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Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands

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(1) A complete separation of *myo*-inositol 1,4,5-[4,5-^{32}P]trisphosphate prepared from human erythrocytes, and *myo*-[2-^{3}H]inositol 1,3,4-trisphosphate prepared from carbachol-stimulated rat parotid glands [Irvine, Letcher, Lander & Downes (1984) *Biochem. J.* **223**, 237–243], was achieved by anion-exchange high-performance liquid chromatography. This separation technique was then used to study the metabolism of these two isomers of inositol trisphosphate in carbachol-stimulated rat parotid glands. (2) Fragments of glands were pre-labelled with *myo*-[2-^{3}H]inositol, washed, and then stimulated with carbachol. At 5s after stimulation a clear increase in inositol 1,4,5-trisphosphate was detected, with no significant increase in inositol 1,3,4-trisphosphate. (3) After this initial lag however, inositol 1,3,4-phosphate rose rapidly; by 15s it predominated over inositol 1,4,5-trisphosphate, and continued to rise so that after 15min it was at 10–20 times the radiolabelling level of the 1,4,5-isomer. (4) In contrast, after the initial rapid rise (maximal within 15s), inositol 1,4,5-trisphosphate levels declined to near control levels after 1min and then rose again very gradually over the next 15min. (5) When a muscarinic blocker (atropine) was added after 15min of carbachol stimulation, inositol 1,4,5-trisphosphate levels dropped to control levels within 2–3min, whereas inositol 1,3,4-trisphosphate levels took at least 15min to fall, consistent with the kinetics observed earlier for total parotid inositol trisphosphates [Downes & Wusteman (1983) *Biochem. J.* **216**, 633–640]. (6) Phosphatidylinositol bisphosphate (PtdInsP_2) from stimulated and control cells were degraded chemically to inositol trisphosphate to seek evidence for ^{3}H-labelled PtdIns(3,4)P_2. No evidence could be obtained that a significant proportion of PtdInsP_2 was this isomer; in control tissues it must be $<5\%$ of the total PtdInsP_2 radiolabelled by *myo*-[2-^{3}H]inositol. (7) These data indicate that, provided that inositol 1,4,5-trisphosphate is studied independently of inositol 1,3,4-trisphosphate, the former shows metabolic characteristics consistent with its proposed role as a second messenger for calcium mobilization. The metabolic profile of inositol 1,3,4-trisphosphate is entirely different, and its function and source remain unclear.

Stimulation of a wide range of tissues by many agonists causes a selective enhancement in inositide metabolism (see Michell, 1975) including a stimulated phosphodiesteratic cleavage of PtdInsP_2 to form the second messenger, diacylglycerol (Nishizuka, 1984), and InsP_3 (Michell *et al.*, 1981; Berridge, 1984; Berridge & Irvine, 1984). Berridge (1983) proposed that Ins(1,4,5)P_3 is also a second messenger whose principal effect is calcium mobilization, and considerable experimental evidence in support of this proposal has been obtained (Streb *et al.*, 1983; Berridge & Irvine, 1984).

In order to demonstrate that this hypothesis applies to the mode of action of Ca^{2+}-mobilizing

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Abbreviations used: Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns, PtdIns*P*, PtdInsP_2, phosphatidylinositol and its mono- and bis-phosphates; Gro*P*Ins, Gro*P*Ins*P* and Gro*P*InsP_2, glycerophosphoinositol and its mono- and bis-phosphates.

receptors in rat parotid glands, one necessary objective is to assess the rate and amount of Ins(1,4,5)P_3 accumulation in the stimulated gland. Initial support for these proposals came from the demonstration that stimulation of muscarinic cholinergic, α_1-adrenergic, or substance P receptors results in a substantial and rapid accumulation of ^{3}H-labelled InsP_3 in parotid gland preparations (Berridge *et al.*, 1983; Downes & Wusteman, 1983; Aub & Putney, 1984). The mode of action of these receptors is known to involve the mobilization of Ca^{2+} from a common intracellular pool (Putney, 1977, 1982). In contrast, activation of parotid gland β-adrenergic receptors involves the activation of adenylate cyclase without Ca^{2+} mobilization (Schramm & Selinger, 1977) and does not cause accumulation of inositol phosphates (Berridge *et al.*, 1982).

However, our present understanding is complicated by the discovery that in stimulated rat parotid glands there are two InsP_3 isomers, Ins(1,4,5)P_3 and Ins(1,3,4)P_3 (Irvine *et al.*, 1984). Furthermore, the source and function of the novel inositol trisphosphate, Ins(1,3,4)P_3, are as yet unknown. A complete separation of these two InsP_3 isomers, which could lead to a study of the metabolism of each of them in stimulated tissues, is therefore an essential first step in clarifying the picture, and it is to this aim that the present study was directed.

Materials and methods

Radioisotopes

myo-[^{3}H]Inositol was purchased from New England Nuclear or Amersham International.

Rat parotid glands

Rat parotid gland slices were prepared and prelabelled with *myo*-[2-^{3}H]inositol exactly as described by Downes & Wusteman (1983). The final incubations of prelabelled slices each contained 0.46–0.72mg of protein, the exact value varying from one preparation to another.

Preparation of samples for h.p.l.c.

Incubations were terminated by addition of trichloroacetic acid followed by washing with diethyl ether as described previously (Downes & Wusteman, 1983). The samples were neutralized by adding portions of Tris base. Mannitol (100μl of 50mM) was added to each sample (to aid recovery of the inositol trisphosphates after freeze drying) and the samples were then freeze-dried. Samples were finally dissolved in 2ml of 1mM-EDTA, pH7.0, or water for injection onto the h.p.l.c. column.

Separation of InsP_3 isomers

This was initially achieved using [^{3}H]InsP_3 from rat parotid glands stimulated with carbachol for 15min (which is predominantly the 1,3,4 isomer; Irvine *et al.*, 1984), and Ins(1,[4,5-^{32}P])P_3 from human erythrocytes (Downes *et al.*, 1982; Irvine *et al.*, 1984). The chromatography column was a 0.46cm × 25cm Partisil SAX 10 high pressure anion exchange column (packed by Technicol, Stockport, Cheshire SK1 3HS, U.K.). In preliminary trials we used adenine nucleotide markers (adenine, AMP, ADP and ATP), and followed their elution by their absorption at 254nm; we tried in particular to avoid using phosphate in the eluting medium so that non-radiolabelled inositol phosphates could be analysed. In our hands however, really sharp peak profiles of nucleotides, in turn indicative of entire separation of the InsP_3 isomers, were only achieved by including some phosphate in the eluting medium.

The final conditions of elution were as follows. Samples in water were loaded onto the column after routine spiking with AMP, ADP and ATP and approx. 300d.p.m. of Ins(1,[4,5-^{32}P])P_3. Water was then allowed to flow through for 6min at 1.25ml/min (and this flow rate was maintained throughout for elution). Then, over 24min a linear gradient was passed through the column, rising from water to 100% 1.0M-ammonium formate buffered to pH3.7 with orthophosphoric acid (i.e. P_i content approx. 0.5M). The formate/phosphate buffer was passed through for a further 5min, and then over the following 2min the eluant was returned linearly to water. Finally, a further 10min of water elution was employed before the column was ready for the next injection.

Most samples of inositol phosphates from parotid glands were 2.0ml in volume. The routine spiking with nucleotides helped to ensure that each sample was behaving normally (by following the A_{254}), and the inclusion of Ins(1[4,5-^{32}P])P_3 ensured that the location of this compound was always unambiguous; there was no significant spill-over of ^{32}P radioactivity into the ^{3}H channel. Routinely, we collected 32 × 0.25min samples, starting 3min before ATP was eluted, and finishing 5min afterwards; Ins(1,3,4)P_3 was eluted with, or very close to, ATP, and Ins(1,4,5)P_3 was eluted shortly afterwards (see below). The 0.31ml samples were diluted with 0.5ml of water and 0.5ml of methanol, and then 5ml of scintillant was added.

Although this elution regime was specifically designed for analysis of InsP_3 isomers and was routinely used for this, on some occasions we did measure other inositol phosphates by collecting 1

or 0.5 min samples throughout the gradient up until just before ATP was eluted, and then changed to 0.25 min samples. Elution times were found to vary slightly between different columns, but the overall pattern is reproducible; a typical separation is shown in Fig. 1. In this sample there are negligible counts in Gro*P*Ins and Gro*P*InsP_2. These two were found to be eluted just before ADP and 2–3 ml before Ins(1,3,4)P_3 respectively (marked on Fig. 1); the separation of Gro*P*Ins*P* from InsP_2 is not very wide, and for a detailed study of these two a change in the gradient may be necessary.

Chemical degradation of inositides to inositol phosphates

^{3}H-labelled lipids from parotid fragments (Downes & Wusteman, 1983) were deacylated exactly as described by Clarke & Dawson (1981). The glycerol moiety was then removed essentially as described by Brown & Stewart (1966), that is, by limited digestion with 0.1 M- or 0.01 M-periodate (trial and error with radioactive samples, or following the A_{260} with Gro*P*InsP_2 from ox brain, lead us to use 90 min at room temperature, which completely removed the glycerol from Gro*P*InsP_2 while leaving the InsP_3 structure substantially intact). This was followed by quenching with ethylene glycol and removal of the remaining aldehyde from the 1-phosphate with 1′,1′dimethylhydrazine (Brown & Stewart, 1966). The dimethylhydrazine was removed by filtering through Dowex W50 beads, and finally the solution was neutralized with NH_3 before being loaded on to the h.p.l.c. column. For the h.p.l.c. analysis of these samples, the collection of fractions from the column was started earlier than usual, so that any Gro*P*InsP_2 remaining after the periodate treatment was also collected. In this way we were able to check routinely that >95% of the glycerol moiety had been removed.

Results

Separation of Ins(1,3,4)P_3 and Ins(1,4,5)P_3

Fig. 2 shows a typical elution profile of a separation of the two InsP_3 isomers by anion-

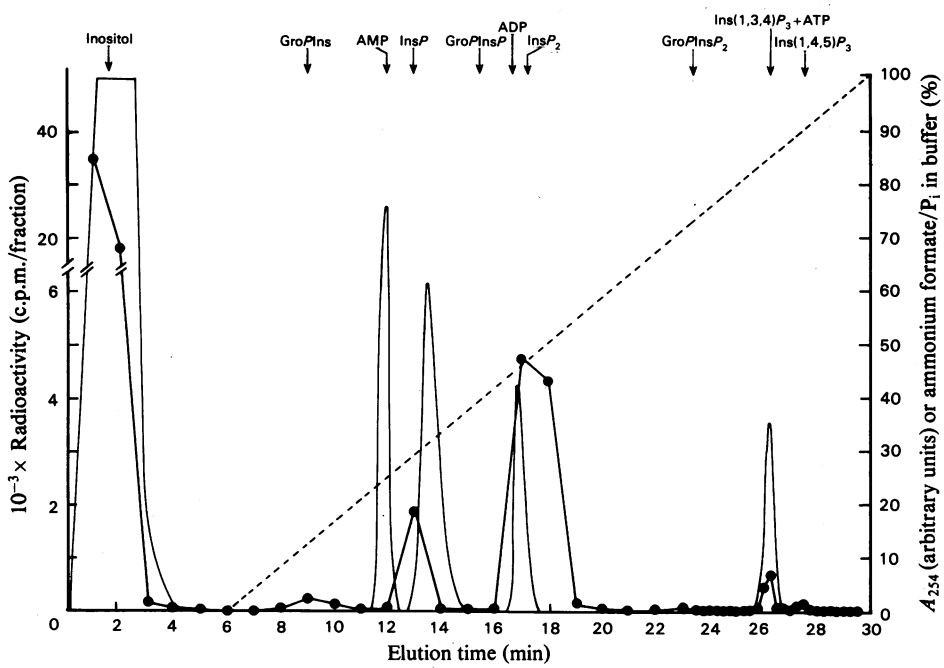

Fig. 1. *Analysis of inositol phosphates by h.p.l.c.*

For experimental details see the Materials and methods section. Inositol phosphates from carbachol-stimulated parotid glands pre-labelled with *myo*-[2-^{3}H]inositol and stimulated with carbachol for 15 min were loaded onto the column with AMP, ADP and ATP markers. The gradient profile eluting from the column (----) is superimposed on the A_{254} (——) and radioactivity (●—●). Note the unidentified absorbing materials eluting with water, and between AMP and ADP. The identification of the inositol phosphates is based on comparison with standards isolated by conventional Dowex anion-exchange chromatography, and has not been supported by further characterization (cf. Berridge *et al.*, 1983). The approximate elution positions of Gro*P*Ins*P* and Gro*P*InsP_2 are marked.

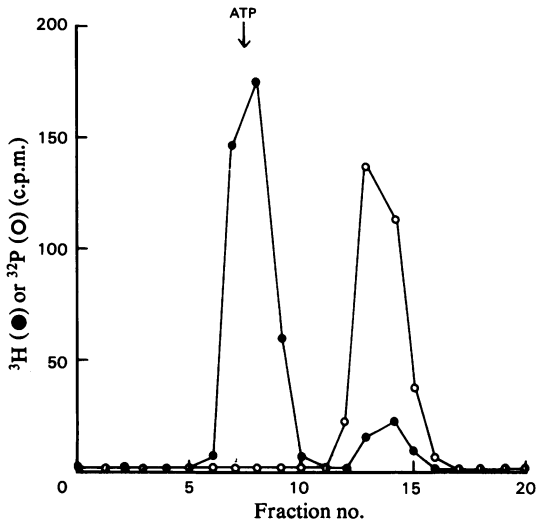

Fig. 2. *Separation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 by high-performance ion exchange chromatography*
For experimental details see the Materials and methods section. The column was loaded with [^{32}P]InsP_3 from red blood cells, and *myo*-[^{3}H]inositol-labelled InsP_3 from carbachol-stimulated parotid glands. The arrow indicates where the peak of ATP marker eluted from the column. ●, ^{3}H; ○, ^{32}P.

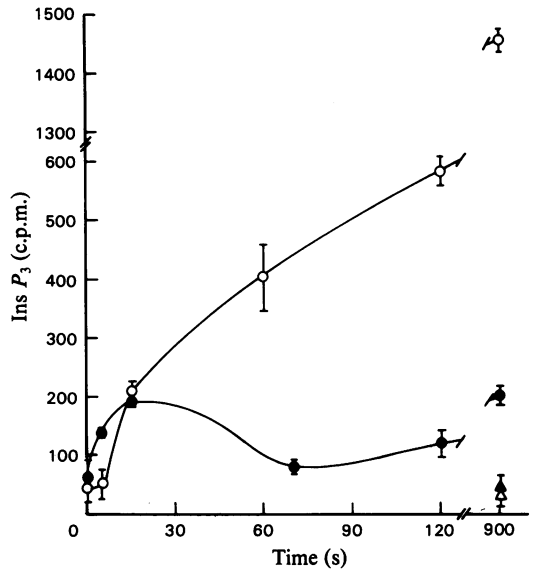

Fig. 3. *Rate of appearance of inositol trisphosphate in carbachol-stimulated rat parotid glands*
Rat parotid gland fragments were prelabelled with *myo*-[2-^{3}H]inositol, washed and stimulated with carbachol as described in the Materials and methods section. After quenching with trichloroacetic acid, the acid-soluble fraction was analysed by anion-exchange h.p.l.c. Control samples (no carbachol added) incubated for 15min showed no significant increase in either InsP_3 isomer (triangles). The data represent the means for triplicate incubations ± S.E.M. The data are all derived from one experiment, but very similar data were obtained in three independent experiments. ●, Ins(1,4,5)P_3; ○, Ins(1,3,4)P_3.

exchange h.p.l.c. It is evident that there is a complete baseline separation of the two isomers, enabling us to study them independently of each other. In some Ins(1,4,5)P_3 preparations we detected another ^{32}P peak (5–10%) coincident with Ins(1,3,4)P_3; this was not ATP, as we analysed two such batches of [^{32}P]InsP_3 by ionophoresis and their [^{32}P]ATP content was 0.01%. This second peak is also not for the most part Ins(1,3,4)P_3 because when it was isolated, desalted and analysed by ionophoresis at pH 3.6 (Dawson & Clarke, 1972) or pH 9.0 (Clarke & Dawson, 1981) at least 80% of the radioactivity migrated faster than an internal marker of Ins(1,4,5)P_3. Ins(1,3,4)P_3 has an identical ionophoretic mobility to Ins(1,4,5)P_3 in these two buffers (Irvine *et al.*, 1984). Some samples of Ins(1,4,5)P_3 (such as the one used for the experiment in Fig. 2) contained no detectable second peak of ^{32}P-labelled material. At present we have no explanation for the occasional and unpredictable appearance of this other compound.

Rates of appearance of inositol trisphosphates in parotid glands

The separation of the two isomers enabled us to study their relative rates of appearance in an extensive series of experiments using carbachol-stimulated parotid glands. Fig. 3 shows a typical result over the first 15min of stimulation. It should be noted in passing that over a number of experiments the absolute values of radioactivity varied considerably, despite the same [^{3}H]inositol addition and weight of tissue. The four experiments of which one is represented in Fig. 3 gave very similar quantitative data, but in two other experiments the radiolabelling was much lower. In these latter experiments the kinetics of Ins(1,3,4)P_3 were similar to Fig. 3, but insufficient radioactivity was present to study Ins(1,4,5)P_3. The reason for this variation in tissue labelling is not known.

Within the first 1 min of stimulation the kinetics of the two inositol trisphosphates are clearly very different. In particular, at the earliest times examined Ins(1,4,5)P_3 is predominant, and may be produced with no apparent time lag (within the resolution time of these experiments). Ins(1,4,5)P_3 reaches its maximal level by about 15s, and then it declines, although it remains above control levels at least up to 15min. This marked peak of Ins(1,4,5)P_3 production, followed by a trough and

then a gradual rise, was reproducible over several experiments.

Ins(1,3,4)P_3 follows a very different pattern. There is a clear and reproducible lag before any increase is detectable. Once its formation begins, its synthesis is very rapid, though we must emphasize that until we know more about where it comes from we cannot assume that it has the same specific radioactivity as Ins(1,4,5)P_3, and so we cannot draw any firm conclusions about the absolute rate of synthesis of Ins(1,3,4)P_3 relative to that of Ins(1,4,5)P_3. Ins(1,3,4)P_3 continues to rise after the first 1 min of stimulation and, as shown previously by total InsP_3 measurements (Downes & Wusteman, 1983; Aub & Putney, 1984) reaches a steady state after about 15 min. At this time it predominates by about 10-fold over Ins(1,4,5)P_3 (Irvine *et al.*, 1984).

Disappearance of inositol trisphosphates

Previous experiments have attempted to examine the rate of catabolism of inositol phosphates in carbachol-stimulated parotid glands by blocking the activation with antagonists (Downes & Wusteman, 1983; Aub & Putney, 1984). The existence of two InsP_3 isomers clearly complicates the interpretation of these data, and Fig. 4 shows data from an experiment designed to repeat these receptor-blocking experiments, but examining the disappearance of the two InsP_3 isomers separately. It is apparent that whereas the decline of Ins(1,3,4)P_3 is, not surprisingly, similar to that of total InsP_3 in earlier experiments (Downes & Wusteman, 1983; Aub & Putney, 1984), the proportional rate of disappearance of Ins(1,4,5)P_3 is very much faster. The low level of counts in this isomer measured in the continuing presence of high levels of Ins(1,3,4)P_3 is pushing the h.p.l.c. separation to its limit, and so exact quantification is difficult, but it is clear that Ins(1,4,5)P_3 has returned to near control levels, probably within 2–3 min of receptor blocking. We emphasize that these experiments were designed to measure specifically the disappearance of Ins(1,4,5)P_3, and the curve showing the decline of Ins(1,3,4)P_3 is only approximate; more detailed kinetics on total InsP_3 [which after the first 3 min is entirely Ins(1,3,4)P_3] can be found in Downes & Wusteman (1983) and in Aub & Putney (1984).

PtdInsP_2 in parotid cells

A possible source of Ins(1,3,4)P_3 is PtdIns(3,4)P_2 (Irvine *et al.*, 1984), so in one experiment we looked for evidence for this lipid in parotid fragments labelled with [^{3}H]inositol as in Downes & Wusteman (1983), before or after 15 min stimulation with carbachol. In triplicate samples of control and stimulated glands we were

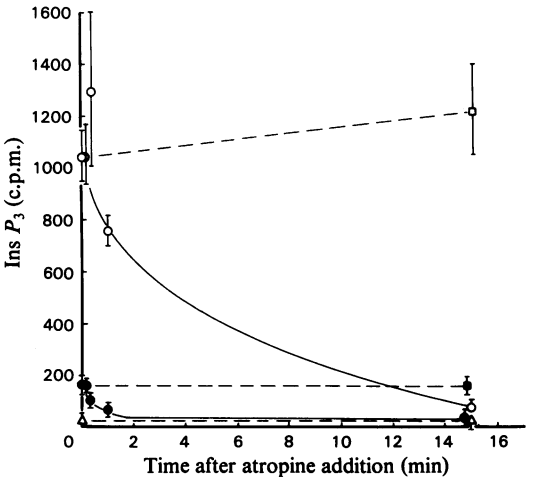

Fig. 4. *Disappearance of inositol trisphosphates following receptor blocking*

Parotid glands were radiolabelled and stimulated with carbachol for 15 min as described in the legend to Fig. 1. Then 10 μM-atropine was added (Downes & Wusteman, 1983) and samples were taken at timed intervals after that. Filled symbols, Ins(1,4,5)P_3; open symbols, Ins(1,3,4)P_3. Samples which received no carbachol, either during the first 15 min or the subsequent incubation, are marked as triangles. Samples treated with carbachol for 15 min but with no atropine added (i.e. incubated a further 15 min with carbachol alone) are marked as squares. Data are from one experiment, but similar data were obtained in an identical experiment.

unable to detect any PtdIns(3,4)P_2; that is, at least 95% of the InsP_3 derived from PtdInsP_2 (see the Materials and methods section) was Ins(1,4,5)P_3. There may be very small amounts of radiolabelled PtdIns(3,4)P_2 which we cannot detect because our samples contained insufficient radioactivity, especially after stimulation (which decreases PtdInsP_2 levels), but from our data we can state with confidence that in these experiments at least 95% of the [^{3}H]inositol-labelled PtdInsP_2 in control tissue was PtdIns(4,5)P_2.

Discussion

Ins(1,4,5)P_3 as a second messenger in parotid gland

The results described here throw light on both of the isomers of InsP_3 under consideration. Firstly, by removing Ins(1,3,4)P_3 from the Ins(1,4,5)P_3 we have been able to study unambiguously for the first time the latter isomer's metabolism in a stimulated tissue. Having done so, it is clear that its metabolism is entirely consistent with the proposed role as a second messenger (Berridge, 1983; Streb *et al.*, 1983; Berridge & Irvine, 1984), in that it is produced very rapidly on stimulation with

little apparent time lag. Berridge *et al.* (1984) have used more sophisticated methods for studying rapid InsP_3 kinetics in 5-hydroxytryptamine-stimulated salivary glands from *Calliphora*, and have shown that the time lag of InsP_3 production is less than 1 s, whereas the lag before any appearance of a Ca^{2+}-mediated response is more than 1 s. A preliminary examination of [^{3}H]InsP_3 from blowfly glands has shown that InsP_3 in this tissue is mostly Ins(1,4,5)P_3 (M. J. Berridge, J. P. Heslop & R. F. Irvine, unpublished work) and the kinetics of Ins(1,4,5)P_3 in the present experiments suggest that the metabolism of this compound in parotid gland is very similar to that in the blowfly salivary gland.

The rate of disappearance of Ins(1,4,5)P_3 is also consistent with its proposed messenger role. The physiological responses of the parotid gland (such as rubidium efflux) which are believed to be mediated by Ca^{2+}, return within 3–4 min to control levels on blocking of the receptor (Poggioli & Putney, 1982). Previous kinetic observations on the decline of InsP_3 in parotid glands (Downes & Wusteman, 1983; Aub & Putney, 1984) appeared to be at odds with this, in that InsP_3 took 15 or more minutes to return to control levels. The experiments in Fig. 4 show that the decline of Ins(1,4,5)P_3 is more rapid, consistent with its proposed role in calcium mobilization. They also show that previous calculated rates of InsP_2 formation and hydrolysis (Downes & Wusteman, 1983; Aub & Putney, 1984) are complicated by the different kinetics of disappearance of the two InsP_3 isomers, and so the previous conclusion of these workers (that not all of the InsP_2 could be derived from InsP_3, and therefore that some phosphodiesteratic hydrolysis of PtdIns4P is occurring), must now be re-interpreted; this question remains open.

Source of Ins(1,3,4)P_3

While the data above are consistent with the suggested role of Ins(1,4,5)P_3 in cellular metabolism, they still leave open some essential questions about Ins(1,3,4)P_3. Our inability to detect radioactive PtdIns(3,4)P_2 before stimulation (limit of unequivocal detection is 5%) is interesting in view of the rapid formation (after the initial lag) of Ins(1,3,4)P_3 in stimulated glands. This result could imply that Ins(1,3,4)P_3 is produced by isomerization of Ins(1,4,5)P_3, but if that is so, then the isomerase can only be active some several seconds after stimulation (for example it might be activated by calcium).

An alternative suggestion is that PtdIns(3,4)P_2 is indeed the precursor of Ins(1,3,4)P_3 but that it is only formed on stimulation (at the location of phosphodiesterase action), so that it is immediately hydrolysed. Several groups (De Chaffoy de Courcelles *et al.*, 1984; Taylor *et al.*, 1984; Halenda & Feinstein, 1984) have reported a phorbol ester-stimulated rise in PtdIns$P$ and PtdInsP_2 radiolabelling which they interpret as a probable protein kinase C-mediated stimulation of PtdIns kinase and PtdIns$P$ kinase (and possibly PtdIns synthetase), presumably by a phosphorylation of these enzymes. If those data represent in part either the activation of different enzymes or allosteric modification of inositide synthetases (so that their catalytic properties are altered), to form some PtdIns(3,4)P_2, then the present results would be explained. At present the simplest suggestion for Ins(1,3,4)P_3 formation in these glands is the stimulation of a PtdIns(4)P-3-kinase, but the other possibilities discussed above remain open.

Function of Ins(1,3,4)P_3

The function of Ins(1,3,4)P_3 remains unknown; the time lag before it appears, and its comparatively slow catabolism as discussed above, make it unlikely that it has anything to do with acute Ca^{2+} homeostasis in the cell, though the evidence for that is purely circumstantial. It may be physiologically inactive yet functional, because it could represent a form of desensitization of one branch of inositide messenger function [that using Ca^{2+} via Ins(1,4,5)P_3] without altering the other branch (that using kinase C via diacylglycerol). If we assume similar specific radioactivities of the two InsP_3 isomers, then the data in Figs. 3 and 4 suggest similar rates of production of the two isomers (once a steady state has been reached) and thus there may be a significant increase of the diacylglycerol-to-Ca^{2+} stoichiometry, caused by the production of Ins(1,3,4)P_3.

An alternative to this hypothesis would be that Ins(1,3,4)P_3 is a second messenger in its own right with intracellular targets distinct from Ins(1,4,5)P_3 and diacylglycerol. Whichever of these is the correct explanation, the identification of both Ins(1,3,4)P_3 and Ins(1,4,5)P_3 in stimulated parotid glands provides another example of the considerably versatility of the inositol phospholipid-dependent receptor signalling system.

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