# 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 $0.3\mu M$). $^{45}Ca^{2+}$ 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 $1.6\mu M$, $1.6\mu M$ and $20\mu M$, but not by inositol 1,4-bisphosphate ($50\mu M$). 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).

## 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 130nM) and ATP (2mM) for 20min to reach equilibrium. The inositol phosphates under study were added, and after 5min 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 0.1M-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.

Abbreviations used: $InsP_3$, inositol trisphosphate; $InsP_2$, inositol bisphosphate; $GroPInsP_2$, glycerophosphoinositol bisphosphate.

*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 30 min of death) by the method of Folch (1949), and analysis by t.l.c. showed that it contained phosphatidyl-serine, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate. A sample of this fraction, containing 10 mg of phosphorus, was treated with 12 ml of 1 M-KOH and boiled under reflux for 30 min. It was then cooled and adjusted to pH 3 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 (1½ cm × 0.3 cm) of Dowex 1 X8-400 (formate form). This column was then successively eluted with 15 ml of 0.1 M-formic acid/0.2 M-ammonium formate, to remove inositol monophosphates and $P_i$ (Ellis et al., 1963; Downes & Michell, 1981), 0.1 M-formic acid/0.4 M-ammonium formate, to remove inositol bisphosphates, and 0.1 M-formic acid/1.0 M-ammonium formate, to remove inositol trisphosphates. The Ins$P_2$ fraction was freeze-dried overnight and the Ins$P_3$ 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 Ins$P_2$ fraction was chromatographed for 7 days, and three phosphate-containing compounds were detected. One very slow-running compound was found to be Ins$P_3$ contamination. The other two components migrated approx. 5 and 9 cm 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 Ins$P_3$ fraction gave two components migrating 3 cm and 6 cm 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 pH 7 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% Ins$P_3$ 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 Ins$P_3$ 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 10 $\mu$M concentration. We have now increased the concentration of Ins(1,4)$P_2$ to 50 $\mu$M, 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 20 $\mu$M and 2 mM 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)$P_3$ (1 $\mu$M) | 552 ± 80 (n = 6) |
| | 2,3-Phosphoglycerate (2 mM) | 1007 ± 180 (n = 6) |
| | Ins(1,4,5)$P_3$ (1 $\mu$M) + 2,3-phosphoglycerate (2 mM) | 533 ± 105 (n = 6) |
| 2 | Control | 1676 ± 530 (n = 3) |
| | Ins(1,4,5)$P_3$ (1 $\mu$M) | 712 ± 77 (n = 3) |
| | Ins(1,4,5)$P_3$ (1 $\mu$M) + Ins(1,4)$P_2$ (10 $\mu$M) | 694 ± 29 (n = 3) |
| 3 | Control | 1677 ± 258 (n = 4) |
| | Phytic acid (20 $\mu$M) | 1635 ± 234 (n = 6) |
| | Gro$P$Ins(4,5)$P_2$ (20 $\mu$M) | 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. $0.3\mu M$) 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 $GroPIns(4,5)P_2$], increases the apparent $K_m$ to about $1.6\mu M$, 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. $0.35mM$) of the inositol trisphosphatase in red-blood-cell membranes (Downes et al., 1982). The fact that $2mM$-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.

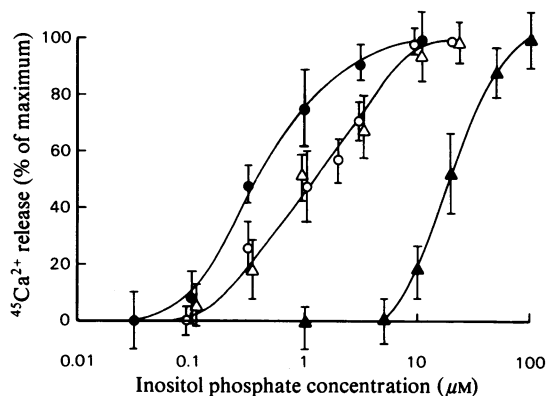Fig. 1. *Dose–response curves of inositol phosphate-stimulated release of $Ca^{2+}$ from permeabilized Swiss-mouse 3T3 cells*

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 $\pm$ 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. ●, $Ins(1,4,5)P_3$; ○, $Ins(2,4,5)P_3$; △, $GroPIns(4,5)P_2$; ▲, $Ins(4,5)P_2$.

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