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Specificity of inositol trisphosphate-induced calcium release from permeabilized Swiss-mouse 3T3 cells

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Swiss-mouse 3T3 cells permeabilized with saponin were used to study the specificity of the inositol trisphosphate-induced release of $^{45}Ca^{2+}$ from their intracellular stores. Inositol 1,4,5-trisphosphate was the most potent compound studied (dose giving half-maximal effect 0.3 μM). $^{45}Ca^{2+}$ was also released by inositol 2,4,5-trisphosphate, glycerophosphoinositol 4,5-bisphosphate and inositol 4,5-bisphosphate, with doses giving half-maximal effect of respectively 1.6 μM, 1.6 μM and 20 μM, but not by inositol 1,4-bisphosphate (50 μM). These data suggest that the *trans*-vicinal phosphates on the 4- and 5-positions are essential for the Ca^{2+}-mobilizing effect of inositol trisphosphate, and that in addition there is a requirement for a phosphate group on the opposite side of the molecule, with a preference for the 1-position.

D-*myo*-Inositol 1,4,5-trisphosphate has been proposed as the intracellular second messenger for the mobilization of Ca^{2+} from intracellular stores, in particular the endoplasmic reticulum (Berridge, 1983; Streb *et al.*, 1983; Berridge & Irvine, 1984; Dawson & Irvine, 1984; Burgess *et al.*, 1984; Joseph *et al.*, 1984; Prentki *et al.*, 1984). Preliminary observations showed that this response was specific for inositol trisphosphate, rather than inositol, inositol monophosphate or inositol 1,4-bisphosphate (Streb *et al.*, 1983; Burgess *et al.*, 1984). Also, randomization of the three phosphate groups by acid treatment caused a marked decrease in potency, suggesting that their position on the inositol ring was important (Streb *et al.*, 1983). However, no quantitative examination of the Ca^{2+}-mobilizing activity of inositol phosphates closer to Ins(1,4,5)P_3 in structure has yet been reported. In order to investigate a structure–activity relationship, we have prepared pure samples of those inositol phosphates readily derived from ox brain inositides, and studied their potency on a saponin-permeabilized Swiss-mouse 3T3-cell preparation which we described previously (Berridge *et al.*, 1984).

Abbreviations used: InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; Gro*P*InsP_2, glycerophosphoinositol bisphosphate.

Materials and methods

$^{45}Ca^{2+}$ release from permeabilized cells

This procedure is described in detail by Berridge *et al.* (1984). In brief, Swiss-mouse 3T3 cells grown to confluency were permeabilized with saponin, and incubated with $^{45}Ca^{2+}$ (buffered with EGTA to a free Ca^{2+} concentration of 130 nM) and ATP (2 mM) for 20 min to reach equilibrium. The inositol phosphates under study were added, and after 5 min to attain a new steady state (Berridge *et al.*, 1984) the incubation medium was removed and the cells were washed with five changes of fresh medium. $^{45}Ca^{2+}$ was extracted from the cells with 0.1 M-HCl and counted for radioactivity. In general, quadruplicate readings were used for each inositol phosphate concentration. Maximal Ca^{2+} release was 40–60% of the total taken up, depending on the experiment; we found that, in direct comparative experiments, maximal doses of the various effective inositol phosphates released very similar amounts of Ca^{2+}. Therefore, for Fig. 1, in order to standardize results over a number of independent experiments, the results are expressed as percentages of maximal release achieved in any given experiment.

Preparation of Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,4)P_2 and Ins(4,5)P_2

The preparation and identification of these compounds is based on the work of Ballou and co-workers (Grado & Ballou, 1961; Tomlinson & Ballou, 1961). A phosphoinositide fraction was isolated from ox brain (removed within 30min of death) by the method of Folch (1949), and analysis by t.l.c. showed that it contained phosphatidylserine, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate. A sample of this fraction, containing 10mg of phosphorus, was treated with 12ml of 1M-KOH and boiled under reflux for 30min. It was then cooled and adjusted to pH3 by the addition of formic acid. Fatty acids were removed by two hexane washes, and then pH was re-adjusted to 8 with saturated ammonia solution. The solution was diluted with water to 2 litres, and passed through a column ($1\frac{1}{2}$cm × 0.3cm) of Dowex 1 X8-400 (formate form). This column was then successively eluted with 15ml of 0.1M-formic acid/0.2M-ammonium formate, to remove inositol monophosphates and P_i (Ellis *et al.*, 1963; Downes & Michell, 1981), 0.1M-formic acid/0.4M-ammonium formate, to remove inositol bisphosphates, and 0.1M-formic acid/1.0M-ammonium formate, to remove inositol trisphosphates. The InsP_2 fraction was freeze-dried overnight and the InsP_3 fraction desalted as described by Downes *et al.* (1982) and Burgess *et al.* (1984). Both fractions were then chromatographed on Whatman no. 1 paper in propan-2-ol/saturated ammonia/water (7:1:2, by vol.). The InsP_2 fraction was chromatographed for 7 days, and three phosphate-containing compounds were detected. One very slow-running compound was found to be InsP_3 contamination. The other two components migrated approx. 5 and 9cm after 7 days. Both were confirmed as inositol bisphosphates by Dowex column chromatography as above and high-voltage electrophoresis (Berridge *et al.*, 1983). From their relative mobilities and the preparative methods used, we can, by comparison with the work of Grado & Ballou (1961) and Tomlinson & Ballou (1961), unambiguously identify them as Ins(1,4)P_2 (slow-running) and Ins(4,5)P_2 (fast-running).

The InsP_3 fraction gave two components migrating 3cm and 6cm after 14 days chromatography; both were confirmed as inositol trisphosphates by Dowex column chromatography and high-voltage electrophoresis, and again, by comparison with the work of Ballou and colleagues, can be identified as Ins(1,4,5)P_3 and Ins(2,4,5)P_3 respectively.

All four inositol phosphates were eluted from the papers overnight with water, passed down a Dowex 50 (H^+ form) column to remove contaminating cations and neutralized to pH7 with KOH (and were therefore predominantly the K^+ salts). Finally, they were dried at 40°C *in vacuo* and then phosphate was determined as described by Rouser *et al.* (1970).

Glycerophosphoinositol 4,5-bisphosphate

This was prepared by transesterification (Clarke & Dawson, 1981) of the Folch inositide fraction (as described above) after an acid wash to remove most bivalent cations, followed by separation of Gro*P*Ins(4,5)P_2 on Dowex (formate form) columns (Ellis *et al.*, 1963; Downes & Michell, 1981). The Gro*P*Ins(4,5)P_2 was analysed by high-voltage electrophoresis and found to contain less than 0.5% InsP_3 contamination.

Results and discussion

The ability of Ins(1,4,5)P_3 to mobilize Ca^{2+} from intracellular stores suggests the existence of a specific receptor. In order to obtain a structure–activity relationship for this putative InsP_3 receptor, a wide range of compounds was tested. We found previously that fructose 1,6-bisphosphate, inositol, inositol 2-phosphate, inositol 1,2-cyclic monophosphate and inositol 1,4-bisphosphate were ineffective at mobilizing Ca^{2+} in permeabilized 3T3 cells (Berridge *et al.*, 1984) at 10μM concentration. We have now increased the concentration of Ins(1,4)P_2 to 50μM, at which it still has no significant effect on $^{45}Ca^{2+}$ mobilization (results not shown), and also we have extended the above list to show that phytic acid and 2,3-phosphoglycerate are inactive at 20μM and 2mM respectively (Table 1).

Table 1. *Sugar phosphates inactive in mobilizing Ca^{2+}*

For experimental details see the Materials and methods section. Data show three typical experiments establishing the lack of effect of 2,3-phosphoglycerate (Expt. 1), Ins(1,4)P_2 (Expt. 2) and phytic acid (Expt. 3) on Ca^{2+} mobilization from permeabilized Swiss-mouse 3T3 cells. Data are expressed as means ± S.E.M.

Expt. no.	Compound added	$^{45}Ca^{2+}$ in cells (d.p.m.)
1	Control	946 ± 86 ($n = 6$)
	Ins(1,4,5)P_3 (1μM)	552 ± 80 ($n = 6$)
	2,3-Phosphoglycerate (2mM)	1007 ± 180 ($n = 6$)
	Ins(1,4,5)P_3 (1μM) + 2,3-phosphoglycerate (2mM)	533 ± 105 ($n = 6$)
2	Control	1676 ± 530 ($n = 3$)
	Ins(1,4,5)P_3 (1μM)	712 ± 77 ($n = 3$)
	Ins(1,4,5)P_3 (1μM) + Ins(1,4)P_2 (10μM)	694 ± 29 ($n = 3$)
3	Control	1677 ± 258 ($n = 4$)
	Phytic acid (20μM)	1635 ± 234 ($n = 6$)
	Gro*P*Ins(4,5)P_2 (20μM)	647 ± 66 ($n = 4$)

Three inositol phosphates that we prepared did show appreciable Ca^{2+}-mobilizing activity, and their potency as compared with Ins(1,4,5)P_3 (apparent K_m approx. 0.3 μM) is shown in Fig. 1. Moving the 1-phosphate group of Ins(1,4,5)P_3 to the 2-position [to form Ins(2,4,5)P_3], or esterifying it with a glycerol moiety [as Gro*P*Ins(4,5)P_2], increases the apparent K_m to about 1.6 μM, i.e. causes an approx. 5-fold loss in potency. A much larger decrease in potency (60-fold) is caused by removal of the 1-phosphate [to form Ins(4,5)P_2]. A negative charge remote from the 4,5-bisphosphate grouping is therefore necessary for optimal activity, and the lower potency of Ins(2,4,5)P_3 suggests that a fairly precise orientation of that negative charge is required. The decrease in potency caused by the presence of a glycerol group is presumably the result either of steric hindrance, or of the decreased charge caused by the conversion of the monoester phosphate group into a phosphodiester.

Cells are likely to have at least two binding sites for inositol trisphosphate. The structure–activity studies described above were concerned with the putative receptor responsible for releasing Ca^{2+}, but another binding site probably exists on the enzyme which degrades inositol trisphosphate (Downes *et al.*, 1982). It was therefore decided to study the effect of 2,3-phosphoglycerate, which contains vicinal phosphate groups and is a competitive inhibitor (K_i approx. 0.35 mM) of the inositol trisphosphatase in red-blood-cell membranes (Downes *et al.*, 1982). The fact that 2 mM-2,3-phosphoglycerate cannot inhibit the Ca^{2+}-releasing properties of Ins(1,4,5)P_3 (Table 1) implies that the inositol trisphosphatase is probably distinct from the putative receptor responsible for Ca^{2+} mobilization. Ins(1,4)P_2 also shows no competitive inhibition of Ins(1,4,5)P_3-stimulated release (Table 1), suggesting that the high concentrations of this product of Ins(1,4,5)P_3 catabolism that are attained in stimulated tissues (Berridge *et al.*, 1983; Rebecchi & Gershengorn, 1983) are unlikely to have much effect on Ca^{2+} mobilization.

The establishment of a messenger role for Ins(1,4,5)P_3 rests on fulfilling a variety of criteria, one of which is that it should have a specific 'receptor' for its Ca^{2+}-mobilizing activity. Although the present experiments have used only a few analogues of this compound, the results described above strongly suggest that the Ca^{2+}-mobilizing ability of inositol phosphates does require a very precise molecular configuration, and that, of naturally occurring compounds, Ins(1,4,5)P_3 fits that configuration most closely.

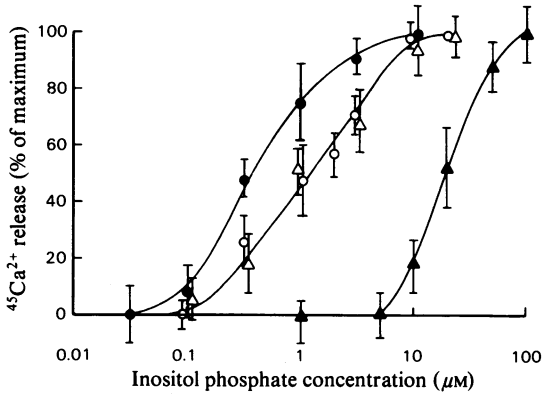

Fig. 1. *Dose–response curves of inositol phosphate-stimulated release of Ca^{2+} from permeabilized Swiss-mouse 3T3 cells*

For details of the experimental method see the text. The data are plotted as concentration of inositol phosphate versus percentage of maximal response for that inositol phosphate, and are expressed as means ± S.E.M. Most points are means for six individual determinations, and the data in the Figure are the combined data from five separate experiments. ●, Ins(1,4,5)P_3; ○, Ins(2,4,5)P_3; △, Gro*P*Ins(4,5)P_2; ▲, Ins(4,5)P_2.

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