Inositol trisphosphates in carbachol-stimulated rat parotid glands

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Carbachol stimulation of rat parotid gland fragments prelabelled with *myo*-[³H]-inositol results in a large accumulation after 15 min of [³H]inositol trisphosphate. Only some of this is the D-1,4,5 isomer which would be expected to be derived from the known phosphatidylinositol bisphosphate. The predominant inositol trisphosphate is not susceptible to hydrolysis by human erythrocyte membranes. It yields altritol after periodate treatment followed by reduction and dephosphorylation, and, from partial dephosphorylation experiments, does not have a phosphate in the 2 position; the most likely structure of this inositol trisphosphate is therefore (D/L)-*myo*-inositol 1,3,4-trisphosphate. The possible origin and significance of this compound are discussed.

Several recent studies have shown that, in a variety of tissues, stimulation by agonists results in a rapid appearance of the inositol phosphates *InsP*₂ and *InsP*₃ (Agranoff *et al.*, 1983; Berridge, 1983; Berridge *et al.*, 1983; Rebecchi & Gershengorn, 1983; Downes & Wusteman, 1983), suggesting that an early event in the activation of these tissues may be the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate (Michell *et al.*, 1981; Creba *et al.*, 1983). Phosphoinositides have been implicated in calcium mobilization (Michell *et al.*, 1981; but see Hawthorne, 1983), and evidence has been presented that *Ins*(1,4,5)*P*₃ probably functions as an intracellular message for the release of Ca²⁺ from intracellular stores (Berridge, 1983; Streb *et al.*, 1983; Burgess *et al.*, 1984; Joseph *et al.*, 1984; Berridge & Irvine, 1984; Dawson & Irvine, 1984). We previously identified [³H]*InsP*₃ in carbachol-stimulated parotid glands, firstly on the basis of its behaviour on Dowex ion-exchange columns, and secondly by its ionophoretic mobility in a pH 3.6 pyridine/acetic acid buffer (Berridge *et al.*, 1983); because of the known structure of *PtdIns*(4,5)*P*₂ (Tomlinson & Ballou, 1961; Brown & Stewart, 1966), we assumed that it was D-*Ins*(1,4,5)*P*₃. We now show that although *Ins*(1,4,5)*P*₃ is indeed present, the predominant

[³H]*InsP*₃ in rat parotid glands prelabelled with [³H]inositol and then stimulated with carbachol for 15 min is not *Ins*(1,4,5)*P*₃. We also describe some experiments to determine its structure.

Materials and methods

Materials

Radioisotopes. *myo*-[2-³H]Inositol was purchased from New England Nuclear, and *myo*-[U-¹⁴C]-inositol was purchased from Amersham International.

Polyols. D-Glucitol, *myo*-inositol, xylitol, D-arabitol, and ribitol were purchased from BDH, Sigma and Aldrich. D-Altritol, D-iditol and D-gulitol were prepared from their corresponding hexoses (purchased from Sigma) by NaBH₄ reduction followed by removal of the NaBH₄ and deionization on mixed-bed ion-exchange columns essentially as described by Grado & Ballou (1961).

Inositol phosphates. *Ins*(1)*P* was prepared by enzymic hydrolysis (using a rat brain supernatant) of phosphatidylinositol, followed by ionophoretic separation (Dawson & Clarke, 1972; Irvine *et al.*, 1979). *Ins*(2)*P*, the principal *InsP* formed by acid hydrolysis of phytic acid (Pizer & Ballou, 1959), was purchased from Sigma. A mixture of *Ins*(1)*P*, *Ins*(4)*P* and *Ins*(5)*P* was prepared by alkaline hydrolysis of *Ins*(1,4,5)*P*₃ as described by Tomlinson & Ballou (1961), except that 24–36 h hydrolysis in conc. NH₃ at 110°C was used as that gave a better yield of inositol monophosphates. The latter

Abbreviations used: *InsP*, *myo*-inositol phosphate; *InsP*₂, *myo*-inositol bisphosphate; *InsP*₃, *myo*-inositol trisphosphate; *PtdIns*(4,5)*P*₂, phosphatidylinositol 4,5-bisphosphate.

were partially purified before use by high-voltage ionophoresis (Dawson & Clarke, 1972).

Ins(1,4,5) P_3 and Ins(2,4,5) P_3 were purified by paper chromatography of a crude Ins P_3 fraction prepared from ox brain by the methods of Grado & Ballou (1961) and Tomlinson & Ballou (1961) as modified by R. F. Irvine, M. J. Berridge & K. D. Brown (unpublished work). In brief, the modifications are primarily the adjustment of the alkaline hydrolysate to pH 3 with formic acid followed by removal of fatty acids with hexane, and then readjustment to pH 8 with NH_4OH before extensive dilution (to 1.5 litres) with water, and separation of crude Ins P , Ins P_2 and Ins P_3 fractions on Dowex formate columns (Ellis *et al.*, 1963; Downes & Michell, 1981). ^{32}P -labelled Ins(1,4,5) P_3 was prepared from human erythrocytes essentially by the method of Downes *et al.* (1982) as modified by Irvine *et al.* (1984) and Burgess *et al.* (1984). ^{3}H -Ins P_3 was prepared from rat parotid gland fragments stimulated with carbachol for 15 min (Berridge *et al.*, 1983; Downes & Wusteman, 1983) and was fractionated from other inositol phosphates on Dowex formate columns, and deionized by exactly the same procedure as the ^{32}P -Ins(1,4,5) P_3 . To increase recovery of the small mass amounts of ^{3}H -Ins P_3 extracted, the samples were usually 'spiked' by the addition of 0.2 μmol of Ins(1,4,5) P_3 before purification and deionization. Alkaline phosphatase from calf intestine was purchased from Boehringer Mannheim.

Methods

Paper chromatography of inositol trisphosphates was as described by Grado & Ballou (1961), i.e. using Whatman no. 1 paper and propan-2-ol/conc. NH_3 /water (7:1:2, by vol). Dowex column chromatography was by stepwise elution with formic acid/ammonium formate solutions from Dowex columns as described by Ellis *et al.* (1963) and Downes & Michell (1981). An alternative method employed briefly during our early experiments was elution by a linear gradient (0.02–1.02M-ammonium acetate) from a Pharmacia MonoQ column with Pharmacia FPLC equipment.

Separation of inositol monophosphates by paper chromatography was as described by Pizer & Ballou (1959) and Grado & Ballou (1961) i.e. using the same solvent as for inositol trisphosphates (above), or by chromatography in ethanol/conc. NH_3 (3:2, v/v) (Dawson & Clarke, 1972).

Degradation of inositol trisphosphates to non-cyclic polyols. This followed the method of Grado & Ballou (1961), except that the initial oxidation with NaIO_4 used only 0.5 ml of 0.1 M- NaIO_4 and the rest of the procedure was scaled down accordingly. As the very small quantities of Ins P_3 employed here

did not permit us to determine completion of the reaction, we simply left it to proceed for 5 days at room temperature. The lack of any radioactivity in *myo*-inositol after this treatment (see below) suggests that the destruction of the inositol ring was complete.

Separation and identification of non-cyclic ^{3}H polyols. The principal method of separating polyols that we used was that of Grado & Ballou (1961), i.e. chromatography on Whatman no. 1 paper in ethyl acetate/pyridine/boric acid-saturated water (12:5:4, by vol.). To standardize our conditions we always allowed the boric acid solution to saturate in a water bath at 25°C before making up the solvent; the chromatograms were run at room temperature. Under these conditions the mobilities of the non-cyclic polyols differed from those reported by Grado & Ballou (1961); *myo*-inositol moved about 20 cm from the origin in 30 h, and the approximate relative mobilities of the polyols relative to *myo*-inositol (mobility = 1) were as follows: D-iditol, 0.8; D-glucitol, 1.33; D-gulitol (= L-glucitol), 1.33; D-mannitol, 1.75; xylitol, 1.85; D-altritol, 2.1; D-arabitol, 2.3; ribitol, 2.4. D-Iditol was unique amongst these in that it never ran as a discrete spot, but invariably ran as a 5–7 cm streak just behind inositol.

We experienced difficulty in detecting the polyols because the benzidine-containing sprays employed by Grado & Ballou (1961) are now considered too toxic for general use, and the boric acid in the chromatography solvent interferes with detection by alkaline AgNO_3 (Frahn & Mills, 1959). However, we found that the detection method of Trevalyan *et al.* (1950) as modified by Frahn & Mills (1959) was satisfactory; that is, we included pentaerythritol in the ethanolic NaOH (saturated at 50°C) and omitted water from the same reagent. We also improved detection by dipping the paper in this reagent rather than spraying it, and fixed the papers 30 min later in 10% (w/v) $\text{Na}_2\text{S}_2\text{O}_3$. In this way, we were able to detect satisfactorily 0.2 mg of any non-cyclic polyol. A further problem encountered was that the silver staining procedure could completely remove (or quench) ^3H from the ^{3}H -polyols. Thus, for identifying ^{3}H -polyols we had always to run one radioactive sample plus an internal standard next to the identical standard at the same concentration. The latter strip was excised and developed to visualize the polyol, and the former was cut into 1 cm segments for radioactive counting. This procedure was carried out using in turn standards for all non-cyclic polyols which would be derived from inositol phosphates, and in this way the ^{3}H -polyols were identified.

Because this method of identifying ^{3}H -polyols is necessarily indirect, a second separation technique

was employed which separates these compounds on entirely different principles, and which would therefore widely separate any pairs of polyols which run close to each other using the above paper chromatographic technique. The method we found to be most satisfactory from this standpoint was ionophoresis in 0.1 M-NaOH (Frahn & Mills, 1959). As our ionophoretic apparatus is very different from that of Frahn & Mills (1959), the mobilities of the polyols in our hands are qualitatively and quantitatively different from their published data. After 5 h at 2 kV, all polyols had migrated towards the cathode from the centre of the paper, and the mean distances moved in cm were as follows: D-altritol, 6.5; D-mannitol, 11.0; D-glucitol, 12.5; D-gulitol, 12.5; D-iditol, 14.5; D-arabitol, 15.8; xylitol, 17.5; *myo*-inositol, 18.5; ribitol, 18.5. It is relevant to note, however, that these mobilities are in the reverse order of the mobilities towards the anode reported by Frahn & Mills (1959). As there is no boric acid present, 20 μ g of standard could easily be detected by AgNO_3 and so mixtures of all the standards, with or without [^3H]polyols derived from parotid [^3H]Ins P_3 , could be run side by side, the former strip cut into 0.5 cm strips for counting, and the latter

stained for carbohydrates as described above, but without pentaerythritol (Trevalyan *et al.*, 1950).

Because we employed these two separation techniques which are complementary to each other, our identification of any [^3H]polyol, although indirect, is unequivocal.

Results

General properties of [^3H]Ins P_3 from parotid glands

When [^{32}P]Ins(1,4,5) P_3 from erythrocyte ghosts is added to *myo*-[^3H]inositol-labelled parotid fragments after 15 min stimulation of the latter with carbachol, but immediately before quenching with trichloroacetic acid, the two radioactive isotopes do not exactly co-chromatograph when eluted from a Pharmacia MonoQ column with an acetate elution gradient (Fig. 1). This was our first indication of an Ins P_3 other than Ins(1,4,5) P_3 being present, and it also demonstrates that the various differences described below between the major [^3H]Ins P_3 in parotid glands and Ins(1,4,5) P_3 are not due to any artefact of the preparation process. The methods of preparation of the various radioactive and non-radioactive Ins P_3 samples are essentially very similar in any case, and Fig. 1

Fig. 1. Separation of water-soluble metabolites from the parotid gland by high resolution anion-exchange chromatography. Parotid gland slices were labelled with [^3H]inositol, washed and then stimulated with carbachol (10^{-3} M) for 10 min as described under 'Methods'. Standard compounds {Ins(2) P , Ins(1) P , [^{32}P]Ins(1,4) P_2 and [^{32}P]Ins(1,4,5) P_3 } were added before the incubations were stopped with trichloroacetic acid, so that they were carried right through the extraction procedure. Samples of this extract were subjected to high resolution anion-exchange chromatography on a Pharmacia MonoQ column eluted with an ammonium acetate (0.02–1.02 M) gradient. Each fraction was divided into two for separate analyses of phosphate content and of radioactivity with a dual label program that allowed measurement of ^3H and ^{32}P in the same samples. $\circ$, ^3H ; $\bullet$, ^{32}P ; $\blacktriangle$, increase in phosphate content when Ins(1+2) P standard was present; ---, acetate gradient, 0.02–1.02 M.

serves primarily to eliminate trichloroacetic acid quenching, or the presence of parotid tissue fragments, as possible causes of the differences. We could not achieve a complete separation of the two InsP_3 samples by this method, so all subsequent experiments were performed on a total $[\text{H}]\text{-InsP}_3$ fraction eluted from Dowex columns as described by Berridge *et al.* (1983).

Total dephosphorylation of the presumed $[\text{H}]\text{-InsP}_3$ from parotid glands was carried out either by prolonged treatment with alkaline phosphatase or by hydrolysis in 6M-HCl at 120°C for 18 h. Recovery from acid hydrolysis was poor (30–40%), probably because of some destruction of the inositol ring; however, more than 85% of the radioactivity was recovered after dephosphorylation with alkaline phosphatase. Paper chromatography of the products from both procedures in propan-1-ol/ethyl acetate/water (24:13:7, by vol.) for 18 h with an internal *myo*-inositol standard showed that all of the radioactivity was in inositol. This indicates that the ^3H -labelled compound (or compounds, see below) that we are studying here are not byproducts of inositol metabolism (for example, derivatives of glucuronic acid). It is conceivable that the inositol ring is not *myo*-inositol, but is another inositol instead; the most likely source of another inositol would be a contamination of the *myo*- $[\text{H}]\text{inositol}$. To eliminate this possibility, we performed one double-labelling experiment with *myo*- $[\text{H}]\text{inositol}$ and *myo*- $[\text{C}]\text{inositol}$ (which would not be contaminated in the same way, as they are prepared by entirely different methods). The $^3\text{H}/^{14}\text{C}$ ratio did not differ outside the experimental error (10%) between the free inositol added and the InsP , InsP_2 and InsP_3 recovered from the carbachol-stimulated tissue. This rules out contamination of the *myo*- $[\text{H}]\text{inositol}$ with another $[\text{H}]\text{inositol}$, although these experiments still cannot, strictly speaking, eliminate metabolic isomerizations within this tissue to form, for example, a *neo*-inositol trisphosphate (cf. Sherman *et al.*, 1978); *D-neo*-inositol 1,4,5-trisphosphate would in fact be consistent with all the data below. All the radioactivity in the $[\text{H}]\text{InsP}_3$ fraction co-chromatographed with $\text{Ins}(1,4,5)\text{P}_3$ when re-run on Dowex formate columns (Downes & Michell, 1981) or when subjected to paper chromatography (see below), and also after ionophoresis in pyridine/acetic acid at pH 3.6 (Dawson & Clarke, 1972), 0.06M-oxalate at pH 1.5 (Agranoff *et al.*, 1983), or ammonium carbamate at pH 9 (Clarke & Dawson, 1981).

A clear difference between $[\text{P}]\text{Ins}(1,4,5)\text{P}_3$ from erythrocytes and $[\text{H}]\text{InsP}_3$ from parotid fragments is seen in their relative stabilities to the enzyme in human erythrocyte membranes that specifically removes the 5-phosphate from

$\text{Ins}(1,4,5)\text{P}_3$ (Downes *et al.*, 1982). It is possible with extensive treatment (see legend to Fig. 2) to hydrolyse virtually all of the $[\text{P}]\text{Ins}(1,4,5)\text{P}_3$ (Fig. 2), but although a varying amount, depending on the individual parotid preparation, of the $[\text{H}]\text{-InsP}_3$ is hydrolysed rapidly, the remainder is remarkably resistant. Although the design of these experiments does not yield quantitative kinetics, the data in Fig. 2, and data from many experiments

Fig. 2. Relative stabilities of $[\text{P}]\text{Ins}(1,4,5)\text{P}_3$ from red blood cells, and $[\text{H}]\text{InsP}_3$ from carbachol-stimulated parotid glands, to human erythrocyte membrane InsP_3 phosphatase

$[\text{P}]\text{Ins}(1,4,5)\text{P}_3$ and $[\text{H}]\text{InsP}_3$, prepared as described under 'Methods', were mixed together so as to give approximately equal amounts of ^3H and ^{32}P radioactivity, and incubated with 0.12 mg of protein/ml (final volume 0.25 ml) of human erythrocyte membranes (pH 7.5, 2 mM- Mg^{2+}). To circumvent denaturation of the phosphatase and to ensure a near complete hydrolysis of the $[\text{P}]\text{Ins}(1,4,5)\text{P}_3$, additional portions (50 μl , containing 0.12 mg of protein) of erythrocyte membranes were added at 30 min and 60 min. The hydrolysis was stopped by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid, 1 ml of water and 0.05 ml of 5% (w/v) bovine serum albumin. The trichloroacetic acid was removed from the supernatant by four washes with diethyl ether (Irvine *et al.*, 1977) and the supernatant was neutralized, diluted to 10 ml and applied to Dowex columns in the formate form. InsP (+ P_1), InsP_2 and InsP_3 were eluted as described under 'Methods', and the InsP_3 fraction was counted for ^{32}P and ^3H . All radioactivity lost from InsP_3 could be accounted for by the formation of InsP_2 (for ^3H), and inorganic phosphorus and InsP_2 (for ^{32}P ; see Downes *et al.*, 1982). Results are expressed as % of the initial radioactivity for ^{32}P (●) and ^3H (○).

like it, all suggest that $[^3\text{H}]\text{InsP}_3$ in carbachol-stimulated parotid fragments is probably a mixture of two InsP_3 species, one hydrolysed by the 5-phosphatase, and the other not. In three separate preparations of $[^3\text{H}]\text{InsP}_3$ from parotid glands, the proportions of InsP_3 in this 'resistant' form were approx. 95, 85 and 70% of the total.

After paper chromatography for 10 days in propan-2-ol/conc. NH_3 /water (7:1:2, by vol.) (Grado & Ballou, 1961), the $[^3\text{H}]\text{InsP}_3$ co-chromatographed very nearly exactly with $\text{Ins}(1,4,5)\text{P}_3$, though the $[^3\text{H}]\text{InsP}_3$ may run slightly faster as there was some evidence that some of the ^3H label was beginning to separate from the $\text{Ins}(1,4,5)\text{P}_3$. Less than 5% of the radioactivity was recovered with an internal $\text{Ins}(2,4,5)\text{P}_3$ marker which ran ahead of, and distinct from, $\text{Ins}(1,4,5)\text{P}_3$ (Grado & Ballou, 1961; Tomlinson & Ballou, 1961).

Distribution of phosphates in the inositol ring

The principal method for determining the distribution of phosphates around the inositol ring in an inositol trisphosphate is by periodate oxidation (Grado & Ballou, 1961); periodate splits the bond between carbon atoms bearing vicinal hydroxyls, but the presence of a phosphate group on the carbon, or of two phosphates flanking it, protects the bond from attack. Thus, periodate treatment of an inositol trisphosphate followed by reduction and dephosphorylation, and subsequent identification of the resulting non-cyclic polyol, gives considerable insight into the parent InsP_3 submitted to such analysis. When we carried out this procedure with the $[^3\text{H}]\text{InsP}_3$ from parotid glands, we found that recovery of radioactivity in the polyols derived from $[^3\text{H}]\text{InsP}_3$ varied from 20 to 80%; most of the experiments performed to establish the identity of the polyols used a batch for which the recovery of radioactivity from the starting $[^3\text{H}]\text{InsP}_3$ was 76%. In all polyol preparations, two ^3H -labelled compounds were detected.

A minority of the label was found to be in iditol. In two separate parotid preparations the quantity of this minority (5 and 15%) was the same as the percentage of the parent InsP_3 that was hydrolysed rapidly by the erythrocyte membrane phosphatase (see Fig. 2), which suggests that 5–30% of the $[^3\text{H}]\text{InsP}_3$, depending on the individual preparation, is the 1,4,5 isomer (cf. Grado & Ballou, 1961; Tomlinson & Ballou, 1961). Further experiments will be necessary to make this identification unequivocal, as we have excluded neither $\text{Ins}(1,4,6)\text{P}_3$, which would also give D-iditol, nor the L-isomers of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4,6)\text{P}_3$, which would yield L-iditol.

The other ^3H -labelled polyol was identified as altritol. The separation methods used to identify

the $[^3\text{H}]\text{polyols}$ do not separate stereoisomers [D-glucitol and D-gulitol (=L-glucitol) co-chromatograph exactly], so the product may be D- or L-altritol. The only four possible InsP_3 structures (assuming a *myo*-inositol ring) are therefore D- $\text{Ins}(1,3,4)\text{P}_3$, its enantiomer L- $\text{Ins}(1,3,4)\text{P}_3$ [=D- $\text{Ins}(1,3,6)\text{P}_3$] and also D- and L- $\text{Ins}(1,2,4)\text{P}_3$ (see Fig. 3).

The latter pair of inositol trisphosphates, D- or L- $\text{Ins}(1,2,4)\text{P}_3$, can be eliminated or confirmed unequivocally by the demonstration of the presence or absence of $\text{Ins}(2)\text{P}$ as a product of partial dephosphorylation. Two dephosphorylation methods were employed, alkaline phosphatase and conc. NH_3 at 110°C . These are complementary in that the enzyme removes a phosphate group flanked by two free hydroxyls preferentially over one of a pair of vicinal phosphates (Grado & Ballou, 1961), whereas NH_3 should produce random hydrolysis. The disadvantage of the enzymic hydrolysis, as shown by Grado & Ballou (1961), is that the preference of the enzyme for lone phosphates results in very little accumulation of any InsP ; we found in addition that despite varying the time of incubation, hydrolysis by NH_3 also produces only a low yield of InsP . Nevertheless, by trial-and-error dilutions of the alkaline phosphatase, and by varying the times of hydrolysis by NH_3 , we were able to obtain enough InsP for a semiquantitative analysis using both hydrolysis methods. Although radioactivity was recovered in $\text{Ins}(1 \text{ or } 3)\text{P}$ [=D- or L- $\text{Ins}(1)\text{P}$], $\text{Ins}(4 \text{ or } 6)\text{P}$ [=D- or L- $\text{Ins}(4)\text{P}$], and $\text{Ins}(5)\text{P}$, in no experiments was any radioactive $\text{Ins}(2)\text{P}$ detected. These findings eliminate D- or L- $\text{Ins}(1,2,4)\text{P}_3$ as possible structures for the InsP_3 under examination.

In summary, only D- or L- $\text{Ins}(1,3,4)\text{P}_3$ as likely structures of the predominant InsP_3 , are consistent with all the above data. Resolution of these two enantiomers must await further experiments using chiral separation techniques.

Discussion

In this analysis of InsP_3 in parotid glands we have throughout been dealing with trace amounts of ^3H -labelled compounds. Thus, all of our conclusions are based solely on co-chromatography of radioactivity with authentic compounds, and clearly the identification of any structure by such indirect criteria cannot be absolute and various other possibilities are mentioned above. Nevertheless, within the limitations of these techniques we can be reasonably certain in our identification of the major InsP_3 in stimulated parotid glands as D- or L- $\text{Ins}(1,3,4)\text{P}_3$. There was also some of what our preliminary identification described above suggests is D- $\text{Ins}(1,4,5)\text{P}_3$; this yields iditol on perio-

Fig. 3. Derivation of alritrol from *myo*-inositol trisphosphates
Abbreviation used: (P), phosphate.

date degradation, and is apparently hydrolysed by the InsP_3 5-phosphatase in erythrocyte cell membranes (Fig. 2). We should perhaps note in passing that these data are in themselves preliminary evidence that erythrocyte- $[\text{^3H}] \text{InsP}_3$ is indeed D-Ins(1,4,5)P_3 , a fact which cannot, strictly speaking, be taken as rigorously established yet. Unequivocal identification of $\text{D-}[\text{^3H}] \text{Ins(1,4,5)P}_3$ in parotid glands must await further data, but in the light of the known structure of the major phosphatidylinositol bisphosphate, D-PtdIns(4,5)P_2 (Tomlinson & Ballou, 1961; Brown & Stewart, 1966), it seems highly likely that this InsP_3 is indeed D-Ins(1,4,5)P_3 , and that it is formed by phosphodiesteratic cleavage of PtdIns(4,5)P_2 ; its probable function as a second messenger for calcium mobilization is discussed in the Introduction.

There are two possible sources of the other InsP_3 , Ins(1,3,4)P_3 . Firstly, it may be formed by isomerization of Ins(1,4,5)P_3 , though that seems less likely than the second possibility, which is as a product of the phosphodiesteratic cleavage of another phosphatidylinositol bisphosphate, $\text{D- or L-PtdIns(3,4)P}_2$. Such a compound has not yet been described; it need only be a small proportion of the total PtdInsP_2 , and because of the resistance

of the Ins(1,3,4)P_3 to Ins(1,4,5)P_3 5-phosphatase (Fig. 2), it may be that a disproportionate amount of Ins(1,3,4)P_3 builds up after prolonged cell stimulation. Heterogeneity within the cell of polyphosphoinositides, and of the enzymes making and degrading them, has been described, and a multiplicity of functions suggested (see Irvine, 1982; Downes & Michell, 1982; Hawthorne, 1983, for reviews), so the possibility that some of these differences may be the result of two different phosphatidylinositol bisphosphates with different cellular functions is an intriguing one. It may be that a more detailed study of the rates of appearance of Ins(1,4,5)P_3 versus Ins(1,3,4)P_3 in carbachol-stimulated parotid fragments could clarify the latter's likely route of synthesis, and such an analysis may also explain why over several apparently similar parotid preparations there was some considerable degree of variation in how much InsP_3 was in the 1,3,4 form.

Whatever the route of synthesis, the existence of Ins(1,3,4)P_3 is not without significance. Its ability to mobilize Ca^{2+} [or to antagonize Ins(1,4,5)P_3 -induced mobilization] is as yet unknown. If it is not active, then if produced by an isomerization of Ins(1,4,5)P_3 , this could act as a possible route of inactivation of the latter compound. If Ins(1,3,4)P_3

is active in this respect, either positively or negatively, then its metabolic stability compared with $\text{Ins}(1,4,5)P_3$ has important implications for calcium homeostasis. In this context, it should be emphasized that its stability is only comparative; on cessation of agonist stimulation of parotid fragments, all $\text{Ins}P_3$ is rapidly catabolized (Downes & Wusteman, 1983). More pertinently, if $\text{Ins}(1,3,4)P_3$ has no effect on calcium homeostasis, any attempts to correlate calcium mobilization with $[^3\text{H}]\text{Ins}P_3$ levels in stimulated tissues will need to be tempered by the reservation that some (sometimes most) of the $[^3\text{H}]\text{Ins}P_3$ is not the 1,4,5 isomer. Finally, it may be that $\text{Ins}(1,3,4)P_3$ is a cellular messenger in its own right with distinct functions, and this is a possibility that requires further investigation.

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