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# Two dephosphorylation pathways of inositol 1,4,5-trisphosphate in homogenates of the cellular slime mould *Dictyostelium discoideum*

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*Dictyostelium discoideum* homogenates contain phosphatase activity which rapidly dephosphorylates Ins(1,4,5) $P_3$  (D-*myo*-inositol 1,4,5-trisphosphate) to Ins (*myo*-inositol). When assayed in  $Mg^{2+}$ , Ins(1,4,5) $P_3$  is dephosphorylated by the soluble *Dictyostelium* cell fraction to 20% Ins(1,4) $P_2$  (D-*myo*-inositol 1,4-bisphosphate) and 80% Ins(4,5) $P_2$  (D-*myo*-inositol 4,5-bisphosphate). In the particulate fraction Ins(1,4,5) $P_3$  5-phosphatase is relatively more active than the Ins(1,4,5) $P_3$  1-phosphatase.  $CaCl_2$  can replace  $MgCl_2$  only for the Ins(1,4,5) $P_3$  5-phosphatase activity. Ins(1,4) $P_2$  and Ins(4,5) $P_2$  are both further dephosphorylated to Ins4P (D-*myo*-inositol 4-monophosphate), and ultimately to Ins.  $Li^+$  ions inhibit Ins(1,4,5) $P_3$  1-phosphatase, Ins(1,4) $P_2$  1-phosphatase, Ins4P phosphatase and L-Ins1P (L-*myo*-inositol 1-monophosphate) phosphatase activities; Ins(1,4,5) $P_3$  1-phosphatase is 10-fold more sensitive to  $Li^+$  (half-maximal inhibition at about 0.25 mM) than are the other phosphatases (half-maximal inhibition at about 2.5 mM). Ins(1,4,5) $P_3$  5-phosphatase activity is potently inhibited by 2,3-bisphosphoglycerate (half-maximal inhibition at 3  $\mu M$ ). Furthermore, 2,3-bisphosphoglycerate also inhibits dephosphorylation of Ins(4,5) $P_2$ . These characteristics point to a number of similarities between *Dictyostelium* phospho-inositol phosphatases and those from higher organisms. The presence of an hitherto undescribed Ins(1,4,5) $P_3$  1-phosphatase, however, causes the formation of a different inositol bisphosphatase isomer [Ins(4,5) $P_2$ ] from that found in higher organisms [Ins(1,4) $P_2$ ]. The high sensitivity of some of these phosphatases for  $Li^+$  suggests that they may be the targets for  $Li^+$  during the alteration of cell pattern by  $Li^+$  in *Dictyostelium*.

## INTRODUCTION

The pivotal role of Ins(1,4,5) $P_3$  as the second messenger for receptor-mediated  $Ca^{2+}$  mobilization has been firmly established in a wide variety of systems (reviews: Downes & Michell, 1985; Berridge, 1987). In the best studied mammalian systems, such as human erythrocytes, platelets, rat brain, liver, pancreas and parotid gland, the Ins(1,4,5) $P_3$  response is attenuated by a specific phosphatase which removes the phosphate from the 5-position to yield Ins(1,4) $P_2$  (Downes *et al.*, 1982; Storey *et al.*, 1984; Connolly *et al.*, 1985; Erneux *et al.*, 1986; Shears *et al.*, 1987). Ins(1,4) $P_2$  is then further dephosphorylated to Ins4P in rat liver and brain and calf brain (Delvaux *et al.*, 1987a; Ackermann *et al.*, 1987; Inhorn *et al.*, 1987; Ragan *et al.*, 1988), and finally to Ins. The Ins formed in this way can then be re-used for the synthesis of inositol phospholipids, thus closing the cyclic metabolic pathway characteristic for this signalling system.

Dephosphorylation of Ins(1,4) $P_2$  and InsP has been shown to be sensitive to  $Li^+$  ions (Hallcher & Sherman, 1980; Storey *et al.*, 1984; Takimoto *et al.*, 1985; Delvaux *et al.*, 1987a,b; Gee *et al.*, 1988), and it has been suggested that the pharmacological effect of  $Li^+$  as a drug

against manic-depressive illness might be due to inhibition of these enzymes by  $Li^+$  (Drummond, 1987).

In the cellular slime mould *Dictyostelium discoideum*, which is frequently used as a model for studying signal transduction and differentiation, a similar second-messenger function has been proposed for Ins(1,4,5) $P_3$ ; Ins(1,4,5) $P_3$  can elicit  $Ca^{2+}$  release from non-mitochondrial  $Ca^{2+}$  stores in saponin-permeabilized *Dictyostelium* cells (Europe-Finner & Newell, 1986), and more recently it was shown that the chemoattractant cyclic AMP can trigger the accumulation of intracellular Ins $P_3$  *in vivo* (Europe-Finner & Newell, 1987). Furthermore,  $Li^+$  ions can alter the pattern in *Dictyostelium* slugs and direct differentiation to the stalk pathway (Maeda, 1970; Sakai, 1973; Van Lookeren Campagne *et al.*, 1988).

Very little is known about the enzymes involved in the turnover of inositol phospholipids and inositol phosphates in *D. discoideum*. The only enzymes that have been described are the CDP-diacylglycerol: inositol phosphatidyltransferase and the  $Mn^{2+}$ -catalysed phosphatidylinositol:*myo*-inositol exchange activity (Machon *et al.*, 1980), a kinase which phosphorylates phosphatidylinositol to phosphatidylinositol 4-phosphate (Varela *et al.*, 1987) and a kinase which phos-

Abbreviations used: Ins, *myo*-inositol; Ins1P, D-*myo*-inositol 1-phosphate; L-Ins1P, L-*myo*-inositol 1-phosphate; Ins4P, D-*myo*-inositol 4-phosphate; Ins(1,4) $P_2$ , D-*myo*-inositol 1,4-bisphosphate; Ins(4,5) $P_2$ , D-*myo*-inositol 4,5-bisphosphate; Ins(1,5) $P_2$ , D-*myo*-inositol 1,5-bisphosphate; Ins(1,4,5) $P_3$ , D-*myo*-inositol 1,4,5-trisphosphate; InsP, Ins $P_2$  and Ins $P_3$ , D-*myo*-inositol phosphates without specification of the phosphate position(s).

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phorylates diacylglycerol to phosphatidic acid (Jimenez *et al.*, 1988). Phosphatidylinositol 4-phosphate kinase is also present in *Dictyostelium* (M. M. Van Lookeren Campagne, unpublished work), but no phospholipase C activity has been demonstrated up to now (Irvine *et al.*, 1980). It was not known whether *Dictyostelium* cells have enzymes which dephosphorylate Ins(1,4,5) $P_3$ . Here we report that Ins(1,4,5) $P_3$  can be dephosphorylated to Ins in *D. discoideum* homogenates. Furthermore, we show that this dephosphorylation can occur by two different routes, with as intermediates either Ins(1,4) $P_2$  or Ins(4,5) $P_2$ , which are both dephosphorylated, through Ins4P, to Ins.

## MATERIALS AND METHODS

### Materials

[2- $^3$ H]Ins(1,4,5) $P_3$  (1.0 Ci/mmol) and L-[U- $^{14}$ C]Ins1P (55 mCi/mmol) were from Amersham International. [2- $^3$ H]Ins(1,4) $P_2$  (2.0 Ci/mmol), [4,5- $^{32}$ P]Ins(1,4,5) $P_3$  (130 Ci/mmol), [2- $^3$ H]Ins1P (5.4 Ci/mmol) and [2- $^3$ H]-Ins4P (1.5 Ci/mmol) were from New England Nuclear. Dowex 1 (200–400 mesh) and 2,3-bisphosphoglycerate were from Sigma. The h.p.l.c. columns were from Waters ( $\mu$ Bondapak NH $_2$ ; 30 cm  $\times$  0.39 cm), Whatman (Partisil SAX; 25 cm  $\times$  0.49 cm) and Chrompack (LiChrosorb 10RP18; 25 cm  $\times$  0.49 cm).

### Organism and culture conditions

*Dictyostelium discoideum* strain NC-4(H) was grown in association with *Escherichia coli* 281 on glucose/peptone agar as described previously (Van Lookeren Campagne *et al.*, 1986). Amoebae were harvested in 10 mM-phosphate buffer, pH 6.5, and freed from bacteria by repeated centrifugation. Cells were then plated on non-nutrient agar plates at a density of  $10^7$  cells/cm $^2$  and incubated overnight at 6 °C to induce full aggregation-competence (Konijn, 1970).

### Homogenate

Aggregation-competent cells were harvested in 10 mM-phosphate buffer, pH 6.5, washed once in ice-cold buffer A (20 mM-Hepes/NaOH, 0.5 mM-EDTA, 200 mM-sucrose, pH 7.0), and resuspended to  $2 \times 10^9$  cells/ml in the same buffer. Homogenates were made by passing the cells through a Nucleopore filter (3  $\mu$ m pore size) (Das & Henderson, 1983). Lysates were then centrifuged for 3 min at 10000 g. The particulate cell fraction was prepared by washing the pellet once in buffer A and resuspending it in the same buffer to the original volume of the homogenate. The soluble fraction was prepared by re-centrifuging the 10000 g supernatant for 5 min in a Beckman Airfuge at 150000 g. Soluble and particulate fractions thus obtained were immediately used for the phosphatase assay.

### Phosphatase assay

Dephosphorylation of [2- $^3$ H]Ins(1,4,5) $P_3$ , [4,5- $^{32}$ P]Ins(1,4,5) $P_3$ , [2- $^3$ H]Ins(1,4) $P_2$ , [2- $^3$ H]Ins(4,5) $P_2$ , [4,5- $^{32}$ P]Ins(4,5) $P_2$  and/or L-[U- $^{14}$ C]Ins1P was assayed in buffer A, in the presence of either 5 mM-MgCl $_2$  or 2.5 mM-CaCl $_2$ ,

at 22 °C. Incubations were started by adding 5  $\mu$ l of either the soluble or particulate fraction of the *Dictyostelium* homogenate to 15  $\mu$ l assay mixture, containing 1000–3000 c.p.m. of radiolabelled substrate. Reactions were stopped after 5–30 min by adding 0.5 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.). Phases were separated by adding 200  $\mu$ l of water. After vigorous shaking and centrifugation (1 min, 10000 g), the aqueous phase was applied to 0.5 ml Dowex-1 anion-exchange columns (formate form). The different reaction products were separated by stepwise elution with: (1) 10 ml of water (Ins); (2) 10 ml of 150 mM-ammonium formate/5 mM-Na $_2$ B $_4$ O $_7$  (InsP and P $_i$ ); (3) 10 ml of 300 mM-ammonium formate/100 mM-formic acid (InsP $_2$ ); and (4) 10 ml of 750 mM-ammonium formate/100 mM-formic acid (InsP $_3$ ). Radioactivity of the fractions was measured by liquid-scintillation counting after adding 13 ml of Instagel (Packard).

When it was necessary to separate Ins4P from Ins1P and P $_i$  reaction products (usually with the internal standard of L-[U- $^{14}$ C]Ins1P) were separated by anion-exchange h.p.l.c. as described in the Figure legends. Ins(1,4,5) $P_3$  5-phosphatase activity from human erythrocyte membranes was assayed as described previously (Erneux *et al.*, 1986). Enzyme activities were approximately linear with time and enzyme concentrations, provided that not more than about 25 % of the substrate was utilized. The s.d. of the phosphatase assay was about 10 %. Experiments were performed at least three times with similar results; the analysis of the InsP isomers by h.p.l.c. was performed twice with identical results.

### Preparation and purification of Ins(4,5) $P_2$

Aggregation-competent *Dictyostelium* cells were lysed in buffer B [50 mM-Tris/HCl, pH 7.2, 10 % (v/v) glycerol, 10 mM-dithiothreitol, leupeptin (6.5  $\mu$ g/ml), 100  $\mu$ M-phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor (50  $\mu$ g/ml) and 5 mM-benzamidine]. The high-speed supernatant from  $3 \times 10^9$  cells was chromatographed on a DEAE-cellulose column (10 ml; 8 cm  $\times$  1.3 cm), which was equilibrated and eluted in buffer B. The Ins(1,4,5) $P_3$  5-phosphatase activity binds to the column, whereas the Ins(1,4,5) $P_3$  1-phosphatase is eluted from the column between 1.5 and 2 column vol. (P. J. M. Van Haastert & E. Rovers, unpublished work).

A mixture of  $^{32}$ P- and  $^3$ H-labelled Ins(4,5) $P_2$  was prepared in an incubation (100  $\mu$ l) containing 50 nCi of [4,5- $^{32}$ P]Ins(1,4,5) $P_3$ , 100 nCi of [2- $^3$ H]Ins(1,4,5) $P_3$ , 5 mM-MgCl $_2$ , buffer A and 40  $\mu$ l of enzyme from the DEAE-cellulose column. After 60 min the incubation was terminated by the addition of 100  $\mu$ l of 0.1 M-tributylammonium phosphate, pH 6.5. The sample was centrifuged immediately for 5 min at 10000 g, and the supernatant was chromatographed by h.p.l.c. on a reversed-phase LiChrosorb 10RP18 column, which was eluted isocratically with 1.5 mM-tributylammonium phosphate / 4.5 mM-triethylammonium formate / 16 % (v/v) methanol, pH 6.5, at a flow rate of 1.2 ml/min. Fractions of volume 0.6 ml were collected; the radioactivity of 6  $\mu$ l samples was determined by using a dual-label program. Peak fractions were combined and concentrated to dryness under reduced pressure at 10 °C. [ $^3$ H]Ins(4,5) $P_2$  was prepared in parallel from 100 nCi of [ $^3$ H]Ins(1,4,5) $P_3$ .

**Table 1. Relative  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity in soluble and particulate cell fractions measured under different conditions**

Soluble or particulate cell fractions, derived from  $5 \times 10^7$  cells/ml in the assay, were incubated with 5 nCi of  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  ( $0.25 \mu\text{M}$ ) in the presence of either  $\text{MgCl}_2$  or  $\text{CaCl}_2$  and the phosphatase inhibitors  $\text{LiCl}$  and 2,3-bisphosphoglycerate as indicated. The phosphatase activity data are expressed as percentages (not standardized for protein) relative to the value measured in the soluble fraction with  $\text{MgCl}_2$ . When standardized for protein content, phosphatase activity assayed with 5 mM- $\text{MgCl}_2$  in soluble and particulate fractions was 9.8 and 9.2 pmol/min per mg of protein respectively.

Assay with:	Activity (%)		
	No addition	25 mM- $\text{LiCl}$	0.25 mM-2,3-Bisphosphoglycerate
Soluble			
5 mM- $\text{MgCl}_2$	100	21	82
2.5 mM- $\text{CaCl}_2$	19	17	1
Particulate			
5 mM- $\text{MgCl}_2$	21	11	13
2.5 mM- $\text{CaCl}_2$	11	10	1

## RESULTS

### General properties of $\text{Ins}(1,4,5)\text{P}_3$ dephosphorylation in *Dictyostelium*

$\text{Ins}(1,4,5)\text{P}_3$  can be rapidly dephosphorylated by a *D. discoideum* homogenate to  $\text{InsP}_2$ . Activity is optimal at pH 7.0 and 5 mM- $\text{MgCl}_2$  (results not shown) and is located predominantly in the soluble cell fraction (Table 1).  $\text{LiCl}$  and 2,3-bisphosphoglycerate, which are known inhibitors of phospho-inositol phosphatases (Downes *et al.*, 1982; Storey *et al.*, 1984; Delvaux *et al.*, 1987a) have differential potencies in the soluble and particulate fractions,  $\text{Li}^+$  being a more potent inhibitor of the soluble  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity and 2,3-bisphosphoglycerate being more potent on the particulate activity (Table 1). The particulate enzyme is probably not located on the cell surface, because intact cells express little enzyme activity (results not shown).

$\text{CaCl}_2$  (2.5 mM) can replace  $\text{MgCl}_2$  to a certain extent, more so in the particulate fraction (to about 50%) than in the soluble fraction (to about 20%). Furthermore, replacement of  $\text{Ca}^{2+}$  for  $\text{Mg}^{2+}$  changes the sensitivity towards  $\text{Li}^+$  and 2,3-bisphosphoglycerate,  $\text{Li}^+$  becoming ineffective and 2,3-bisphosphoglycerate almost completely inhibiting all phosphatase activity (Table 1).

### Identification of the first phosphate group removed by $\text{Ins}(1,4,5)\text{P}_3$ phosphatase

The studies with the  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase inhibitors  $\text{Li}^+$  and 2,3-bisphosphoglycerate suggest that in 5 mM- $\text{Mg}^{2+}$   $\text{Ins}(1,4,5)\text{P}_3$  is dephosphorylated by a route different from that in 2.5 mM- $\text{Ca}^{2+}$ . To see which phosphate group is removed first under the different conditions, we measured the hydrolysis of a mixture of  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ . The distribution of  $^{32}\text{P}$  label between the 4- and 5-phosphates of

**Table 2. Ratio between  $^{32}\text{P}$  and  $^3\text{H}$  radioactivity in  $\text{InsP}_2$  produced by hydrolysis of a mixture of  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$** 

$\text{InsP}_3$  phosphatase activity was assayed in the soluble fraction from  $5 \times 10^7$  cells/ml with 0.5 nCi of  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and 2.5 nCi of  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  under the conditions indicated and described in the Materials and methods section. Incubations were terminated after 10 min when assayed in  $\text{MgCl}_2$  or after 20 min when assayed in  $\text{CaCl}_2$  and chromatographed on Dowex columns. The ratio between the  $^{32}\text{P}$  and  $^3\text{H}$  radioactivity in the  $\text{InsP}_2$  fractions was determined by liquid-scintillation counting, with a dual-label program. The  $^{32}\text{P}/^3\text{H}$  ratios in  $\text{InsP}_2$  relative to that in  $\text{Ins}(1,4,5)\text{P}_3$  are given in parentheses. The incubation with human erythrocyte membranes, which contains only 5-phosphatase, revealed that the  $^{32}\text{P}$  label was distributed over the 4- and 5-positions in the ratio 12:88. A low  $^{32}\text{P}:^3\text{H}$  ratio in  $\text{InsP}_2$  therefore indicates 5-phosphatase activity, whereas a high ratio indicates 1- and/or 4-phosphatase activity.

	$^{32}\text{P}/^3\text{H}$ ratio
$\text{Ins}(1,4,5)\text{P}_3$ substrate	0.74 (1.00)
$\text{InsP}_2$ formed in 5 mM- $\text{MgCl}_2$	
No additions	0.61 (0.82)
25 mM- $\text{LiCl}$	0.12 (0.16)
0.25 mM-2,3-bisphosphoglycerate	0.68 (0.92)
$\text{InsP}_2$ formed in 2.5 mM- $\text{CaCl}_2$	
No additions	0.11 (0.15)
$\text{InsP}_2$ formed by 5-phosphatase from human erythrocyte membranes	0.09 (0.12)

the commercial  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  was 12% at the 4- and 88% at the 5-position (as determined with specific 5-phosphatase in human erythrocyte membranes; Downes *et al.*, 1982). After incubation of this  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  mixture with the *D. discoideum* soluble cell fraction, and subsequent fractionation of the inositol phosphates formed on Dowex columns, the ratio of  $^{32}\text{P}$  label recovered with respect to the  $^3\text{H}$  label in the  $\text{InsP}_2$  column fraction was determined. As the  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  is labelled predominantly in the 5-position, a low  $^{32}\text{P}:^3\text{H}$  ratio in  $\text{InsP}_2$  indicates 5-phosphatase activity, whereas a high  $^{32}\text{P}:^3\text{H}$  ratio in  $\text{InsP}_2$  indicates 1- and/or 4-phosphatase activity.

Table 2 shows that the  $^{32}\text{P}:^3\text{H}$  ratio in  $\text{InsP}_2$  is 82% of the original value in the  $\text{Ins}(1,4,5)\text{P}_3$  substrate, when dephosphorylation is assayed in 5 mM- $\text{Mg}^{2+}$ . In the presence of 2,3-bisphosphoglycerate the ratio even approaches the original ratio in  $\text{Ins}(1,4,5)\text{P}_3$  (92%). This suggests that, in 5 mM- $\text{Mg}^{2+}$ ,  $\text{Ins}(1,4,5)\text{P}_3$  is dephosphorylated predominantly at the 1- and/or the 4-position, thus yielding  $\text{Ins}(1,5)\text{P}_2$  and/or  $\text{Ins}(4,5)\text{P}_2$ . In contrast, the  $\text{Li}^+$ -insensitive part of the activity in  $\text{Mg}^{2+}$  dephosphorylates  $\text{Ins}(1,4,5)\text{P}_3$  at the 5-position, as the  $^{32}\text{P}:^3\text{H}$  ratio is similar to that found for the 5-phosphatase from erythrocyte membrane (Table 2). The same is the case for the activity measured in  $\text{Ca}^{2+}$ .

From this we conclude that in the soluble fraction of *Dictyostelium* homogenates, assayed for  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase in  $\text{Mg}^{2+}$ , about 80% of the activity is accounted for by either a 1- and/or a 4-phosphatase, which is sensitive to  $\text{Li}^+$ , and about 20% of the activity

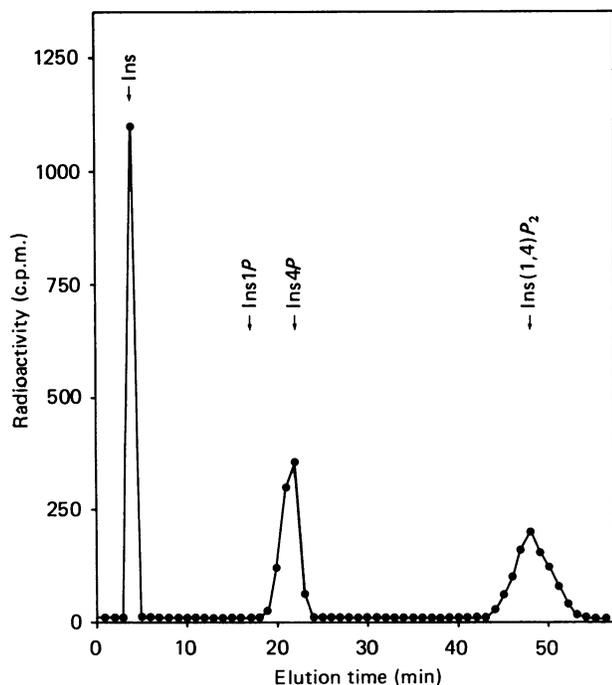


Fig. 1. H.p.l.c. analysis of the products of  $\text{Ins}(1,4)\text{P}_2$  dephosphorylation

$[2\text{-}^3\text{H}]\text{Ins}(1,4)\text{P}_2$  (10 nCi;  $0.25\ \mu\text{M}$ ) was dephosphorylated by incubation with *Dictyostelium* soluble cell fraction for 10 min, as described in the Materials and methods section in the presence of  $5\ \text{mM-MgCl}_2$ . After filtration on a Centricon TM microconcentrator, a sample was loaded on a  $\mu\text{Bondapak NH}_2$  column. Separation was carried out by isocratic elution for 10 min with  $20\ \text{mM-ammonium acetate/acetic acid, pH 4.0}$ , followed by a 60 min linear gradient to  $1\ \text{M-ammonium acetate/acetic acid, pH 4.0}$ . Fractions were collected every 1 min (flow rate  $1\ \text{ml/min}$ ), and radioactivity was determined by liquid-scintillation spectroscopy. The arrows indicate the elution times of commercial  $[2\text{-}^3\text{H}]\text{Ins}$ , commercial  $[2\text{-}^3\text{H}]\text{Ins1P}$  and  $[2\text{-}^3\text{H}]\text{Ins4P}$  (prepared by dephosphorylation of  $[2\text{-}^3\text{H}]\text{Ins}(1,4)\text{P}_2$  with rat brain soluble fraction; Delvaux *et al.*, 1987b).

by a 5-phosphatase, which is sensitive to 2,3-bis-phosphoglycerate.  $\text{Ca}^{2+}$  can only replace  $\text{Mg}^{2+}$  for the 5-phosphatase. In the particulate fraction the 5-phosphatase and 1- and/or 4-phosphatase are about equally active.

Analysis of  $^{32}\text{P}:^3\text{H}$  ratios does not give a clear-cut answer about whether the  $\text{Li}^+$ -sensitive  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity dephosphorylates  $\text{Ins}(1,4,5)\text{P}_3$  at the 1- or at the 4-position, as the fraction of  $^{32}\text{P}$  label in the 4-position is too small for accurate analysis. As we do know that the  $\text{InsP}_2$  thus formed still contains the 5-phosphate, the two possible isomers of this  $\text{InsP}_2$  are  $\text{Ins}(4,5)\text{P}_2$  or  $\text{Ins}(1,5)\text{P}_2$ , and it is therefore termed  $\text{Ins}(x,5)\text{P}_2$ .

#### Dephosphorylation of $\text{InsP}_2$

The two distinct pathways for the first step in  $\text{Ins}(1,4,5)\text{P}_3$  dephosphorylation, measured in  $5\ \text{mM-MgCl}_2$ , have been shown above to yield  $\text{Ins}(x,5)\text{P}_2$  (80%) and  $\text{Ins}(1,4)\text{P}_2$  (20%). We used  $[2\text{-}^3\text{H}]\text{Ins}(1,4)\text{P}_2$  as a

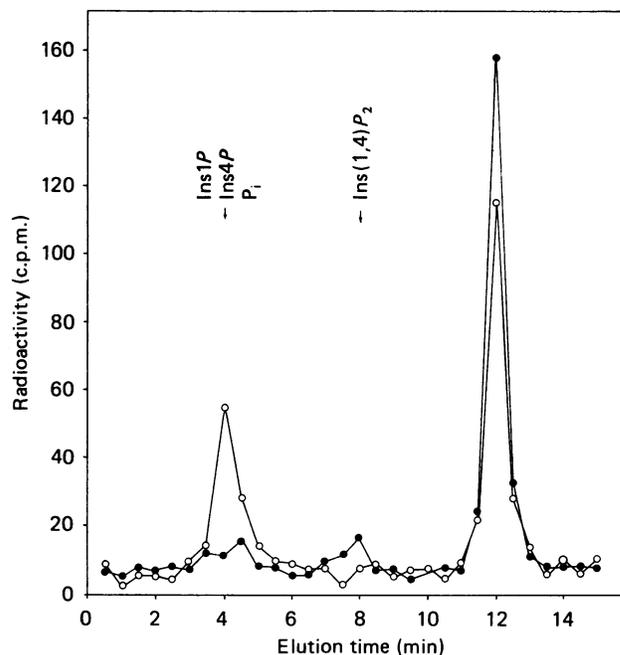


Fig. 2. Purification by h.p.l.c. of a  $^{32}\text{P}/^3\text{H}$ -labelled mixture of  $\text{Ins}(x,5)\text{P}_2$  synthesized from  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  with a partially purified phosphatase

A mixture of  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  was incubated with a partially purified phosphatase from *Dictyostelium* as described in the Materials and methods section. The reaction mixture was chromatographed on a LiChrosorb reversed-phase column, and 0.6 ml fractions were collected; the radioactivity in  $6\ \mu\text{l}$  was determined with a dual-label program. The three fractions eluted around 12 min were combined. The  $^{32}\text{P}/^3\text{H}$  ratio in the substrate  $\text{Ins}(1,4,5)\text{P}_3$  was 0.60, and that in the product  $\text{Ins}(x,5)\text{P}_2$  was 0.61. The elution of commercial standards is indicated by the arrows;  $\text{Ins}(1,4,5)\text{P}_3$  is eluted later than 15 min.  $\circ$ ,  $^{32}\text{P}$  radioactivity;  $\bullet$ ,  $^3\text{H}$  radioactivity.

substrate to characterize the second dephosphorylation step of the latter pathway. H.p.l.c. analysis of the products formed after incubating  $\text{Ins}(1,4)\text{P}_2$  with the *Dictyostelium* soluble cell fraction shows that the  $\text{InsP}$  formed from  $\text{Ins}(1,4)\text{P}_2$  is exclusively  $\text{Ins4P}$  (Fig. 1).

To study the dephosphorylation of  $\text{Ins}(x,5)\text{P}_2$  to  $\text{InsP}$ , we synthesized and purified a  $^{32}\text{P}/^3\text{H}$ -labelled mixture of  $\text{Ins}(x,5)\text{P}_2$  from  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  (see the Materials and methods section and Fig. 2). The  $^{32}\text{P}/^3\text{H}$  ratio of the  $\text{Ins}(1,4,5)\text{P}_3$  substrate was 0.60; the ratio in the  $\text{Ins}(x,5)\text{P}_2$  product was 0.61. This purified  $^{32}\text{P}/^3\text{H}$  mixture of  $\text{Ins}(x,5)\text{P}_2$  was incubated with *Dictyostelium* soluble cell fraction, and the dephosphorylation products were subsequently analysed by h.p.l.c. (Fig. 3). The  $\text{InsP}$  formed is co-eluted with  $\text{Ins4P}$ , is not co-eluted with  $\text{Ins1P}$ , and has a low  $^{32}\text{P}/^3\text{H}$  ratio (0.05) compared with the synthesized  $\text{Ins}(x,5)\text{P}_2$  substrate (0.61), indicating that the 5-phosphate has been removed. From this we infer that the  $\text{InsP}$  formed is  $\text{Ins4P}$ , and thus the synthesized  $\text{Ins}(x,5)\text{P}_2$  must have been  $\text{Ins}(4,5)\text{P}_2$  [and not  $\text{Ins}(1,5)\text{P}_2$ ]. In summary, we can conclude that  $\text{Ins}(1,4,5)\text{P}_3$  is dephosphorylated by *Dictyostelium* soluble cell fraction, through  $\text{Ins}(1,4)\text{P}_2$  and predominantly  $\text{Ins}(4,5)\text{P}_2$ , to  $\text{Ins4P}$ .

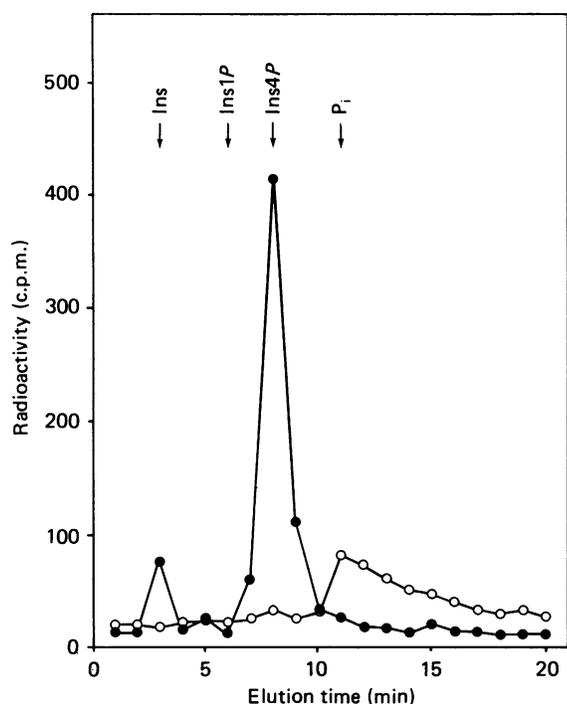


Fig. 3. H.p.l.c. analysis of the  $\text{InsP}$  isomer(s) formed after dephosphorylation of a  $^{32}\text{P}/^3\text{H}$ -labelled mixture of  $\text{Ins}(x,5)\text{P}_2$  synthesized from  $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$  and  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$

H.p.l.c.-purified  $[^{32}\text{P}, ^3\text{H}]\text{Ins}(x,5)\text{P}_2$  (see Fig. 2) was incubated with *Dictyostelium* soluble cell fraction for 60 min in the presence of 5 mM- $\text{MgCl}_2$ . The incubation was terminated by adding 0.5 ml of ice-cold 150 mM-ammonium acetate/acetic acid (pH 4.0) and immediate filtration on a Centricon TM microconcentrator. The reaction products were separated on a Partisil SAX column by isocratic elution with 150 mM-ammonium acetate/acetic acid, pH 4.0. Fractions were collected every 1 min (flow rate 1.5 ml/min), and radioactivity was determined by liquid-scintillation spectrometry. The  $^{32}\text{P}/^3\text{H}$  ratio in the  $\text{InsP}$  peak is low (0.05, relative to 0.61 in substrate), indicating that it does not contain a 5-phosphate group. Co-elution with authentic  $\text{Ins4P}$  and absence of a 5-phosphate in the  $\text{InsP}$  identifies the product as  $\text{Ins4P}$ , and the  $\text{Ins}(x,5)\text{P}_2$  substrate as  $\text{Ins}(4,5)\text{P}_2$ .  $\circ$ ,  $^{32}\text{P}$  radioactivity;  $\bullet$ ,  $^3\text{H}$  radioactivity.

#### Inhibition of $\text{Ins}(1,4,5)\text{P}_3$ phosphatase by $\text{Li}^+$

As shown in Table 1, the inhibitor-sensitivity of  $\text{Ins}(1,4,5)\text{P}_3$  dephosphorylation is rather complex. We have therefore studied the sensitivity of the different dephosphorylation reactions in more detail. In mammalian tissues,  $\text{Li}^+$  does not affect the 5-phosphatase activity acting on  $\text{Ins}(1,4,5)\text{P}_3$  (Downes *et al.*, 1982; Storey *et al.*, 1984; Connolly *et al.*, 1985; Erneux *et al.*, 1986), but is a potent uncompetitive inhibitor of 1- and 4-phosphatase activities acting on  $\text{Ins}(1,4)\text{P}_2$ ,  $\text{Ins4P}$  and  $\text{Ins1P}$  (Hallcher & Sherman, 1980; Inhorn & Majerus, 1987; Gee *et al.*, 1988). As shown in Fig. 4,  $\text{Li}^+$  effectively inhibits  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity to a maximum of about 80%, if measured in 5 mM- $\text{Mg}^{2+}$  with 0.25  $\mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ . Half-maximal inhibition of this activity occurs at about 0.25 mM- $\text{LiCl}$ . When phosphatase activity was measured in  $\text{Ca}^{2+}$ ,  $\text{Li}^+$  had no effect (Fig. 4).

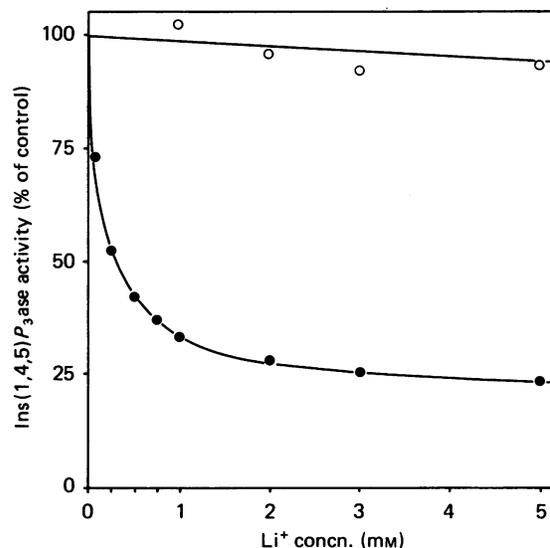


Fig. 4. Effect of  $\text{Li}^+$  on  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity in the soluble cell fraction of *Dictyostelium* homogenates

Dephosphorylation of  $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  (5 nCi; 0.25  $\mu\text{M}$ ) was assayed in the presence of different  $\text{LiCl}$  concentrations as described in the Materials and methods section, with either 5 mM- $\text{MgCl}_2$  ( $\bullet$ ) or 2.5 mM- $\text{CaCl}_2$  ( $\circ$ ). The phosphatase activity data are expressed as percentages of the control without  $\text{LiCl}$ .

#### Inhibition of $\text{Ins}(1,4,5)\text{P}_3$ phosphatase by 2,3-bisphosphoglycerate

2,3-Bisphosphoglycerate is a potent competitive inhibitor of 5-phosphatase activities from erythrocyte membranes (Downes *et al.*, 1982) and soluble and particulate rat brain fraction (Delvaux *et al.*, 1987a). Furthermore, concentrations of up to 1 mM have no effect on  $\text{Ins}(1,4)\text{P}_2$  and  $\text{Ins1P}$  phosphatases (Delvaux *et al.*, 1987a).

In the *Dictyostelium* soluble cell fraction 2,3-bisphosphoglycerate inhibits the  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity biphasically, when measured with 0.25 mM- $\text{Ins}(1,4,5)\text{P}_3$  in 5 mM- $\text{Mg}^{2+}$  (Fig. 5). Under these conditions, about 20% of the activity is inhibited with high sensitivity, whereas the remaining 80% is inhibited only at high concentrations (above 0.5 mM) of 2,3-bisphosphoglycerate. When measured in  $\text{Ca}^{2+}$ , however, all the activity can be inhibited with high sensitivity, half-maximal inhibition occurring at about 3  $\mu\text{M}$  (Fig. 5).

The combination of the effects of  $\text{Li}^+$  and 2,3-bisphosphoglycerate on the *Dictyostelium*  $\text{Ins}(1,4,5)\text{P}_3$  phosphatase activity (assuming that the inhibitor-sensitivities of the *Dictyostelium* and rat brain enzymes are similar) supplements the evidence presented above that in  $\text{Mg}^{2+}$   $\text{Ins}(1,4,5)\text{P}_3$  is dephosphorylated by two enzymes: 20% of the activity is due to a 5-phosphatase, as this 20% of the activity is insensitive to  $\text{Li}^+$  and highly sensitive to 2,3-bisphosphoglycerate, and 80% of the activity is due to a 1-phosphatase, as this activity is less sensitive to 2,3-bisphosphoglycerate and highly sensitive to  $\text{Li}^+$ .  $\text{Ca}^{2+}$  apparently can only replace  $\text{Mg}^{2+}$  in that part of the activity which is sensitive to 2,3-bisphosphoglycerate, so in  $\text{Ca}^{2+}$  all activity is apparently due to a 5-phosphatase which has some similarities to the 5-phosphatase from higher organisms.

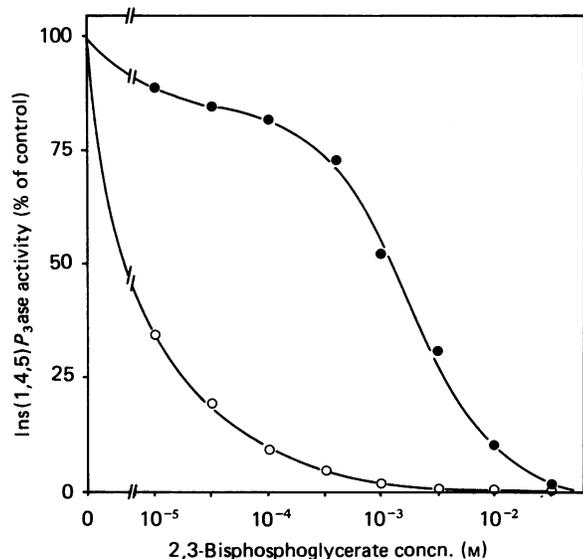


Fig. 5. Effect of 2,3-bisphosphoglycerate on Ins(1,4,5) $P_3$  phosphatase activity in the soluble cell fraction of *Dictyostelium homogenates*

Dephosphorylation of [ $2\text{-}^3\text{H}$ ]Ins(1,4,5) $P_3$  (5 nCi; 0.25  $\mu\text{M}$ ) was assayed in the presence of different 2,3-bisphosphoglycerate concentrations as described in the Materials and methods section, with either 5 mM-MgCl $_2$  (●) or 2.5 mM-CaCl $_2$  (○). The phosphatase activity data are expressed as percentages of the control without 2,3-bisphosphoglycerate.

#### Sensitivity of Ins $P_2$ dephosphorylation to Li $^+$ and 2,3-bisphosphoglycerate

Dephosphorylation of Ins(1,4) $P_2$  to Ins4P has been shown to be Li $^+$ -sensitive and 2,3-bisphosphoglycerate-insensitive in rat brain (Inhorn *et al.*, 1987; Delvaux *et al.*, 1987a). The same is the case for [ $2\text{-}^3\text{H}$ ]Ins(1,4) $P_2$  dephosphorylation in *Dictyostelium*; Li $^+$  inhibits the 1-phosphatase activity with half-maximal inhibition at about 2.5 mM (Fig. 6), and 0.25 mM-2,3-bisphosphoglycerate has no effect on this activity (results not shown).

To measure the sensitivities of Ins(4,5) $P_2$  dephosphorylation to Li $^+$  and 2,3-bisphosphoglycerate, we synthesized and purified [ $2\text{-}^3\text{H}$ ]Ins(4,5) $P_2$  from [ $2\text{-}^3\text{H}$ ]Ins(1,4,5) $P_3$  (see the Materials and methods section) and incubated the compound with the high-speed supernatant from *Dictyostelium*: 25 mM-LiCl inhibits the dephosphorylation of [ $2\text{-}^3\text{H}$ ]Ins(4,5) $P_2$  by only 7%, whereas 0.25 mM-2,3-bisphosphoglycerate inhibits the dephosphorylation by 76% (results not shown).

#### Li $^+$ -sensitivity of Ins4P and L-Ins1P dephosphorylation

*myo*-Inositol monophosphates can be derived from two different sources: (1) L-Ins1P formed from isomerization of D-glucose 6-phosphate catalysed by L-*myo*-inositol 1-phosphate synthase, which is required for Ins synthesis *de novo*, and (2) InsP in the D-conformation, formed through the action of phospholipase C on phosphatidylinositol, or through dephosphorylation of the D-*myo*-inositol polyphosphates. In high organisms, InsP is dephosphorylated by a phosphatase which is not very specific; the enzyme can hydrolyse all InsP isomers with an equatorial phosphate group, as well as 2'-AMP

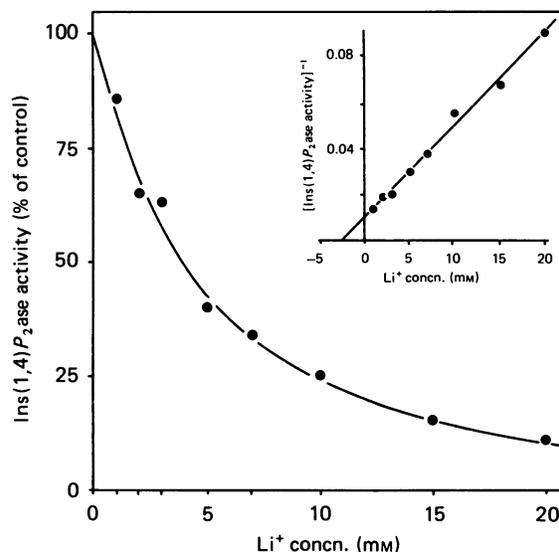


Fig. 6. Effect of Li $^+$  on Ins(1,4) $P_2$  1-phosphatase activity in the soluble cell fraction of *Dictyostelium homogenates*

Dephosphorylation of [ $2\text{-}^3\text{H}$ ]Ins(1,4) $P_2$  (5 nCi; 0.125  $\mu\text{M}$ ) was assayed in the presence of different LiCl concentrations as described in the Materials and methods section. The phosphatase activity data are expressed as percentages of the control without LiCl. The inset shows a Dixon plot of the same data.

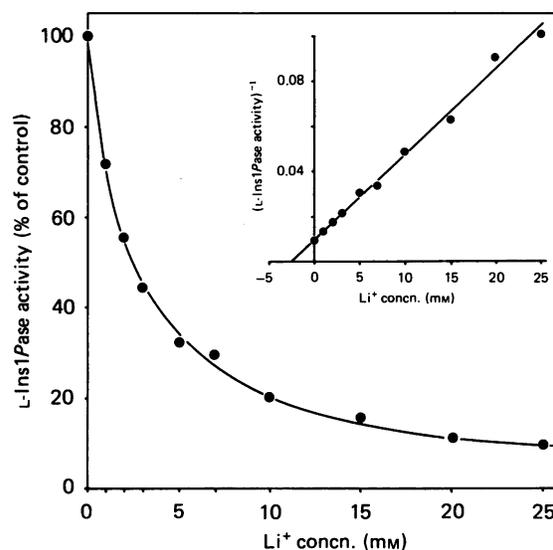


Fig. 7. Effect of Li $^+$  on L-Ins1P phosphatase activity in the soluble cell fraction of *Dictyostelium homogenates*

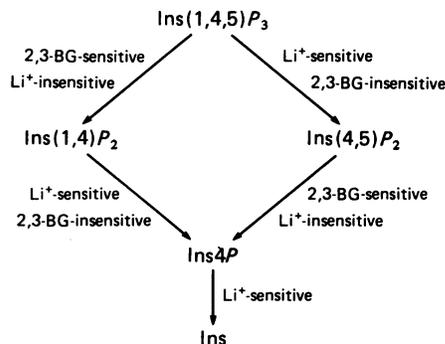
Dephosphorylation of L-[U- $^{14}\text{C}$ ]Ins1P (6.3 nCi; 5.7  $\mu\text{M}$ ) was assayed as described for Ins $P_2$  phosphatase in Fig. 6.

and (–)-*chiro*-Ins(3)P [but not Ins(1,4,5) $P_3$ ] and does not discriminate between the two enantiomeric conformations of Ins1P (Eisenberg, 1967; Hallcher & Sherman, 1980; Ackermann *et al.*, 1987). Furthermore, Li $^+$  is a potent inhibitor of InsP phosphatase activities (Naccarato *et al.*, 1974; Hallcher & Sherman, 1980; Sherman *et al.*, 1984; Ackermann *et al.*, 1987; Delvaux *et al.*, 1987b; Gee *et al.*, 1988).

In *Dictyostelium* homogenates, L-Ins1P and Ins4P are dephosphorylated under conditions similar to those described for the above systems. Li<sup>+</sup> inhibits the dephosphorylation of L-[U-<sup>14</sup>C]Ins1P in the soluble fraction of these homogenates, with half-maximal inhibition at about 2.5 mM (Fig 7). Furthermore, when [2-<sup>3</sup>H]Ins(1,4)P<sub>2</sub> is incubated with this cell fraction until almost no substrate is left, subsequent addition of Li<sup>+</sup> inhibits the further dephosphorylation of the Ins4P thus formed: by 51% with 4 mM-LiCl and by 74% with 20 mM-LiCl.

## DISCUSSION

Our results show that *Dictyostelium discoideum* homogenates possess phosphatases which can rapidly dephosphorylate Ins(1,4,5)P<sub>3</sub>. The two presumptive dephosphorylation pathways can be summarized by Scheme 1.



Scheme 1.

Abbreviation: 2,3-BG, 2,3-bisphosphoglycerate.

Although at first sight the phosphatase activities appear to be very different from those in higher organisms, on closer inspection many similarities can be found. *Dictyostelium* contains an Ins(1,4,5)P<sub>3</sub> 5-phosphatase activity which is Mg<sup>2+</sup>-dependent, sensitive to 2,3-bisphosphoglycerate, insensitive to Li<sup>+</sup> and present in both particulate and soluble cell fractions. This is similar to the 5-phosphatase from rat liver and brain (Erneux *et al.*, 1986; Shears *et al.*, 1987), which also contains both soluble and particulate 5-phosphatase activities. Further dephosphorylation of Ins(1,4)P<sub>2</sub> by the *Dictyostelium* soluble cell fraction is also similar to the rat or bovine brain systems; Ins(1,4)P<sub>2</sub> is dephosphorylated in both systems by a 1-phosphatase to form Ins4P, which is then dephosphorylated to Ins (Inhorn *et al.*, 1987; Delvaux *et al.*, 1987b; Ragan *et al.*, 1988). Furthermore, the Ins(1,4)P<sub>2</sub> 1-phosphatase and the Ins4P phosphatases of both systems are sensitive to Li<sup>+</sup>, with half-maximal inhibition at about 2.5 mM-LiCl, and are 10–100 times less sensitive to 2,3-bisphosphoglycerate than are their Ins(1,4,5)P<sub>3</sub> 5-phosphatase activities (Delvaux *et al.*, 1987a,b). The *Dictyostelium* 5-phosphatase enzyme differs from these systems in that Ca<sup>2+</sup> can replace Mg<sup>2+</sup>. In platelets and erythrocytes Ca<sup>2+</sup> cannot replace Mg<sup>2+</sup>, and Ca<sup>2+</sup> inhibits the Mg<sup>2+</sup>-activated activity with high affinity (K<sub>i</sub> = 70 μM) (Downes *et al.*, 1982; Connolly *et al.*, 1985). We have used Ca<sup>2+</sup> as a tool to elucidate the dephosphorylation pathway of Ins(1,4,5)P<sub>3</sub> in *Dictyos-*

*stelium*, but have no indication for its physiological importance.

The major difference between Ins(1,4,5)P<sub>3</sub> dephosphorylation in *Dictyostelium* and that of higher organisms is, however, that in *Dictyostelium* most of the Ins(1,4,5)P<sub>3</sub> phosphatase activity is due to a predominantly soluble 1-phosphatase, which is highly Li<sup>+</sup>-sensitive [10-fold more sensitive than Ins(1,4)P<sub>2</sub> and L-Ins1P 1-phosphatase activities]. This shows that, although many of the *Dictyostelium* Ins(1,4,5)P<sub>3</sub>-dephosphorylating enzymes could be very similar to those of higher organisms, the metabolic pathway of Ins(1,4,5)P<sub>3</sub> dephosphorylation is very different, and a new InsP<sub>2</sub> isomer, Ins(4,5)P<sub>2</sub>, is formed.

It is now important to investigate which InsP<sub>2</sub> is present *in vivo*. Nothing is known about the identity of any of the isomers of the inositol phosphates that are present *in vivo* in *Dictyostelium*, but this can be investigated by h.p.l.c. The high sensitivity of Ins(1,4,5)P<sub>3</sub> dephosphorylation for Li<sup>+</sup> *in vitro* makes it interesting to see whether the dramatic effects that Li<sup>+</sup> has on the determination of cell differentiation and pattern formation *in vivo* (Maeda, 1970; Sakai, 1973; Van Lookeren Campagne *et al.*, 1988) are mediated through interference with the metabolism of the inositol (poly)phosphates.

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