Inositol 1,2-cyclic 4,5-trisphosphate is not a product of muscarinic receptor-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis in rat parotid glands

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We have employed a neutral-pH extraction technique to look for inositol 1,2-cyclic phosphate derivatives in [^{3}H]inositol-labelled parotid gland slices stimulated with carbachol. The incubations were terminated by adding cold chloroform/methanol (1:2, v/v), the samples were dried under vacuum and inositol phosphates were extracted from the dried residues by phenol/chloroform/water partitioning. Water-soluble inositol metabolites were separated by h.p.l.c. at pH 3.7. ^{32}P-labelled inositol phosphate standards (inositol 1-phosphate, inositol 1,2-cyclic phosphate, inositol 1,4,5-trisphosphate and inositol 1,2-cyclic 4,5-trisphosphate) were quantitively recovered through both extraction and chromatography steps. Treatment of inositol cyclic phosphate standards with 5% (w/v) $HClO_4$ for 10 min prior to chromatography resulted in formation of the expected non-cyclic compounds. [^{3}H]Inositol 1-phosphate and [^{3}H]inositol 1,4,5-trisphosphate were both present in parotid gland slices and both increased during stimulation with 1 mM-carbachol. There was no evidence for significant quantities of [^{3}H]inositol 1,2-cyclic phosphate or [^{3}H]inositol 1,2-cyclic 4,5-trisphosphate in control or carbachol-stimulated glands. Parotid gland homogenates rapidly converted inositol 1,4,5-trisphosphate to inositol bisphosphate and inositol tetrakisphosphate, but metabolism of the inositol cyclic trisphosphate was much slower. The results suggest that inositol 1,4,5-trisphosphate, but not inositol 1,2-cyclic 4,5-trisphosphate, is the water-soluble product of muscarinic receptor-stimulated phospholipase C in rat parotid glands.

INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is a membrane-bound phospholipid that is the source of at least two intracellular signal molecules. Activation of a variety of cell surface receptors, such as the muscarinic cholinergic receptor of rat parotid glands (Schramm & Selinger, 1975), stimulates a phosphoinositidase C (PIC) whose primary substrate appears to be PtdIns(4,5)P_2 (Michell *et al.*, 1981; Berridge, 1983; Downes & Wusteman, 1983; Aub & Putney, 1984; Hawkins *et al.*, 1986). The lipid product of this reaction is 1,2-diacylglycerol and there is general agreement that its accumulation in the plasma membrane causes translocation of protein kinase C from an inactive form in the cytosol to give an active membrane-associated complex (Nishizuka, 1984). However, there is now considerable controversy regarding the precise nature of the water-soluble product of PIC-catalysed PtdIns(4,5)P_2 breakdown.

Both membrane-bound and soluble PIC activities have been documented in mammalian cells (Allan & Michell, 1978; Downes & Michell, 1981, 1985; Irvine, 1982), though it is not yet known to what extent each of these enzymes contributes to receptor-controlled PtdIns(4,5)P_2 hydrolysis. The relatively high activity and ease of study of the soluble activity have, however, meant that most detailed work has utilized this form of the enzyme(s). The early experiments of Dawson & Clarke (1972) demonstrated that the rat brain cytosol enzyme(s) could degrade phosphatidylinositol (PtdIns) to 1,2-diacylglycerol and a mixture of inositol 1-phosphate (Ins1P) and inositol 1,2-cyclic phosphate (Ins1,2cyc$P$), with the proportions of these two water-soluble products depending upon the pH of the incubation medium. Irvine *et al.* (1984*a*) later found that the soluble enzyme could attack phosphatidylinositol 4-phosphate (PtdIns4P) and PtdIns(4,5)P_2 as well as PtdIns, with the preferred substrate being determined by the precise conditions of the assay, but they did not assess whether cyclic phosphate derivatives of the inositol bis- and tris-phosphates were products of these reactions.

Majerus and his colleagues have since made two important contributions to the study of soluble PIC. First, they confirmed, using a purified enzyme from ram seminal vesicles, the multiple substrate specificity noted above (Wilson *et al.*, 1984), and secondly, they found two water-soluble products when PtdIns(4,5)P_2 was the substrate (Wilson *et al.*, 1985*a*). These products were Ins(1,4,5)P_3 and inositol 1,2-cyclic 4,5-trisphosphate [Ins(1,2cyc4,5)P_3].

The previous studies, which pointed to PtdIns(4,5)P_2 as the primary target for receptor-dependent PIC, used acid-extraction techniques that would have destroyed the cyclic phosphodiester bond, so it was not known to what extent the cyclic and non-cyclic forms of inositol

Abbreviations used: Ins$P$, InsP_2, InsP_3, InsP_4, inositol mono-, bis-, tris- and tetrakis-phosphates; locants where specified are placed in parentheses, e.g. Ins(1,2cyc4,5)P_3 is inositol 1,2-cyclic 4,5-trisphosphate; the D isomers are assumed throughout; PtdIns, PtdIns4P, PtdIns(4,5)P_2, phosphatidylinositol and its 4-mono- and 4,5-bis-phosphates; PIC, phosphoinositidase C.

trisphosphate were generated during agonist stimulation. If they were produced within cells, either or both of these compounds could have second messenger functions, since Ins(1,4,5)P_3 has been shown to release Ca^{2+} from the intracellular stores of many different cells (Berridge & Irvine, 1984) and the cyclic derivative has similar activity and potency to Ins(1,4,5)P_3 when added to permeabilized platelets and when injected into *Limulus* photoreceptors (Wilson *et al.*, 1985*b*). We have now used a neutral-pH extraction technique to assess whether inositol cyclic phosphates are formed during muscarinic cholinergic stimulation of parotid gland slices.

MATERIALS AND METHODS

Materials

Materials and animals were of the type and from the source specified previously (Downes *et al.*, 1986*a*; Hawkins *et al.*, 1986). Phenol (analytical grade reagent) was purchased from BDH. Isoamyl alcohol (3-methylbutan-1-ol, 98%) was purchased from Aldrich.

Preparation and incubation of parotid slices

Rat parotid slices were prepared and incubated with [^{3}H]inositol (3–4 μCi/250 μl) for 90 min at 37 °C, as described by Downes *et al.* (1986*a*). Small incubations (250 μl) were then challenged with either carbachol (10 μl, 1 mM final concn.) or Krebs/Hepes buffer (Downes *et al.*, 1986*a*) (10 μl) for various lengths of time, in the continued presence of [^{3}H]inositol in the medium.

Extraction of inositol phosphates

The method used was based on that described by Slater *et al.* (1973) for the extraction of adenine nucleotides from mitochondria.

Incubations of [^{3}H]inositol-labelled parotid slices (250 μl) were terminated by addition of chloroform/methanol (1:2, v/v; 0.93 ml) cooled on solid CO_2. The samples were then thawed to between 0 and 4 °C, a portion of ^{32}P-labelled inositol phosphate standards was added {containing a mixture of [^{32}P]Ins1*P*, [^{32}P]Ins(1,2cyc)*P*, [^{32}P]Ins(1,4,5)P_3 and [^{32}P]Ins(1,2cyc4,5)P_3}, and the samples were dried *in vacuo*. Water (250 μl) and 10 mM-Na_2EDTA/5 mM-KH_2PO_4, pH 6.8 (750 μl) were added to the dried residue followed by phenol/chloroform/isoamyl alcohol (38:24:1, by vol.; 1.0 ml). (Phenol was added as a liquid after being heated gently to its melting point.) The samples were mixed vigorously and the resulting two phases were separated by bench centrifugation. Aliquots of the upper phase were then taken for the analysis of inositol phosphates by h.p.l.c. (see below) either directly or after acid-catalysed hydrolysis of any inositol cyclic phosphates present (see below).

The above procedure extracted between 90 and 98% of the following radioactive standards when they were added to unlabelled parotid slice incubations immediately after addition of cold chloroform/methanol: [^{14}C]Ins3*P* (sold as L-Ins1*P* by Amersham International), [^{32}P]Ins(1,4)P_2, [^{32}P]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4.

In a small number of experiments (see the Results section), incubations of ^{3}H-labelled parotid slices (250 μl) were terminated by addition of 10% (v/v) $HClO_4$ (250 μl) on ice. $HClO_4$ was then removed from these samples by extraction with tri-n-octylamine and the [^{3}H]inositol phosphates were analysed by anion-exchange chromatography on AG-1 (X8, 200–400 mesh, formate form) columns, as described by Downes *et al.* (1986*a*).

Anion-exchange chromatography of inositol phosphates

In experiments where analysis of inositol phosphate isomers and their cyclic phosphate derivatives was required (see the Results section), inositol phosphates were separated by h.p.l.c. on Whatman Partisil 10 SAX, using a gradient of increasing concentration of ammonium formate/phosphoric acid (pH 3.7), as described by Batty *et al.* (1985) and Hawkins *et al.* (1986). In the remaining experiments (see the Results section), inositol phosphates were separated on small AG-1 (X8, 200–400 mesh, formate form) columns using batch elution with increasing concentrations of ammonium formate/formic acid (Downes *et al.*, 1986*a*).

Acid-catalysed hydrolysis of cyclic inositol phosphates to their non-cyclic derivatives

The samples containing inositol cyclic phosphates were incubated with 5% (v/v) $HClO_4$ at 25 °C for 10 min. Incubations were neutralized by addition of an equal volume of tri-n-octylamine/Freon (in the proportion 1:1, v/v) (Downes *et al.*, 1986*a*). After vigorous mixing, the resulting three phases were separated by bench centrifugation and an aliquot of the upper phase (pH approx. 7) taken for analysis by anion-exchange h.p.l.c. (see above).

Preparation of ^{32}P-labelled erythrocyte membranes

Human erythrocytes were incubated with [^{32}P]P_i as described by Downes *et al.* (1982) and Hawkins *et al.* (1986). ^{32}P-labelled membranes were prepared from these cells by lysis and washing in 20 mM-Tris/HCl (pH 7.2)/1 mM-EDTA (Downes & Michell, 1981).

Preparation of radioactive inositol phosphate standards

[^{32}P]Ins(1,4,5)P_3 and [^{32}P]Ins(1,2cyc4,5)P_3 were prepared from ^{32}P-labelled erythrocyte membranes by Ca^{2+}-activation of the endogenous PIC (Downes & Michell, 1981). PIC was activated by incubating packed membranes (50 ml) in 20 mM-Tris/acetate (pH 5.5)/1 mM-$CaCl_2$ for 30 min at 37 °C. The incubation was terminated by addition of 4 vol. of ice-cold 2.0 mM-EDTA (pH 7.0) and the membranes were pelleted by centrifugation (15 min at 12000 *g*). The supernatant was applied to two 0.6 ml AG-1 (X8, 200–400 mesh, formate form) columns and InsP_3 was separated from InsP_2 and P_i by elution with batches of increasing concentrations of ammonium formate/formic acid (Downes *et al.*, 1986*a*). The column eluate containing InsP_3 was neutralized by addition of triethylamine and the ammonium formate was removed by a LiCl desalting technique (Grado & Ballou, 1961). This involved addition of a 1:5 dilution (in water) of the eluate to a 200 μl Ag-1 (X8, 200–400 mesh, hydroxide form) column, with InsP_3 being eluted with 1 M-LiCl (3 ml). The LiCl eluate was dried *in vacuo* and the LiCl removed by repeated washing of the dried residue with dry ethanol. The Li^+ salts of the inositol cyclic and non-cyclic trisphosphates were then separated by anion-exchange chromatography on a preparative h.p.l.c. column (25 cm × 2.2 cm), as described above; the minor peak (retention time of 23.25 min, 8–12%) being

[^{32}P]Ins(1,2cyc4,5)P_3 and the major peak (retention time of 25.0 min, 88–92%) being [^{32}P]Ins(1,4,5)P_3.

[^{32}P]Ins1P and [^{32}P]Ins(1,2cyc)P were prepared by PIC-catalysed hydrolysis of [^{32}P]PtdIns (Dawson *et al.*, 1971; Dawson & Clarke, 1972). The [^{32}P]PtdIns was obtained by lipid extraction of parotid slices that had been incubated with [^{32}P]P_i (20 μCi/ml) for 30 min in the presence of 1 mM-carbachol. [^{32}P]PtdIns was partially purified from other phospholipids by ethanol precipitation (Folch, 1949), emulsified with 10 mM-Tris/maleate buffer (pH 5.5), and incubated at 37 °C for 60 min with 1 mM-$CaCl_2$ and a rat liver supernatant (200000 *g*, 10 min) which had been dialysed against this buffer overnight. The incubation was terminated with 3.75 vol. of chloroform/methanol (1:2, v/v), additional chloroform and water were added to give two phases (Bligh & Dyer, 1959), and the water-soluble products in the upper phase were separated by high voltage paper electrophoresis in pyridine/acetic acid (pH 3.5). Two ^{32}P-labelled compounds with the appropriate mobilities relative to P_i of Ins1P and Ins(1,2cyc)P (Dawson *et al.*, 1971) were obtained. Confirmation of the identities of these two compounds was achieved by acid-catalysed hydrolysis of the [^{32}P]Ins(1,2cyc)P (see above); analysis of the products by h.p.l.c. demonstrated the disappearance of [^{32}P]Ins(1,2cyc)P, with the appearance of a single ^{32}P-labelled peak that co-chromatographed with [^{32}P]Ins1P (see the Results section). The structure of Ins(1,2cyc)P was originally deduced from the observation that acid-catalysed hydrolysis of this compound yielded both Ins1P and Ins2P (Dawson *et al.*, 1971). It appears therefore, that our h.p.l.c. system is incapable of separating these two inositol monophosphate isomers.

Identification and characterization of the [^{32}P]Ins(1,2cyc4,5)P_3 standard

When the endogenous PIC of ^{32}P-labelled erythrocyte membranes was activated by Ca^{2+}, [^{32}P]Ins(1,2cyc4,5)P_3 was released together with [^{32}P]Ins(1,4,5)P_3. The proportion of [^{32}P]Ins(1,2cyc4,5)P_3 was greater when the activation by Ca^{2+} was carried out at pH 5.5 (8–12%) rather than at pH 7.4 (< 1%). The peak retention time of [^{32}P]Ins(1,2cyc4,5)P_3 on anion-exchange h.p.l.c. was 1.75 min less than that of Ins(1,4,5)P_3 (see the Results section) and it ran ahead of Ins(1,4,5)P_3 on high voltage electrophoresis in pyridine/acetic acid (pH 3.5) (result not shown).

Treatment of Ins(1,2cyc4,5)P_3 with 1 M-HCl for 2 min at 25 °C (result not shown), or 5% (v/v) $HClO_4$ for 10 min at 25 °C, yielded a compound that co-chromatographed with Ins(1,4,5)P_3 on anion-exchange h.p.l.c. (see the Results section), high voltage electrophoresis and paper chromatography (R. F. Irvine, personal communication; Irvine *et al.*, 1986). Furthermore, h.p.l.c. analysis of acid-treated [^{32}P]Ins(1,2cyc4,5)P_3 always demonstrated a late-eluting shoulder on the peak of [^{32}P]Ins(1,4,5)P_3 produced (see the Results section). This showed a retention time of 1–1.5 minutes after that of Ins(1,4,5)P_3, and amounted to approx. 13% of the recovered radioactivity. Such a retention time corresponds to that expected for Ins(2,4,5)P_3, obtained after alkaline hydrolysis of PtdIns(4,5)P_2 (R. F. Irvine, personal communication). The appearance on acid treatment of both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 provides strong confirmation of the structure of our Ins(1,2cyc4,5)P_3 standard.

Preparation of parotid homogenates

The parotid glands from two male rats (180–200 g) were dissected and homogenized in 8 ml of ice-cold 250 mM-sucrose/50 mM-Tris/HCl (pH 7.4)/1 mM-EGTA, containing 50 μM-phenylmethanesulphonyl fluoride, 1 μg of leupeptin/ml and 10 mg of bovine serum albumin/ml, as described by Hawkins *et al.* (1986). The homogenate was stored on ice and used within 30 min of preparation.

Measurement of the rate of hydrolysis of Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 by a parotid homogenate

Samples of ^{32}P-labelled Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 were incubated, in parallel, with 70 mM-potassium glutamate/40 mM-NaCl/10 mM-Hepes/NaOH (pH 7.2)/4 mM-$MgSO_4$ and a 2.5-fold dilution of a parotid homogenate, prepared as described above. Incubations were at 30 °C, and were terminated after various lengths of time (see the Results section) by addition of an equal volume of ice-cold 10% $HClO_4$. $HClO_4$ was removed by extraction with tri-n-octylamine/Freon (Downes *et al.*, 1986*a*) and the [^{32}P]inositol phosphates were separated by chromatography on small AG-1 columns (Downes *et al.*, 1986*a*).

Measurement of the rate of phosphorylation of Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 by a parotid homogenate

Samples of ^{32}P-labelled Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 were incubated with a 3.3-fold dilution of a parotid homogenate in an identical manner to that described above for measurement of InsP_3 hydrolysis, except that 4 mM-$MgSO_4$ was replaced with 10 mM-MgATP. Incubations were terminated and the [^{32}P]inositol phosphates analysed as described immediately above.

RESULTS

Neutral-pH extraction of inositol phosphates

The purpose of this study was to look for the stimulated accumulation of acid-labile inositol cyclic phosphates in parotid glands. The first step was, therefore, the development of a method for the extraction of inositol phosphates from tissues which avoided the use of low pH. We chose a method based on the mixing of tissue residues with phenol (see the Materials and methods section), since it is widely used as an effective method for the extraction of polar nucleotides (Slater *et al.*, 1973). Phenol extraction was compared with conventional $HClO_4$ extraction in its ability to extract [^{3}H]inositol phosphates from [^{3}H]inositol-labelled parotid slices. The slices were left unstimulated or were stimulated with 1 mM-carbachol for 20 s or 10 min. Identical stimulated increases in the levels of radioactivity in [^{3}H]Ins$P$, [^{3}H]InsP_2, [^{3}H]InsP_3 and [^{3}H]InsP_4 were seen by using both extraction methods (results not shown). However, the levels of radioactivity in the [^{3}H]inositol phosphates, particularly InsP_2 and InsP_3, were consistently higher in phenol extracts of unstimulated slices than in acid extracts of unstimulated slices (results not shown). The reasons for these differences are unknown, although they appear to be related to the addition of chloroform/methanol to slice incubations rather than to the use of phenol, since acid extraction of chloroform/methanol-terminated incuba-

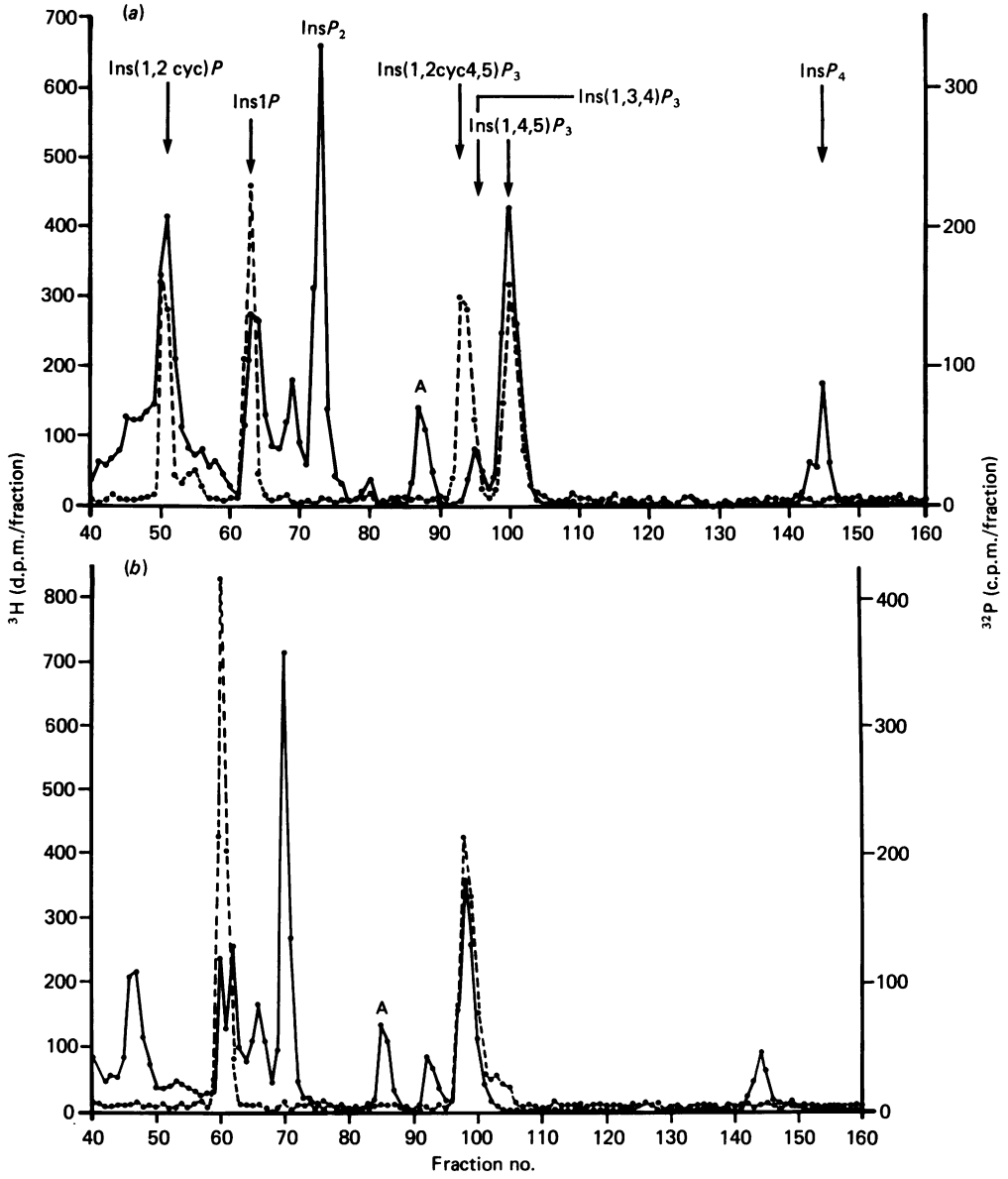

Fig. 1. H.p.l.c. separation of [^{3}H]inositol-labelled water-soluble compounds extracted from rat parotid gland slices

Incubations of [^{3}H]inositol-labelled parotid slices were terminated after a 5 s stimulation with 1 mM-carbachol by addition of cold chloroform/methanol (see the Materials and methods section). ^{32}P-labelled inositol phosphate standards were then added and the tissue was extracted with phenol, as described in the Materials and methods section. Equal aliquots were taken from the upper phase of the phenol extraction and separated either directly (Fig. 1*a*), or after acid-catalysed hydrolysis of any inositol cyclic phosphates present (Fig. 1*b*), by h.p.l.c. on a Whatman Partisil 10 SAX column (see the text). Fractions were collected every 0.25 min and levels of ^{3}H (●——●) and ^{32}P (●---●) radioactivity in each were determined by liquid-scintillation counting.

tions yields the same high unstimulated values for [^{3}H]inositol phosphates (results not shown). It is perhaps worth noting that some of these differences may be explained, in part, by the extraction of novel ^{3}H-labelled compounds; see the discussion of Figs. 1(*a*) and 1(*b*).

Chromatography of inositol phosphate standards by anion-exchange h.p.l.c.

Compounds containing the inositol 1,2-cyclic phosphate structure are known to be acid-labile (Dawson & Clarke, 1972) and Wilson *et al.* (1985*b*) separated cyclic and non-cyclic inositol phosphates by anion-exchange h.p.l.c. using ammonium formate gradients at pH 6.25. Our previously published work described the separation of non-cyclic inositol derivatives on Partisil 10 SAX eluted with an ammonium formate/phosphoric acid gradient at pH 3.7 (Irvine *et al.*, 1985; Hawkins *et al.*, 1986). We therefore assessed the stability of cyclic inositol phosphate standards during chromatography on h.p.l.c. columns eluted exactly as described previously (see Hawkins *et al.*, 1986). The results were identical with those shown in Fig. 1(*a*) in which samples of the same standards were added to [^{3}H]inositol-labelled parotid gland slices and carried through both the extraction and chromatographic procedures. A mixture containing approx. 2000 c.p.m. of each ^{32}P-labelled standard was

Table 1. Carbachol stimulation of Ins1*P* and Ins(1,4,5)P_3 in rat parotid gland slices: effect of acid treatment of neutral extracts

[^{3}H]Inositol-loaded parotid slices were incubated at 37 °C without and with 1 mM-carbachol for 5 s or 5 min, with the inositol phosphates being extracted at neutral pH as described in the Materials and methods section. Aliquots were taken for analysis of inositol phosphates by h.p.l.c. either directly or after acid treatment (see the text). Recoveries of Ins1*P* and Ins(1,4,5)P_3 were calculated from standards included in each of the 18 h.p.l.c. runs (see the text for details). Values are means ± S.E.M. ($n = 3$).

Condition	^{3}H (d.p.m.) in: Ins1*P*	Ins(1,4,5)P_3
Control	282 ± 64	533 ± 60
Control and acid treatment	378 ± 8	436 ± 153
Stimulation (5 s)	293 ± 44	834 ± 84
Stimulation (5 s) and acid treatment	333 ± 70	853 ± 99
Stimulation (5 min)	3077 ± 331	1134 ± 165
Stimulation (5 min) and acid treatment	3979 ± 695	1315 ± 126

injected onto the columns and these were eluted as four discrete peaks with the following recoveries: Ins(1,2cyc)*P* (100%), Ins1*P* (104%), Ins(1,2cyc4,5)P_3 (98%) and Ins(1,4,5)P_3 (106%). This result clearly demonstrates that cyclic inositol phosphates are stable during h.p.l.c. analysis at pH 3.7, a result that might have been anticipated considering the stability of Ins(1,2cyc)*P* when analysed by high voltage electrophoresis at pH 3.5 (Dawson *et al.*, 1971).

H.p.l.c. analysis of inositol phosphates in neutral extracts of parotid gland slices

[^{3}H]Inositol-labelled parotid gland slices were incubated with a supramaximal dose of carbachol (1 mM) for 5 s or 5 min. The incubations were terminated by addition of cold chloroform/methanol (1:2, v/v) as described in the Materials and methods section. ^{32}P-labelled inositol phosphate standards were added at this point and the final tissue extracts, containing [^{3}H]inositol-labelled parotid gland metabolites and ^{32}P-labelled standards, were divided into two equal portions. One portion of each sample was analysed directly by anion-exchange h.p.l.c. and the other portion was acid-treated before analysis (see the Materials and methods section).

Fig. 1(*a*) shows the h.p.l.c. profile obtained for a single sample that had been stimulated with carbachol for 5 s and includes four ^{32}P-labelled peaks with the appropriate retention times for Ins(1,2cyc)*P*, Ins1*P*, Ins(1,2cyc4,5)P_3 and Ins(1,4,5)P_3 (in order of elution). Both cyclic and non-cyclic standards were quantitatively recovered through the extraction and chromatography steps. The average recovery for all standards from nine tissue samples and 18 h.p.l.c. runs was 88 ± 5% (acid-treated samples) and 106 ± 3% (not acid-treated).

[^{3}H]Inositol-labelled compounds that co-chromatographed precisely with standard Ins1*P* and Ins(1,4,5)P_3 were observed in 18 separate h.p.l.c. runs. By contrast, none of the samples contained a ^{3}H-labelled compound that corresponded to Ins(1,2cyc4,5)P_3. This is illustrated in Fig. 1(*a*) where the centre of the ^{32}P-labelled Ins(1,2cyc4,5)P_3 peak corresponds to background radioactivity in the ^{3}H window. We estimate that [^{3}H]Ins(1,2cyc4,5)P_3 would have been detected if it amounted to more than 5% of the peak value for [^{3}H]Ins(1,4,5)P_3. The occurrence of [^{3}H]Ins(1,2cyc)*P* was more difficult to analyse because the standard was eluted in a region of the chromatogram that contained a complex ^{3}H peak, and it was not possible to say whether this peak contained a compound with identical chromatographic properties to the standard. Other peaks marked on the Figure have been identified in previous studies (Irvine *et al.*, 1984*b*; Hawkins *et al.*, 1986). Of particular relevance to the present experiments, Ins(1,3,4)P_3 was identified as the major trisphosphate in parotid glands after prolonged stimulation with carbachol (Irvine *et al.*, 1984*b*). This compound elutes approx. 1.5 min before Ins(1,4,5)P_3 and partially overlaps the Ins(1,2cyc4,5)P_3 standard. However, it is clearly distinguishable from the cyclic trisphosphate because its peak elutes 0.5 min (two fractions) after the ^{32}P-labelled standard and because it is not affected by acid treatment (see below and Fig. 1*b*).

Samples that were acid-treated before h.p.l.c. analysis (Fig. 1*b*) were characterized by the occurrence of only two major ^{32}P-labelled peaks, corresponding to the Ins1*P* and Ins(1,4,5)P_3 peaks in Fig. 1(*a*). Acid-catalysed hydrolysis of the 1,2-cyclic phosphate derivatives would be expected to yield inositol phosphates with phosphate groups in the 1 or 2 positions. This appears to be the case for the cyclic trisphosphate (see the Materials and methods section), but a single inositol monophosphate peak was obtained with the retention time of Ins1*P*. We suggest that Ins1*P* and Ins2*P* are not separated under these chromatographic conditions. There was, however, very little difference between the [^{3}H]inositol-labelled profiles in Figs. 1(*a*) and 1(*b*), indicating that inositol cyclic phosphate derivatives make little or no contribution to the water-soluble inositol metabolites in parotid glands.

Some ^{3}H-labelled peaks remain unidentified at present, but may correspond to compounds such as Ins4*P*, which has been identified as a product of Ins(1,4)P_2 metabolism (Storey *et al.*, 1985), and Ins(3,4)P_2 and Ins(1,3)P_2, which we have found to be products of Ins(1,3,4)P_3 hydrolysis is parotid gland homogenates (P. T. Hawkins, A. Morris & C. P. Downes, unpublished work). Of particular note is the unidentified ^{3}H-labelled peak marked A in Figs. 1(*a*) and 1(*b*). This compound is not seen in $HClO_4$ extracts of ^{3}H-labelled parotid slices (compare with Hawkins *et al.*, 1986), but appears in extracts prepared from slices terminated by addition of neutral chloroform/methanol and extracted with either phenol (see the Materials and methods section) or a standard Bligh & Dyer (1959) phase partition (results not shown). It runs approx. 1 min later than [^{32}P]glycerophosphoinositol 4,5-bisphosphate on our h.p.l.c. system (results not shown), it is not acid-labile (compare Figs. 1*a* and 1*b*) and its levels appear unaffected by stimulation of parotid slices with 1 mM-carbachol.

Table 1 shows the combined results for control and carbachol-stimulated samples in which we determined the levels of [^{3}H]Ins1*P* and [^{3}H]Ins(1,4,5)P_3. We have concentrated on these two compounds because they are the only ones for which there is a corresponding ^{32}P-labelled standard in each chromatogram. As noted in previous studies (Irvine *et al.*, 1985), [^{3}H]Ins(1,4,5)P_3

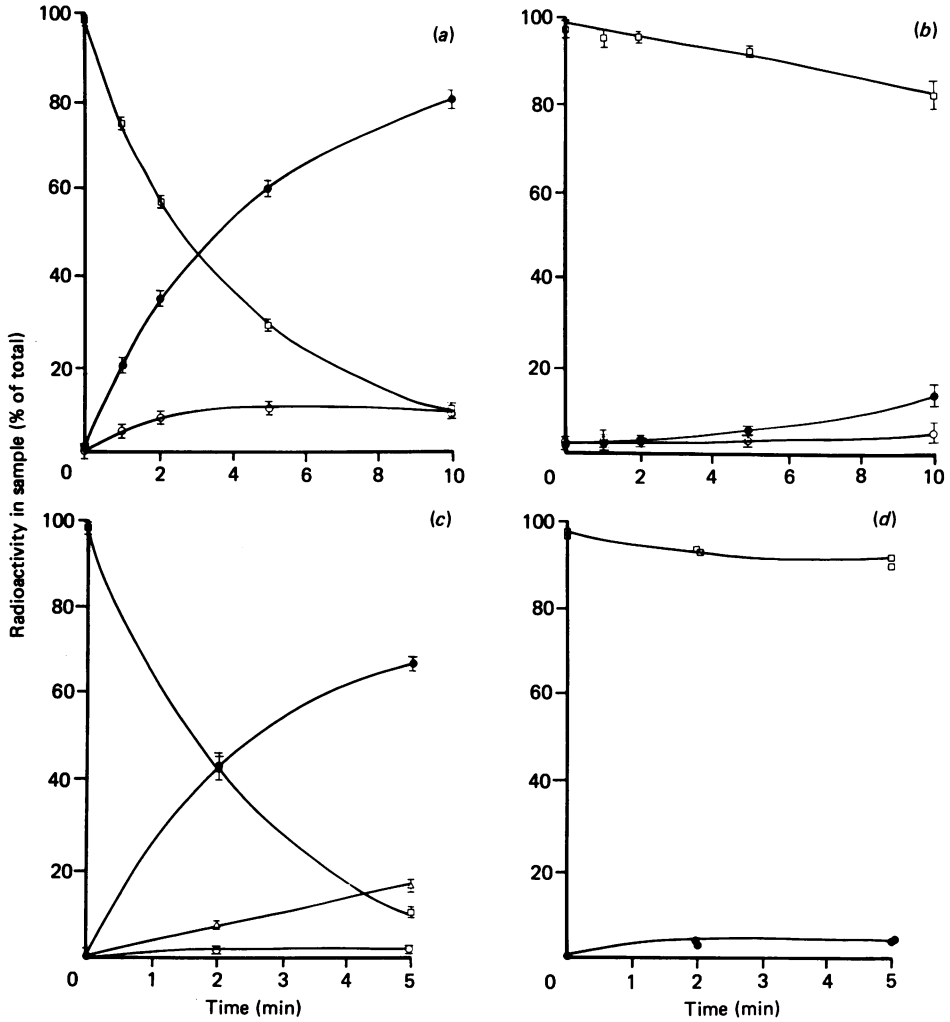

Fig. 2. Metabolism of Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 by a parotid homogenate

Aliquots of ^{32}P-labelled Ins(1,2cyc4,5)P_3 and Ins(1,4,5)P_3 were incubated, in parallel, with samples of parotid homogenate at 30 °C. The assays of InsP_3 hydrolysis (*a* and *b*) were carried out in the presence of 2 mM-Mg^{2+}; the assays of InsP_3 phosphorylation (*c* and *d*) were carried out in the presence of 10 mM-Mg ATP (see the text). The incubations were terminated after various lengths of time by adding $HClO_4$ and the neutralized extracts were separated by anion-exchange chromatography on small AG-1 (X8, 200–400 mesh, formate form) columns. All data are expressed as the means ± S.E.M. ($n = 3$) of, or individual determinations of, the amount of radioactivity in the appropriate column eluant (see Downes *et al.*, 1986*a*). (*a*) Hydrolysis of [^{32}P]Ins(1,4,5)P_3 (430 c.p.m./sample, 3.7 μM): InsP_3 (□), InsP_2 (○), P_i+Ins$P$ (●). (*b*) Hydrolysis of [^{32}P]Ins(1,2cyc4,5)P_3 (400 c.p.m./sample, 3.5 μM): InsP_3 (□), InsP_2 (○), P_i+Ins$P$ (●). (*c*) Phosphorylation of [^{32}P]Ins(1,4,5)P_3 (234 c.p.m./sample, 2.3 μM): InsP_3 (□), InsP_4 (●), InsP_2 (○), P_i+Ins$P$ (△). (*d*) Phosphorylation of [^{32}P]Ins(1,2cyc4,5)P_3 (212 c.p.m./sample, 2.1 μM): InsP_3 (□), InsP_4 (●).

increased sharply within 5 s of carbachol stimulation and remained elevated at 5 min. Ins1P, on the other hand, showed no change after 5 s, but had increased substantially after 5 min exposure to carbachol. This demonstrates that the carbachol-induced effects on the levels of these compounds are not an artefact caused by the use of acid extraction conditions.

Not only was there no evidence for Ins(1,2cyc4,5)P_3 in samples that had been stimulated for 5 s (Figs. 1*a* and 1*b*), but there was also no evidence for the occurrence of this compound in control samples nor for its accumulation during prolonged (5 min) exposure to the agonist. This is illustrated in Table 1 by the failure of acid treatment to increase significantly the amount of radioactivity associated with the peak of Ins(1,4,5)P_3. Acid treatment did cause a small, apparently insignificant, increase in the [^{3}H]Ins1P peak and the error associated with this measurement means that we cannot eliminate the possibility that any Ins(1,2cyc)P could have contributed $\leqslant 10\%$ to the Ins1P peak.

Metabolism of inositol cyclic phosphates by parotid gland homogenates

If inositol cyclic phosphate derivatives are metabolized more rapidly than their non-cyclic counterparts they will be less likely to accumulate in stimulated cells [compare the accumulation of rapidly metabolized Ins(1,4,5)P_3 with that of the more slowly metabolized Ins(1,3,4)P_3 in Irvine *et al.*, (1985)]. We therefore directly compared the rates of metabolism of Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 by phosphatase and kinase activities present in parotid gland homogenates (see Hawkins *et al.*, 1986). The

^{32}P-labelled inositol trisphosphates used in these studies had the same specific radioactivity, because they were both products of PIC attack upon the same preparation of erythrocyte membrane [^{32}P]PtdIns(4,5)P_2. In the absence of ATP, Ins(1,4,5)P_3 was rapidly dephosphorylated with a half-life of approx. 2.5 min to give mainly InsP_2 and P_i (Fig. 2*a*). When ATP was present, some dephosphorylation still occurred, but the majority of Ins(1,4,5)P_3 was converted into labelled InsP_4 (Fig. 2*c*). By contrast, Ins(1,2cyc4,5)P_3 was metabolized very slowly compared with the non-cyclic compound, whether or not ATP was present in the incubations (Figs. 2*b* and 2*d*). This suggests that parotid gland homogenates contain kinase and phosphatase activities that utilize Ins(1,4,5)P_3 as a substrate, but are much less active against its cyclic counterpart. Assuming the expression of these enzyme activities under the conditions employed in the assays reasonably reflects the situation in intact cells, our failure to detect significant quantities of [^{3}H]Ins(1,2cyc4,5)P_3 in parotid slices is not due to its rapid metabolism. Indeed, if the cyclic trisphosphate was being generated at a significant rate it would be expected to accumulate to much higher levels than Ins(1,4,5)P_3 itself. This is clearly not the case in carbachol-stimulated parotid gland slices.

DISCUSSION

We have demonstrated that inositol 1,2-cyclic phosphate standards can be efficiently extracted from tissue slices at neutral pH and quantitatively eluted from Partisil 10 SAX columns using a salt gradient at pH 3.7. The cyclic phosphate esters were stable at pH 3.7, but were rapidly hydrolysed in 1 M-HCl (results not shown) or 5% $HClO_4$ to give the expected non-cyclic compounds. Thus these methods are likely to be suitable to assess the importance of Ins(1,2cyc4,5)P_3 in a variety of tissues and cells which show agonist-dependent hydrolysis of inositol phospholipids.

Experiments using parotid gland slices or dispersed acinar cells have demonstrated that PtdIns(4,5)P_2 is likely to be the primary target for the muscarinic receptor-activated PIC and that a similar mechanism is likely to operate for both α_1 and substance P receptors present on these cells (Downes & Wusteman, 1983; Aub & Putney, 1984). The present work indicates that Ins(1,4,5)P_3 is the only detectable, water-soluble product of this reaction. Many of the other [^{3}H]inositol phosphates found in agonist-stimulated parotid glands can be produced, directly or indirectly, from Ins(1,4,5)P_3 by kinase or phosphomonoesterase activities (Hawkins *et al.*, 1986), and their accumulation to much higher levels than Ins(1,4,5)P_3 can be accounted for by their much slower rates of metabolism (Downes & Wusteman, 1983; Irvine *et al.*, 1985).

Dixon & Hokin (1985) have identified Ins(1,2cyc)P in caerulein-stimulated pancreatic mini-lobules. This compound appeared to accumulate at a slow rate yet, even after prolonged stimulation, comprised only a small proportion of the total inositol phosphates. Such levels of the cyclic monophosphate would not have been detected in our experiments because of the presence of acid-stable [^{3}H]inositol-containing compounds that eluted in the same part of the gradient as Ins(1,2cyc)P. As suggested by Dixon & Hokin (1985), their results may indicate a relatively slow rate of hydrolysis of PtdIns to Ins1P and Ins(1,2cyc)P in exocrine pancreas, but the significance of such a reaction as a response of the glands to agonists is unknown, since it would not be expected to contribute significantly to diacylglycerol formation and no function has yet been found for the cyclic or non-cyclic monophosphate.

An essential feature of second messengers is that they should be rapidly metabolized so that their potent intracellular effects can be efficiently reversed. Indeed, the discovery of an enzyme that specifically dephosphorylated Ins(1,4,5)P_3 to Ins(1,4)P_2 was an important clue to the likely importance of Ins(1,4,5)P_3 (Downes *et al.*, 1982). More recently a second route for metabolism of Ins(1,4,5)P_3, involving ATP-dependent phosphorylation to Ins(1,3,4,5)P_4, has been described (Irvine *et al.*, 1986; Hawkins *et al.*, 1986). Both pathways are catalysed by parotid gland homogenates, but Ins(1,2cyc4,5)P_3 is a very poor substrate for endogenous phosphatases or kinases compared with the rapid metabolism of the non-cyclic compound. These results contrast with previous observations showing that Ins(1,2cyc4,5)P_3 is a substrate for a phosphatase in a platelet cytosol fraction (Connolly *et al.*, 1986) and for a kinase in rat brain cytosol (Irvine *et al.*, 1986). However, both these groups used high concentrations of Ins(1,4,5)P_3 and Ins(1,2cyc4,5)P_3 and may not have detected differences in their rates of metabolism due to a difference in the K_m values of the relevant enzymes for the cyclic and non-cyclic substrates.

Both cytosolic (Dawson & Clarke, 1972) and membrane-bound PICs (see the Material and methods section and Irvine *et al.*, 1986) can generate inositol cyclic phosphates during hydrolysis of inositol phospholipids *in vitro*, but the cyclic derivatives do not contribute significantly to the large increases in water-soluble inositol metabolites in carbachol-stimulated parotid glands. As noted in the Materials and methods section, 8–12% of the InsP_3 obtained when human erythrocyte membranes were incubated with Ca^{2+} at pH 5.5 was Ins(1,2cyc4,5)P_3. However, it should be noted that the Ca^{2+} concentration used to activate the erythrocyte enzyme *in vitro* was approx. 1000 times greater than the Ca^{2+} concentration attained in carbachol-stimulated parotid gland acinar cells (Downes *et al.*, 1986*b*). Furthermore, low pH is known to favour the formation of Ins(1,2cyc)P from PtdIns (Dawson & Clarke, 1972) but the pH within healthy cells is more likely to be close to 7.4 rather than 5.5.

The situation is further complicated by the occurrence of multiple forms of PIC in some tissues (Low & Weglicki, 1983: Hirasawa *et al.*, 1982), raising the possibility that some forms of PIC, but not others, could yield inositol cyclic phosphates. There is a clear need to examine the products generated by these and other forms of PIC in a wide variety of tissues, and to compare these results with the spectrum of inositol phosphates that accumulate during agonist stimulation. An important goal will be the elucidation of assay conditions that accurately reflect the control of PIC in the cell by receptors and GTP-dependent regulatory proteins (see Downes & Michell, 1985, for review).

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