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Receptor-mediated metabolism of the phosphoinositides and phosphatidic acid in rat lacrimal acinar cells

Philip P. GODFREY and James W. PUTNEY, Jr.*
Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

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The metabolism of the inositol lipids and phosphatidic acid in rat lacrimal acinar cells was investigated. The muscarinic cholinergic agonist methacholine caused a rapid loss of 15% of [^{32}P]phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and a rapid increase in [^{32}P]phosphatidic acid (PtdA). Chemical measurements indicated that the changes in ^{32}P labelling of these lipids closely resembled changes in their total cellular content. Chelation of extracellular Ca^{2+} with excess EGTA caused a significant decrease in the PtdA labelling and an apparent loss of PtdIns(4,5)P_2 breakdown. The calcium ionophores A23187 and ionomycin provoked a substantial breakdown of [^{32}P]PtdIns(4,5)P_2 and phosphatidylinositol 4-phosphate (PtdIns4P); however, a decrease in [^{32}P]PtdA was also observed. Increases in inositol phosphate, inositol bisphosphate and inositol trisphosphate were observed in methacholine-stimulated cells, and this increase was greatly amplified in the presence of 10mM-LiCl; α-adrenergic stimulation also caused a substantial increase in inositol phosphates. A23187 provoked a much smaller increase in the formation of inositol phosphates than did either methacholine or adrenaline. Experiments with excess extracellular EGTA and with a protocol that eliminates intracellular Ca^{2+} release indicated that the labelling of inositol phosphates was partially dependent on the presence of extracellular Ca^{2+} and independent of intracellular Ca^{2+} mobilization. Thus, in the rat lacrimal gland, there appears to be a rapid phospholipase C-mediated breakdown of PtdIns(4,5)P_2 and a synthesis of PtdA, in response to activation of receptors that bring about an increase in intracellular Ca^{2+}. The results are consistent with a role for these lipids early in the stimulus–response pathway of the lacrimal acinar cell.

In many cell types there is a close association between agonist-induced elevation of cytosol Ca^{2+} concentrations and enhanced inositol lipid metabolism (Berridge, 1981; Putney, 1981; Michell & Kirk, 1981). Michell (1975) originally suggested that phospholipase C-mediated breakdown of PtdIns may be the initial event in receptor-stimulated Ca^{2+} mobilization. The evidence for this hypothesis is the ubiquitous association of PtdIns turnover with receptors that utilize Ca^{2+} as second messenger, and the demonstration that, for most systems, PtdIns turnover is not a Ca^{2+}-mediated event (Michell, 1979; Michell *et al.*, 1981). However, exceptions have been noted (Cockcroft, 1981; Hawthorne, 1982; Prpic *et al.*, 1982).

Recently there has been increased interest in the phosphorylated derivatives of PtdIns, PtdIns4P and PtdIns(4,5)P_2. Rapid agonist-induced breakdown of the polyphosphoinositides has been found in a variety of tissues (Kirk *et al.*, 1981; Billah & Lapetina, 1982*a*,*b*, 1983; Soukup & Schanberg, 1982; Weiss *et al.*, 1982*b*; Creba *et al.*, 1983; Imai *et al.*, 1983; Putney *et al.*, 1983; Rhodes *et al.*, 1983), and it has been suggested that breakdown of PtdIns(4,5)P_2 may precede the previously reported PtdIns response (Michell, 1982).

The products of the phospholipase C-mediated PtdIns and polyphosphoinositide breakdown are

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdA, phosphatidic acid; PtdCho, phosphatidylcholine.

* To whom reprint requests should be addressed.

the inositol phosphates (Berridge *et al.*, 1982, 1983) and diacylglycerol, which is then rapidly phosphorylated to PtdA (Lapetina *et al.*, 1981; Imai *et al.*, 1982; Weiss *et al.*, 1982*a*; Lapetina, 1983). Possible roles for diacylglycerol, PtdA and inositol phosphates have been suggested. Diacylglycerol is an activator of a phospholipid-dependent Ca^{2+}-activated protein kinase, and this enzyme may be involved in the physiological responses to Ca^{2+}-mobilizing hormones (Kaibuchi *et al.*, 1982). PtdA has ionophoretic properties *in vitro*, and it has been suggested that PtdA derived from the inositol lipids might mediate inward Ca^{2+} movement by acting as an endogenous Ca^{2+} ionophore (Putney, 1982). Inositol phosphates could act as second messengers linking receptor activation to release of Ca^{2+} from internal stores (Berridge, 1984).

In the present study we have used a variety of methods to investigate the inositol lipid turnover in the rat lacrimal cell, and the role of Ca^{2+} in this process.

Experimental

Exorbital lacrimal glands were removed from anaesthetized (sodium pentobarbital) male Sprague–Dawley rats (140–220g), and dispersed acinar cells were prepared as previously described (Parod *et al.*, 1980). The medium used had 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 was titrated with HCl (about 10mM-Cl^- final concentration) to pH 7.4 at 37°C. The gas phase was 100% O_2. In some experiments, ionized Ca^{2+} was decreased to $<1\,\mu M$ by the addition of 2mM-EGTA (neutralized with NaOH). The cells were 80–95% viable as judged by Trypan Blue exclusion.

Measurement of phospholipids

Cells were equilibrated in the above medium for 20min, at which time $10\,\mu M$-[^{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 $10\,\mu M$ concentration provided a radioactivity concentration of about $5\,\mu Ci$/ml. At various times thereafter, drugs were added to the media and portions of the cell suspension were taken for analysis. The portions (0.6ml) were rapidly homogenized in 2.25ml of chloroform/methanol (1:2, v/v)+$20\,\mu l$ of 6M-HCl; after a 30min extraction period, 0.75ml of chloroform and 0.75ml of 2M-KCl were added, and the samples were mixed and centrifuged (2000*g*-min). The lower phase was removed and the upper phase was washed with 1.4ml of chloroform, which was collected and combined with the original extract. The combined lower phase was then washed with methanol/1M-HCl (1:1, v/v) and dried *in vacuo*. This method provided a consistent and reproducible extraction of polyphosphoinositides, though we do not know whether all the lipids were extracted or simply a very constant proportion. However, when purified [^{32}P]polyphosphoinositides were re-extracted from t.l.c. plates with this procedure, the recovery was over 90%. The phospholipids were separated by t.l.c. on 10cm plates coated with 0.25mm-thick silica gel 60 (EM Science, Cincinnati, OH, U.S.A.) and were detected by autoradiography. The area corresponding to the required phospholipid was scraped into a scintillation vial, 5ml of water was added and radioactivity was quantified from Čerenkov radiation in a liquid-scintillation counter. The migration of all the phospholipids was verified by the use of appropriate standards.

PtdIns4*P* and PtdIns(4,5)P_2 were separated from the other phospholipids by a one-dimensional chromatographic system as described by Schacht (1978); the solvent system was chloroform/methanol/conc. NH_3 (sp.gr. 0.880)/water (90:90:7:20, by vol.). In experiments where PtdIns(4,5)P_2 was measured chemically, eight identical samples were run concurrently; the corresponding spots were scraped into a borosilicate test tube and digested in 70% (v/v) $HClO_4$ (0.2ml) at 200°C for 2h. After digestion, volumes were made up to 2.0ml and the samples were centrifuged (2000*g*-min) to sediment the silica gel. Samples (0.5ml) were taken for radioactivity determination as above, and for P_i determination by the method of Itaya & Ui (1966).

For measurement of radioactivity in PtdA, PtdIns and PtdCho, the chromatograms were developed twice in the same dimension in a solvent system of chloroform/methanol/acetic acid/water; in the first development the proportions (by vol.) were 40:10:10:1 and in the second development they were 120:46:19:3. For chemical determination of PtdIns and PtdCho the solvent system that yielded the best separation was a one-dimension system of chloroform/methanol/acetic acid/water (100:60:16:5, by vol.). For reproducible separation of PtdA from other contaminants for chemical analysis, it was necessary to use a two-dimensional system. The first dimension was chloroform/methanol/methylamine (17:6:1, by vol.); the plate was then neutralized for 3–5min in fuming HCl vapour; the second dimension was chloroform/methanol/acetic acid/water (900:140:80:3, by vol.). Measurement of phospholipid phosphorus was the same as described for PtdIns(4,5)P_2.

The protein content of each sample was determined from the cellular precipitate obtained from the interface between the organic and aqueous phases during lipid extraction. The precipitates were solubilized in 1M-NaOH and analysed for protein by the method of Lowry *et al.* (1951).

Measurement of inositol phosphates

Formation of labelled inositol phosphates was determined with techniques previously described (Downes & Michell, 1981; Berridge *et al.*, 1983). The cells were equilibrated in the above medium for 20min, at which time *myo*-[2-^{3}H]inositol was added to a radioactivity concentration of about 5μCi/ml. The cells were incubated for an additional 90min, at which time they were washed and resuspended in unlabelled medium. At various times thereafter drugs were added and portions of the cell suspension were taken for analysis. LiCl (10mM), a potent inhibitor of the enzyme *myo*-inositol 1-phosphatase (Berridge *et al.*, 1982) was added to some of the samples 5min before drug addition. After the incubations were completed, 0.3ml portions were added to 0.6ml of ice-cold 4.5% $HClO_4$ and the samples were vortex-mixed and then centrifuged (1000*g*-min). A portion (0.7ml) of the supernatant was then neutralized by a sufficient volume of 0.5M-KOH/9mM-$Na_2B_4O_7$ to give pH8–9. The samples were centrifuged (1000*g*-min) and the supernatants were applied to anion-exchange columns containing 1ml of Dowex-1 (AG 1-X8, 200–400 mesh; formate form; Bio-Rad, Richmond, CA, U.S.A.). Inositol and the inositol phosphates were eluted by stepwise addition of solutions containing increasing concentrations of formate (Downes & Michell, 1981; Berridge *et al.*, 1982, 1983). Free *myo*-inositol was eluted with 20ml of water; *myo*-inositol 1-phosphate (and glycerophosphoinositol) was eluted with 25ml of 5mM-sodium tetraborate plus 150mM-ammonium formate. This was followed by 25ml of 0.4M-ammonium formate plus 0.1M-formic acid, and then by 20ml of 1.0M-ammonium formate plus 0.1M-formic acid, each of which removes more-polar inositol phosphates (Downes & Michell, 1981), namely inositol bisphosphate and inositol trisphosphate respectively (Berridge *et al.*, 1983). Each fraction was counted for radioactivity after addition of an equal volume of scintillant (Aquassure; New England Nuclear, Boston, MA, U.S.A.).

Expression of results

Experiments were generally repeated three to five 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.

Materials

The enzymes used for cell dispersion, acetyl-β-methylcholine, adrenaline, propranolol, isoprenaline and lipid standards were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phentolamine was from Ciba–Geigy Corp., Summit, NJ, U.S.A.; A23187 was from Eli Lilly Co., Indianapolis, IN, U.S.A.; ionomycin was kindly given by Mr. S. J. Lucania, Squibb Institute for Medical Research, Princeton, NJ, U.S.A.; [^{32}P]P_i and [^{3}H]inositol were purchased from New England Nuclear.

Results

The time course of accumulation of [^{32}P]P_i into various lipids from rat lacrimal cells is summarized in Fig. 1. PtdIns(4,5)*P*$_2$, PtdIns4*P* and PtdA were rapidly labelled, with similar kinetics; the labelling reached near steady-state by 90min, with a half-time of approx. 25min. The labelling of PtdIns was

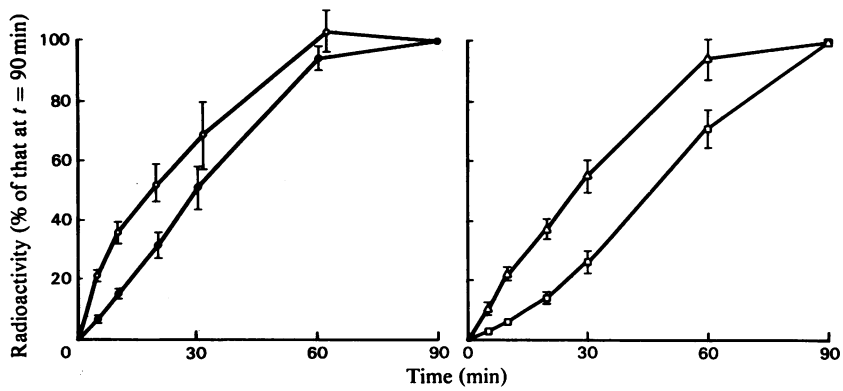

Fig. 1. *Time course of* [^{32}*P*]P_i *incorporation into phospholipids in rat lacrimal acinar cells*
Data from four experiments were expressed as percentages of the 90min value. Values are means±S.E.M. ○, PtdIns4*P*; ●, PtdIns(4,5)*P*$_2$; △, PtdA; □, PtdIns. Radioactivities (c.p.m.) at $t=90$min are: PtdIns(4,5)*P*$_2$, 13222±1879; PtdIns4*P*, 2825±483; PtdIns, 7724±469; PtdA, 2426±336.

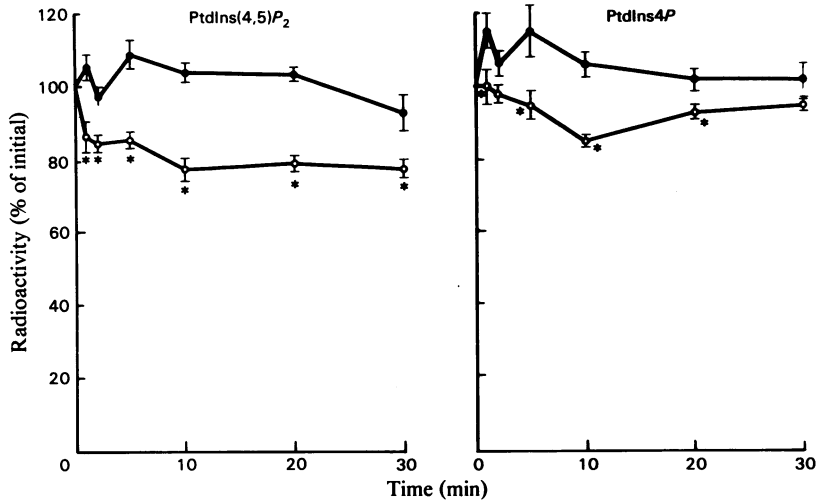

Fig. 2. *Effect of 0.1 mM-methacholine on ^{32}P-labelled polyphosphoinositides*
Cells were preincubated with [^{32}P]P_i for 90 min, and samples were taken just before ($t = 0$) and at various times after methacholine addition. ●, Controls; ○, plus methacholine. The data are expressed as percentages of the $t = 0$ value. Results are means ± S.E.M. for five experiments. * Significantly different from control value ($P < 0.05$), by analysis of variance.

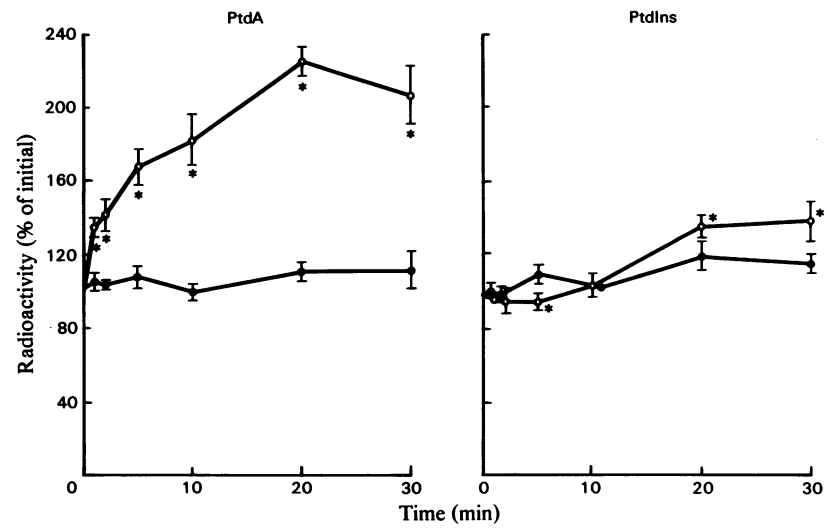

Fig. 3. *Effect of 0.1 mM-methacholine on ^{32}P-labelled PtdA and PtdIns*
Methods and symbols are the same as Fig. 2. Results are means ± S.E.M. for five experiments.

considerably slower and had not reached steady-state by 90 min. This suggests that it was the 4- and 5-position monoester phosphates of PtdIns4*P* and PtdIns(4,5)P_2 that were being labelled to equilibrium.

Addition of the muscarinic cholinergic agonist methacholine to cells labelled for 90 min caused a rapid disappearance of labelled PtdIns(4,5)P_2, approx. 15% in 1 min (Fig. 2). The radioactivity remained at the decreased value for the 30 min experimental period. There was a slow decline in labelled PtdIns4*P* during the 10 min after methacholine administration, to approx. 85% of the initial value, but it increased back to control values by 30 min (Fig. 2). Methacholine also caused a rapid synthesis of labelled PtdA (Fig. 3); a 35% increase was seen after 1 min, rising to 70% by 5 min. PtdIns labelling declined slightly during the

Table 1. *Effect of methacholine on the content of various phospholipids in isolated lacrimal cells*
Lacrimal cells were preincubated for 90min with [^{32}P]P_i and samples were taken just before ($t = 0$) and 5min after addition of 0.1mM-methacholine. Results are means ± S.E.M. [$n = 7$ for PtdIns(4,5)P_2; $n = 5$ for PtdA, PtdIns and PtdCho]. *Results significantly different from controls ($P < 0.05$) by paired Student's t-test.

	Lipid concn. (nmol/mg of protein)		Lipid radioactivity (% of value at $t = 0$)		Specific radioactivity (c.p.m./nmol of lipid, % of value at $t = 0$)	
Lipid	$t = 0$	$t = 5$min	$t = 0$	$t = 5$min	$t = 0$	$t = 5$min
PtdIns(4,5)P_2	0.60±0.11	0.47±0.07*	100	93.1±4.2*	100	121±16.4
PtdA	1.09±0.20	1.94±0.41*	100	199±23*	100	102±10.4
PtdIns	29.5±3.9	29.4±2.9	100	98.2±4.7	100	96.8±4.0
PtdCho	105±6	99±7	100	103±3.4	100	110±3.8

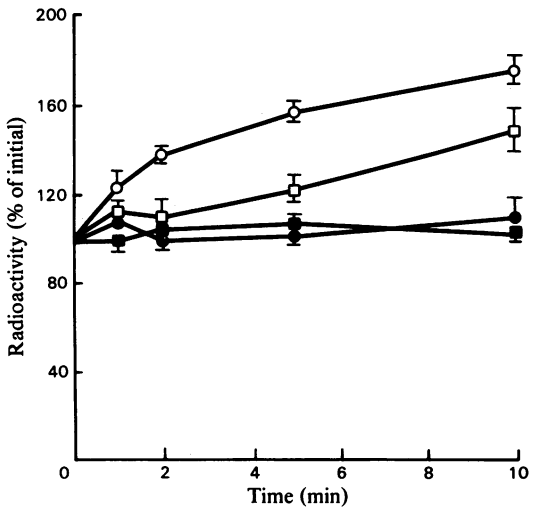

Fig. 4. *Effect of Ca^{2+} deprivation on [^{32}P]PtdA synthesis induced by methacholine*
Samples were taken just before, and at various times after, addition of 0.1mM-methacholine. In the Ca^{2+}-depleted samples, (□, ■) 2mM-EGTA was added 5min before methacholine. ○, □, plus methacholine; ●, ■, controls. The data are expressed as percentages of the value at $t = 0$. Results are means ± S.E.M. for four experiments.

5min after methacholine addition and then increased to 40% above the initial value by 30min (Fig. 3).

Chemical measurement of the phospholipids was undertaken 5min after methacholine stimulation, and compared with zero-time controls. PtdIns(4,5)P_2 and PtdA were present at very low concentrations compared with the other phospholipids (Table 1); both are <1% of the PtdCho concentration and are only 2–4% of that of PtdIns. Although the cellular content of PtdIns(4,5)P_2 was variable, a decrease in PtdIns(4,5)P_2 was observed in each of seven experiments, along with a decline in the radioactivity; both changes were statistically significant (Table 1). PtdA labelling and content were both increased about 90% 5min after methacholine administration; the increase in PtdA content was considerably larger than the decrease in PtdIns(4,5)P_2, suggesting either that the PtdIns(4,5)P_2 pool turns over several times with replenishment from the larger pool of PtdIns, or that a small and statistically undetectable amount of the PtdIns is also being broken down.

The role of Ca^{2+} in the observed lipid changes was investigated in experiments summarized in Figs. 4 and 5. Lowering Ca^{2+} to <1 μM by the addition of 2mM-EGTA 5min before methacholine decreased, but did not completely block, the stimulation of PtdA synthesis by methacholine (Fig. 4). The net breakdown of polyphosphoinositides was, however, no longer statistically significant (results not shown).

Two Ca^{2+} ionophores, A23187 and ionomycin, were also examined for their effects on phospholipid metabolism. Concentrations were chosen which have been previously shown to activate Ca^{2+}-mediated responses similar to those of methacholine (Parod & Putney, 1980; Poggioli *et al.*, 1982). The data in Fig. 5 show that both ionophores caused substantial loss of both labelled polyphosphoinositides, though the kinetics of this breakdown were slower than with the hormone stimulus. Unlike the effect of methacholine, labelled PtdA also decreased with ionophore treatment. There was no effect detected on PtdIns or PtdCho (results not shown).

The loss of the PtdIns(4,5)P_2-breakdown response in Ca^{2+}-deficient medium could mean either that hydrolysis was blocked or that accelerated breakdown was accompanied by accelerated synthesis, resulting in no net change in cellular content. Thus the rate of formation of soluble inositol phosphates, the putative products of phosphodiesterase cleavage of phosphoinositides, was examined.

The content of inositol phosphates increased considerably after methacholine stimulation

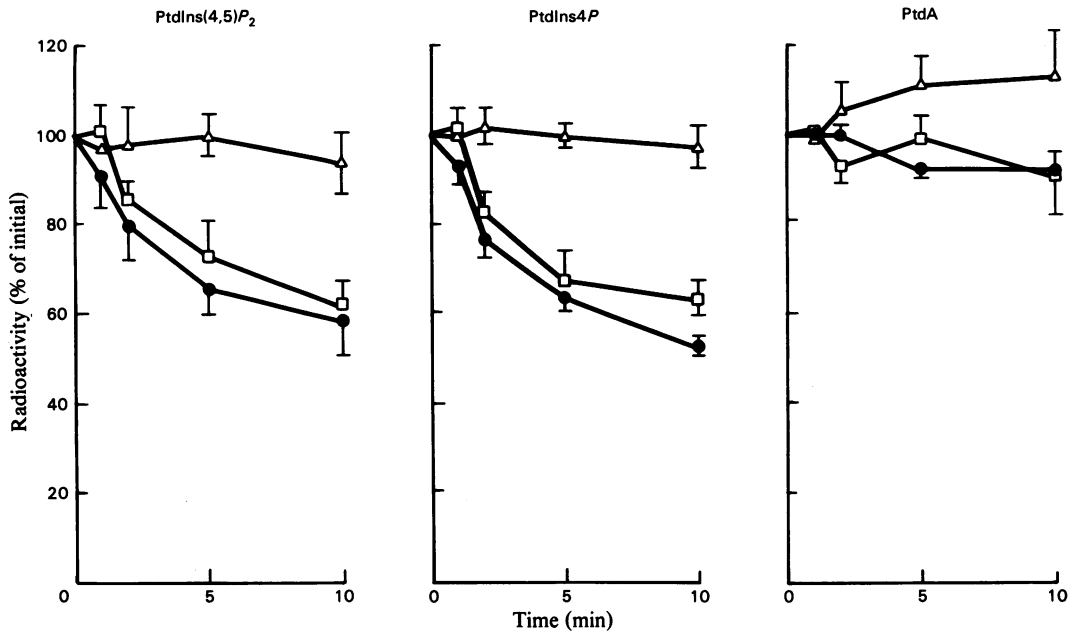

Fig. 5. *Effects of ionophores on metabolism of PtdIns(4,5)P_2, PtdIns4P and PtdA*
Samples were taken just before, and at various times after, addition of 2.67 μM-ionomycin (●), 10 μM-A23187 in 0.5% dimethyl sulphoxide (□), or 0.5% dimethyl sulphoxide (△). Results are means ± S.E.M. ($n = 5$ for A23187 and dimethyl sulphoxide; $n = 3$ for ionomycin).

Table 2. *Effects of Li^+ and methacholine on the production of [^{3}H]inositol phosphates*
Cells were preincubated with [^{3}H]inositol for 90 min and then washed and resuspended in unlabelled medium; 10 mM-LiCl was added to the appropriate sample 5 min before the addition of 0.1 mM-methacholine. The incubations were terminated, after 30 min, by the addition of $HClO_4$. Results are means ± S.E.M. ($n = 3$, without LiCl; $n = 8$, with LiCl).

	Radioactivity (c.p.m.)	
	Control	0.1 mM-Methacholine
Control	163 ± 52	238 ± 39
10 mM-LiCl	240 ± 33	2051 ± 501

(Table 2). If 10 mM-LiCl was included in the incubations, the inositol phosphates were increased greatly compared with samples without LiCl. Li^+ is a potent and specific inhibitor of *myo*-inositol 1-phosphatase (Sherman *et al.*, 1981; Berridge *et al.*, 1982), and its addition leads to an accumulation of inositol 1-phosphate (Berridge *et al.*, 1982).

Various agents known to activate secretory responses in the lacrimal gland were tested for their ability to stimulate formation of inositol phosphates; the results are summarized in Fig. 6. Adrenaline was used together with 10 μM-propranolol to ensure activation of only the α-adrenergic pathway. Methacholine caused the greatest effect, an 800% increase in inositol phosphates after

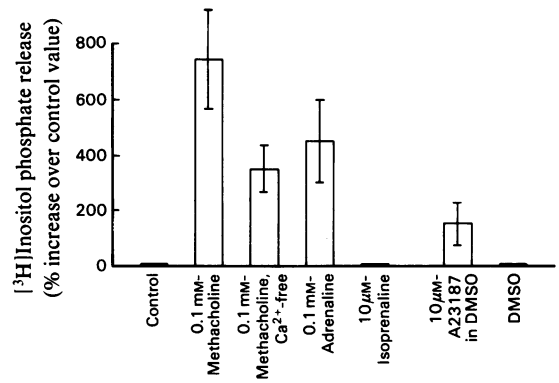

Fig. 6. *Effects of various agonists on the formation of [^{3}H]inositol phosphates*
Drugs were added to the cells 5 min after the addition of 10 mM-LiCl, and the incubations were terminated after a further 30 min. Results are expressed as the percentage increase over the paired control value. Results are means ± S.E.M.: $n = 8$ for control, methacholine; $n = 5$ for methacholine Ca^{2+}-free; $n = 4$ for adrenaline; $n = 3$ for isoprenaline, A23187 in 0.5% dimethyl sulphoxide (DMSO), and 0.5% DMSO.

Table 3. *Effect of ionophore A23187 on formation of inositol phosphates*
Methods are the same as for Table 2. Results are means ± S.E.M. ($n = 3$).

	[^{3}H]Inositol phosphates (c.p.m.)		
	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
10 μM-A23187 in 0.5% dimethyl sulphoxide	310 ± 66	120 ± 18	53 ± 13
0.5% dimethyl sulphoxide	116 ± 31	63 ± 13	39 ± 12

Table 4. *Effect of sequential stimulation in low-Ca^{2+} medium on the formation of inositol phosphates*
Cells were preincubated with [^{3}H]inositol for 90 min in medium containing 1 mM-Ca^{2+}, washed, and then divided into equal samples. The cells were then treated sequentially with 0.1 mM-adrenaline, 10 μM-phentolamine and 0.1 mM-methacholine, as described in the text; 10 mM-LiCl was added 5 min before methacholine, and 2 mM-EGTA was added, as required, 5 min before adrenaline. Results are means ± S.E.M. for four experiments.

	[^{3}H]Inositol phosphates (c.p.m.)		
	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
Control, 1 mM-Ca^{2+}	192 ± 25	29 ± 17	15 ± 7
0.1 mM-Methacholine, 1 mM-Ca^{2+}	4717 ± 1225	436 ± 103	450 ± 98
Control, 1 mM-Ca^{2+} + 2 mM-EGTA	287 ± 51	15 ± 3	11 ± 1
0.1 mM-Methacholine, 1 mM-Ca^{2+} + 2 mM-EGTA	2513 ± 886	343 ± 116	245 ± 95

30 min. The response to α-adrenergic activation was considerably smaller, a 4-fold increase. The β-adrenoreceptor agonist isoprenaline had no effect on inositol phosphate formation. Addition of the Ca^{2+} ionophore A23187 at a concentration that gives a maximal secretory response (Parod & Putney, 1980) caused a small, but significant, increase in inositol phosphates (Fig. 6). If the individual phosphates were measured, only inositol phosphate and inositol bisphosphate were increased; the inositol trisphosphate content was not significantly different from controls (Table 3).

Lowering the external Ca^{2+} concentration to less than 1 μM (2 mM-EGTA was added 5 min before the methacholine) caused a 50% decrease in the stimulated inositol phosphate formation compared with samples containing 1 mM-Ca^{2+} (Fig. 6). By using a phenomenon previously described as 'cross-receptor inactivation' (Putney, 1977), the role of intracellular Ca^{2+} release, known to occur in the absence of extracellular Ca^{2+} (Putney *et al.*, 1981), was investigated. In these experiments, cells were treated with 0.1 mM-adrenaline for 10 min in Ca^{2+}-containing or Ca^{2+}-deficient media. Then 10 μM-phentolamine was added to block the response. After 5 min, 10 mM-LiCl was added, followed by 0.1 mM-methacholine 5 min later. The contents of the three inositol phosphates were measured 30 min after the methacholine addition (Table 4). The protocol in which the adrenaline and phentolamine treatment are in a Ca^{2+}-deficient medium causes release of the receptor-regulated intracellular Ca^{2+} pool, such that no intracellular Ca^{2+} release occurs when methacholine is subsequently added (Parod & Putney, 1979). However, methacholine still caused a large increase in all three inositol phosphates in the Ca^{2+}-deficient cells, though the accumulated amounts were less than in the corresponding Ca^{2+}-containing cells. More significantly, perhaps, the elimination of internal Ca^{2+} release did not decrease inositol phosphate formation beyond that obtained from simply removing external Ca^{2+} (Fig. 6). A similar effect on PtdA synthesis was seen in a concurrent experiment with ^{32}P-labelled cells (results not shown).

Discussion

The secretion of water, electrolytes and protein by the rat lacrimal gland is stimulated by three known receptor pathways: muscarinic cholinergic, α-adrenergic (Putney, 1979) and β-adrenergic (Friedman *et al.*, 1981). Both the muscarinic and α-adrenergic receptors appear to act by increasing the cytosolic Ca^{2+} concentrations via two mechanisms: an enhancement of Ca^{2+} influx (an increased membrane permeability to Ca^{2+}) and release of bound Ca^{2+} from intracellular stores (Putney *et al.*, 1981).

Stimulated PtdIns turnover in the lacrimal gland, in response to muscarinic and α-adrenergic stimuli, has been previously reported (Jones *et al.*, 1979); the data shown here indicate that activation of these receptors also causes a rapid breakdown of PtdIns(4,5)P_2 and synthesis of PtdA. It has been suggested that breakdown of PtdIns(4,5)P_2 may be the initial event in receptor-activated phosphoino-

sitide turnover and that the disappearance of PtdIns is due to resynthesis of the polyphosphoinositides (Michell, 1982). The presence of increased amounts of inositol trisphosphate in the stimulated cells (Table 4) provides good evidence for a phospholipase C-mediated breakdown of the PtdIns(4,5)P_2, since there is no other known mechanism for formation of this molecule. The inositol bisphosphate and inositol phosphate could be formed either by phosphatase activity on the inositol trisphosphate or by direct breakdown of PtdIns4*P* and PtdIns. The present experiments do not distinguish between the two pathways.

Downes & Michell (1982) have suggested that PtdIns(4,5)P_2 breakdown may have a role in receptor-activated Ca^{2+} mobilization. Thus it is important to determine whether the effects on phospholipid metabolism are secondary to the increase in intracellular Ca^{2+}, or result more directly from receptor activation. Depleting the cells of Ca^{2+} with EGTA and artificially elevating the intracellular [Ca^{2+}] with ionophores provide useful answers. In the parotid gland (Weiss *et al.*, 1982*b*), exocrine pancreas (Putney *et al.*, 1983) and platelet (Billah & Lapetina, 1982*b*), the PtdIns(4,5)P_2 breakdown is not Ca^{2+}-dependent, though a Ca^{2+}-dependence was found in the iris smooth muscle (Akhtar & Abdel-Latif, 1978). In the liver some workers have shown PtdIns(4,5)P_2 breakdown to be Ca^{2+}-dependent (Rhodes *et al.*, 1983) and others found it Ca^{2+}-independent (Kirk *et al.*, 1981). A possible solution to this discrepancy was suggested (Creba *et al.*, 1983; Thomas *et al.*, 1983) where it was argued that the lipid breakdown was not a Ca^{2+}-activated process, but that a part of the process had a requirement for a minimal amount of Ca^{2+}.

On the basis of experiments measuring net lipid changes, one might be led to the conclusion that, in the lacrimal gland, phosphoinositide breakdown is Ca^{2+}-mediated. No net loss of PtdIns(4,5)P_2 was seen in Ca^{2+}-deficient medium, and PtdIns(4,5)P_2 decreased after addition of Ca^{2+} ionophores. Measurement of formation of inositol phosphates, however, seemed to clarify this apparent inconsistency with previous findings. Thus the relatively small amount of inositol phosphates formed with A23187 suggest that the ionophore induces PtdIns(4,5)P_2 loss by a mechanism other than phospholipase C. This is consistent with the failure of A23187 or ionomycin to cause PtdA synthesis. The decline could result from either phospholipase A_2 activation or inhibition of synthesis owing to ATP depletion, as previously suggested for the exocrine pancreas (Putney *et al.*, 1983).

For methacholine, the substantial accumulation of inositol phosphates accompanying a small net breakdown of PtdIns(4,5)P_2 suggests that breakdown by phospholipase C is the primary pathway involved. These data also serve to clarify the apparent Ca^{2+}-dependency of the lipid breakdown. The experiments summarized in Fig. 6 and Table 4 show clearly that the inhibitory effect of Ca^{2+} omission is only partial. Apparently, when breakdown is partially inhibited, resynthesis can keep pace, and no net change in PtdIns(4,5)P_2 concentration can be detected. Whether or not this situation is relevant to other reported cases of Ca^{2+}-dependent PtdIns(4,5)P_2 breakdown (Rhodes *et al.*, 1983) remains to be determined. Further, the use of the 'cross-receptor inactivation' protocol for experiments summarized in Table 4 shows that elimination of internal Ca^{2+} release does not result in additional inhibition beyond that seen when external Ca^{2+} is removed. This shows that the effect of Ca^{2+} omission on these reactions may reflect an undefined role for extracellular Ca^{2+} or membrane-bound Ca^{2+} in coupling receptors to subsequent enzyme reactions. However, the activation of phosphoinositide breakdown apparently occurs independently of changes in intracellular ionized Ca^{2+}.

Thus, in summary, these data indicate that, in the rat exorbital lacrimal gland, activation of Ca^{2+}-mobilizing receptors results in breakdown of PtdIns(4,5)P_2 and synthesis of PtdA. These changes are due, at least in part, to phospholipase C cleavage of PtdIns(4,5)P_2. Direct breakdown of other inositol lipids may occur as well, but this is not yet proved. Inositide breakdown and PtdA synthesis depend partially on the presence of external Ca^{2+}, but do not appear to result from the receptor-induced increase in intracellular ionized Ca^{2+}. It is more likely that inositol lipid breakdown is an early, receptor-activated, event and could thus play a role in the mechanism of cellular Ca^{2+} mobilization in the lacrimal acinar cell.

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