Rapid formation of inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands may both result indirectly from receptor-stimulated release of inositol 1,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate

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1. Addition of 1 mM-carbachol to [^3H]inositol-labelled rat parotid slices stimulated rapid formation of [^3H]inositol 1,3,4,5-tetrakisphosphate, the accumulation of which reached a peak 20 s after stimulation, and then declined rapidly towards a new steady state. The initial rate of formation of inositol 1,3,4,5-tetrakisphosphate was slower than that for inositol 1,4,5-trisphosphate. 2. The radioactivity in [^3H]inositol 1,3,4,5-tetrakisphosphate fell quickly in carbachol-stimulated and then atropine-blocked parotid slices, suggesting that it is rapidly metabolized during stimulation. 3. Parotid homogenates rapidly dephosphorylated inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate and, less rapidly, inositol 1,3,4-trisphosphate. Inositol 1,3,4,5-tetrakisphosphate was specifically hydrolysed to a compound with the chromatographic properties of inositol 1,3,4-trisphosphate. 4. The only ^3H -labelled phospholipids that we could detect in parotid slices labelled with [^3H]inositol for 90 min were phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. 5. Parotid homogenates synthesized inositol tetrakisphosphate from inositol 1,4,5-trisphosphate. This activity was dependent on the presence of ATP. 6. We suggest that, during carbachol stimulation of parotid slices, the key event in inositol lipid metabolism is the activation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase C. The inositol 1,4,5-trisphosphate thus liberated is metabolized in two distinct ways; by direct hydrolysis of the 5-phosphate to form inositol 1,4-bisphosphate and by phosphorylation to form inositol 1,3,4,5-tetrakisphosphate and hence, by hydrolysis of this tetrakisphosphate, to form inositol 1,3,4-trisphosphate.

INTRODUCTION

In a variety of tissues, stimulation by agonists causes rapid accumulation of InsP_3 , InsP_2 and InsP . During stimulation there is also depletion of PtdIns , $\text{PtdIns}(4,5)\text{P}_2$ and sometimes of $\text{PtdIns}4\text{P}$, suggesting that the formation of inositol phosphates stems from activation of one or more of the appropriate inositol phospholipid-specific phospholipases C. Agonists which stimulate inositol metabolism in this way all act at the cell surface and share at least two intracellular messages; diacylglycerol, which activates protein kinase C, and $\text{Ins}(1,4,5)\text{P}_3$, which releases Ca^{2+} from cell-associated stores (for reviews see Nishizuka, 1984; Berridge & Irvine, 1984; Downes & Michell, 1985).

Recently, however, a more detailed investigation of the inositol phosphates formed during stimulation has revealed hitherto unforeseen complications. As well as the expected $\text{Ins}(1,4,5)\text{P}_3$ isomer, there is also accumulation of $\text{Ins}(1,3,4)\text{P}_3$ in a number of tissues stimulated by different agonists (Irvine *et al.*, 1984, 1985; Burgess *et al.*, 1985). Furthermore, Batty *et al.* (1985) and Downes *et al.* (1986) have now shown accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ in brain and parotid slices stimulated with carbachol. The

origin and fate of these two novel inositol phosphates are unknown and are particularly intriguing, since we have no proof of the existence of any parent lipid from which they may be derived [i.e. a putative $\text{PtdIns}(3,4)\text{P}_2$ and a putative $\text{PtdIns}(3,4,5)\text{P}_3$]. We have attempted to solve this apparent paradox by studying the receptor-activated formation and the routes of metabolism of $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ in carbachol-stimulated rat parotid slices and in rat parotid homogenates and by exploring the possible existence of novel inositol-containing phospholipids.

MATERIALS AND METHODS

Materials

Materials and animals were, unless stated otherwise, of the type and from the source specified in the accompanying paper (Downes *et al.*, 1986). *myo*-[2- ^3H]inositol was obtained from New England Nuclear (Du Pont, Stevenage, Herts., U.K.). [^{32}P] P_i and L-[1- ^{14}C] $\text{Ins}1\text{P}$ were obtained from Amersham International (Amersham, Bucks., U.K.). Phenylmethanesulphonyl fluoride, atropine, carbachol and disodium ATP were obtained

Abbreviations used: InsP_4 , InsP_3 , InsP_2 and InsP , inositol tetrakisphosphate, trisphosphate, bisphosphate and monophosphate (isomeric forms unspecified); $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}1\text{P}$, inositol 1,3,4,5-tetrakisphosphate, 1,4,5-trisphosphate, 1,3,4-trisphosphate, 1,4-bisphosphate and 1-phosphate; $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}4\text{P}$ and PtdIns , phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-monophosphate and phosphatidylinositol; $\text{GroPIns}(4,5)\text{P}_2$, $\text{GroPIns}4\text{P}$ and GroPIns , glycerophosphoinositol 4,5-bisphosphate, glycerophosphoinositol 4-phosphate and glycerophosphoinositol.

from Sigma Chemical Co. (Poole, Dorset, U.K.). EGTA and leupeptin hemisulphate were from Fluka A.G. (Buchs, Switzerland). Methylamine was purchased as a 25% (w/v) solution in water (laboratory-grade reagent) from BDH Chemicals (Poole, Dorset, U.K.).

Preparation and incubation of parotid slices

Rat parotid slices were prepared and incubated with [^3H]inositol for 90 min at 37 °C, as described in the accompanying paper (Downes *et al.*, 1986). Slices were then stimulated with 1 mM- or 0.1 mM-carbachol in the continued presence of [^3H]inositol in the medium.

Preparation and incubation of parotid homogenates

The parotid glands from two rats were dissected and cleaned from connective tissue. Pieces of parotid, roughly 2 mm $\times$ 2 mm, were then homogenized in bulk with 8 ml of ice-cold 250 mM-sucrose/50 mM-Tris/HCl (pH 7.3)/1 mM-EGTA, containing 50 μM -phenylmethanesulphonyl fluoride, 1 μg of leupeptin/ml and 10 mg of bovine serum albumin/ml. The homogenization was performed with five up-and-down strokes of a loose-fitting glass/Teflon homogenizer. The homogenate was stored on ice and used within 30 min of preparation.

Homogenates prepared in this manner were diluted 2.5-fold with 70 mM-potassium glutamate/40 mM-NaCl/4 mM-MgSO₄/1 mM-EGTA/10 mM-Hepes, pH 7.2, and incubated with various ^{32}P - and ^3H -labelled inositol phosphates at 30 °C.

Extraction of inositol phosphates

All tissue incubations concerned in the preparation of inositol phosphate standards, the metabolism of inositol phosphates by homogenates or experiments with intact parotid slices were terminated by addition of an equal volume of ice-cold 10% (v/v) HClO₄. Perchlorate was then removed from these samples by extraction with tri-*n*-octylamine, as described by Downes *et al.* (1986). These neutralized extracts were diluted approx. 5-fold before analysis by anion-exchange chromatography.

Chromatographic separation of inositol phosphates

Inositol phosphates were separated by two forms of anion-exchange chromatography.

In cases where examination of inositol phosphate isomers was required, separation was by h.p.l.c. We used a 20 cm-long main column with a 5 cm-long guard column, both packed with Whatman Partisil 10 SAX. The sample was filtered (Millipore HV; 0.45 μm pore size) before loading in a 2 ml injection loop. Elution was with a gradient of ammonium formate and phosphate, as described by Batty *et al.* (1985), with a flow rate of 1.25 ml/min. Over appropriate regions of the gradient 0.25 min fractions were collected directly into scintillation vials. These fractions were solubilized by addition of 0.5 ml of methanol, 0.5 ml of water and 3 ml of Insta-gel (Packard) and their radioactivity was determined by scintillation counting.

In the remaining cases, separation was by small (2 cm $\times$ 6 mm) gravity-fed columns, packed with Bio-Rad AG-1 (X8, 200–400 mesh; formate form) resin beads. Routinely, inositol phosphates were eluted with 3 $\times$ 3 ml fractions of the appropriate concentration of ammonium formate/formic acid (see Batty *et al.*, 1985; Downes *et al.*, 1986). Each 3 ml sample was solubilized by addition of Insta-gel (18 ml) and its radioactivity determined

by scintillation counting. This method achieved excellent separation of InsP, InsP₂, InsP₃ and InsP₄ (as characterized by use of radioactive standards prepared as described below).

Preparation of inositol phosphates

^{32}P -labelled Ins(1,4,5)P₃ was prepared from ^{32}P -labelled human erythrocytes as described by Downes *et al.* (1982), except that erythrocytes were labelled with [^{32}P]P_i in the presence of citrate as the major extracellular anion (the removal of chloride allows faster equilibration of P_i across the erythrocyte membrane; Mollison *et al.*, 1958).

^{32}P -labelled Ins(1,4)P₂ was prepared via specific human erythrocyte-membrane-catalysed removal of the 5-phosphate from [^{32}P]Ins(1,4,5)P₃ (Downes *et al.*, 1982). Ins(1,4)P₂ was recovered by separation on a small AG-1 (formate) column; see above.

^3H -labelled Ins(1,3,4)P₃ was prepared as follows. Parotid slices were labelled with [^3H]inositol for 60 min and then stimulated for 10 min with 1 mM-carbachol. The incubation was terminated by addition of HClO₄ and an InsP₃ fraction obtained by separation on a small AG-1 (formate) column as described above. An InsP₃ fraction prepared in this manner contains approx. 90% of the radioactivity in Ins(1,3,4)P₃ and 10% in Ins(1,4,5)P₃ (Irvine *et al.*, 1984; see also Fig. 1 in the Results and discussion section). This InsP₃ fraction was then incubated with human erythrocyte membranes at 37 °C [approx. 2 mg of protein/ml for 80 min; see Downes *et al.* (1982) for details of incubation conditions], a treatment designed to hydrolyse essentially all Ins(1,4,5)P₃ present but only a small proportion of Ins(1,3,4)P₃ (Irvine *et al.*, 1984). The remaining Ins(1,3,4)P₃ was recovered by separation on a small AG-1 (formate) column; see above.

^3H -labelled Ins(1,3,4,5)P₄ was prepared from parotid slices incubated with [^3H]inositol for 90 min, and stimulated with 1 mM-carbachol for 20 s, precisely as described by Downes *et al.* (1986).

The last stage in the preparation of all of the above inositol phosphates is recovery in a substantial quantity (approx. 10 ml) of concentrated ammonium formate (0.4–1.2 M). Before use in any of the experiments described here, these samples were desalted by consecutive treatment with AG-1 (X8; 200–400 mesh; OH[−] form) and AG-50W (X8; 200–400 mesh; H⁺ form) resins, as described by Downes *et al.* (1986).

Analysis of inositol-containing phospholipids

The purpose of analysing phospholipids in this study was to search for novel inositol-containing phospholipids, in particular a putative PtdIns(3,4,5)P₃ (see the Results and discussion section). For this reason, we adopted a procedure which avoided any potential losses owing to failure of extraction from tissue precipitates. Tissue incubations were terminated by addition of chloroform and methanol to give a conventional Bligh & Dyer (1959) phase split. At this point the lower phase, interfacial tissue-pellet and upper phase were deacylated with methylamine, see below. The resulting glycerophosphoesters were then analysed by h.p.l.c. Incubations were terminated by the addition of neutral chloroform/methanol rather than the more usual acid mixtures, since this ensured that most of the PtdIns4P and PtdIns(4,5)P₂ remained bound to the tissue pellet and thus acted as internal controls for the efficacy of deacylation of this

pellet. We also analysed the aqueous and chloroform fractions by the same procedure (i.e. by lipid deacylation) to check the possibility that the putative $\text{PtdIns}P_3$ was more water-soluble than $\text{PtdIns}(4,5)P_2$ and $\text{PtdIns}4P$.

Four 40 μl samples of parotid slices were each incubated with approx. 50 μCi of $[^3\text{H}]$ inositol in a total volume of 250 μl , for 90 min. Two incubations were treated with 1 mM-carbachol for 20 s and two others were left untreated. All four incubations were terminated by addition of 0.94 ml of chloroform/methanol (1:2, v/v), followed by 0.31 ml of chloroform and 0.31 ml of 5 mM-EDTA/1 mM-inositol to give two phases (Bligh & Dyer, 1959). The upper phase was removed from each sample and the lower phase plus interfacial pellet washed with 3×1 ml of a 10:9 (v/v) mixture of methanol and 5 mM-EDTA/1 mM-inositol. These washings were combined with the original upper phase and dried *in vacuo*. The lower phase was then removed from the pellet, together with two 0.5 ml chloroform washes, and dried *in vacuo*. The remaining tissue pellet was stored overnight at -20°C . Immediately before drying or storage at -20°C , each of the above samples received a small portion of ^{32}P -labelled erythrocyte lipids (approx. 3000 d.p.m., prepared as described by Downes & Michell, 1981).

One upper-phase, tissue pellet and lower-phase sample derived from control slices and one of each from carbachol-stimulated slices were deacylated with methylamine (see below). The remaining samples were taken through the deacylation procedure, except that methylamine [25% (w/v) in water] was replaced by an equal volume of water.

Deacylation of phospholipids

Lipid deacylation was by transacylation to methylamine and was applied to dried samples of lipids or directly to tissue pellets (see above). The method used was slightly modified from that of Clarke & Dawson (1981). The modification concerned the composition of the methylamine reagent, which contained aq. methylamine (25%), methanol and butan-1-ol in the proportions 4:4:1 respectively. Deacylation was carried out at 53°C for 45 min. When this procedure was applied to the deacylation of ^{32}P -labelled erythrocyte lipids, approx. 97% of lipid radioactivity was recovered in water-soluble form. When these ^{32}P -labelled esters were separated by h.p.l.c., radioactivity was found in only three compounds, with the expected mobilities of glycerophosphate, $\text{GroPIns}4P$ and $\text{GroPIns}(4,5)P_2$.

The above method of deacylation was chosen since its use avoided formation of unidentified side-products of $\text{PtdIns}4P$ and $\text{PtdIns}(4,5)P_2$ deacylation. These side-products (usually 1–5% of the appropriate glycerophosphoester) arise from use of conventional deacylation procedures, which utilize mixtures of methanol and NaOH (P. T. Hawkins, unpublished work; see also Clarke & Dawson, 1981).

RESULTS AND DISCUSSION

Analysis of $[^3\text{H}]$ inositol-labelled tissue extracts by h.p.l.c.

Typical profiles describing the h.p.l.c. separation of $[^3\text{H}]$ inositol-labelled extracts prepared from control parotid slices and from slices stimulated with 1 mM-

Fig. 1. H.p.l.c. profiles of aqueous $[^3\text{H}]$ inositol-labelled extracts prepared from control (○) and carbachol-stimulated (●) parotid slices

Rat parotid slices were incubated with $[^3\text{H}]$ inositol (2 $\mu\text{Ci}/250 \mu\text{l}$) and aqueous extracts prepared from control slices or slices incubated with 1 mM-carbachol for 20 s, as described in the Materials and methods section. These extracts were then analysed by h.p.l.c. Immediately before injection on to the column the following standards were added to each sample: L- $[^{14}\text{C}]$ Ins1P (1500 d.p.m.), $[^{32}\text{P}]$ Ins(1,4) P_2 (200 d.p.m.) and $[^{32}\text{P}]$ Ins(1,4,5) P_3 (1000 d.p.m.); the data shown (□) are for the standards added to the carbachol-stimulated sample.

Fig. 2. Accumulation of $[^3\text{H}]\text{inositol}$ -labelled inositol phosphates in carbachol-stimulated parotid slices

Rat parotid slices were incubated with $[^3\text{H}]\text{inositol}$ ($2 \mu\text{Ci}/250 \mu\text{l}$) for 90 min and then treated with either 1 mM-carbachol or an equal volume of Krebs buffer. At various times the incubations were terminated by addition of HClO_4 , and neutral aqueous extracts prepared as described in the Materials and methods section. These extracts were analysed by h.p.l.c. and the radioactivity in the appropriate inositol phosphate fractions was determined (see Fig. 1). (a) Accumulation of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (●) and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (□). (b) Accumulation of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ (○) and $[^3\text{H}]\text{InsP}_2$ (■). Control incubations, containing no added carbachol, showed no significant change in inositol phosphates up to 10 min (results not shown). The data presented are means $\pm$ S.E.M. ($n = 3$) from a single experiment. One further experiment has been performed, which gave essentially identical results.

carbachol for 20 s are shown in Fig. 1. The use of ^{14}C - and ^{32}P -labelled inositol phosphate standards enabled us to identify three of the ^3H -labelled compounds as InsP , InsP_2 and $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 1). Two further compounds have been identified previously as $\text{Ins}(1,3,4)\text{P}_3$ (peak A in Fig. 1) and $\text{Ins}(1,3,4,5)\text{P}_4$ (peak B in Fig. 1) (Irvine *et al.*, 1984, 1985; Downes *et al.*, 1986). The remaining ^3H -labelled compounds shown in Fig. 1 remain unidentified. During stimulation with carbachol for 20 s, radioactivity accumulated above control values in InsP_2 , $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Fig. 1).

The h.p.l.c. methodology employed appears quite capable of separating isomers of InsP_3 (1,3,4 and 1,4,5; see Fig. 1). However, since we do not understand the chemical basis for this separation, we cannot predict whether isomers of other inositol phosphates would be equally well separated. For this reason, we cannot unequivocally identify ^3H -labelled InsP and InsP_2 in parotid extracts as InsP and $\text{Ins}(1,4)\text{P}_2$, even though they appear to co-chromatograph with the appropriate standards (Fig. 1). In this respect, we have sometimes noticed a very small shoulder to the $[^3\text{H}]\text{InsP}_2$ peak (more retained than the major peak and $\leq 5\%$ of the total InsP_2 radioactivity) in extracts prepared from slices stimulated with carbachol for over 3 min (results not shown). It is possible that this reflects the emergence of a second InsP_2 isomer at longer times of stimulation.

There have been reports of the occurrence of cyclic inositol phosphates as products of phospholipase C-catalysed hydrolysis of purified PtdIns (Dawson *et al.*, 1971), $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (Wilson *et al.*, 1985). We do not know whether any inositol phosphate esters

Fig. 3. Rate of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ depletion in carbachol-stimulated atropine-blocked parotid slices

Rat parotid slices were incubated with $[^3\text{H}]\text{inositol}$ ($2 \mu\text{Ci}/250 \mu\text{l}$) for 90 min and then either stimulated with 0.1 mM-carbachol or left untreated. After a further 20 s of incubation $10 \mu\text{M}$ -atropine was added to every sample. Incubations were terminated at various times by addition of HClO_4 , and neutral aqueous extracts were prepared as described in the Materials and methods section. These extracts were then analysed by chromatography on small AG-1 (formate) columns (see the Materials and methods section). Atropine was added at zero time; ○, control slices; ●, carbachol-stimulated slices. The data presented are means $\pm$ S.E.M. ($n = 3$) from a single experiment, one of two that gave essentially the same result.

containing the 1:2-cyclic phosphodiester bond were present in our parotid slices. The extracts analysed in the present paper were usually prepared from acid-quenched slices and, as such, any cyclic inositol phosphates would have been acid-hydrolysed to their non-cyclic derivatives (Dawson *et al.*, 1971; Wilson *et al.*, 1985).

Time course for the accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ in carbachol-stimulated parotid slices

Downes *et al.* (1986) demonstrated that $\text{Ins}(1,3,4,5)\text{P}_4$ was present in carbachol-stimulated parotid slices. Here we looked at the time course for its stimulated accumulation in relation to those for other inositol phosphates (Fig. 2).

The relative rates of carbachol-stimulated accumulation of ^3H radioactivity in $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and InsP_2 were similar to those reported previously (Downes & Wusteman, 1983; Aub & Putney, 1984). The radioactivity in $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ rose very rapidly, reaching a new steady state by 5 s, the first time of sampling (Fig. 2). In contrast, the radioactivity in $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[^3\text{H}]\text{InsP}_2$ rose steadily throughout the 3 min period of stimulation, with an initial short time lag of approx. 5 s for $\text{Ins}(1,3,4)\text{P}_3$, as reported previously (Irvine *et al.*, 1985).

In these experiments, $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was virtually undetectable in control slices. However, on exposure of slices to 1 mM-carbachol the radioactivity in $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ rose quickly, reaching a maximum value after 20 s of stimulation. The radioactivity in $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ then fell, equally rapidly, towards a new steady state, but still remained above control. The maximum value attained by $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was approximately twice that attained by $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ at 20 s.

The results shown in Fig. 2 suggest that, during stimulation, $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ reaches a new steady state much more rapidly than do $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[^3\text{H}]\text{InsP}_2$, but, importantly, less rapidly than $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$. Also, the markedly biphasic nature of the time course for $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ accumulation suggests that some form of control must operate to change the relative activities of enzymes involved in the formation and/or removal of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ during the first 1 min of stimulation.

We believe a more detailed analysis of these results is unwarranted, because the experimental protocol employed did not exclude the possibility of small changes occurring in specific radioactivity, as well as in chemical concentrations, of the inositol phosphates (e.g. see Downes & Wusteman, 1983). Clearly, any such changes in specific radioactivity would be greater with the longer periods of stimulation.

Rate of removal of $\text{Ins}(1,3,4,5)\text{P}_4$ in parotid slices during carbachol stimulation

In principle, a study of the rate of change in the concentration of each inositol phosphate on stimulation should lead to an understanding of the chemical flux through each inositol phosphate pool. However, in practice, methodological difficulties would render our data unsuitable for such a detailed analysis (see the previous section). Therefore, in order to obtain a more direct measure of the flux through $\text{Ins}(1,3,4,5)\text{P}_4$ during stimulation, we employed the following protocol. $[^3\text{H}]\text{Inositol}$ -labelled parotid slices were stimulated with

Fig. 4. Hydrolysis of inositol phosphates by parotid homogenates

Homogenates were prepared from rat parotids and then incubated with various ^{32}P or ^3H -labelled inositol phosphates at 30°C , as described in the Materials and methods section. At various times incubations were terminated by addition of HClO_4 , and a neutral extract was prepared and analysed by chromatography on small AG-1 (formate) columns, as described in the Materials and methods section. ●, $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$; △, $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$; □, $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$. The data shown are individual incubations from a single experiment.

a slightly sub-maximal dose of carbachol (0.1 mM; Jacobson *et al.*, 1985) for 20 s, a treatment which leads to substantial accumulation of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (see Fig. 2a). Carbachol was then rapidly displaced from muscarinic receptors by addition of 10 μM -atropine (Downes & Wusteman, 1983). The rate of decline of the accumulated $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was then measured.

On addition of atropine, the radioactivity in $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ fell very quickly, with a $t_{1/2}$ of approx. 15 s (Fig. 3). This rate of $\text{Ins}(1,3,4,5)\text{P}_4$ depletion is similar to that previously reported for $\text{Ins}(1,4,5)\text{P}_3$ and much faster than for $\text{Ins}(1,3,4)\text{P}_3$ and InsP_2 (see Downes & Wusteman, 1983; Aub & Putney, 1984; Irvine *et al.*, 1985). Since the radioactivity in $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ returned to the control value within 1 min after atropine blockade, this suggests that the substantial increase in its value 20 s after carbachol stimulation does not involve a substantial increase in its specific radioactivity.

In order to interpret the depletion shown in Fig. 3 in terms of the rate of depletion of $\text{Ins}(1,3,4,5)\text{P}_3$ during stimulation, two basic assumptions must be made. The first is that stimulated formation of $\text{Ins}(1,3,4,5)\text{P}_4$ is instantaneously blocked by addition of atropine. The second is that the changing intracellular conditions

Fig. 5. H.p.l.c. analysis of the products of $\text{Ins}(1,3,4,5)P_4$ hydrolysis

Samples of $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ were incubated with parotid homogenates at 30°C , as described in the Materials and methods section. Incubations were terminated after (a) 0, (b) 1 or (c) 5 min by addition of HClO_4 , and neutral extracts were obtained and then analysed by h.p.l.c. (●) (see the Materials and methods section). Immediately before injection on to the column, each sample was spiked with $[^{32}\text{P}]\text{Ins}(1,4,5)P_3$ (○). The data shown are traces from individual incubations. This experiment has been repeated once with essentially identical results.

during atropine blockade (e.g. changes in cytosolic Ca^{2+}) do not significantly affect the rate of removal of $\text{Ins}(1,3,4,5)P_4$. With these reservations in mind, the results shown in Fig. 3, when combined with those in Fig. 2(a), indicate that there must be substantial flux through $\text{Ins}(1,3,4,5)P_4$ during stimulation. This suggestion is also consistent with the observed rapid changes, both up and down, in the radioactivity in $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ during stimulation. Furthermore, the efficacy with which atropine induced the depletion of $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ suggests that either there is a receptor-controlled enzyme which manufactures $\text{Ins}(1,3,4,5)P_4$ directly, or there is an intermediate in the formation of $\text{Ins}(1,3,4,5)P_4$ which is itself very short-lived.

Hydrolysis of $\text{Ins}(1,3,4,5)P_4$, $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,4,5)P_3$ by parotid homogenates

The rates of hydrolysis of $\text{Ins}(1,3,4,5)P_4$, $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,4,5)P_3$ by a parotid homogenate are shown in Fig. 4. The hydrolysis rates of $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,4,5)P_3$ were very rapid, with the hydrolysis of $\text{Ins}(1,3,4)P_3$ less so. The substantial difference between the rates of hydrolysis of $\text{Ins}P_3$ isomers [i.e. $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$], as seen in carbachol-stimulated atropine-blocked slices (Fig. 3), suggests that we are studying substrate-specific, physiologically relevant, enzyme activities.

Parotid homogenates specifically catalysed the hydrolysis of $\text{Ins}(1,3,4,5)P_4$ to an $\text{Ins}P_3$ which chromatographed on h.p.l.c. with the mobility of $\text{Ins}(1,3,4)P_3$ (Fig. 5). It appears, therefore, that parotid homogenates contain an active $\text{Ins}(1,3,4,5)P_4$ 5-phosphomonoesterase similar to that observed in human erythrocytes (Batty *et al.*, 1985; Downes *et al.*, 1986). This result strongly suggests that the $\text{Ins}(1,3,4)P_3$ which accumulates in parotid slices during carbachol stimulation is formed via the production and subsequent hydrolysis of $\text{Ins}(1,3,4,5)P_4$. This notion is consistent with the slow accumulation of $\text{Ins}(1,3,4)P_3$, relative to $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ in carbachol-stimulated parotid slices (Fig. 2b; Irvine *et al.*, 1985), and also the slower accumulation of $\text{Ins}(1,3,4)P_3$ than of $\text{Ins}(1,4,5)P_3$ in fMet-Leu-Phe-stimulated dimethyl sulphoxide-differentiated HL60 cells (Burgess *et al.*, 1985) and angiotensin-stimulated hepatocytes (Burgess *et al.*, 1985). It is also consistent with the observation that there is considerable flux through $\text{Ins}(1,3,4,5)P_4$ during stimulation (see the previous section).

Hydrolysis of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ resulted in the formation of $\text{Ins}P_2$ isomers which were chromatographically distinct on our h.p.l.c. system (peaks C and D, Fig. 6b). We have not yet determined the phosphate distribution of these two $\text{Ins}P_2$ isomers, though on the basis of previous work we would expect the product of $\text{Ins}(1,4,5)P_3$ degradation to be $\text{Ins}(1,4)P_2$ (Downes *et al.*, 1982; Connolly *et al.*, 1985). Peak B in Fig. 6(b) has the chromatographic properties of P_1 . Peak A in Fig. 6(b) is unidentified, though we suspect it is $\text{Ins}4P$ formed by removal of the 1-phosphate of $\text{Ins}(1,4)P_2$ (any $\text{Ins}1P$ formed would not be detected since the 1-phosphate is not labelled); see Storey *et al.* (1984), who have described a similar activity in rat liver.

Analysis of inositol phospholipids in parotid slices

The discovery of $\text{Ins}(1,3,4,5)P_4$ accumulation in brain and parotid slices suggested the possible existence of a

Fig. 6. H.p.l.c. analysis of the products of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ hydrolysis

Samples of both $[\text{}^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[\text{}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ were incubated together with a parotid homogenate at 30°C , as described in the Materials and methods section. Incubations were terminated after (a) 0 or (b) 5 min by addition of HClO_4 , and neutral extracts were prepared and analysed by h.p.l.c. (see the Materials and methods section); $\bullet$, $[\text{}^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$; $\circ$, $[\text{}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$. The peaks designated A–D in (b) are referred to in the text. The data shown are from a single incubation. This experiment has been repeated twice with essentially identical results.

novel parent lipid, $\text{PtdIns}(3,4,5)\text{P}_3$ (Batty *et al.*, 1985). However, there have been no confirmed reports of the presence of $\text{PtdIns}(3,4,5)\text{P}_3$ in any tissue. We thought that the failure to detect $\text{PtdIns}(3,4,5)\text{P}_3$ might be due to difficulties in its extraction from tissue precipitates, since difficulties in extraction of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ have been well documented in many tissues (see Downes & Michell, 1982). One might predict that these difficulties would be accentuated with an even more polar lipid such as $\text{PtdIns}(3,4,5)\text{P}_3$. We avoided this problem by using a novel method for the analysis of phospholipids which did not use a conventional lipid extraction from tissue precipitates (see Materials and methods section). This method involved direct deacylation of the proteolipid tissue pellet obtained by addition of chloroform and methanol to parotid slices. This method had the added virtue of producing water-soluble glycerophosphoesters which could be analysed by h.p.l.c. [a method that would be certain of detecting a putative $\text{GroPIIns}(3,4,5)\text{P}_3$, by analogy with its ability to separate InsP_3 and InsP_4].

This methodology was applied to $[\text{}^3\text{H}]\text{inositol}$ -labelled parotid slices incubated with or without 1 mM-carbachol for 20 s. A comparison of the h.p.l.c. chromatograms of deacylated versus non-deacylated samples derived from each of the aqueous, chloroform and tissue pellet fractions from these slices (see the Materials and methods section) allowed us to identify which ^3H -labelled compounds were derived from ^3H -labelled lipids and which were genuine water-soluble compounds, e.g. inositol phosphates.

Such a comparison showed that each of the aqueous, chloroform and pellet fractions from control slices contained only three ^3H -labelled compounds which could be derived from lipids (i.e. which appeared on deacylation). These were one compound with the expected mobility on h.p.l.c. of GroPIIns (peak A, Fig. 7), and two compounds which co-chromatographed precisely on h.p.l.c. with $[\text{}^{32}\text{P}]\text{GroPIIns}4\text{P}$ and $[\text{}^{32}\text{P}]\text{GroPIIns}(4,5)\text{P}_2$ (only the results for the pellet fraction are shown; Fig. 7). As expected, most of the $[\text{}^3\text{H}]\text{GroPIIns}$ (92%) was

Fig. 7. H.p.l.c. analysis of ^3H -labelled glycerophosphoesters isolated from [^3H]inositol-labelled parotid slices

Parotid slices were incubated with [^3H]inositol ($10\ \mu\text{Ci}/250\ \mu\text{l}$) for 90 min, terminated by additions of chloroform and methanol, and 'tissue-pellet' fractions were obtained, precisely as described in the Materials and methods section. At this stage the ^3H -labelled samples were spiked with ^{32}P -labelled erythrocyte lipids. Two h.p.l.c. profiles of the ^3H radioactivity are shown: one of an extract prepared from a tissue-pellet deacylated by methylamine (—) and one of an extract prepared from a tissue-pellet left untreated (---) (see the Materials and methods section). Immediately before injection on to the column, both deacylated and undeacylated samples were spiked with [^{32}P]Ins(1,4,5) P_3 . This compound chromatographed identically in the two parallel runs shown; its peak position is indicated in the diagram. The peak positions of [^{32}P]GroP, [^{32}P]GroPIns4P and [^{32}P]GroPIns(4,5) P_2 in the deacylated sample are indicated in the diagram; no ^{32}P radioactivity, other than that in Ins(1,4,5) P_3 , was found in the undeacylated sample. Peak A has the expected mobility of GroPIns; peak B is referred to later in the text. The h.p.l.c. separation was carried out with a gradient of ammonium formate/phosphate as described in the Materials and methods section, except that the gradient was extended to include a linear step of 20 min from 1.7 M-ammonium formate (pH 3.7 with H_3PO_4) to 3.5 M-ammonium formate (pH 3.7 with H_3PO_4); 0.25 min fractions were collected throughout the gradient.

found in the chloroform fraction and most of the [^3H]GroPIns(4,5) P_2 (83%) was in the pellet fraction, but somewhat surprisingly, most of the [^3H]GroPIns4P (85%) was found in the aqueous fraction. It appears that, under neutral extraction conditions, GroPIns4P has the appropriate polarity to partition into the aqueous phase, but GroPIns(4,5) P_2 retains substantial affinity for the proteolipid pellet.

An analysis of those fractions obtained from slices stimulated with carbachol for 20 s gave qualitatively identical results (results not shown). Again, the only ^3H -labelled lipid-derived compounds present were [^3H]GroPIns, [^3H]GroPIns4P and [^3H]GroPIns(4,5) P_2 , but in these samples a major difference was the proportion of GroPIns(4,5) P_2 , which was approx. 50% of its value in control slices. This result is consistent with the ability of carbachol to cause rapid depletion of PtdIns(4,5) P_2 in parotid slices (Weiss *et al.*, 1982).

These results strongly suggest that the only inositol phospholipids in parotid slices that are acutely labelled with [^3H]inositol are PtdIns, PtdIns4P and PtdIns(4,5) P_2 , i.e. [^3H]PtdIns(3,4,5) P_3 is very unlikely to be the precursor of [^3H]Ins(1,3,4,5) P_4 during stimulation.

Ins(1,4,5) P_3 kinase in parotid homogenates

Parotid homogenates showed an ATP-dependent capacity to convert [^{32}P]Ins(1,4,5) P_3 into a compound which co-chromatographed on h.p.l.c. with [^3H]Ins(1,3,4,5) P_4 (Figs. 8 and 9). We estimate that, in the presence of ATP, approx. 15% of the Ins(1,4,5) P_3 was converted into Ins P_4 and 75% into Ins P_2 plus Ins P and P_i . It appears therefore that parotid homogenates possess an active Ins(1,4,5) P_3 kinase.

The existence of this kinase activity is consistent with the formation of Ins(1,3,4,5) P_4 from Ins(1,4,5) P_3 and may explain why the former is formed more slowly than the latter in stimulated parotid slices (Fig. 2a). This hypothesis is, however, far from proven: we do not yet know which isomer of Ins P_4 is formed by parotid homogenates [we have learned of a similar activity in brain homogenates which can convert Ins(1,4,5) P_3 into Ins(1,3,4,5) P_4 ; Irvine *et al.*, 1986], nor do we yet have information about the ability of these homogenates to phosphorylate other inositol phosphates. There have been previous reports of enzyme activities capable of phosphorylating Ins P_3 to Ins P_5 in avian erythrocytes

(Chakrabarti & Majumder, 1978), Ins to InsP_6 in plant seeds (see Biswas *et al.*, 1978, for access to the literature) and Ins to InsP in bovine liver and *Escherichia coli* (Dietz & Albersheim, 1965). Perhaps we have overlooked an important physiological role for inositol phosphate kinases during acute stimulation of animal cells by hormones and neurotransmitters.

In principle, it is also possible that $\text{Ins}(1,3,4,5)\text{P}_4$ arises from hydrolysis of an inositol pentakisphosphate [$\text{Ins}(1,3,4,5,6)\text{P}_5$ is present in avian erythrocytes; Johnson & Tate, 1969]. We have detected a ^3H -labelled compound in parotid extracts heavily labelled with [^3H]inositol, which has a mobility on h.p.l.c. expected of inositol pentakisphosphate (peak B in Fig. 7). In control slices, this compound contained a similar amount of radioactivity to that found in $\text{Ins}(1,3,4,5)\text{P}_4$ (Fig. 7). However, in slices stimulated with carbachol for 20 s, its radioactivity did not significantly increase, whereas the

Fig. 8. ATP-dependent phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to InsP_4 by parotid homogenates

Samples of [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ were incubated with parotid homogenates at 30 °C as described in the Materials and methods section, except that some incubations included 6 mM- MgCl_2 and 5 mM- Na_2ATP . Incubations were terminated at various times by addition of HClO_4 , and neutral extracts were prepared and analysed by chromatography on small AG-1 (formate) columns (see the Materials and methods section); ●, InsP_3 ; △, InsP_2 ; ○, InsP_4 . The data shown are from individual incubations in a single experiment. One further experiment has been performed with essentially identical results.

Fig. 9. H.p.l.c. analysis of the products of [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ metabolism

A sample of [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ was incubated with a parotid homogenate in the presence of 6 mM- MgCl_2 and 5 mM- Na_2ATP , at 30 °C, as described in the Materials and methods section. After 10 min, the incubation was terminated by addition of HClO_4 , and a neutral extract was obtained and analysed by h.p.l.c. (●). Immediately before injection on to the column, the sample was spiked with [^3H] $\text{Ins}(1,3,4)\text{P}_3$ and [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ (○). Since the samples of [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ and parotid homogenate used in this experiment were identical with those used in Fig. 6, Fig. 6(a) acts as zero-time control and Fig. 6(b) as a no-ATP control to the data presented here. The peaks designated A–C are equivalent to peaks A–C in Fig. 6(b). This experiment was repeated twice with essentially identical results.

Scheme 1. Metabolic pathways to formation of inositol phosphates in agonist-stimulated parotid glands

The interaction of an agonist (A; e.g. acetylcholine) with an appropriate receptor (R; e.g. the muscarinic receptor) stimulates a phospholipase C (phosphoinositidase C, PIC; Downes & Michell, 1985) that may be specific for hydrolysis of PtdIns(4,5)P₂. Ins(1,4,5)P₃ is the only water-soluble product of this agonist-dependent reaction, and accumulation of Ins(1,4,5)P₃ leads indirectly to formation of Ins(1,3,4,5)P₄ and Ins(1,4)P₂ by the actions of a specific kinase and a specific phosphatase respectively. Ins(1,3,4)P₃ is formed by the 5-specific dephosphorylation of Ins(1,3,4,5)P₄. The rapid formation and subsequent hydrolysis of both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ are consistent with these molecules having important functions and contrasts with the slower rates of turnover of Ins(1,4)P₂ and Ins(1,3,4)P₃. Abbreviation: DG, diacylglycerol.

radioactivity in Ins(1,3,4,5)P₄ rose approx. 5-fold (results not shown). Therefore we consider it unlikely that this compound is the precursor of Ins(1,3,4,5)P₄ during stimulation.

CONCLUSION

A qualitative interpretation of the results presented here suggest a simple mechanism for the accumulation of inositol phosphates during stimulation (Scheme 1). In this model, the key event is receptor-controlled activation of PtdIns(4,5)P₂-specific phospholipase C. The activity of this enzyme releases Ins(1,4,5)P₃ into the cytosol of the cell, whereupon it is metabolized in two ways: firstly, by removal of the 5-phosphate to form Ins(1,4)P₂, and secondly, by phosphorylation to form Ins(1,3,4,5)P₄. Ins(1,4)P₂ and Ins(1,3,4,5)P₄ are then hydrolysed, via inositol phosphate intermediates, to free inositol (Storey *et al.*, 1984). We do not know whether there is additional formation of InsP and InsP₂ via activation of PtdIns- and PtdIns4P-specific phospholipase(s) C. However, it is clear that, in principle, rapid formation of all the inositol phosphates seen during stimulation can be derived from an initial receptor-dependent generation of Ins(1,4,5)P₃.

Finally, since cells possess enzymes which catalyse rapid formation and degradation of Ins(1,3,4,5)P₄ during stimulation, it is possible that this compound has an

important physiological role in parotid acinar cells and in tissues where it is also rapidly produced upon stimulation [e.g. brain slices (Batty *et al.*, 1985) and hepatocytes (Palmer *et al.*, 1986)]. Since Ins(1,3,4,5)P₄ may be produced by phosphorylation of Ins(1,4,5)P₃ and is specifically degraded without re-forming Ins(1,4,5)P₃, we suggest that its function is likely to be distinct from that of Ins(1,4,5)P₃-catalysed Ca²⁺ release.

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