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Inositol 1,3,4,5-tetrakisphosphate and not phosphatidylinositol 3,4-bisphosphate is the probable precursor of inositol 1,3,4-trisphosphate in agonist-stimulated parotid gland

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When [³H]inositol-prelabelled rat parotid-gland slices were stimulated with carbachol, noradrenaline or Substance P, the major inositol trisphosphate produced with prolonged exposure to agonists was, in each case, inositol 1,3,4-trisphosphate. Much lower amounts of radioactivity were present in the inositol 1,4,5-trisphosphate fraction separated by anion-exchange h.p.l.c. Analysis of the inositol trisphosphate head group of phosphatidylinositol bisphosphate in [³²P]P_i-labelled parotid glands showed the presence of phosphatidylinositol 4,5-bisphosphate, but no detectable phosphatidylinositol 3,4-bisphosphate. Carbachol-stimulated [³H]inositol-labelled parotid glands contained an inositol polyphosphate with the chromatographic properties and electrophoretic mobility of an inositol tetrakisphosphate, the probable structure of which was determined to be inositol 1,3,4,5-tetrakisphosphate. Since an enzyme in erythrocyte membranes is capable of degrading this tetrakisphosphate to inositol 1,3,4-trisphosphate, it is suggested to be the precursor of inositol 1,3,4-trisphosphate in parotid glands.

INTRODUCTION

Many neurotransmitters and hormones acting at cell-surface receptors have been shown to stimulate the hydrolysis of a membrane lipid, phosphatidylinositol bisphosphate (PtdInsP_2), to give inositol trisphosphate (InsP_3) and diacylglycerol. PtdInsP_2 in brain has monoester phosphates on the 4- and 5-positions in the *myo*-inositol ring (Grado & Ballou, 1961), and therefore inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is the expected InsP_3 arising from phospholipase C attack on PtdInsP_2. Both diacylglycerol and Ins(1,4,5)P_3 have been shown to have second-messenger functions, with diacylglycerol activating protein kinase C and Ins(1,4,5)P_3 causing release of Ca^{2+} from a membrane-associated pool in the endoplasmic reticulum (for reviews, see Nishizuka, 1984; Berridge & Irvine, 1984; Downes and Michell, 1985). Detection and measurement of InsP_3 in agonist-stimulated tissues is therefore of considerable importance to an understanding of the control of cellular physiology through activation of receptors at the cell surface.

In [³H]inositol-prelabelled parotid-gland slices stimulated with cholinergic agonists, most of the InsP_3 that accumulates after 10 min exposure to the agonists is inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3], with only about 10% of the expected Ins(1,4,5)P_3 isomer (Irvine *et al.*, 1984, 1985). Ins(1,4,5)P_3 is formed immediately on stimulation and is rapidly degraded after atropine blockade of previously activated muscarinic receptors, in keeping with its proposed second-messenger role. Ins(1,3,4)P_3 is only formed after an initial delay of about 5 s and is more slowly metabolized than is the 1,4,5-isomer (Irvine *et al.*, 1985). Very similar results have been obtained by using [³H]inositol-labelled isolated hepatocytes stimulated with angiotensin (Burgess *et al.*, 1985). Both the role and the source of Ins(1,3,4)P_3 require further investigation.

We have now determined the inositol trisphosphates present in [³H]inositol-labelled parotid glands stimulated with noradrenaline and substance P, which, like cholinergic agonists, stimulate hydrolysis of PtdInsP_2 (Weiss *et al.*, 1982). We have also demonstrated that the likely source of Ins(1,3,4)P_3 is not phosphatidylinositol 3,4-bisphosphate, but a novel inositol polyphosphate, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], as described in cholinergically stimulated rat brain slices (Batty *et al.*, 1985).

MATERIALS AND METHODS

Materials

Carbachol and atropine were obtained from Sigma. Freon (1,1,2-trichlorotrifluoroethane) and tri-n-octylamine were from BDH. Pre-packed 25 cm-long Partisil 10 SAX columns were supplied by Technicol, Stockport, U.K. AG1 and AG50 ion-exchange resins were from Bio-Rad. [³H]Inositol was purchased from New England Nuclear, and [³²P]P_i was supplied by Amersham. All reagents and chemicals were of the highest grade commercially available.

Tissue incubations

Parotid-gland slices were prepared as described previously (Downes *et al.*, 1983), except that the incubation medium contained 20 mM-Hepes (pH 7.4) in place of $NaHCO_3$ and was gassed with O_2 rather than O_2/CO_2 (19:1).

For experiments involving labelling with [³H]inositol, 50 μl portions of washed parotid-gland slices (0.75–1 mg

Abbreviations used: Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; InsP_3, inositol trisphosphate (isomeric form unspecified); PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdInsP_2, phosphatidylinositol bisphosphate (isomeric form unspecified).

of protein) were transferred to Beckman Biovials containing 200 μl of incubation medium and 1–2 μCi of [^{3}H]inositol (10–20 Ci/mmol). The slices were labelled for 90 min at 37 °C in a gently shaking water bath before addition of agonists as detailed in the legends to Figures. For experiments involving labelling with [^{32}P]P$_i$, parotid-gland slices (50 μl) were transferred to Beckman Biovials containing 200 μl of incubation medium (lacking phosphate) and 20 μCi of [^{32}P]P$_i$ (carrier-free). The slices were labelled for 60 min (sufficient to label the monoester phosphates of PtdIns$P$ and PtdInsP_2 close to isotopic equilibrium with the cellular ATP pool; see Weiss *et al.*, 1982), before addition of agonists as detailed in the legends to Figures.

For analysis of [^{3}H]inositol-labelled water-soluble components, the incubations were terminated by addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid. Trichloroacetic acid was removed and the samples were prepared for chromatographic procedures exactly as described previously (Irvine *et al.*, 1985). An alternative method, essentially that of Sharpes & McCarl (1982), involved terminating the incubations by addition of an equal volume of ice-cold 10% (v/v) $HClO_4$. After 10 min on ice, the tissue pellets were centrifuged for 5 min at 2000 rev./min in a Beckman bench-top centrifuge, and 400 μl of the supernatant from each sample was transferred to a separate tube containing 100 μl of 10 mM-EDTA (pH 7.0). The samples were neutralized by adding 300 μl of a 1:1 (v/v) mixture of Freon and tri-n-octylamine, followed by vigorous mixing of the separate phases on a vortex mixer. After centrifugation for 1 min at 2000 rev./min, three phases are obtained. The lower phase is Freon plus any tri-n-octylamine that had not reacted; the middle phase is tri-n-octylamine perchlorate; and the upper phase is the neutralized sample plus all water-soluble components. A 400 μl portion of the upper phase was removed for subsequent analysis; see below.

Lipid extractions were carried out on tissue incubations that were terminated by addition of 0.94 ml of chloroform/methanol/HCl (40:80:1, by vol.). Dried lipid extracts were prepared exactly as described previously (Downes & Wusteman, 1983).

Preparation of standards

^{32}P-labelled Ins(1,4,5)P_3 was prepared from erythrocyte membranes essentially as described previously (Downes *et al.*, 1982). Standard samples recovered from AG1 columns in ammonium formate/formic acid mixtures were desalted as follows. The column eluate (8 ml) was diluted 5-fold with distilled water and then applied to a column (4 mm internal diameter) containing 0.2 ml of AG1 resin (formate form). This column was first eluted with 1.5 ml of 0.5 M-NaOH, which converts most of the resin into the OH^- form, yet removes less than 2% of bound inositol phosphates; 90% recovery of InsP_3 was then obtained by elution in 4 ml of 2 M-NaOH. NaOH was removed from this sample by passing it down a column containing 9 ml of Bio-Rad AG50 (H^+ form; 200–400 mesh) and completing the elution of inositol phosphates with 1 vol. of distilled water. This eluate was generally about pH 4, owing to traces of formate that remained on the AG1 column. The samples were therefore neutralized with triethylamine before drying *in vacuo*.

Separation of inositol phosphates by ion exchange chromatography

[^{3}H]Inositol-labelled acid-soluble compounds in tissue extracts were separated on small anion-exchange resin columns by a modification of our previously published methods (Downes & Wusteman, 1983) as described by Batty *et al.* (1985). Neutralized tissue extracts prepared as described above were applied to columns (6 mm internal diameter) containing 0.6 ml (wet bed vol.) of Bio-Rad AG1 X8 (200–400 mesh; formate form). [^{3}H]Inositol-labelled compounds were eluted by sequential addition of ammonium formate/formic acid mixtures of increasing ionic strength (see Figure legends for details).

Measurement of radioactive InsP_3 isomers, analysis of lipid head groups (see below) and confirmation of the homogeneity of standard compounds were accomplished by anion-exchange h.p.l.c. on a Partisil 10 SAX column eluted with ammonium formate/phosphoric acid gradients as described previously (Irvine *et al.*, 1985; Batty *et al.*, 1985). Further details are given in the accompanying paper (Hawkins *et al.*, 1986).

Identification of PtdInsP_2 head group

^{32}P-labelled parotid-gland phospholipids were deacylated as described by Clarke & Dawson (1981) and the glycerol moieties were then removed by the method of Brown & Stewart (1966), exactly as described previously (Irvine *et al.*, 1985). This procedure results in $>90\%$ yield of InsP_3 from glycerophosphorylinositol bisphosphate (see Irvine *et al.*, 1985). ^{32}P-labelled inositol trisphosphates obtained by this procedure were analysed by anion-exchange h.p.l.c. (see above).

RESULTS

Previous studies demonstrated that prolonged stimulation of parotid-gland slices with carbachol, noradrenaline or Substance P leads to substantial accumulation of inositol mono-, bis- and tris-phosphates (Berridge *et al.*, 1983). However, most of the InsP_3 that accumulates in carbachol-stimulated slices is Ins(1,3,4)P_3 and not Ins(1,4,5)P_3. Table 1 shows the results of an experiment

Table 1. Inositol trisphosphates in agonist-stimulated parotid-gland slices

Parotid-gland slices were labelled with [^{3}H]inositol as described in the Materials and methods section (1 μCi per incubation). After 90 min, agonists were added in 10 μl volumes of incubation medium to give the final concentrations indicated. The vials were re-gassed with O_2 and capped, and 10 min later the incubations were terminated by addition of trichloroacetic acid and the radioactivity in inositol trisphosphates was determined after their separation by anion-exchange h.p.l.c. The results are means ± S.E.M. for triplicate incubations.

	Radioactivity in inositol trisphosphates (d.p.m.)	
Agonist	Ins(1,3,4)P_3	Ins(1,4,5)P_3
Control	77 ± 20	280 ± 111
Carbachol (1 mM)	5989 ± 1403	803 ± 157
Noradrenaline (0.1 mM)	2743 ± 194	274 ± 51
Substance P (1 μM)	1089 ± 151	206 ± 34

in which [^{3}H]inositol-labelled parotid-gland slices were stimulated for 10 min with carbachol, noradrenaline or Substance P, and then the radioactivity associated with each of the InsP_3 isomers was determined after they were separated by anion-exchange h.p.l.c. Each of the agonists produced a substantial increase in radioactive Ins(1,3,4)P_3, the order of efficacy being carbachol > noradrenaline > Substance P, in keeping with the previous observations that did not separate this isomer from Ins(1,4,5)P_3. By contrast, only carbachol produced a significant increase in Ins(1,4,5)P_3 after 10 min stimulation.

Determination of the head-group structure of parotid-gland PtdInsP_2

One possible mechanism whereby agonists could induce accumulation of Ins(1,3,4)P_3 is by stimulating phospholipase C attack on a novel lipid, i.e. phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2]. Since we have developed a chromatographic system that separates Ins(1,3,4)P_3 from Ins(1,4,5)P_3 (Irvine *et al.*, 1985) and because it was not certain that existing methods for separation of phospholipids would resolve PtdIns(4,5)P_2 from the putative PtdIns(3,4)P_2, we elected to derive the phospholipid headgroups from parotid-gland PtdInsP_2 and analyse them by anion-exchange h.p.l.c. Any Ins(1,3,4)P_3 in the head-group preparation would indicate the existence of PtdIns(3,4)P_2 as its parent lipid.

Parotid-gland slices were heavily labelled with [^{32}P]P$_i$ to generate substantial amounts of [^{32}P]PtdInsP_2, so that the occurrence of as little as 1% of the PtdInsP_2 pool as the putative PtdIns(3,4)P_2 could be detected. Fig. 1 shows the h.p.l.c. profile of such a head-group preparation; 98–99% of the radioactivity in this portion of the chromatograms was eluted as a single symmetrical peak in the position expected for Ins(1,4,5)P_3, indicating that the great majority of the parotid gland's PtdInsP_2 has the expected PtdIns(4,5)P_2 structure. Fig. 1 also indicates the presence of a very low amount of radioactivity in a compound that is eluted from the h.p.l.c. column just before Ins(1,4,5)P_3. It amounted to approx. 1% of the Ins(1,4,5)P_3 peak and was present in the same proportion in all the chromatograms that we ran for the various incubation conditions described in Table 2. However, this compound was not Ins(1,3,4)P_3, because it was routinely eluted about two fractions before the expected position for Ins(1,3,4)P_3 [note that out of more than 100 chromatographic separations of InsP_3 isomers, the relative positions of the Ins(1,3,4)P_3 and Ins(1,4,5)P_3 peaks have remained extremely consistent]. It most probably arises as a side-product of the reaction that removes the glycerol moiety (i.e. oxidation by atmospheric O_2 of the aldehyde group formed by periodate, to convert it into a carboxy group which is not eliminated by 1,1-dimethylhydrazine); this possibility is supported by the observation that, if the removal of the glycerol moiety is performed under N_2, the amount of the unknown compound is decreased substantially (result not shown). The effect of its presence in these experiments is simply to decrease the sensitivity of our assay for phospholipid-derived Ins(1,3,4)P_3, since any of this compound in these preparations would overlap with the unknown compound when separated by h.p.l.c. Nevertheless we can be confident that PtdIns(4,5)P_2 is the predominant form of this phospholipid in ^{32}P-labelled

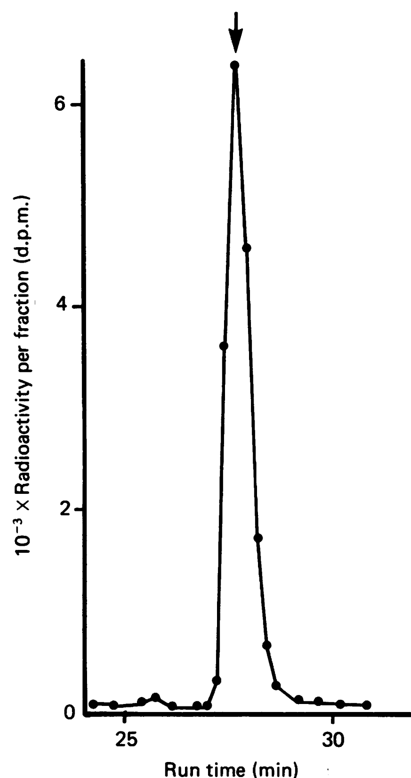

Fig. 1. H.p.l.c. analysis of the head groups obtained from PtdInsP_2 of [^{32}P]P$_i$-labelled parotid-gland slices

Parotid-gland slices were labelled with [^{32}P]P$_i$ and phospholipids were extracted as described in the Materials and methods section. The phospholipid head groups were obtained by deacylation and removal of the glycerol moiety and the resulting samples were applied to a 25 cm Partisil 10 SAX h.p.l.c. column. Inositol trisphosphates were separated with a complex gradient of ammonium formate and H_3PO_4 as described previously (Irvine *et al.*, 1985). The Figure shows only the portion of the chromatogram in which inositol trisphosphates are eluted, and the data shown are for a single control incubation identical with that in Table 1. The arrow marks the position of the centre of the peak of a [^{32}P]Ins(1,4,5)P_3 standard that was eluted in a parallel chromatographic separation on the same column. The centre of the Ins(1,3,4)P_3 peak in most separations is 1–1.25 min before the Ins(1,4,5)P_3 peak.

parotid glands and that any PtdIns(3,4)P_2 must comprise less than 1% of the total PtdInsP_2 pool.

We also tested the possibility that PtdIns(3,4)P_2 could be formed during agonist stimulation, by analysing the head-group profile of PtdInsP_2 after prolonged stimulation with carbachol and during the recovery phase after atropine blockade of carbachol-stimulated slices. The results are shown in Table 2. Carbachol treatment induces a rapid and sustained decrease in steady-state labelled PtdInsP_2 (Weiss *et al.*, 1982; Downes & Wusteman, 1983) which was reproduced in this experiment. However, there was no detectable Ins(1,3,4)P_3 in the head-group preparations from carbachol-stimulated slices. PtdInsP_2 rapidly returned to control amounts within 2 min of adding atropine to carbachol-stimulated slices. None of this newly synthesized PtdInsP_2 was detected as Ins(1,3,4)P_3 in the head-group preparations. Finally, treatment with tumour-promoting phorbol esters

Table 2. Analysis of PtdInsP_2 head groups obtained from ^{32}P-labelled parotid-gland slices

Parotid-gland slices were labelled for 60 min with [^{32}P]P_i as described in the Materials and methods section. The stimulants indicated in the Table were added and incubations continued for the times shown. The incubations were finally terminated by addition of chloroform/methanol/HCl (40:80:1, by vol.), phospholipids were extracted and head groups were prepared as described in the Materials and methods section. Inositol trisphosphates were separated by anion-exchange h.p.l.c. and radioactivity was determined by scintillation counting. The results are means ± S.E.M. for triplicate incubations; N.D., not detected.

Stimulus	Radioactivity in inositol trisphosphates obtained from PtdInsP_2 (d.p.m.)	
	Ins(1,3,4)P_3	Ins(1,4,5)P_3
Control	N.D.	15731 ± 1336
Carbachol (1 mM) for 10 min	N.D.	7580 ± 1570
Carbachol for 10 min, then atropine (10 μM) for 2 min	N.D.	15547 ± 3173
Tetradecanoylphorbol acetate (100 nM) for 10 min	N.D.	14900 ± 1528

such as tetradecanoylphorbol acetate, which directly activate cellular protein kinase C, leads to enhanced steady-state amounts of PtdInsP_2 in thymocytes (Taylor *et al.*, 1984). We speculated that activation of protein kinase C, in the absence of receptor-activated phospholipase C, might cause accumulation of the putative PtdIns(3,4)P_2. However, treatment with tetradecanoylphorbol acetate had no significant effect on steady-state PtdInsP_2 content in the parotid gland, and the head-group preparation obtained from slices treated with tetradecanoylphorbol acetate contained no detectable Ins(1,3,4)P_3 (Table 2).

Detection of an inositol tetrakisphosphate in carbachol-stimulated parotid glands

Batty *et al.* (1985) have reported the occurrence of Ins(1,3,4,5)P_4 in carbachol-stimulated brain slices, and suggested that a similar compound was also present in rat parotid glands. This is confirmed in Fig. 2(*a*), which shows the separation of [^{3}H]inositol-labelled acid-soluble compounds from parotid glands stimulated for 20 s with a large dose of carbachol on AG1 (formate) columns. This elution profile is very similar to that reported previously (Berridge *et al.*, 1983), except that the inositol trisphosphate fraction was quantitatively eluted with 0.8 M-ammonium formate/0.1 M-formic acid (rather than 1 M-ammonium formate/0.1 M-formic acid as used previously) as described by Batty *et al.* (1985). When the eluent concentration was increased to 1.2 M-ammonium formate/0.1 M-formic acid, another peak emerged that was barely present in the samples from control (not stimulated) slices. This final peak was not contaminated with inositol trisphosphates, because it contained no detectable ^{32}P-labelled Ins(1,4,5)P_3 standard that was added to the tissue extracts immediately before application to the column. InsP_3 isomers are not resolved on these small AG1 columns, but Ins(1,4,5)P_3 is retained on such columns marginally longer than is Ins(1,3,4)P_3 (result not shown).

This quantitative separation of a highly polar inositol phosphate ester from inositol trisphosphates was used to prepare a highly radioactive sample of the novel compound for structure determination. Ten 50 μl portions of parotid-gland slices were incubated in Beckman Biovials containing 250 μl of incubation medium and 10 μCi of [^{3}H]inositol. The slices were labelled for 90 min at 37 °C before addition of carbachol (final concn. 1 mM). The incubations were terminated 20 s later by addition of an equal volume of 10% (v/v) $HClO_4$ and the inositol phosphate fraction was obtained as described in the Materials and methods section. The neutralized inositol phosphate fraction was applied to a small AG1 (formate) column, and inositol phosphates, up to and including inositol trisphosphates, were eluted in 12 ml of 0.8 M-ammonium formate/0.1 M-formic acid. The novel phosphate ester was then eluted in 8 ml of 1.2 M-ammonium formate/0.1 M-formic acid, and this sample was desalted as described in the Materials and methods section. This procedure yielded about 80000 d.p.m. of the novel phosphate ester for structural analysis. It contained only a single radioactive peak on anion-exchange h.p.l.c. that was well resolved from [^{32}P]Ins(1,4,5)P_3 (Fig. 2*b*) and had chromatographic properties identical with the Ins(1,3,4,5)P_4 identified in brain slices by Batty *et al.* (1985).

The procedures that have been used to identify the novel labelled compound in parotid-gland extracts are the same as those described by Batty *et al.* (1985). The chromatographic and electrophoretic systems used for identification of polyols, inositol phosphates and free inositol are described by Irvine *et al.* (1984) and Batty *et al.* (1985). It is an inositol tetrakisphosphate because it yielded free inositol after extensive treatment with alkaline phosphatase and because it ran with the inositol tetrakisphosphate fraction, obtained by partial dephosphorylation of phytic acid, on paper chromatography (Desjobert & Petek, 1956) and ionophoresis in 0.1 M-sodium oxalate, pH 1.5 (Seiffert & Agranoff, 1965). Treatment of the labelled compound with erythrocyte ghosts yielded an InsP_3 which co-chromatographed precisely with Ins(1,3,4)P_3 on anion-exchange h.p.l.c. and which gave altritol after periodate oxidation, dephosphorylation and subsequent reduction. The parent InsP_4 was not susceptible to periodate oxidation. This leaves only Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 as possible structures (D- or L-), but, in view of its chromatographic identity with the Ins(1,3,4,5)P_4 isolated from carbachol-stimulated rat brain slices, by far the most likely structure for the InsP_4 obtained from stimulated parotid glands is Ins(1,3,4,5)P_4.

DISCUSSION

The occurrence of substantial quantities of Ins(1,3,4)P_3 in carbachol-stimulated parotid glands has had a number of important implications for studies intended to examine the role of Ins(1,4,5)P_3 as an intracellular second messenger. Firstly, it was necessary to devise a chromatographic system for separation of these InsP_3 isomers in order to study their metabolism in

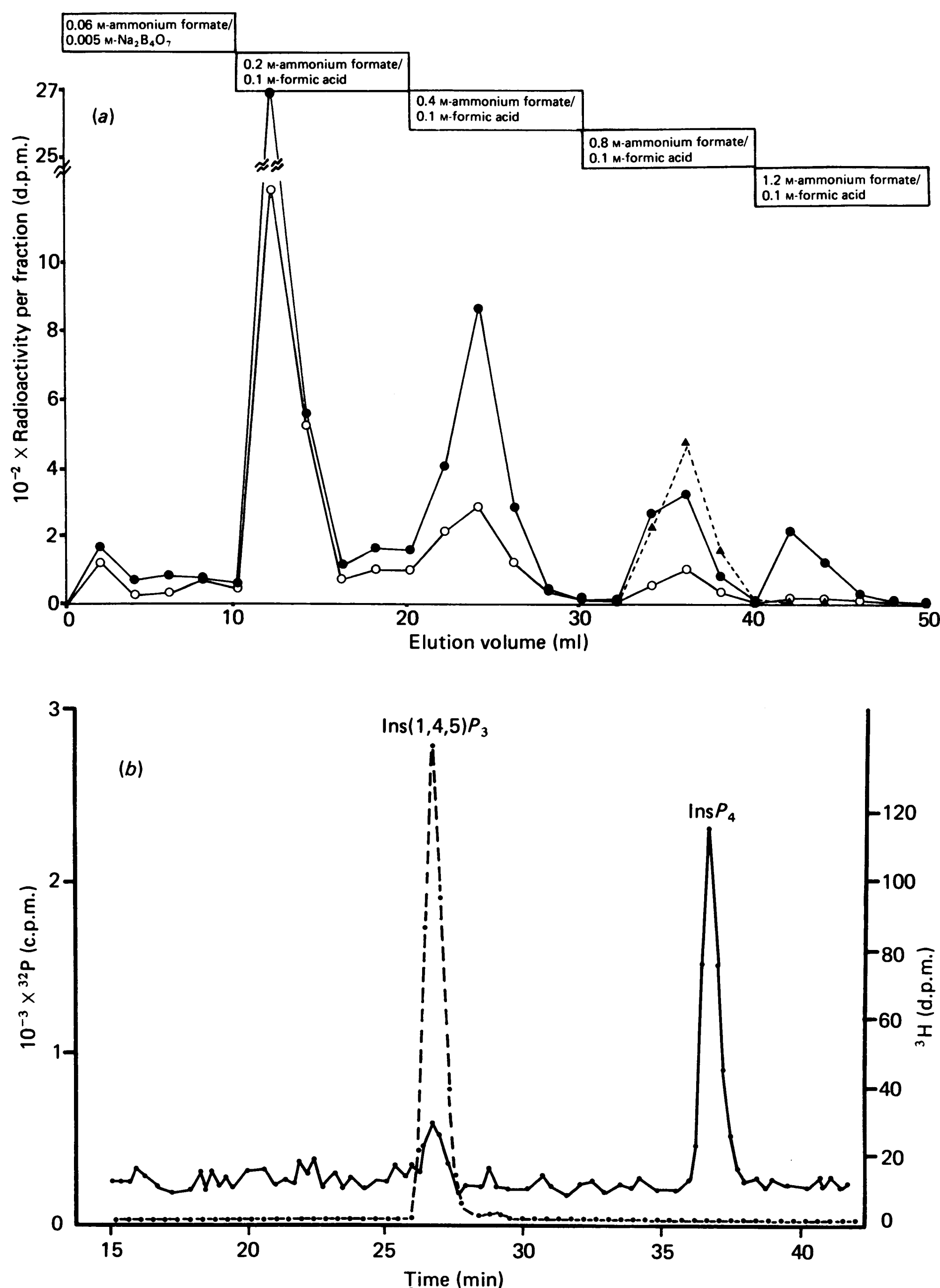

Fig. 2. Separation of an unknown polar inositol phosphate ester from inositol trisphosphates by anion-exchange chromatography

(*a*) Parotid-gland slices were labelled with [^{3}H]inositol, and the labelled acid-soluble compounds, spiked with [^{32}P]Ins(1,4,5)P_3, were eluted from small AG1 (formate) columns with the eluents indicated on the Figure; 2 ml fractions were collected and counted for radioactivity by scintillation counting by using a dual-label programme. ○, Control incubation; ●, incubated with carbachol (1 mM) for 20 s before addition of $HClO_4$; ▲, [^{32}P]Ins(1,4,5)P_3 standard. (*b*) A highly radioactive sample of the unknown polar inositol phosphate ester was prepared from [^{3}H]inositol-labelled parotid-gland slices that had been stimulated with carbachol (1 mM) for 20 s. It was separated from other labelled compounds by chromatography on AG1 (formate) as described above and the sample was desalted as in the Materials and methods section. A small proportion of this preparation was mixed with [^{32}P]Ins(1,4,5)P_3 and separated on a 25 cm Partisil 10 SAX column by using the gradient described by Batty *et al.* (1985); 0.31 ml fractions were collected and counted for radioactivity by scintillation counting by using a dual-label programme. ●——●, ^{3}H-labelled unknown preparation; ●---●, [^{32}P]Ins(1,4,5)P_3.

stimulated cells independently from one another (Irvine *et al.*, 1985). Such studies have revealed that Ins(1,4,5)P_3 is formed rapidly (within 5 s) in both carbachol-stimulated rat parotid glands (Irvine *et al.*, 1985) and angiotensin-stimulated guinea-pig hepatocytes (Burgess *et al.*, 1985), and that it is rapidly metabolized when agonist activity is terminated by addition of a large excess of a competitive antagonist (Irvine *et al.*, 1985). These properties of Ins(1,4,5)P_3 are consistent with its proposed role as an intracellular second messenger. By contrast, Ins(1,3,4)P_3 is formed only after an initial time-lag of a few seconds after stimulation, and is degraded relatively slowly, so

that its amount continues to rise over several minutes, by which time it greatly exceeds the amount of Ins(1,4,5)P_3.

The generation of a large Ins(1,3,4)P_3 signal is not a phenomenon only of muscarinic-receptor activation, because activation of all three inositol phospholipid-coupled receptors increased Ins(1,3,4)P_3 in [^{3}H]inositol-labelled parotid-gland slices. However, with the prolonged stimulations with noradrenaline and Substance P shown in Table 1, there was no significant change in Ins(1,4,5)P_3 amounts, although both of these agents raise intracellular Ca^{2+} concentration in this tissue primarily by releasing Ca^{2+} from intracellular, non-mitochondrial, stores (Putney, 1977; Poggioli & Putney, 1982). More detailed studies, employing different times of stimulation (particularly short periods of a few seconds), are required to resolve this apparent paradox.

Secondly, the head-group structure of polyphosphoinositides had, until recently, only been determined for such lipids extracted from brain. Our previous studies (Irvine *et al.*, 1984, 1985) have confirmed that the InsP_3 obtained from human erythrocyte phospholipids by activation of the erythrocyte membrane phospholipase C also has the Ins(1,4,5)P_3 structure. Thus InsP_3 obtained by controlled hydrolysis of brain or human erythrocyte PtdInsP_2 represents a suitable standard for characterizing chromatographic systems, can be used in studies of the second-messenger role of inositol trisphosphates (see Streb *et al.*, 1983; Berridge & Irvine, 1984), and can be used to examine the metabolism of Ins(1,4,5)P_3 by subcellular fractions (Downes *et al.*, 1982; Storey *et al.*, 1984; Connolly *et al.*, 1985). The results in the present paper confirm that the only detectable PtdInsP_2 in [^{32}P]P_i-labelled parotid-gland slices has the PtdIns(4,5)P_2 structure and that a novel phospholipid is very unlikely to be the source of Ins(1,3,4)P_3 in this and, presumably, other tissues.

The discovery of Ins(1,3,4,5)P_4 in carbachol-stimulated brain slices and its conversion into Ins(1,3,4)P_3 by human erythrocyte membranes led Batty *et al.* (1985) to propose that InsP_4 is likely to be the precursor for Ins(1,3,4)P_3 in brain. They also noted the occurrence of a compound with similar properties to InsP_4 in carbachol-stimulated parotid glands. We have extended their initial observations in confirming that parotid glands also contain Ins(1,3,4,5)P_4. Further, more detailed results, describing the kinetics of InsP_4 formation in stimulated glands, its metabolism by parotid-gland homogenates and the source of its production are presented in the accompanying paper (Hawkins *et al.*, 1986).

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