Two dephosphorylation pathways of inositol 1,4,5-trisphosphate in homogenates of the cellular slime mould Dictyostelium discoideum
Lookeren Campagne, Michiel M. van; Erneux, Cristophe; Eijk, Ronald van; Haastert, Peter J.M. van

Published in:
FEMS Microbiology Letters

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1988

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Two dephosphorylation pathways of inositol 1,4,5-trisphosphate in homogenates of the cellular slime mould Dictyostelium discoideum

Michiel M. VAN LOOKEREN CAMPAGNE,*† Cristophe ERNEUX,† Ronald VAN EIJK* and Peter J. M. VAN HAASTERT*

*Cell Biology and Genetics Unit, Zoological Laboratory, University of Leiden, Kazerstraat 63, NL-2311 GP Leiden, The Netherlands, and †Institute of Interdisciplinary Research (IRIBHN), School of Medicine, Free University of Brussels (U.L.B.), Campus Erasme (Bât. C), Route de Lennik 808, B-1070 Brussels, Belgium

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 Ins$_4$P (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, Ins$_4$P phosphatase and t-Ins$_1$P (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 μM). Furthermore, 2,3-bisphosphoglycerate also inhibits dephosphorylation of Ins(4,5)P$_2$. These characteristics point to a high degree 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 Ins$_4$P 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 Ins$_4$P has been shown to be sensitive to Li$^+$ ions (Hallcher & Sherman, 1980; Storey et al., 1984; Takimoto et al., 1985; Delvaux et al., 1987a; 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 chemooattractant cyclic AMP can trigger the accumulation of intracellular Ins$_4$P 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 (Maceron et al., 1980), a kinase which phosphorylates phosphatidylinositol to phosphatidylinositol 4-phosphate (Varela et al., 1987) and a kinase which phospho-

Abbreviations used: Ins, myo-inositol; Ins$_1$P, l-myo-inositol 1-phosphate; t-Ins$_1$P, l-myo-inositol 1-phosphate; Ins$_4$P, 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; Ins$P$, Ins$_2$P and Ins$_3$P, d-myo-inositol phosphates without specification of the phosphate position(s).

† Present address: Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032, U.S.A.
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**

$[^2\text{H}]$Ins(1,4,5)P$_3$ (1.0 Ci/mmol) and l-[U-14C]Ins1P (55 mCi/mmol) were from Amersham International. $[^2\text{H}]$Ins(1,4)P$_2$ (2.0 Ci/mmol), $[^4,5-\text{sp}]$Ins(1,4,5)P$_3$ (130 Ci/mmol), $[^2\text{H}]$Ins1P (5.4 Ci/mmol) and $[^3\text{H}]$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 × 0.39 cm), Whatman (Partisil SAX; 25 cm × 0.49 cm) and Chrompack (LiChrosorb 10RP18; 25 cm × 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 × 10$^8$ cells/ml in the same buffer. Homogenates were made by passing the cells through a Nucleopore filter (3 µm pore size) (Das & Henderson, 1983). Lysates were then centrifuged for 3 min at 10,000 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 recentrifuging the 10,000 g supernatant for 5 min in a Beckman Airfuge at 150,000 g. Soluble and particulate fractions thus obtained were immediately used for the phosphatase assay.

**Phosphatase assay**

Dephosphorylation of $[^2\text{H}]$Ins(1,4,5)P$_3$, $[^4,5-\text{sp}]$Ins(1,4,5)P$_3$, $[^2\text{H}]$Ins(1,4)P$_2$, $[^2\text{H}]$Ins(4,5)P$_2$, $[^4,5-\text{sp}]$Ins(4,5)P$_2$ and/or l-[U-14C]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 µl of the soluble or particulate fraction of the *Dictyostelium* homogenate to 15 µl 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 µl of water. After vigorous shaking and centrifugation (1 min, 10,000 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 (Ins$_4$P); and (4) 10 ml of 750 mM-ammonium formate/100 mM-formic acid (Ins$_2$P$_2$). 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-14C]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 µg/ml), 100 µM-phenylmethylsulphonyl fluoride, soya-bean trypsin inhibitor (50 µg/ml) and 5 mM-benzamidine]. The high-speed supernatant from 3 × 10$^9$ cells was chromatographed on a DEAE-cellulose column (10 ml; 8 cm × 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 µl) containing 50 nCi of $[^4,5-\text{sp}]$Ins(1,4,5)P$_3$, 100 nCi of $[^2\text{H}]$Ins(1,4,5)P$_3$, 5 mM-MgCl$_2$, buffer A and 40 µl of enzyme from the DEAE-cellulose column. After 60 min the incubation was terminated by the addition of 100 µl of 0.1 M-tributylammonium phosphate, pH 6.5. The sample was centrifuged immediately for 5 min at 10,000 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-tributylammonium 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 µl samples was determined by using a dual-label program. Peak fractions were combined and concentrated to dryness under reduced pressure at 10°C. $[^3\text{H}]$Ins(4,5)P$_2$ was prepared in parallel from 100 nCi of $[^3\text{H}]$Ins(1,4,5)P$_3$.
Table 1. Relative Ins(1,4,5)P$_3$ phosphatase activity in soluble and particulate cell fractions measured under different conditions

<table>
<thead>
<tr>
<th>Assay with:</th>
<th>No addition</th>
<th>25 mM LiCl</th>
<th>0.25 mM 2,3-Bisphosphoglycerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM-MgCl$_2$</td>
<td>100</td>
<td>21</td>
<td>82</td>
</tr>
<tr>
<td>2.5 mM-CaCl$_2$</td>
<td>19</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Particulate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM-MgCl$_2$</td>
<td>21</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>2.5 mM-CaCl$_2$</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

RESULTS

General properties of Ins(1,4,5)P$_3$ dephosphorylation in Dictyostelium

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

CaCl$_2$ (2.5 mM) can replace 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 Ca$^{2+}$ for Mg$^{2+}$ changes the sensitivity towards Li$^+$ and 2,3-bisphosphoglycerate, Li$^+$ becoming ineffective and 2,3-bisphosphoglycerate almost completely inhibiting all phosphatase activity (Table 1).

Identification of the first phosphate group removed by Ins(1,4,5)P$_3$ phosphatase

The studies with the Ins(1,4,5)P$_3$ phosphatase inhibitors Li$^+$ and 2,3-bisphosphoglycerate suggest that in 5 mM-Mg$^{2+}$ Ins(1,4,5)P$_3$ is dephosphorylated by a route different from that in 2.5 mM-Ca$^{2+}$. To see which phosphate group is removed first under the different conditions, we measured the hydrolysis of a mixture of [4,5-$^{32}$P]Ins(1,4,5)P$_3$ and [2-$^3$H]Ins(1,4,5)P$_3$. The distribution of $^{32}$P label between the 4- and 5-phosphates of the commercial [4,5-$^{32}$P]Ins(1,4,5)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-$^{32}$P]Ins(1,4,5)P$_3$ and [2-$^3$H]Ins(1,4,5)P$_3$ mixture with the D. discoideum soluble cell fraction, and subsequent fractionation of the inositol phosphates formed on Dowex columns, the ratio of $^{32}$P to $^3$H and $^{32}$P to $^3$H radioactivity in the InsP$_2$ fraction was determined. As the [4,5-$^{32}$P] and [2-$^3$H]Ins(1,4,5)P$_3$ is labelled predominantly in the 5-position, a low $^{32}$P/$^3$H ratio in InsP$_2$ indicates 5-phosphatase activity, whereas a high $^{32}$P/$^3$H ratio in InsP$_2$ indicates 1- and/or 4-phosphatase activity.

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

<table>
<thead>
<tr>
<th>Assay with:</th>
<th>$^{32}$P/$^3$H ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ins(1,4,5)P$_3$</td>
<td>0.74 (1.00)</td>
</tr>
<tr>
<td>InsP$_2$ formed in 5 mM-MgCl$_2$</td>
<td>0.61 (0.82)</td>
</tr>
<tr>
<td>No additions</td>
<td>0.61 (0.82)</td>
</tr>
<tr>
<td>25 mM-LiCl</td>
<td>0.12 (0.16)</td>
</tr>
<tr>
<td>0.25 mM-2,3-bisphosphoglycerate</td>
<td>0.68 (0.92)</td>
</tr>
<tr>
<td>InsP$_2$ formed in 2.5 mM-CaCl$_2$</td>
<td>0.11 (0.15)</td>
</tr>
<tr>
<td>No additions</td>
<td>0.11 (0.15)</td>
</tr>
<tr>
<td>InsP$_2$ formed by 5-phosphatase from human erythrocyte membranes</td>
<td>0.09 (0.12)</td>
</tr>
</tbody>
</table>

The ratio between the $^{32}$P and $^3$H radioactivity in InsP$_2$ is similar to that found for the 5-phosphatase from erythrocyte membrane (Table 2). The same is the case for the activity measured in Ca$^{2+}$.
Fig. 1. H.p.l.c. analysis of the products of Ins(1,4)P₂ dephosphorylation

[2-³H]Ins(1,4)P₂ (10 nCi; 0.25 µM) was dephosphorylated by incubation with Dicyostelium soluble cell fraction for 10 min, as described in the Materials and methods section in the presence of 5 mM-MgCl₂. After filtration on a Centricon TM microconcentrator, a sample was loaded on a µBondapak NH₂ column. Separation was carried out by isocratic elution for 10 min with 20 mM-ammonium acetate/acetic acid, pH 4.0, followed by a 60 min linear gradient to 1 M-ammonium acetate/acetic acid, pH 4.0. Fractions were collected every 1 min (flow rate 1 ml/min), and radioactivity was determined by liquid-scintillation spectroscopy. The arrows indicate the elution times of commercial [2-³H]Ins, commercial [2-³H]Ins1P and [2-³H]Ins4P (prepared by dephosphorylation of [2-³H]Ins(1,4)P₂ with rat brain soluble fraction; Delvaux et al., 1987b). 

by a 5-phosphatase, which is sensitive to 2,3-bisphosphoglycerate. Ca²⁺ can only replace Mg²⁺ for the 5-phosphatase. In the particulate fraction the 5-phosphatase and 1- and/or 4-phosphatase are about equally active.

Analysis of ³²P/³H ratios does not give a clear-cut answer about whether the Li⁺-sensitive Ins(1,4,5)P₃ phosphatase activity dephosphorylates Ins(1,4,5)P₃ at the 1- or at the 4-position, as the fraction of ³²P label in the 4-position is too small for accurate analysis. As we do know that the InsP₁ thus formed still contains the 5-phosphate, the two possible isomers of this InsP₂ are Ins(4,5)P₂ or Ins(1,5)P₂, and it is therefore termed Ins(x,5)P₂.

Dephosphorylation of InsP₂

The two distinct pathways for the first step in Ins-(1,4,5)P₃ dephosphorylation, measured in 5 mM-MgCl₂, have been shown above to yield Ins(x,5)P₂ (80%) and Ins(1,4)P₂ (20%). We used [2-³H]Ins(1,4)P₂ as a substrate to characterize the second dephosphorylation step of the latter pathway. H.p.l.c. analysis of the products formed after incubating Ins(1,4)P₂ with the Dicyostelium soluble cell fraction shows that the InsP formed from Ins(1,4)P₂ is exclusively Ins4P (Fig. 1).

To study the dephosphorylation of Ins(x,5)P₂ to InsP, we synthesized and purified a ³²P/³H-labelled mixture of Ins(x,5)P₂ from [4,5-³²P]Ins(1,4,5)P₃ and [2-³H]Ins(1,4,5)P₃ (see the Materials and methods section and Fig. 2). The ³²P/³H ratio of the Ins(1,4,5)P₂ substrate was 0.60; the ratio in the Ins(x,5)P₂ product was 0.61. This purified ³²P/³H mixture of Ins(x,5)P₂ was incubated with Dicyostelium soluble cell fraction, and the dephosphorylation products were subsequently analysed by h.p.l.c. (Fig. 3). The InsP formed is co-eluted with Ins4P, is not co-eluted with Ins1P, and has a low ³²P/³H ratio (0.05) compared with the synthesized Ins(x,5)P₂ substrate (0.61), indicating that the 5-phosphate has been removed. From this we infer that the InsP Formed is Ins4P, and thus the synthesized Ins(x,5)P₂ must have been Ins(4,5)P₂ [and not Ins(1,5)P₂]. In summary, we can conclude that Ins(1,4,5)P₃ is dephosphorylated by Dicyostelium soluble cell fraction, through Ins(1,4)P₂ and predominantly Ins(4,5)P₂, to Ins4P.
Dephosphorylation of inositol trisphosphate in Dictyostelium

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

H.p.l.c.-purified [$^{32}$P,$^3$H]Ins($x,5$)$_2$ (see Fig. 2) was incubated with Dictyostelium soluble cell fraction for 60 min in the presence of 5 mM-MgCl$_2$. The incubation was terminated by adding 0.5 ml of ice-cold 150 mM-ammonium acetate/acet 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/acet 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 $^{32}$P/$^3$H ratio in the 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 Ins4P and absence of a 5-phosphate in the InsP identifies the product as Ins4P, and the Ins($x,5$)$_2$ substrate as Ins(4,5)$_2$. $^{32}$P radioactivity; $^3$H radioactivity.

Inhibition of Ins(1,4,5)$_3$ P$_3$ phosphatase by Li$^+$

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

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

Dephosphorylation of [2-$^3$H]Ins(1,4,5)$_3$ P$_3$ (5 nCi; 0.25 µM) was assayed in the presence of different LiCl 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 LiCl.

Inhibition of Ins(1,4,5)$_3$ 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 Ins(1,4)$_2$ and Ins1P phosphatases (Delvaux et al., 1987a).

In the Dictyostelium soluble cell fraction 2,3-bisphosphoglycerate inhibits the Ins(1,4,5)$_3$ P$_3$ phosphatase activity biphasically, when measured with 0.25 mM-Ins(1,4,5)$_3$ P$_3$ in 5 mM-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 Ca$^{2+}$, however, all the activity can be inhibited with high sensitivity, half-maximal inhibition occurring at about 3 µM (Fig. 5).

The combination of the effects of Li$^+$ and 2,3-bisphosphoglycerate on the Dictyostelium Ins(1,4,5)$_3$ 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 Mg$^{2+}$ Ins(1,4,5)$_3$ 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 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 Li$^+$. Ca$^{2+}$ apparently can only replace Mg$^{2+}$ in that part of the activity which is sensitive to 2,3-bisphosphoglycerate, so in 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-H]Ins(1,4,5)P$_3$ (5 nCi; 0.25 $\mu$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 InsP$_2$ 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-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-H]Ins(4,5)P$_2$ from [2-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-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 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).

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-H]Ins(1,4)P$_2$ (5 nCi; 0.125 $\mu$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-14C]Ins1P (6.3 nCi; 5.7 $\mu$M) was assayed as described for InsP$_2$ phosphatase in Fig. 6.
DISCUSSION

Our results show that Dictyostelium discoideum homogenates possess phosphatases which can rapidly dephosphorylate Ins(1,4,5)P₃. The two presumptive dephosphorylation pathways can be summarized by Scheme 1.

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)P₃ 5-phosphatase activity which is Mg⁺⁺-dependent, sensitive to 2,3-bisphosphoglycerate, insensitive to Li⁺ 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)P₂ by the Dictyostelium soluble cell fraction is also similar to the rat or bovine brain systems; Ins(1,4)P₂ 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)P₂ 1-phosphatase and the Ins4P phosphatases of both systems are sensitive to Li⁺, 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)P₃ 5-phosphatase activities (Delvaux et al., 1987a,b). The Dictyostelium 5-phosphatase enzyme differs from these systems in that Ca²⁺ can replace Mg²⁺. In platelets and erythrocytes Ca²⁺ cannot replace Mg²⁺, and Ca²⁺ inhibits the Mg²⁺-activated activity with high affinity ($K_i = 70 \mu M$) (Downes et al., 1982; Connolly et al., 1985). We have used Ca²⁺ as a tool to elucidate the dephosphorylation pathway of Ins(1,4,5)P₃ in Dictyostelium, but have no indication for its physiological importance.

The major difference between Ins(1,4,5)P₃ dephosphorylation in Dictyostelium and that of higher organisms is, however, that in Dictyostelium most of the Ins(1,4,5)P₃ phosphatase activity is due to a predominantly soluble 1-phosphatase, which is highly Li⁺-sensitive [10-fold more sensitive than Ins(1,4)P₂ and 1-Ins₁P 1-phosphatase activities]. This shows that, although many of the Dictyostelium Ins(1,4,5)P₃-dephosphorylating enzymes could be very similar to those of higher organisms, the metabolic pathway of Ins(1,4,5)P₃ dephosphorylation is very different, and a new Ins₅P₂ isomer, Ins(4,5)P₃, is formed.

It is now important to investigate which Ins₅P₂ 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)P₃ dephosphorylation for Li⁺ in vitro makes it interesting to see whether the dramatic effects that Li⁺ 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.

We thank Pim Janssens and Theo Konijn for stimulating discussions and for critically reading the manuscript. This work was supported in part by a grant from Duphar (The Netherlands) and under contract from the Ministère de la Politique Scientifique (Belgium) to C.E., and in part by a grant from the C. and C. Huygens Fund, which is subsidized by the Netherlands Organization for Scientific Research, to P.J.M.V.H.

REFERENCES

Eisenberg, F., Jr. (1967) J. Biol. Chem. 242, 1375–1382

Received 15 January 1988/12 April 1988; accepted 18 April 1988

---