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Breakdown of polyphosphoinositides and not phosphatidylinositol accounts for muscarinic agonist-stimulated inositol phospholipid metabolism in rat parotid glands

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The molecular mechanisms underlying the ability of muscarinic agonists to enhance the metabolism of inositol phospholipids were studied using rat parotid gland slices prelabelled with tracer quantities of [^{3}H]inositol and then washed with 10mM unlabelled inositol. Carbachol treatment caused rapid and marked increases in the levels of radioactive inositol 1-phosphate, inositol 1,4-bisphosphate, inositol 1,4,5-trisphosphate and an accumulation of label in the free inositol pool. There were much less marked changes in the levels of [^{3}H]phosphatidylinositol, [^{3}H]phosphatidylinositol 4-phosphate and [^{3}H]phosphatidylinositol 4,5-bisphosphate. At 5s after stimulation with carbachol there were large increases in [^{3}H]inositol 1,4-bisphosphate and [^{3}H]inositol 1,4,5-trisphosphate, but not in [^{3}H]inositol 1-phosphate. After stimulation with carbachol for 10min the levels of radioactive inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate greatly exceeded the starting level of radioactivity in phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate respectively. When carbachol treatment was followed by addition of sufficient atropine to block all the muscarinic receptors the radioactive inositol phosphates rapidly returned towards control levels. The carbachol-evoked changes in radioactive inositol phosphate and phospholipid levels were blocked in the presence of 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation). The results suggest that muscarinic agonists stimulate a polyphosphoinositide-specific phospholipase C and that these lipids are continuously replenished from the labelled phosphatidylinositol pool. [^{3}H]Inositol 1-phosphate in the stimulated glands probably arises via hydrolysis of inositol 1,4-bisphosphate and not directly from phosphatidylinositol.

A variety of agonists act on cell-surface receptors to cause an increase in the cytosol Ca^{2+} concentration. These same agonists also induce the hydrolysis of inositol phospholipids, which has been suggested to be an essential response that couples receptor activation to Ca^{2+}-mobilization (Michell, 1975; Berridge, 1981; Putney, 1981; Michell *et al.*, 1981). Acceptance of this idea has been limited by the lack of information concerning the precise molecular mechanism underlying agonist-stimulated phospholipid metabolism. For example, we can only determine the intracellular location of the response when we know the characteristics and in particular the substrate specificity of the enzyme(s) involved. Many Ca^{2+}-mobilizing agonists can cause the disappearance of PtdIns and therefore it was suggested that agonists could activate a PtdIns-specific phospholipase C (Michell, 1975; Michell *et al.*, 1977; Jones *et al.*, 1979; Irvine *et al.*, 1982). More recently it has become clear that the same group of agonists can provoke the disappearance of the polyphosphoinositides PtdIns4*P* and PtdIns(4,5)P_2 (Durrell *et al.*, 1968; Abdel-Latif *et al.*, 1977; Akhtar & Abdel-Latif, 1980; Kirk *et al.*, 1981; Weiss *et al.*, 1982; Creba *et al.*, 1983;

Abbreviations used: PtdIns, phosphatidylinositol; KRB, Krebs–Ringer bicarbonate; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Gro*P*Ins, glycerophosphoinositol; Gro*P*Ins4*P*, glycerophosphoinositol 4-phosphate; Gro*P*Ins(4,5)P_2, glycerophosphoinositol 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; DNP, 2,4-dinitrophenol; TCA, trichloroacetic acid.

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Billah & Lapetina, 1983; Rhodes *et al.*, 1983; Thomas *et al.*, 1983). We recently identified Ins1*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3 as products after stimulating Ca^{2+}-mobilizing receptors in the blowfly salivary gland, rat parotid gland slices and rat brain slices (Berridge *et al.*, 1983). Thus receptor activation stimulates a phospholipidase C that degrades polyphosphoinositides. However, these previous experiments did not exclude the possibility of direct hydrolysis of PtdIns by the same enzyme(s). To resolve this question we have used rat parotid gland slices, prelabelled *in vitro* with [^{3}H]inositol, to provide a more detailed examination of the molecular mechanism underlying the enhanced metabolism of inositol phospholipids resulting from muscarinic receptor activation.

Methods

Rat parotid gland slices were prepared as described previously except that the collagenase treatment was omitted (Hanley *et al.*, 1980). Precise details of the incubation conditions for prelabelling parotid gland slices with [^{3}H]inositol have been given in a previous publication (Berridge *et al.*, 1983). Briefly, the slices were incubated with [^{3}H]inositol (0.32μM; 12.5Ci/mmol) for 90min at 37°C and then rinsed several times with KRB containing 10mM unlabelled inositol. They were then incubated for a further 60min before rinsing again with the cold inositol/KRB. The prelabelled slices were allowed to settle under gravity and 40μl portions were transferred to Beckman Biovials containing 200μl of unlabelled inositol/KRB. Drugs were added (10μl) from stock solutions in KRB. All incubations were stopped by adding 200μl of ice-cold 15% (w/v) TCA.

Analysis of [^{3}H]inositol phosphates

TCA-treated samples were briefly centrifuged and 350μl of the supernatant was transferred to separate tubes. TCA was removed by diethyl ether extraction and analysis of [^{3}H]inositol-labelled water-soluble compounds was by anion-exchange chromatography on Dowex-1 (formate) columns exactly as described previously (Berridge *et al.*, 1983). The following fractions were collected directly into 20ml counting vials: free inositol (2 × 6ml of water); Gro*P*Ins (2 × 6ml of 60mM-ammonium formate/5mM-disodium tetraborate); Ins1*P* (2 × 6ml of 200mM-ammonium formate/100mM-formic acid); Ins(1,4)P_2 (3 × 6ml of 400mM-ammonium formate/100mM-formic acid); Ins(1,4,5)P_3 (4 × 3ml of 1000mM-ammonium formate/100mM-formic acid). Radioactivity in the Ins(1,4,5)P_3 fractions was determined by scintillation counting in the liquid phase using 80% (v/v) Aquasol (New England Nuclear). Radioactivity in all other fractions was determined by scintillation counting in the gel phase using 60% (v/v) Aquasol.

Analysis of [^{3}H]inositol phospholipids

After removing samples of TCA-soluble material the remaining TCA was discarded. The precipitate was washed once with 1ml of 5% TCA containing 1mM-EDTA and once with water (Creba *et al.*, 1983). Chloroform/methanol/12M-HCl (40:80:1; 940μl) was then added and phospholipids were extracted for 10min at room temperature. Chloroform (310μl) and 560μl of 0.1M-HCl were then added and two phases were obtained by brief centrifugation. The lower phase was removed and dried under a stream of N_2.

The dried lipid samples were dissolved in 0.5ml of chloroform and the phospholipids were deacylated using a method based on that of Ellis *et al.* (1963) as described previously (Creba *et al.*, 1983). The resulting glycerophosphoryl esters were then separated by anion-exchange chromatography on the same columns that were used for separating inositol phosphates. The following fractions were collected directly into 20ml counting vials: Gro*P*Ins, the deacylation product of PtdIns (2 × 6ml of 60mM-ammonium formate/5mM-disodium tetraborate); Gro*P*Ins4*P*, the deacylation product of PtdIns4*P* (2 × 6ml of 400mM-ammonium formate/100mM-formic acid); Gro*P*Ins(4,5)P_2, the deacylation product of PtdIns(4,5)P_2 (4 × 3ml of 1000mM-ammonium formate/100mM-formic acid). Radioactivity was determined by scintillation counting in the gel phase (Gro*P*Ins, Gro*P*Ins4*P*) or the liquid phase [Gro*P*Ins(4,5)P_2].

Results

Distribution of [^{3}H]inositol amongst acid-soluble and acid-insoluble components of parotid glands

The TCA-soluble extract obtained from control or carbachol-stimulated parotid gland slices contained five [^{3}H]inositol-labelled components that were separated by ion-exchange chromatography. These compounds were previously identified as inositol, Gro*P*Ins, Ins1*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3 (Berridge *et al.*, 1983). Acid chloroform/methanol extracts of the tissue pellets formed after treating the slices with TCA contained only three radioactive components that were identified after deacylation and separation of the resulting glycerophosphoryl esters on the same ion-exchange columns that were used for separating the acid-soluble compounds. These compounds were eluted in the positions expected for Gro*P*Ins, Gro*P*Ins4*P* and Gro*P*Ins(4,5)P_2 as described previously for ^{32}P-labelled hepatocyte lipids (Creba *et al.*, 1983).

Table 1 shows the distribution of radioactivity amongst the [^{3}H]inositol-containing components of

Table 1. *Distribution of [^{3}H]inositol amongst components of rat parotid glands and the effects of carbachol treatment on these components*

Rat parotid gland slices were prelabelled with [^{3}H]inositol and then washed with KRB containing 10mM-inositol as described in the Methods section. TCA-soluble and -insoluble fractions were obtained and radioactivity in the different components of these fractions was determined and expressed as a percentage of the total recovered radioactivity. The effect of carbachol treatment was determined using samples of prelabelled parotid gland slices incubated in groups of three with or without carbachol (1mM) for 10min and the results are expressed as a percentage of the control values. The results are means ± S.E.M. for three separate parotid gland preparations and each preparation contained the glands from three rats. Statistical significance was assessed using a paired *t*-test.

Component	Radioactivity incorporated (% of total recovered)	Effect of carbachol on radioactivity incorporated (% of control)
Inositol	55.0 ± 9	125 ± 1.9*
Gro*P*Ins	0.25 ± 0.05	131 ± 12
Ins1*P*	0.48 ± 0.14	371 ± 46*
Ins(1,4)P_2	0.15 ± 0.07	2142 ± 514*
Ins(1,4,5)P_3	0.18 ± 0.009	992 ± 134*
PtdIns	41 ± 9	84.1 ± 4.9
PtdIns4*P*	1.4 ± 0.03	78.1 ± 2.7*
PtdIns(4,5)P_2	1.3 ± 0.25	46.0 ± 2.1*

* Significantly different from control incubations at $P < 0.05$.

parotid gland slices after the labelling and cold-washing cycle described in the Methods section. Most of the radioactivity was found in free inositol and PtdIns. The glands retained approx. 5% of the total label in the original incubations as free inositol despite extensive washing of the tissue slices in cold inositol/KRB. This retention of substantial levels of [^{3}H]inositol by the washed glands may indicate the existence of an active uptake process for inositol. This would be one explanation for the very high levels of *myo*-inositol in many tissues by comparison with plasma as suggested by Dawson & Freinkel (1961). PtdIns4*P* and PtdIns(4,5)P_2 accounted for only 3.2% and 2.9% respectively of the radioactivity in the TCA-insoluble fraction, presumably reflecting the much lower concentrations of these lipids in parotid glands compared with their precursor, PtdIns (assuming the three lipids are in isotopic equilibrium with one another, as discussed below). In control incubations only very low levels of radioactivity were found in the inositol phosphate and Gro*P*Ins fractions.

The labelled phospholipids and inositol phosphates clearly do not reach isotopic equilibrium with the added [^{3}H]inositol during the initial incubation period. However, it seems likely that the inositol phospholipids will reach isotopic equilibrium with one another extremely rapidly because the monoester phosphate groups of the polyphosphoinositides show metabolic turnover times of only a few minutes in a number of tissues (see Downes & Michell, 1982, for a review). Furthermore, rapid interconversions between the labelled PtdIns and the polyphosphoinositides are demonstrated for the parotid gland in the results discussed below.

Effects of carbachol on the distribution of [^{3}H]-inositol amongst parotid-gland components

Exposure of prelabelled parotid-gland slices to carbachol for 10min resulted in large changes in the levels of the different radioactive compounds (Table 1, final column). Most noticeable were the increases in the levels of each of the [^{3}H]inositol phosphates. The magnitude of the response varied between experiments, but the relative increases in each fraction remained very constant. The largest increase was always seen in the [^{3}H]Ins(1,4)P_2 fraction. In the series of experiments reported previously (Berridge *et al.*, 1983) carbachol treatment for 10min caused an approx. 40-fold increase in the [^{3}H]Ins(1,4)P_2 fraction, whereas in the present series of experiments, which were done under identical conditions with those reported previously, the increases varied between 12-fold and 29-fold. The reasons for these variations between experiments remain uncertain. In addition to these changes in [^{3}H]inositol phosphate levels there was an accumulation of [^{3}H]inositol after stimulation with carbachol. This accumulation was small in percentage terms, but accounted for a large proportion of net redistribution of radioactivity. As discussed below, this accumulation of substantial amounts of [^{3}H]inositol may be due to sequential hydrolysis of inositol phosphates by a series of inositol phosphomonoesterases.

Carbachol treatment caused a reduction of the label in PtdIns4*P* and PtdIns(4,5)P_2, but the apparent decrease in [^{3}H]PtdIns was not statistically significant ($P > 0.05$). PtdIns disappearance has been detected previously in parotid glands stimulated with muscarinic agonists (Jones & Michell, 1974). However, it is important that carbachol did not stimulate inositol phospholipid labelling in the glands washed with unlabelled inositol because the changes in [^{3}H]inositol phosphate levels can be interpreted as an increase in mass and not simply a change in their specific radioactivity. Similarly since the carbachol-induced decrease in [^{3}H]polyphosphoinositides is much greater than the apparent decrease in [^{3}H]-PtdIns this implies that there is a net reduction in polyphosphoinositide levels during stimulation with carbachol. The decrease in [^{3}H]PtdIns(4,5)P_2 is very

similar to that reported previously by Weiss *et al.* (1982), who used parotid acinar cells in which polyphosphoinositides were prelabelled with [^{32}P]P_i. However, we also detected a small decrease in [^{3}H]PtdIns4*P* after incubating with carbachol, whereas Weiss *et al.* (1982) reported no change in ^{32}P-labelled PtdIns4*P*. The mechanism that generates large accumulations of inositol phosphates with only modest falls in the steady-state levels of their radioactive parent lipids is discussed in the next section.

Examination of the data in Tables 1 and 2 suggests that the total increase in the TCA-soluble pool of ^{3}H is twice the decrease in the TCA-insoluble pool. Comparison of the data from individual experiments demonstrated that this apparent discrepancy was not statistically significant ($P > 0.05$).

Comparison of carbachol-induced changes in labelled lipids and inositol phosphates

Table 2 shows the results of a single experiment in order to compare directly the levels of radioactivity in each labelled component after carbachol treatment. The most important point to note is that the accumulation of [^{3}H]Ins(1,4)P_2 and [^{3}H]-Ins(1,4,5)P_3 greatly exceeds even the starting level of radioactivity in their parent lipids. Thus exposure to carbachol for 10min caused an accumulation of 7160d.p.m. in the Ins(1,4)P_2 fraction yet PtdIns4*P* accounted for only 3160d.p.m. in control incubations and only fell to 2020d.p.m. in the incubations with carbachol. There is a similar though less marked discrepancy between the accumulation of [^{3}H]Ins(1,4,5)P_3 and the fall in [^{3}H]-PtdIns(4,5)P_2. The only simple explanation for this result is that the labelled polyphosphoinositides are constantly replenished, presumably from the labelled PtdIns pool.

When carbachol treatment was followed by adding sufficient atropine to displace the agonist from all the available muscarinic receptors, the radioactivity that had accumulated in each of the inositol phosphate fractions declined (Table 3). Furthermore there was a significant increase in [^{3}H]PtdIns(4,5)P_2 back towards the control level. Thus receptor blockade leads to a rapid resynthesis of [^{3}H]PtdIns(4,5)P_2. Furthermore, atropine treatment reveals the existence of an inositol phosphomonoesterase pathway that presumably generates free inositol from each of the inositol phosphates and may account for the accumulation of [^{3}H]inositol in these experiments (the specificity of the putative enzymes involved in such a pathway is not yet certain and this point is considered in the Discussion section). The probable existence of such a pathway has been suggested previously (Akhtar & Abdel-Latif, 1980; Downes *et al.*, 1982; Berridge *et al.*, 1983). Assuming this pathway is active during incubations with muscarinic agonists, the measured accumulation of [^{3}H]inositol phosphates represents a gross underestimate of the metabolic flux through the polyphosphoinositides during stimulation. The whole labelled pool of these lipids must, therefore, break down and be resynthesized from PtdIns several times during a 10min incubation with carbachol.

Time courses of inositol phosphate formation and hydrolysis

Because measurements of the accumulation and hydrolysis of inositol phosphates, rather than the corresponding changes in their parent lipids, give a

Table 2. *Effects of carbachol and subsequent atropine treatment on [^{3}H]inositol-labelled components of rat parotid glands*

The conditions of prelabelling, extraction and separation were exactly as described for Table 1. The incubations of prelabelled slices were for 20min without additions (control), 10min without additions followed by 10min with 1mM-carbachol (carbachol) and 10min with 1mM-carbachol followed by 10min with 10μM-atropine to displace carbachol from muscarinic receptors (carbachol/atropine). Results are means ± S.E.M. for groups of three incubations using a single preparation of parotid-gland slices, the tissue being obtained from three rats. These results were reproduced in two additional experiments employing similar conditions. Statistical significance was assessed using Student's *t*-test. *, Significantly different from control at $P < 0.05$. **, Significantly different from carbachol at $P < 0.05$.

	Radioactivity (d.p.m.)		
Component	Control	Carbachol	Carbachol/atropine
Inositol	101100 ± 5550	124300 ± 13160	136200 ± 2960*
Ins1*P*	1030 ± 63	3550 ± 170*	2290 ± 140**
Ins(1,4)P_2	600 ± 11	7820 ± 390*	2690 ± 170**
Ins(1,4,5)P_3	500 ± 24	3620 ± 124*	1160 ± 73**
PtdIns	134900 ± 4260	117500 ± 11260	115000 ± 4040
PtdIns4*P*	3160 ± 130	2300 ± 210*	2930 ± 270
PtdIns(4,5)P_2	2020 ± 90	910 ± 41*	1620 ± 70**

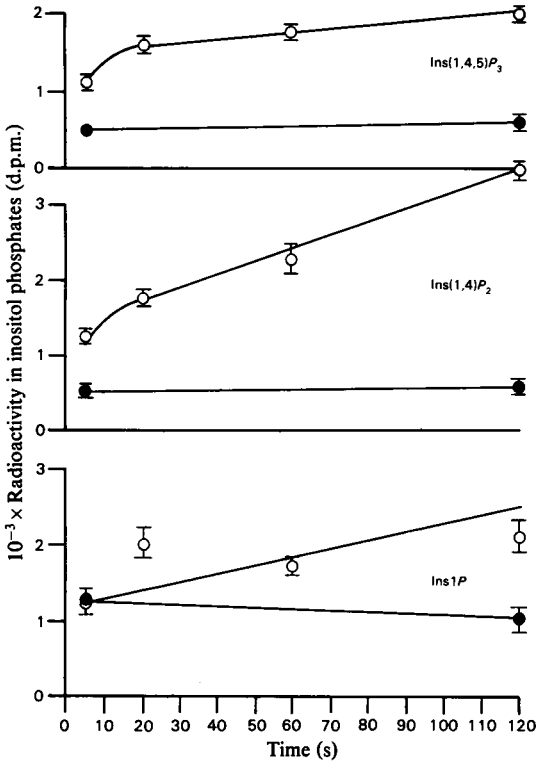

Fig. 1. *Time course of carbachol-stimulated [^{3}H]inositol phosphate formation*

[^{3}H]Inositol-labelled parotid-gland slices were incubated with (○) or without (●) 1 mM-carbachol for the times indicated. The incubations were stopped and [^{3}H]inositol phosphates analysed as described for Table 1. Each point is the mean ± S.E.M. for three samples using a single preparation of parotid glands obtained from three rats. The observations were repeated in two separate experiments (see the text).

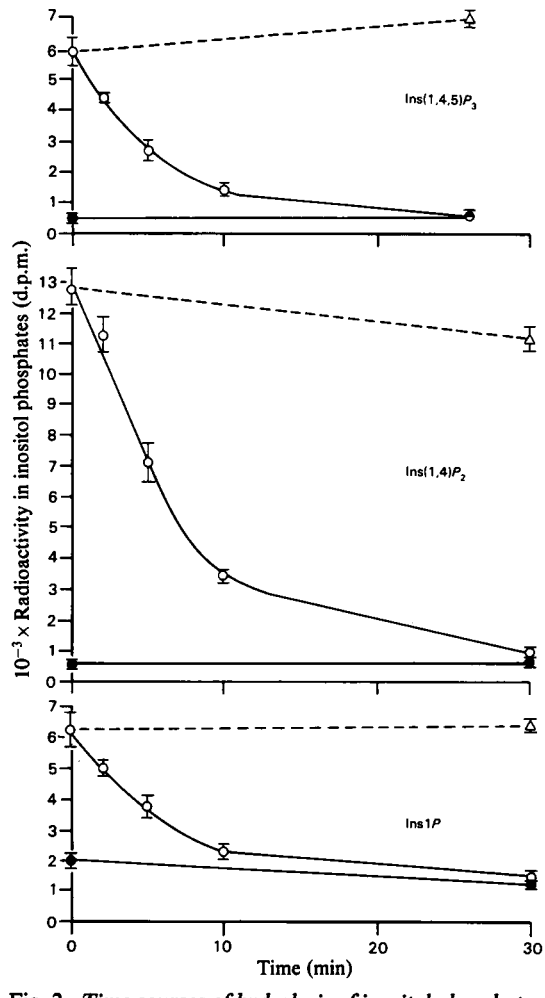

Fig. 2. *Time courses of hydrolysis of inositol phosphates*

[^{3}H]Inositol-labelled parotid-gland slices were incubated for 15 min with (○) or without (●) 1 mM-carbachol. Atropine (final concentration 10 μM) was then added to some of the samples and the incubations were continued for the times shown. A further group of samples that contained carbachol were incubated without further additions (△). The incubations were stopped by adding TCA and radioactive inositol phosphates were analysed. Each point is the mean ± S.E.M. for three samples using a single preparation of parotid glands obtained from three rats. The observations were repeated in one other experiment under identical conditions.

more quantitative understanding of the changes induced by receptor activation, these events were examined in more detail. Fig. 1 shows the time course of inositol phosphate formation after adding carbachol to the parotid-slice preparation. There were large increases in [^{3}H]Ins(1,4)P_2 and [^{3}H]-Ins(1,4,5)P_3 within 5 s, but no detectable change in [^{3}H]Ins1P at this time. Three separate experiments, stimulating with carbachol (1 mM) for 5 s, gave the following results expressed as a percentage of control: Ins(1,4,5)P_3, 191 ± 21; Ins(1,4)P_2, 203 ± 24 Ins1P, 104 ± 4%. [^{3}H]Ins1P increased steadily at the later times measured up to 2 min. This suggests that polyphosphoinositides are the first targets for the receptor-stimulated phospholipase C.

The results in Fig. 2 were obtained by first treating parotid-gland slices with carbachol for 15 min to allow inositol phosphates to accumulate. Muscarinic receptors were then blocked with atropine and the rate of decline of the [^{3}H]inositol phosphates was followed. Each of the inositol phosphates declined rapidly under these conditions, returning to control levels within 30 min. In carbachol-treated slices not exposed to atropine, the increased levels of [^{3}H]inositol phosphates remained relatively constant, suggesting that after 15 min incubation with carba-

Table 3. *Effects of pretreatment with DNP on the carbachol-induced changes in [^{3}H]inositol-labelled components of rat parotid glands*

A single preparation of parotid-gland slices obtained from three rats was prelabelled with [^{3}H]inositol and divided into two portions. Samples of the first portion were incubated with or without 0.5 mM-DNP for 10 min. Carbachol was then added to some of the samples and the incubations were continued for a further 10 min before determining radioactivity in the inositol phosphate and phospholipid fractions. The second portion of prelabelled slices was treated with 0.5 mM-DNP for 20 min and then washed five times with 10 ml of KRB. Samples of this preparation were then incubated for a further 10 min either with or without carbachol (1 mM). Results are means ± S.E.M. for groups of three incubations. The observations were repeated in a second experiment.

	Radioactivity (d.p.m.)					
	No pretreatment		DNP pretreatment		DNP recovery	
Component	Control	Carbachol	Control	Carbachol	Control	Carbachol
Ins1*P*	1230 ± 30	5680 ± 190	1300 ± 20	2020 ± 42	1260 ± 250	5080 ± 260
Ins(1,4)P_2	470 ± 20	13810 ± 540	460 ± 70	1660 ± 44	480 ± 80	10700 ± 660
Ins(1,4,5)P_3	430 ± 2	4650 ± 160	380 ± 20	710 ± 30	470 ± 66	3980 ± 360
PtdIns	162300 ± 7400	121050 ± 1850	143380 ± 7840	125000 ± 5270	152840 ± 19670	122340 ± 6960
PtdIns4*P*	3850 ± 75	3150 ± 170	1920 ± 49	1730 ± 96	4060 ± 390	3290 ± 150
PtdIns(4,5)P_2	3110 ± 320	1550 ± 280	1590 ± 130	1230 ± 67	3270 ± 390	1860 ± 80

chol the rate of inositol phosphate formation and hydrolysis were approx. equal.

Effects of a metabolic inhibitor on carbachol-stimulated inositol phospholipid metabolism

The results in Table 2 demonstrate that polyphosphoinositide resynthesis must occur for maximal generation of inositol phosphates. This could explain the apparent paradox that PtdIns disappearance evoked in platelets by thrombin (Holmsen *et al.*, 1982) was extraordinarily sensitive to treatments that deplete cellular ATP levels. Our results suggest that PtdIns disappears as it is used to resynthesize polyphosphoinositides via two ATP-dependent reactions catalysed by PtdIns kinase and PtdIns4*P* kinase respectively. We therefore examined the effects of an uncoupler of oxidative phosphorylation, DNP, on carbachol-stimulated inositol phospholipid metabolism in the parotid-gland slice preparation.

As shown in Table 3, incubating parotid-gland slices with DNP (0.5 mM) for 20 min caused a large (approx. 50%) decrease in labelled polyphosphoinositides levels with no change in the level of [^{3}H]PtdIns. The most likely explanation for this result is that DNP treatment leads to a rapid decline in cellular ATP and a correspondingly rapid loss of polyphosphoinositides. If this interpretation is correct then the results demonstrate the rapid rate of metabolic turnover of the monoester phosphates of the [^{3}H]inositol-labelled pool of polyphosphoinositides as suggested by their rapid rate of labelling with [^{32}P]P_i (Weiss *et al.*, 1982). Furthermore, when the DNP-treated slices were exposed to carbachol, [^{3}H]inositol phosphate formation was almost abolished. The effects of DNP pretreatment on accumulation of each of the inositol phosphate fractions was similar. In addition, carbachol did not cause any significant further disappearance of [^{3}H]PtdIns4*P* or [^{3}H]PtdIns(4,5)P_2, perhaps because the initial loss of these lipids after DNP treatment (presumably through phosphomonoesterase attack) removes most of the substrate available for the receptor-stimulated phospholipase C. These effects of DNP were not due to irreversible membrane damage bacause the levels of radioactivity in the polyphosphoinositides and the response to carbachol returned to normal when DNP was removed by washing pretreated slices several times with KRB.

Discussion

Previous work has demonstrated that Ca^{2+}-mobilizing agonists acting on blowfly salivary glands, rat brain slices and rat parotid-gland slices can stimulate hydrolysis of polyphosphoinositides by phospholipase C activity. However, the precise substrate specificity of the enzyme(s) involved remains uncertain. Three possibilities seem worth considering. The enzyme(s) could attack PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, leading to direct formation of their respective inositol phosphate headgroups; PtdIns4*P* and PtdIns4*P*(4,5)P_2 could both be attacked directly with Ins1*P* being formed by Ins(1,4)P_2 hydrolysis and not directly from PtdIns; PtdIns(4,5)P_2 could be attacked directly with both Ins(1,4)P_2 and Ins1*P* being formed via hydrolysis of Ins(1,4,5)P_3.

We can now reject the idea of direct formation of Ins1*P* from PtdIns on several grounds. In previous work (Berridge *et al.*, 1983) we failed to detect the formation of inositol 1:2-cyclic phosphate as a product after stimulation of different tissues with Ca^{2+}-mobilizing agonists, suggesting that the PtdIns-specific phospholipase C, which generates a mixture

of Ins1*P* and inositol 1:2-cyclic phosphate as products (see Irvine *et al.*, 1982), is not involved. There was a detectable time-lag for the formation of Ins1*P*, but not for Ins(1,4)P_2 or Ins(1,4,5)P_3 (Fig. 1). Assuming that Ins1*P* is a product of Ins(1,4)P_2 hydrolysis by the phosphatase activity demonstrated in Fig. 2, then this process could account for Ins1*P* formation. DNP pretreatment depleted [^{3}H]-polyphosphoinositides without affecting [^{3}H]PtdIns, but it almost abolished the carbachol-induced formation of Ins1*P*. In this context it is difficult to imagine an ATP requirement for the direct hydrolysis of PtdIns unless the appropriate enzyme is regulated by a protein kinase as suggested by Holmsen *et al.* (1982). As mentioned previously ATP would be required to fuel PtdIns kinase and PtdIns4*P* kinase.

Carbachol is known to provoke extensive disappearance of PtdIns in rat parotid glands (up to 30% in 5 min; Jones & Michell, 1974). For this effect to be explained entirely by polyphosphoinositide hydrolysis these lipids must be hydrolysed and replaced from the PtdIns pool several times during even relatively brief periods of stimulation. This clearly occurs for the [^{3}H]inositol-labelled phospholipid fractions studied in these experiments (Table 3).

Specificity of polyphosphoinositide hydrolysis

The observation that Ca^{2+}-mobilizing agonists acting on parotid acinar cells caused a relatively large decrease in ^{32}P-labelled PtdIns(4,5)P_2 as compared with [^{32}P]PtdIns4*P* led Weiss *et al.* (1982) to the reasonable conclusion that the enzyme involved preferred to attack PtdIns(4,5)P_2. However, the time courses of [^{3}H]Ins(1,4)P_2 and [^{3}H]-Ins(1,4,5)P_3 formation (Fig. 1) appear almost identical, suggesting that both PtdIns4*P* and PtdIns(4,5)P_2 are degraded directly. This conclusion is supported by considering the relative rates of hydrolysis of [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 (Fig. 2). The initial absolute rate of disappearance of [^{3}H]Ins(1,4)P_2 appears faster than that of [^{3}H]-Ins(1,4,5)P_3 [this is an underestimate if Ins(1,4)P_2 is also a product of Ins(1,4,5)P_3 phosphomonoesterase activity]. These results are consistent with the direct formation of Ins(1,4,5)P_3 and Ins(1,4)P_2 from their respective lipid precursors. The larger accumulation of Ins(1,4)P_2 during stimulation with carbachol may occur because it could also be formed by hydrolysis of Ins(1,4,5)P_3. However, conclusions based upon the relative rates of hydrolysis of inositol phosphates must be treated with caution until we know more about the properties and intracellular distribution of the inositol phosphomonoesterases.

Hydrolysis of inositol phosphates

Since a variety of hormones and neurotransmitters appear capable of stimulating the hydrolysis of polyphosphoinositides to yield inositol phosphates there is a need to degrade these products to free inositol. Rat parotid-gland slices appear to possess enzymes capable of hydrolysing Ins1*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3 and the results shown in Fig. 2 are consistent with the existence of the following metabolic pathway:

$$\text{Ins}(1,4,5)P_3 \rightarrow \text{Ins}(1,4)P_2 \rightarrow \text{Ins}1P \rightarrow \text{Inositol}$$

Although the present results cannot be used to determine the precise specificity of the enzymes catalysing this sequence of reactions [for example hydrolysis of Ins(1,4,5)P_3 could proceed by the initial removal of any one of the phosphate groups], results from other tissues suggest that the inositol phosphomonoesterases may have specific substrate requirements. The Ins(1,4,5)P_3 phosphomonoesterase of human erythrocyte membranes removes only the 5-phosphate and is inactive against Ins(1,4)P_2 (Downes *et al.*, 1982). Furthermore, Li^+ specifically inhibits Ins1*P* hydrolysis in a variety of tissues (Hallcher & Sherman, 1980; Sherman *et al.*, 1981; Berridge *et al.*, 1982), suggesting that there is a distinct Ins1*P* phosphomonoesterase in many tissues. Li^+ is used to treat the symptoms of manic depression and it has been argued that Li^+ may exert its therapeutic effect by limiting the availability of free inositol (Sherman *et al.*, 1981; Berridge *et al.*, 1982). If this hypothesis proves to be correct then inhibitors of Ins(1,4)P_2 phosphomonoesterase and Ins(1,4,5)P_3 phosphomonoesterase could be important therapeutic agents not just in the area of affective disorders but perhaps also for other diseases that involve receptors whose functions require inositol phospholipids.

Polyphosphoinositide breakdown and Ca^{2+}-mobilization

Rat parotid gland acinar cells possess a variety of receptors (α_1-adrenoceptors, muscarinic cholinergic and substance P) that control secretion of fluid and protein through an increase in the cytosol Ca^{2+} concentration [Ca^{2+}-mobilization (Oron *et al.*, 1975; Putney, 1981, 1982)]. Activation of this same group of receptors provokes the hydrolysis of inositol phospholipids, but in contrast with the secretory response this effect is not elicited if the receptors are bypassed and cytosol Ca^{2+} concentration is raised using an ionophore (see Putney, 1982, for a review), nor are the changes in phospholipid metabolism abolished by treatment of the tissue with EGTA. These observations, and similar results in many different tissues using a wide variety of agonists, led Michell (1975, 1979) to suggest that PtdIns breakdown might be an essential reaction that couples receptor occupation to Ca^{2+}-mobilization. The present results provide compelling evidence to support

our previous suggestion (Kirk *et al.*, 1981; Michell *et al.*, 1981; Downes & Michell, 1982; Creba *et al.*, 1983) that polyphosphoinositide breakdown should replace PtdIns breakdown in this role. PtdIns probably disappears, as it is used to replenish the polyphosphoinositide pools.

How might phospholipase C attack upon polyphosphoinositides lead to an increase in cytosol Ca^{2+} concentration? One possibility is that conversion of PtdIns(4,5)P_2 (the most polar, acidic phospholipid known) into diacylglycerol (a neutral, hydrophilic lipid) might act as a trigger to release Ca^{2+} from membrane-bound stores. Alternatively the water-soluble products of phospholipase C activity, Ins(1,4,5)P_3 and/or Ins(1,4)P_2, could act as second messengers and themselves catalyse Ca^{2+} release as suggested recently by Berridge (1983). In addition to this putative role for polyphosphoinositide breakdown in cellular Ca^{2+}-mobilization, diacylglycerol formed by phospholipase C activity can act as a trigger for activating protein kinase C (Takai *et al.*, 1981; Kaibuchi *et al.*, 1982). The rate of formation of these potentially important products (inositol phosphates and diacylglycerol) depends upon the activity of the receptor-stimulated phospholipase C and upon the rate at which the polyphosphoinositides can be resynthesized by PtdIns kinase and PtdIns4*P* kinase. These synthesizing enzymes can maintain substantial levels of polyphosphoinositides even when the phospholipase C is activated by high doses of carbachol.

In conclusion, it now seems certain that muscarinic agonists act on the parotid gland by stimulating a polyphosphoinositide-specific phospholipase C. Our previous work suggests the same mechanism may operate for the other Ca^{2+}-mobilizing receptors of the parotid (α_1 and Substance P) and for such receptors in the brain and the blowfly salivary gland (Berridge *et al.*, 1983). The polyphosphoinositide pools labelled by [^{3}H]inositol can be broken down and resynthesized several times within a 10min period of stimulation and yet substantial levels of the lipids are maintained in the membrane. This may imply that there is also a high degree of control (perhaps secondary to control of the phospholipase C by activated receptors) at the level of PtdIns and PtdIns4*P* kinases.

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