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 [3H]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. 22P-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 quantitatively recovered through both extraction and chromatography steps. Treatment of inositol cyclic phosphate standards with 5% (w/v) HClO4 for 10 min prior to chromatography resulted in formation of the expected non-cyclic compounds. [3H]inositol 1-phosphate and [3H]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 [3H]inositol 1,2-cyclic phosphate or [3H]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)P2] 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)P2 (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)P2 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)P2 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 (Ins1.2cycP), with the proportions of these two water-soluble products depending upon the pH of the incubation medium. Irvine et al. (1984a) later found that the soluble enzyme could attack phosphatidylinositol 4-phosphate (PtdIns4P) and PtdIns(4,5)P2 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)P2 was the substrate (Wilson et al., 1985a). These products were Ins(1,4,5)P3 and inositol 1,2-cyclic 4,5-trisphosphate [Ins(1,2cyc4,5)P3]. The previous studies, which pointed to PtdIns(4,5)P2 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: InsP, InsP2, InsP3, InsP4, inositol mono-, bis-, tris- and tetrakis-phosphates; locants where specified are placed in parentheses, e.g. Ins(1,2cyc4,5)P3 is inositol 1,2-cyclic 4,5-trisphosphate; the d isomers are assumed throughout; PtdIns, PtdIns4P, PtdIns(4,5)P2, phosphatidylinositol and its 4-mono- and 4,5-bis-phosphates; PIC, phosphoinositidase C.
triphosphates 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)P₃ has been shown to release Ca²⁺ 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)P₃ when added to permeabilized platelets and when injected into Limulus photoreceptors (Wilson et al., 1985b). 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., 1986a; 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 [³²P]Ins(1,4,5)P₃ (3–4 μCi/250 μl) for 90 min at 37 °C, as described by Downes et al. (1986a). Small incubations (250 μl) were then challenged with either carbachol (10 μl, 1 mM final concn.) or Krebs/Hepes buffer (Downes et al., 1986a) (10 μl) for various lengths of time, in the continued presence of [³²P]Ins(1,4,5)P₃ 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 [³²P]Ins(1,4,5)P₃-labelled parotid slices (250 μl) were terminated by addition of chloroform/methanol (1:2, v/v; 0.93 ml) cooled on solid CO₂. The samples were then thawed to between 0 and 4 °C, a portion of ³²P-labelled inositol phosphate standards was added containing a mixture of [³²P]Ins-1P, [³²P]Ins(1,2cyc)P, [³²P]Ins(1,4,5)P₃ and [³²P]Ins(1,2cyc,4,5)P₄, and the samples were dried in vacuo. Water (250 μl) and 10 mM Na₂EDTA/5 mM KH₂PO₄, 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: [³¹C]Ins-3P (sold as L-Ins-3P by Amersham International), [³²P]Ins(1,4,5)P₃, [³²P]Ins(1,2cyc)P, [³²P]Ins(1,4,5)P₃, [³²P]Ins(1,3,4,5)P₄ and [³²P]Ins(1,3,4,5)P₄.

In a small number of experiments (see the Results section), incubations of ³²H-labelled parotid slices (250 μl) were terminated by addition of 10% (v/v) HClO₄ (250 μl) on ice. HClO₄ was then removed from these samples by extraction with tri-n-octylamine and the [³H]inositol phosphates were analysed by anion-exchange chro-matography on AG-1 (X8, 200–400 mesh, formate form) columns, as described by Downes et al. (1986a).

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., 1986a).

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

The samples containing inositol cyclic phosphates were incubated with 5% (v/v) HClO₄ 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., 1986a). 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 ³²P-labelled erythrocyte membranes

Human erythrocytes were incubated with [³²P]P₁ as described by Downes et al. (1982) and Hawkins et al. (1986). ³²P-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

[³²P]Ins(1,4,5)P₃ and [³²P]Ins(1,2cyc,4,5)P₄ were prepared from ³²P-labelled erythrocyte membranes by Ca⁺⁺-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-CaCl₂ 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 Ins₃P was separated from Ins₅P and P₄ by elution with batches of increasing concentrations of ammonium formate/formic acid (Downes et al., 1986a). The column eluate containing Ins₃P was neutralized by addition of triethylenamine 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 Ins₅P 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 triphosphates 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
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[32P]Ins(1,2cyc4,5)P₃ and the major peak (retention time of 25.0 min, 88–92%) being [32P]Ins(1,4,5)P₃.

[32P]Ins1P and [32P]Ins(1,2cyc)P were prepared by PIP-catalysed hydrolysis of [32P]PtdIns (Dawson et al., 1971; Dawson & Clarke, 1972). The [32P]PtdIns was obtained by lipid extraction of parotid slices that had been incubated with [32P]P, (20 μCi/ml) for 30 min in the presence of 1 mm-carbachol. [32P]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-CaCl₂ and a rat liver supernatant (20 000 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 32P-labelled compounds with the appropriate mobilities relative to P₁ 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 [32P]Ins(1,2cyc)P (see above); analysis of the products by h.p.l.c. demonstrated the disappearance of [32P]Ins(1,2cyc)P, with the appearance of a single 32P-labelled peak that co-chromatographed with [32P]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 [32P]Ins(1,2cyc4,5)P₃ standard

When the endogenous PIC of 32P-labelled erythrocyte membranes was activated by Ca²⁺, [32P]Ins(1,2cyc4,5)P₃ was released together with [32P]Ins(1,4,5)P₃. The proportion of [32P]Ins(1,2cyc4,5)P₃ was greater when the activation by Ca²⁺ was carried out at pH 5.5 (8–12%) rather than at pH 7.4 (< 1%). The peak retention time of [32P]Ins(1,2cyc4,5)P₃ on anion-exchange h.p.l.c. was 1.75 min less than that of Ins(1,4,5)P₃ (see the Results section) and it ran ahead of Ins(1,4,5)P₃ on high voltage electrophoresis in pyridine/acetic acid (pH 3.5) (result not shown).

Treatment of Ins(1,2cyc4,5)P₃ with 1 m-HCl for 2 min at 25 °C (result not shown), or 5% (v/v) HClO₄ for 10 min at 25 °C, yielded a compound that co-chromatographed with Ins(1,4,5)P₃ 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 [32P]Ins(1,2cyc4,5)P₃ always demonstrated a late-eluting shoulder on the peak of [32P]Ins(1,4,5)P₃ produced (see the Results section). This showed a retention time of 1–1.5 minutes after that of Ins(1,4,5)P₃ and amounted to approx. 13% of the recovered radioactivity. Such a retention time corresponds to that expected for Ins(2,4,5)P₃, obtained after alkaline hydrolysis of PtdIns(4,5)P₂ (R. F. Irvine, personal communication). The appearance on acid treatment of both Ins(1,4,5)P₃ and Ins(2,4,5)P₃ provides strong confirmation of the structure of our Ins(1,2cyc4,5)P₃ 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-HCl (pH 7.4)/1 mm-EGTA, containing 50 μm-phenylmethylsulphonyl 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)P₃ and Ins(1,2cyc4,5)P₃ by a parotid homogenate

Samples of 32P-labelled Ins(1,4,5)P₃ and Ins(1,2cyc4,5)P₃ were incubated, in parallel, with 70 mm-potassium glutamate/40 mm-NaCl/10 mm-Hepes/NaOH (pH 7.2)/4 mm-MgSO₄ 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% HClO₄. HClO₄ was removed by extraction with tri-n-octylamine/Fe₂O₃ (Downes et al., 1986a) and the 32Pinositol phosphates were separated by chromatography on small AG-1 columns (Downes et al., 1986a).

Measurement of the rate of phosphorylation of Ins(1,4,5)P₃ and Ins(1,2cyc4,5)P₃ by a parotid homogenate

Samples of 32P-labelled Ins(1,4,5)P₃ and Ins(1,2cyc4,5)P₃ were incubated with a 3.3-fold dilution of a parotid homogenate in an identical manner to that described above for measurement of InsP₃ hydrolysis, except that 4 mm-MgSO₄ was replaced with 10 mm-MgATP. Incubations were terminated and the 32Pinositol 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 HClO₄ extraction in its ability to extract [3H]inositol phosphates from [3H]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 [3H]InsP₂, [3H]InsP₃, and [3H]InsP₄ were seen by using both extraction methods (results not shown). However, the levels of radioactivity in the [3H]inositol phosphates, particularly InsP₂ and InsP₃, 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-
Incubations of [3H]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). 32P-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. 1a), or after acid-catalysed hydrolysis of any inositol cyclic phosphates present (Fig. 1b), 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 3H (○) and 32P (●) radioactivity in each were determined by liquid-scintillation counting. 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 [3H]inositol-labelled parotid gland slices and carried through both the extraction and chromatographic procedures. A mixture containing approx. 2000 c.p.m. of each 32P-labelled standard was...
Table 1. Carbachol stimulation of InsLP and Ins(1,4,5)P3 in rat parotid gland slices: effect of acid treatment of neutral extracts

<table>
<thead>
<tr>
<th>Condition</th>
<th>InsLP (d.p.m.)</th>
<th>Ins(1,4,5)P3 (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>282 ± 64</td>
<td>533 ± 60</td>
</tr>
<tr>
<td>Control and acid treatment</td>
<td>378 ± 8</td>
<td>436 ± 153</td>
</tr>
<tr>
<td>Stimulation (5 s)</td>
<td>293 ± 54</td>
<td>834 ± 84</td>
</tr>
<tr>
<td>Stimulation (5 s) and acid treatment</td>
<td>333 ± 70</td>
<td>853 ± 99</td>
</tr>
<tr>
<td>Stimulation (5 min)</td>
<td>3077 ± 331</td>
<td>1134 ± 165</td>
</tr>
<tr>
<td>Stimulation (5 min) and acid treatment</td>
<td>3979 ± 695</td>
<td>1315 ± 126</td>
</tr>
</tbody>
</table>

injected onto the columns and these were eluted for four discrete peaks with the following recoveries: Ins(1,2cyc4,5)P3 (100%), InsLP (104%), Ins(1,2cyc4,5)P3 (98%) and Ins(1,4,5)P3 (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(1,2cyc4,5)P3 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

[3H]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. [3P]-labelled inositol phosphate standards were added at this point and the final tissue extracts, containing [3H]inositol-labelled parotid gland metabolites and [3P]-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 [3P]-labelled peaks with the appropriate retention times for Ins(1,2cyc4,5)P3, InsLP, Ins(1,2cyc4,5)P3 and Ins(1,4,5)P3 (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).

[3H]Inositol-labelled compounds that co-chromatographed precisely with standard InsLP and Ins(1,4,5)P3 were observed in 18 separate h.p.l.c. runs. By contrast, none of the samples contained a [3H]-labelled compound that corresponded to Ins(1,2cyc4,5)P3. This is illustrated in Fig. 1(a) where the centre of the [3P]-labelled Ins(1,2cyc4,5)P3 peak corresponds to background radioactivity in the [3H] window. We estimate that [3H]Ins(1,2cyc4,5)P3 would have been detected if it amounted to more than 5% of the peak value for [3H]Ins(1,4,5)P3. The occurrence of [3H]Ins(1,2cyc4,5)P3 was more difficult to analyse because the standard was eluted in a region of the chromatogram that contained a complex [3H] 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., 1984b; Hawkins et al., 1986). Of particular relevance to the present experiments, Ins(1,3,4)P3 was identified as the major trisphosphate in parotid glands after prolonged stimulation with carbachol (Irvine et al., 1984b). This compound elutes approx. 1.5 min before Ins(1,4,5)P3 and partially overlaps the Ins(1,2cyc4,5)P3 standard. However, it is clearly distinguishable from the cyclic trisphosphate because its peak elutes 0.5 min (two fractions) after the [3P]-labelled standard and because it is not affected by acid treatment (see below and Fig. 1b). Samples that were acid-treated before h.p.l.c. analysis (Fig. 1b) were characterized by the occurrence of only two major [3P]-labelled peaks, corresponding to the InsLP and Ins(1,4,5)P3 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 InsLP. We suggest that InsLP and Ins2P are not separated under these chromatographic conditions. There was, however, very little difference between the [3H]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 [3H]-labelled peaks remain unidentified at present, but may correspond to compounds such as Ins4P, which has been identified as a product of Ins(1,4)P3 metabolism (Storey et al., 1985), and Ins(3,4)P2 and Ins(1,3)P2, which we have found to be products of Ins(1,3,4)P3 glycerophosphoinositol 4,5-bisphosphate on our h.p.l.c. system (results not shown), it is not acid-labile (compare Figs. 1a and 1b) and its level was not increased 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 [3H]InsLP and [3H]Ins(1,4,5)P3. We have concentrated on these two compounds because they are the only ones for which there is a corresponding [3P]-labelled standard in each chromatogram. As noted in previous studies (Irvine et al., 1985), [3H]Ins(1,4,5)P3

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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., 1986a). (a) Hydrolysis of $[^{32}$P]Ins(1,4,5)P$_3$ (430 c.p.m./sample, 3.7 μM): InsP$_3$ (□), InsP$_3$ (○), P$_1$ + InsP (●). (b) Hydrolysis of $[^{32}$P]Ins(1,2cyc4,5)P$_3$ (400 c.p.m./sample, 3.5 μM): InsP$_3$ (□), InsP$_3$ (○), P$_1$ + InsP (●). (c) Phosphorylation of $[^{32}$P]Ins(1,4,5)P$_3$ (234 c.p.m./sample, 2.3 μM): InsP$_3$ (□), InsP$_3$ (●), InsP$_3$ (○), P$_1$ + InsP (△). (d) Phosphorylation of $[^{32}$P]Ins(1,2cyc4,5)P$_3$ (212 c.p.m./sample, 2.1 μM): InsP$_3$ (□), InsP$_3$ (●).

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. 1a and 1b), 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 ≤ 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$_4$ 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).

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$^{32}$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 Ins$P_2$ and P$_1$ (Fig. 2a). When ATP was present, some dephosphorylation still occurred, but the majority of Ins(1,4,5)$P_3$ was converted into labelled Ins$P_2$ (Fig. 2c). 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. 2b and 2d). 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 $x_1$ 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 carauline-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 Ins1$P$ 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 Ins$P_2$ 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., 1986b). 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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