in experiments shown in Fig. 4. Analysis of variance showed that PtdIns-

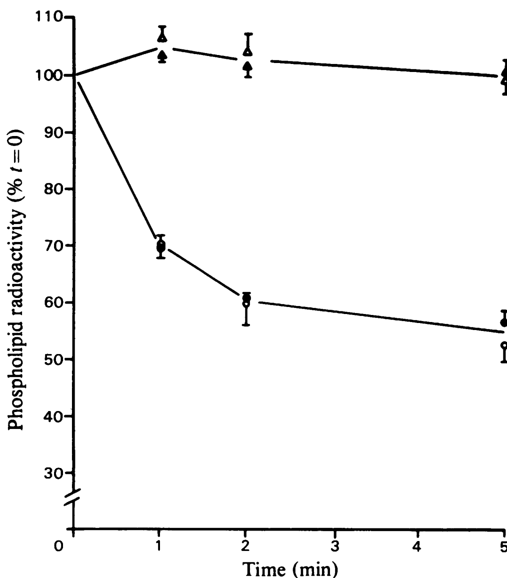

Fig. 2. *Effect of 0.1 mM-methacholine on ^{32}P-labelled polyphosphoinositides*

Cells were pre-incubated with [^{32}P]P$_i$ for 30 (●, ▲) or 60 (○, △) min, and samples were taken just before ($t = 0$), and 1, 2 and 5 min after addition of 0.1 mM-methacholine. ● and ○, PtdIns(4,5)P_2; ▲ and △, PtdIns4P. Results are means ± S.E.M. for four experiments.

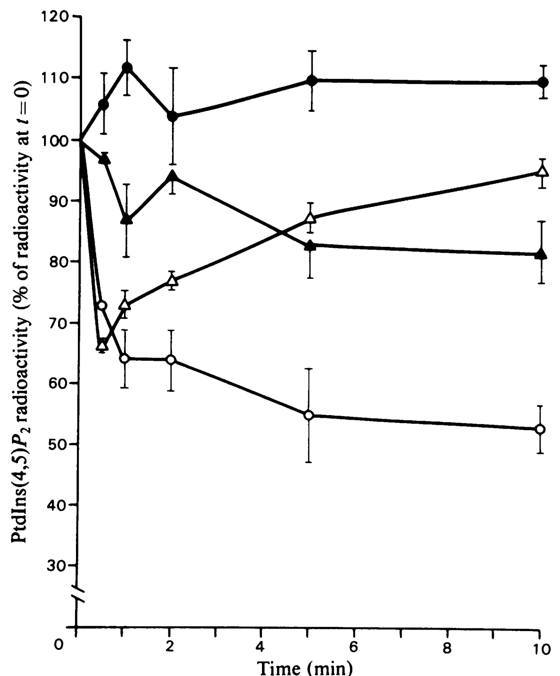

Fig. 3. *Radioactivity in PtdIns(4,5)P_2 (as a percentage of the value at $t = 0$) as affected by various agents*

●, Control (no agonist); ○, 0.1 mM-methacholine; ▲, 0.1 mM-adrenaline + 10 μM-propranolol; △, 0.1 μM-Substance P. Results are means ± S.E.M. for three experiments.

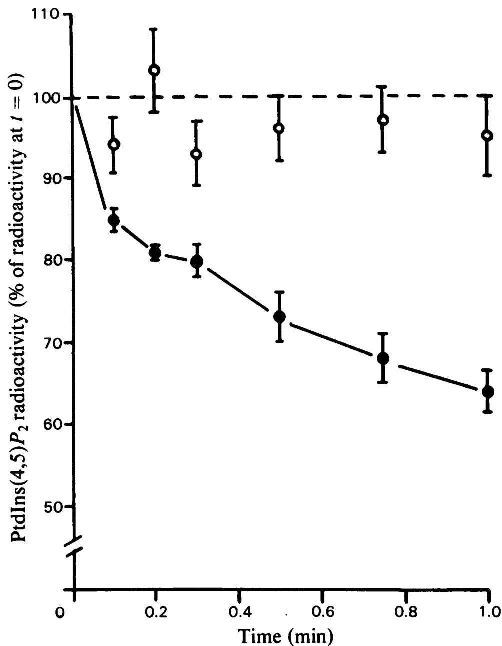

Fig. 4. *Time course of PtdIns(4,5)P_2 breakdown caused by 0.1 mM-methacholine*

Samples were taken every 6s after addition of 0.1 mM-methacholine and data were expressed as percentages of radioactivity before agonist addition. ○, Control (no agonist); ●, 0.1 mM-methacholine. Results are means ± S.E.M. of three (○) or four (●) determinations.

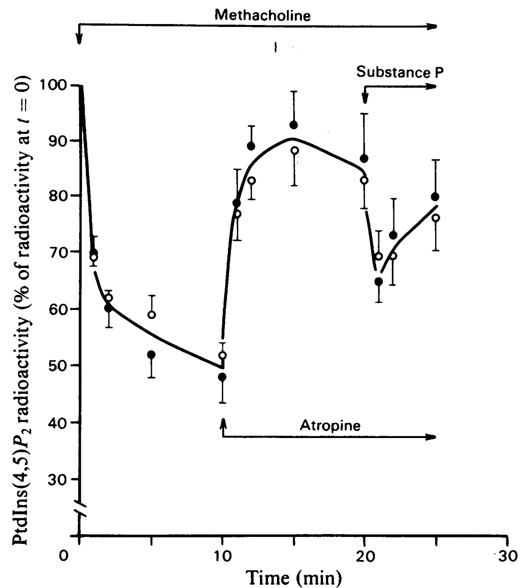

Fig. 5. *Effect of Ca^{2+} omission on changes in PtdIns(4,5)P_2 levels induced by agonists and antagonists*

Methacholine (0.1 mM), atropine (10 μM) and Substance P (0.1 μM) were added as indicated. ●, Experiments carried out in normal Ringer; ○, 2 mM-EGTA was added 5 min before the addition of agonist. Results are means ± S.E.M. for four experiments.

(4,5)P_2 radioactivity differed from the concurrently run controls at all times, including the earliest sample taken 6 s after the addition of methacholine.

The relationship of Ca^{2+}-mobilization to the reeceptor-mediated effects of labelled PtdIns(4,5)P_2 was examined in experiments shown in Fig. 5. In these experiments, methacholine was added to the cells at $t = 0$, atropine, to block the effects of methacholine at 10 min, and substance P at 20 min. Paired experiments were carried out in the presence or absence of external Ca^{2+}. For the low-Ca^{2+} experiments, cells were pre-incubated in normal Ringer for 55 min, at which time (5 min before methacholine addition) 2 mM-EGTA was added, which effectively decreases external [Ca^{2+}] to less than 1 μM. Analysis of variance revealed no significant differences in the pattern of this experiment when Ca^{2+} was absent compared with the pattern in its presence.

The data on PtdIns4P radioactivity from these same experiments are shown in Fig. 6 (note the expanded scale). As before, only minor (and insignificant) changes were observed when agonists were applied, but when atropin was applied after methacholine, a 20–30% increase in PtdIns4P labelling was observed that was statistically significant and roughly paralleled the rise in radioactive PtdIns(4,5)P_2 in Fig. 5.

To obtain an approximation of the PtdIns(4,5)P_2 content of the parotid, a rat was injected subcutaneously with 100 μCi of [^{3}H]inositol. After 18 h, the animal was anaesthetized, the glands were removed, each gland was cut in half and each of the four pieces rapidly homogenized in cold chloroform/methanol (1:2, v/v). The phospholipids were extracted as before and chromatographed on plates with [^{32}P]P_i-labelled specimens in parallel lanes. Autoradiographic localization of PtdIns(4,5)P_2 in the parallel lanes were used to predict the location of the ^{3}H-labelled material. From the replications of the analyses from this single animal, the [PtdIns(4,5)P_2]/[PtdIns] ratio was estimated to be 0.053 ± 0.010. From prior estimates of parotid PtdIns content (12 nmol/mg of protein; Weiss & Putney, 1981), an estimate of PtdIns(4,5)P_2 content of about 0.6 nmol/mg of protein is obtained, although this must be regarded as a rather approximate value.

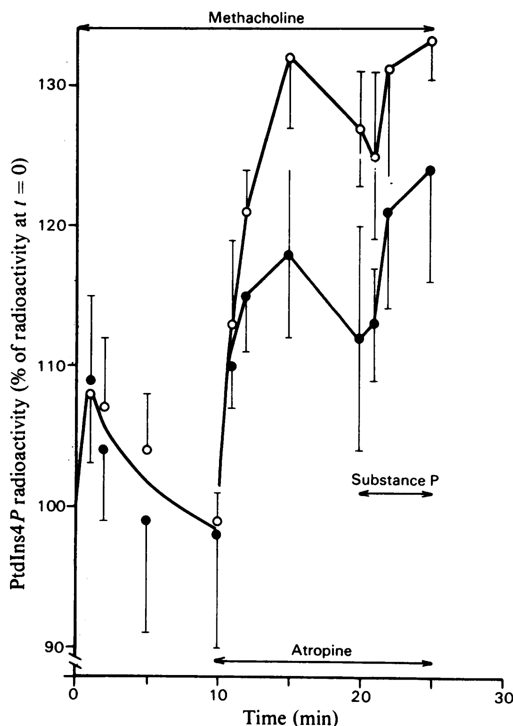

Fig. 6. *Radioactivity in PtdIns4P from the same experiments as shown in Fig. 5*

For explanation of the symbols, see the legend to Fig. 5.

Discussion

The rat parotid gland can be activated by any of four known receptor pathways: β-adrenergic, α-adrenergic, muscarinic (cholinergic) and substance P (Schramm & Selinger, 1975; Putney, 1978). The β-adrenoceptor pathway primarily regulates protein secretion utilizing cyclic AMP as second messenger. The other three pathways (muscarinic, α-adrenergic, Substance P) primarily regulate univalent ion fluxes, which may be involved in secretion of water and electrolytes; apparently Ca^{2+} serves as second messenger for these effects (Putney, 1978). Further, the evidence suggests that these three receptors in the parotid (and probably elsewhere) mobilize Ca^{2+} by two mechanisms: an enhancement of Ca^{2+} influx (increased membrane permeability to Ca^{2+}) and release of bound or sequestered Ca^{2+} from some cellular store (Putney *et al.*, 1981). Indirect evidence suggests that the releasable pool of cellular Ca^{2+} may reside in the plasma membrane (Poggioli & Putney, 1982).

The data obtained in this investigation suggest that breakdown of a specific pool of PtdIns(4,5)P_2 could play a role in the mechanism by which receptor activation effects Ca^{2+}-mobilization. The arguments are similar to those developed originally by Michell (1975, 1979), suggesting such a role for PtdIns breakdown. The data in Fig. 3 show that this reaction is activated by receptors involved in Ca^{2+}-mobilization. However, the effect cannot be provoked through the β-adrenergic receptor, which acts through cyclic AMP. Despite this association with the Ca^{2+} pathway, the data also clearly show that PtdIns(4,5)P_2 loss is not a consequence of Ca^{2+}-mobilization. For example, ionomycin, a Ca^{2+}-ionophore, failed to stimulate PtdIns(4,5)P_2 breakdown. Previous studies have shown that this concentration of ionomycin (2.67 μM) under these experimental conditions provokes Ca^{2+}-mobilization and Ca^{2+}-mediated responses of a magnitude similar to those obtained with muscarinic agonists (Poggioli *et al.*, 1982).

The addition to the cell suspension of EGTA in concentrations sufficient to decrease external [Ca^{2+}] to less than 1 μM failed to affect the PtdIns(4,5)P_2 breakdown response to methacholine (Fig. 5). However, the possibility exists that receptor-mediated intracellular Ca^{2+} release known to occur in Ca^{2+}-deficient media (Haddas *et al.*, 1979) could be acting as catalyst for the PtdIns(4,5)P_2 effect. This possibility is effectively ruled out by the ability of Substance P to cause PtdIns(4,5)P_2 breakdown in the Ca^{2+}-deficient medium. It has previously been shown that this experimental sequence, i.e., muscarinic agonist followed by muscarinic antagonist followed by Substance P, results in a failure of Ca^{2+} release by Substance P due to the prior depletion of the relevant Ca^{2+} pool by the cholinergic agonist (Putney, 1977; Haddas *et al.*, 1979; Poggioli & Putney, 1982). Thus in the experiments of Fig. 5 in Ca^{2+}-deficient medium, Substance P causes PtdIns(4,5)P_2 breakdown in the absence of either Ca^{2+}-influx or Ca^{2+}-release. Taken with the failure of ionomycin to cause PtdIns(4,5)P_2 breakdown, this finding clearly establishes receptor-activated PtdIns(4,5)P_2 breakdown as an event that is not mediated by Ca^{2+}-mobilization. It is noteworthy, however, that these results do not rule out a possible requirement for tightly bound Ca^{2+} for one or more of the enzymes involved in the response. Rather, the point is that the loss of PtdIns(4,5)P_2 that comes about as a consequence of receptor activation does not result from the alterations in cellular [Ca^{2+}] that agonists invoke.

Following Michell's logic as originally applied to PtdIns turnover (Michell, 1975), this would suggest that PtdIns(4,5)P_2 breakdown may be an early event following receptor activation and one preceding Ca^{2+}-mobilization. Conducive to this idea is the demonstrated rapidity of PtdIns(4,5)P_2 breakdown (Fig. 4). Also, the time course of PtdIns(4,5)P_2

breakdown, being essentially complete in 2 min, is consistent with the time course of net $^{45}Ca^{2+}$ release (Poggioli & Putney, 1982). As the polyphosphoinositides are known to bind Ca^{2+} well (Dawson & Hauser, 1979), it is tempting to speculate that Ca^{2+}-release could arise directly from the breakdown of a PtdIns(4,5)P_2–Ca^{2+} complex. Consistent with this idea is the generally held view that the polyphosphoinositides may be localized in the plasma membrane (Michell, 1975), which is believed to be the site of receptor-activated Ca^{2+}-release (Poggioli & Putney, 1982). Studies on the subcellular distribution of the polyphosphoinositides in the parotid gland have not been carried out, however. The roughly estimated PtdIns(4,5)P_2 content of 0.6 nmol/mg of protein would suggest a breakdown in the range of about 0.3 nmol/mg of protein if all of the cellular PtdIns-(4,5)P_2 were labelled equally with [^{32}P]P_i. Estimates of the quantity of Ca^{2+} released after receptor activation are in the range 0.2–0.5 nmol/mg of protein (calculated from data in Poggioli & Putney, 1982). Although there is not sufficient data as yet for rigorous quantitative comparison of these parameters, this treatment does show that one estimate of the small quantity of PtdIns(4,5)P_2 lost may at least be in the range anticipated for Ca^{2+} release.

It is not possible from these data to suggest a specific pathway for loss of PtdIns(4,5)P_2. The term breakdown seems tentatively justified only in the absence of known synthetic pathways utilizing PtdIns(4,5)P_2. Reversal of, or decrease in, synthesis of PtdIns(4,5)P_2, for example by stimulation of a PtdIns(4,5)P_2 phosphomonoesterase or inhibition of PtdIns4P kinase seems unlikely (but not disproven) since PtdIns4P radioactivity is unaltered. This also argues against a secondary effect on PtdIns(4,5)P_2 after PtdIns breakdown, i.e., by decreasing one of the substrates for PtdIns(4,5)P_2 synthesis. It also effectively rules out effects secondary to changes in labelled ATP. In fact, since PtdIns4P is the only known intermediate linking PtdIns and PtdIns(4,5)P_2, there is no readily apparent connection between the observed receptor-mediated breakdown of these two inositides, and we must tentatively consider the possibility that they represent distinct pathways. Since resynthesis of PtdIns(4,5)P_2 after atropine treatment (Fig. 5) was associated with a small increase in PtdIns4P, the route of synthesis could involve the previously described sequential phosphorylation of PtdIns (Michell, 1975). Other tissues have been shown to contain PtdIns(4,5)P_2 phosphodiesterase activity, which could be the pathway involved (Akhtar & Abdel-Latif, 1980; Downes & Michell, 1981). Until breakdown products are identified, however, other mechanisms remain as possibilities. Hopefully, continued research in this area will more clearly define these pathways, and extend our understanding of the roles of phosphoinositide metabolism in mechanisms of Ca^{2+}-mobilization.

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