Specificity of inositol phosphate-stimulated Ca^{2+} mobilization from Swiss-mouse 3T3 cells

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Pure samples of inositol 1,3,4-trisphosphate, inositol 1,3,4,5-tetrakisphosphate and inositol 1,2-cyclic 4,5-trisphosphate were prepared and tested for their ability to mobilize calcium from intracellular stores in a permeabilized Swiss mouse 3T3 cell preparation. In this system inositol 1,4,5-trisphosphate mobilizes Ca^{2+} with a half-maximal dose of 0.3 μM. Inositol 1,2-cyclic 4,5-trisphosphate mobilized Ca^{2+} to the same extent with a half-maximal dose of 0.3 μM, whereas inositol 1,3,4-trisphosphate required a half-maximal dose of approx. 9 μM to give the same effect. Inositol 1,3,4,5-tetrakisphosphate was ineffective up to 20 μM and at that concentration did not antagonize the mobilization induced by inositol 1,4,5-trisphosphate. The relevance of these findings to the function of the inositol tris/tetrakis-phosphate pathway is discussed.

INTRODUCTION

Earlier studies on permeabilized cell preparations of mouse pancreatic acinar cells (Streb *et al.*, 1983), Swiss mouse 3T3 cells (Berridge *et al.*, 1984; Irvine *et al.*, 1984*a*) and guinea-pig hepatocytes (Burgess *et al.*, 1984*a*) have established in a preliminary form the specificity of inositol phosphate-induced Ca^{2+} mobilization from the endoplasmic reticulum (see Berridge & Irvine, 1984; Berridge, 1986, for reviews). However, of the various inositol phosphates tested so far that are active, only one, Ins(1,4,5)P_3, occurs naturally (though glycerophosphoinositol 4,5-bisphosphate may be formed as a result of deacylation of phosphatidylinositol 4,5-bisphosphate). Recently two novel inositol phosphates have been identified in animal tissues from radiolabelling studies, Ins(1,3,4)P_3 (Irvine *et al.*, 1984*b*) and its precursor Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). The latter compound is formed by a specific 3-phosphorylation of Ins(1,4,5)P_3 (Irvine *et al.*, 1986). Also, Wilson *et al.* (1985*a*,*b*) have shown that Ins(1,2cyc4,5)P_3 is formed by phosphatidylinositol 4,5-bisphosphate phosphodiesterase *in vitro*, and that this compound can mobilize Ca^{2+} from permeabilized platelets. As yet, unambiguous evidence for any appreciable formation of Ins(1,2cyc4,5)P_3 in intact cells is not available, but it may well exist in some tissues under certain conditions (see Ishii *et al.*, 1986).

We have prepared sufficient quantities of these three novel inositol phosphates in a pure form to test in the permeabilized Swiss mouse 3T3 cell system used previously for such specificity studies (Berridge *et al.*, 1984; Irvine *et al.*, 1984*a*), so that we can assess the relevance of these recent discoveries to Ca^{2+} mobilization.

MATERIALS AND METHODS

Permeabilized Swiss mouse 3T3 cells

This is the same preparation as described in Berridge *et al.* (1984), and the assay for Ca^{2+} mobilization is as described in that paper. In addition some Ca^{2+} efflux time-course experiments were performed to check qualitatively the effect of the inositol phosphates. All experiments included 5 μM-Ins(1,4,5)P_3 samples to quantify a 100% response (Berridge *et al.*, 1984; Irvine *et al.*, 1984*a*).

Preparation of inositol phosphates

Ins(1,4,5)P_3. This was prepared from the Folch inositide fraction of bovine brain (Folch, 1949) by a modification of the method of Brown & Stewart (1966). The inositide (Folch, 1949) was acid-washed (0.1 M-HCl) twice to remove divalent cations (which interfere with the deacylation; Clarke & Dawson, 1981), dried down thoroughly, resuspended in chloroform and re-dried. To 60 mg P of the lipid was added 300 ml of Clarke & Dawson's (1981) deacylation reagent; this gives a clean removal of the fatty acids with no cleavage of the glycerol–inositol phosphate diester linkage [and so no formation of Ins(2,4,5)P_3; cf. Grado & Ballou, 1961]. After 60 min at 59 °C, the mixture was cooled on ice and then processed exactly as described by Clarke & Dawson (1981) with all volumes increased 100-fold. Phase separations to remove fatty acids were improved by centrifugation. The resulting deacylated phospholipid preparation was thoroughly dried and resuspended in 100 ml of water and the pH checked to be < 6.5. An equal volume of 0.03 M-sodium periodate was added and the A_{254} was monitored; in general 15 min were sufficient to complete the rapid phase of oxidation which is due to removal of the *sn*-1 carbon of the glycerol moiety (Brown & Stewart, 1966). The reaction was quenched with 10 ml of 3% ethylene glycol, and after 20 min, 50 ml of Brown & Stewart's (1966) dimethylhydrazine reagent was added. After 4 h under N_2 (see below) 60 ml of washed Dowex-W (H^+ form) was added and the solution was filtered with Celite 545 prewashed with 0.1 M-formic acid.

To the resulting filtrate was added 10 ml of Dowex 1 X8-400 resin in the formate form to bind all the

Abbreviations used: InsP_3 and InsP_4, inositol tris- and tetrakis-phosphates; locants where specified are in parentheses, e.g. Ins(1,2cyc4,5)P_3 is inositol 1,2-cyclic 4,5-trisphosphate.

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Ins(1,4,5)P_3 [this was checked on several preparations by the inclusion of a ^{32}P-labelled Ins(1,4,5)P_3 spike]. After filtration the Dowex was washed with 20 ml of 0.1 M-formic acid/0.4 M-ammonium formate, and then suspended in 40 ml of 0.1 M-formic acid/1.2 M-ammonium formate and filtered to remove the InsP_3. After 6-fold dilution with water, the solution was loaded onto a 2 ml Dowex formate column, and after washing with 30 ml of 0.1 M-formic acid/0.4 M-ammonium formate, the InsP_3 was eluted with 30 ml of 0.1 M-formic acid/0.8 M-ammonium formate. {N.B. Use of 1.0 M-ammonium formate at this point (cf. Berridge *et al.*, 1983) increased contamination with two unknown compounds. We believe these are (*a*) acetylphospho(1)-4,5-bis(phospho)inositol [formed by oxidation by atmospheric O_2 of the formaldehyde group created by the periodate oxidation stage; the introduction of N_2 over the subsequent stage (see above) reduced the amount of this component greatly] and (*b*) Ins(1,4,5)P_3 which had had its ring split open by periodate; this unknown compound migrates as an InsP_4 on ionophoresis, but does not yield inositol on total dephosphorylation.} The 0.8 M solution was diluted 5-fold, loaded onto a 1 ml Dowex chloride column, and eluted with 4 ml of 1 M-LiCl which was then removed by drying and ethanol washes as described by Grado & Ballou (1961) and Burgess *et al.* (1984*b*).

The Ins(1,4,5)P_3 obtained was > 99% pure by ionophoresis and paper chromatography analysis. In general, from each 60 mg P batch of inositide we obtained 7–10 mg P of Ins(1,4,5)P_3 (= approx. 40 mg of InsP_3).

Ins(1,2cyc4,5)P_3. We have identified this as an earlier eluting peak on our h.p.l.c. separations of ^{32}P-labelled Ins(1,4,5)P_3 made from human red blood cells (Irvine *et al.*, 1985*a*, 1986). We prepared it here by using non-radiolabelled red blood cells exactly as in Irvine *et al.* (1985*a*, 1986), spiking the preparation either with ^{32}P-labelled Ins(1,4,5)P_3 purified by h.p.l.c., or with [^{3}H]Ins(1,4,5)P_3 (Amersham). Fractions corresponding to cyclic InsP_3 (Irvine *et al.*, 1985*a*, 1986) were collected, and after 5-fold dilution and adjustment to a final concentration of 0.1 M-formic acid and 0.15 M-ammonium formate, poured down a 0.5 ml Dowex formate column. Inorganic phosphorus was removed by 10 ml of 0.1 M-formic acid/0.4 M-ammonium formate, and the cyclic InsP_3 was eluted with 0.1 M-formic acid/0.8 M-ammonium formate and desalted as for Ins(1,4,5)P_3 above. Cyclic InsP_3 and non-cyclic InsP_3 separate by ionophoresis (Dawson & Clarke, 1972; see Irvine *et al.*, 1985*a*, 1986) and so we could check the final product for hydrolysis of the cyclic bond during preparation; the samples used in these experiments were > 90% pure.

Ins(1,3,4,5)P_4. This was prepared by phosphorylation of Ins(1,4,5)P_3 by a rat brain supernatant under similar incubation conditions to those of Irvine *et al.* (1986), except that a pH of 9 was used, as at this pH there is negligible InsP_3 or InsP_4 phosphatase activity. Each batch contained 20 μmol of Ins(1,4,5)P_3 and the final volume of the reaction, containing 0.1 M-Tris/maleate, pH 9.0, 20 mM-Mg^{2+} and 10 mM-ATP was 40 ml. After quenching of the reaction, removal of trichloroacetic acid and neutralization (Irvine *et al.*, 1986), the solution (50 ml) was mixed with 40 ml of 0.2 M-glycine/NaOH, pH 8.6, containing 0.1 M-magnesium acetate and poured down a 1.7 cm × 20 cm column of phenyl boronate (Amicon) pre-equilibrated with the glycine/Mg^{2+} buffer (see Rosenberg *et al.*, 1972). Under these conditions we found that > 99% of InsP_3 and InsP_4 pass through the column, and > 99.9% of ATP binds to it. The ATP-free eluate was mixed with 1 volume of 0.2 M-formic acid/0.4 M-ammonium formate and 0.5 volume of water, and inositol phosphates were removed by adding 5 ml of Dowex formate. After that we followed essentially the same procedure as for Ins(1,4,5)P_3 above, except that the final separation of InsP_3 and InsP_4 was achieved on a 2 cm × 0.8 cm Dowex formate column (Batty *et al.*, 1985). Ins(1,3,4,5)P_4 (Batty *et al.*, 1985; Irvine *et al.*, 1986) was obtained in 5–6 μmol yields from 20 μmol of Ins(1,4,5)P_3 and was found to be 99% free from P_i, adenine nucleotide or InsP_3 contamination when examined by ionophoresis (Seiffert & Agranoff, 1965). For some experiments it was necessary to increase the purity of Ins(1,3,4,5)P_4 with regard to Ins(1,4,5)P_3 contamination to > 99%. This was done by h.p.l.c. separation (Batty *et al.*, 1985; Irvine *et al.*, 1986), with the InsP_4 fractions then desalted as for Ins(1,2cyc4,5)P_3, above. We estimate the likely contamination of these samples with Ins(1,4,5)P_3 to be < 0.1%.

Ins(1,3,4)$_3$. This was obtained by incubation of 3 μmol of Ins(1,3,4,5)P_4 with two additions (at zero time and 30 min) of 8 ml of human red cell membranes (Downes *et al.*, 1982) at pH 7.5 and 4 mM-magnesium acetate in 8 ml initial volume (cf. Irvine *et al.*, 1984*b*). This treatment specifically removes the 5-phosphate (Batty *et al.*, 1985) and the InsP_3 resulting was purified and desalted as for the other inositol phosphates (above). The InsP_3 was at least 99% pure by ionophoresis, and from our earlier data on the InsP_3 isomer formed (Batty *et al.*, 1985) we deduce that it must be > 95% the D-1,3,4-isomer. As with Ins(1,3,4,5)P_4, we also purified one batch by h.p.l.c. using a [^{3}H]Ins(1,4,5)P_3 marker to give a > 99.9% pure [with regard to Ins(1,4,5)P_3 contamination] sample.

All amounts of inositol phosphates were quantified by phosphate estimation (Rouser *et al.*, 1970).

RESULTS AND DISCUSSION

Ins(1,2cyc4,5)P_3

Fig. 1 shows the dose–response of Ca^{2+} mobilization for Ins(1,2cyc4,5)P_3 and Ins(1,3,4)P_3. In confirmation of the results of Wilson *et al.* (1985*b*) we find that Ins(1,2cyc4,5)P_3 is an effective Ca^{2+} mobilizer, and is approximately as potent as Ins(1,4,5)P_3 (indistinguishable by our experimental procedures). As Ins(1,2cyc4,5)P_3 is a poor substrate for Ins(1,4,5)P_3 phosphatase (Connolly *et al.*, 1986), it is important to be sure that no preferential hydrolysis of Ins(1,4,5)P_3 is occurring in the Ca^{2+} mobilization assay, so apparently increasing the relative efficacy of Ins(1,2cyc4,5)P_3. However, when Ins(1,4,5)P_3 was tested in the presence and absence of 3 mM-2,3-bisphophoglycerate [an inhibitor of Ins(1,4,5)P_3 phosphatase (Downes *et al.*, 1982)], we found no change in apparent potency (results not shown), indicating that little or no Ins(1,4,5)P_3 hydrolysis is occurring in these experiments. Although Connolly *et al.* (1986) have shown that little hydrolysis of the

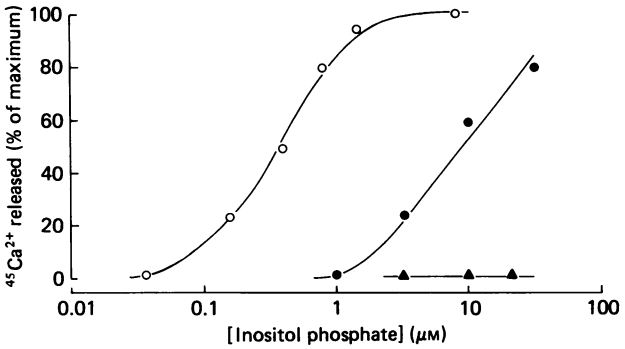

Fig. 1. Dose–response curves of inositol phosphate-stimulated release of Ca^{2+} from permeabilized Swiss mouse 3T3 cells

For experimental details see the text. Most points are the means of four individual determinations and the curves for the three compounds are derived from one experiment for each compound, though in every case at least one independent confirmatory experiment giving very similar data was performed. ○, Ins(1,2,cyc4,5)P_3; ●, Ins(1,3,4)P_3 (h.p.l.c. pure); ▲, Ins(1,3,4,5)P_4 (h.p.l.c. pure).

1,2-cyclic bond occurs in Ins(1,2cyc4,5)P_3 in cell homogenates, we cannot absolutely eliminate the possibility that this is occurring in our experiments, but taken in conjunction with the results of Connolly *et al.* (1986), plus the Ca^{2+}-mobilizing power of Ins(2,4,5)P_3 [5–6-fold less than that of Ins(1,4,5)P_3; Irvine *et al.*, 1984*a*], we suggest that cyclization of the 1-phosphate to the 2-position has little effect on the interaction of Ins(1,4,5)P_3 with its receptor in the endoplasmic reticulum.

Ins(1,3,4,5)P_4

This proved entirely ineffective at mobilizing Ca^{2+} up to a dose of 20 μM (Fig. 1). Preliminary results suggested a small response at this dose, indicating a possible activity about two orders of magnitude less than Ins(1,4,5)P_3, but this could have been accounted for by a 1% contamination with the latter compound. For the experiment recorded in Fig. 1 we prepared a sample of Ins(1,3,4,5)P_4 by h.p.l.c., and then even that small activity disappeared. Furthermore, at this same dose no detectable antagonism of the effect of Ins(1,4,5)P_3 could be seen (Table 1). From this we conclude that Ins(1,3,4,5)P_4 at concentrations likely to occur in intact cells can effectively be discounted with respect to Ca^{2+} mobilization from the endoplasmic reticulum.

Ins(1,3,4)P_3

From its kinetic behaviour in stimulated cells we had predicted (Irvine *et al.*, 1985*a*; Burgess *et al.*, 1985) that Ins(1,3,4)P_3 would be ineffective at mobilizing Ca^{2+}. Yet clearly this is not the case, as it reproducibly mobilized Ca^{2+} but with an efficacy considerably less than that of Ins(1,4,5)P_3. Release of calcium by Ins(1,3,4)P_3 was half-maximal at approx. 9 μM (Fig. 1) whereas the corresponding value for Ins(1,4,5)P_3 was 0.3 μM (Berridge *et al.*, 1984). We can discount contamination with the latter compound, as the Ins(1,3,4)P_3 sample used in these experiments was purified by h.p.l.c. As far as we know Ins(1,3,4)P_3 is the major InsP_3 produced by incubation of Ins(1,3,4,5)P_4 with human red cell ghosts (Batty *et al.*, 1985). We know that Ins(3,4,5)P_3 chromatographs on h.p.l.c. in about the same position as Ins(1,3,4)P_3 (R. F. Irvine & A. J. Letcher, unpublished work), but we have no evidence that red cell membranes form this from Ins(1,3,4,5)P_4 (Batty *et al.*, 1985); besides, it would have to be at a level of about 5% even assuming that Ins(3,4,5)P_3 were exactly as effective as Ins(1,4,5)P_3, which is very unlikely. The observation that Ins(1,3,4)P_3 is a Ca^{2+}-mobilizing compound may have important physiological consequences.

Table 1. Lack of effect of Ins(1,3,4,5)P_4 on Ins(1,4,5)P_3-induced Ca^{2+} mobilization

For experimental methods see the text. This result is typical of a pair of experiments. The Ins(1,3,4,5)P_4 was purified by h.p.l.c. Results are expressed as means ± S.D.

Addition	$^{45}Ca^{2+}$ remaining in tissue (c.p.m.)
Control	930 ± 120
0.5 μM-Ins(1,4,5)P_3	613 ± 42
0.5 μM-Ins(1,4,5)P_3 + 20 μM-Ins(1,3,4,5)P_4	587 ± 57

If we assume that labelling experiments represent relative masses of the two InsP_3 isomers, then there is no doubt that in a number of tissues the mass of Ins(1,3,4)P_3 can rise to at least ten times that of Ins(1,4,5)P_3 (e.g. Irvine *et al.*, 1985*a*; Burgess *et al.*, 1985; Turk *et al.*, 1986; Wollheim & Biden, 1986) and may thus be making a contribution to Ca^{2+} mobilization. However, due to the slow rate at which it increases in most cells, the Ca^{2+} stores could well be empty by the time it reaches significant levels. Ins(1,3,4)P_3 may therefore possibly be considered as a compound which keeps these stores empty, hence lowering the Ca^{2+}-buffering power of the cytoplasm and thus in turn helping to hand over the acute control of Ca^{2+} in the stimulated cell to the plasma membrane. These suggestions may help to explain some experimental observations, for example the Li^+-induced prolonging of contraction in smooth muscle (Rapoport, 1986); Li^+ can specifically cause a buildup of Ins(1,3,4)P_3 (Burgess *et al.*, 1985) and if Ca^{2+} mobilization is still making a contribution to Ca^{2+} homoeostasis in these cells, then Li^+ may be causing its effect by prolonging the presence of Ins(1,3,4)P_3. Ins(1,3,4)P_3 is the major InsP_3 in *Limulus* photoreceptors (Irvine *et al.*, 1985*b*) and is produced in less than 0.3 s (J. E. Brown & R. F. Irvine, unpublished work), so it may there play a role in photoadaptation.

Speculation of this sort can only be confirmed by further experimentation, but we believe the results presented here show essentially two things. Firstly, that Ins(1,4,5)P_3 remains the most potent Ca^{2+}-mobilizing compound despite the discovery of other naturally-occurring inositol phosphates. Secondly, that the InsP_3/InsP_4 pathway to a large extent deactivates the Ca^{2+}-mobilizing power of Ins(1,4,5)P_3, and the discussion above notwithstanding, we suggest that its principal function lies elsewhere than in regulating Ca^{2+} mobilization from intracellular stores.

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