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Nucleus-associated phosphorylation of Ins(1,4,5)P₃ to InsP₆ in Dictyostelium

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Although many cells contain large amounts of InsP₆, its metabolism and function is still largely unknown. In Dictyostelium lysates, the formation of InsP₆ by sequential phosphorylation of inositol via Ins(3,4,6)P₃ has been described [Stevens and Irvine (1990) Nature (London) 346, 580–583], the second messenger Ins(1,4,5)P₃ was excluded as a potential substrate or intermediate for InsP₆ formation. However, we observed that mutant cells labelled in vivo with [³H]inositol showed altered labelling of both [³H]Ins(1,4,5)P₃ and [³H]InsP₆. In this report we demonstrate that Ins(1,4,5)P₃ is converted into InsP₆ in vitro by nucleus-associated enzymes, in addition to the previously described stepwise phosphorylation of inositol to InsP₆ that occurs in the cytosol.

INTRODUCTION

The inositol cycle plays a central role in signal-transduction pathways in many organisms. The key enzyme phospholipase C, activated on receptor stimulation, cleaves PtdIns(4,5)P₂ producing two second messengers: diacylglycerol and Ins(1,4,5)P₃ [1]. Ins(1,4,5)P₃ is metabolized via extended phosphorylation and dephosphorylation reactions [2,3]. A broad spectrum of inositol phosphates has been characterized in many systems including amoebae [4], algae [5], plants [6] and a variety of cultured mammalian cells [7,8]. The function and metabolism of these inositol phosphate isomers is understood to some extent. The second messengers Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ are involved in Ca²⁺ regulation in many systems [9–11]. Ins(1,3,4,5,6)P₅ is involved in the regulation of the affinity of oxygen for avian haemoglobin [12], and InsP₆ is thought to serve as a phosphate buffer in plants [13]. Ins(1,3,4,5,6)P₆ and InsP₆ may also serve as neurotransmitters [14]. Recently an Ins₆-phosphatase protein has been isolated and recognized as the AP2 clathrin-assembly protein [15].

The recent characterization of a new class of inositol phosphatases, the inositol polyphosphate tyrophosphatases, shows that InsP₆ is not a metabolic end point [16]. Evidence for the existence of InsP₆ and InsP₆ arose from the detection of [³H]-labelled compounds more polar than InsP₆, which were formed in Dictyostelium cells labelled with [³H]inositol in vivo [17]. After structural analysis, these compounds were identified as D/L-1-diphosphoinositol pentakisphosphate and D/L-1-bis-(1,4)-diphosphoinositol tetrakisphosphate [18]. These compounds (also detected in mammalian cell types [18–20]) contain high-energy phosphates and have a high metabolic turnover. This feature might allow them to play a role in energy metabolism or regulation of cellular processes by substrate phosphorylation.

Although in Dictyostelium InsP₆ is present at high concentration (about 0.6 mM [21]), no function has been attributed to it yet. A route for InsP₆ formation via stepwise phosphorylation of myo-inositol in Dictyostelium has been described by Stephens and Irvine [22]. They excluded Ins(1,4,5)P₃ as a direct precursor in the InsP₆ synthesis. We have also reported experiments suggesting the absence of Ins(1,4,5)P₃ kinase activity in Dictyostelium lysates [23]. However, when Dictyostelium cells expressing an oncogenic ras gene are labelled with [³H]inositol, they show increased conversion of [³H]PtdIns into [³H]PtdInsP₄ and as a consequence elevated levels of [³H]PtdIns(4,5)P₂ and [³H]Ins(1,4,5)P₃ [24]. The observations that the levels of [³H]InsP₆ were also increased [24,25] suggest a link between [³H]InsP₆ formation and [³H]Ins(1,4,5)P₃ levels. In this study we have further investigated the formation of InsP₆ in Dictyostelium cells, and indeed observed that Ins(1,4,5)P₃ can be phosphorylated to InsP₆ in vitro. The intermediates were identified as Ins(1,3,4,5)P₄ and Ins(1,3,4,5,6)P₅. This enzymatic conversion was observed in a preparation of broken nuclei, whereas the formation of InsP₆ from inositol was detected only in the soluble fraction of a cell lysate.

We conclude that Dictyostelium cells have two metabolic routes to InsP₆: a cytosolic route in which inositol is phosphorylated stepwise as described by Stephens and Irvine [22], and a nucleus-associated route involving PtdIns turnover.

MATERIALS AND METHODS

Materials

Alkakine phosphatase (grade II; calf intestine), hexokinase from yeast [(NH₄)₂SO₄ suspension] and NAD⁺ were from Boehringer-Mannheim. Dialysis tubing with a 12–14 kDa molecular-mass cut-off was obtained from Visking. The Zorbax SAX column was purchased from Chrompack. Polycarbonate filters of 3 µm pore size were from Nucleopore. [¹⁴C]InsP₃, [³H]Ins(1,4,5)P₃, [³H]inositol (20 Ci/mmol), Ins(4-³²P)(1,4,5)P₃ (200 Ci/mmol), Ins(1,4,5)[5-³²P]P₃ (200 Ci/mmol), and [γ-³²P]ATP (3000 Ci/mmol) were from Amersham. [³H]InsP₆, [³H]InsP₄, [³H]Ins(1,4)P₂, [³H]Ins(1,3,4,5)P₄ and [³H]InsP₆ (23 Ci/mmol) were from NEN-Dupont. [³H]Ins(1,3,4,6)P₄ and purified Ins(1,4,5)P₃ 3-kinase were gifts from C. Erneux (IRIBHN, University Libre de Bruxelles); [³H]Ins(3,4,5,6)P₄ and [³H]Ins(1,3,4,5,6)P₅ were kindly provided by B. Hoiting (University of Groningen).

* To whom correspondence should be addressed.
Preparation of \([^{1}H]\text{Ins}(4,5)P_2\), \([^{5-32P}]\text{(1,3,4,5)P}_4\) and \([^{3-32P}]\text{(1,3,4,5)P}_4\)

\([^{1}H]\text{Ins}(4,5)P_2\) was prepared by incubation of \([^{1}H]\text{Ins}(1,4,5)P_2\) with a partially purified Dictyostelium \text{Ins}(1,4,5)P_2 1-phosphatase in the presence of 0.25 mM 2,3-diphosphoglyceric acid and 5 mM MgCl_2 as described [26].

\([^{3-32P}]\text{(1,3,4,5)P}_4\) was prepared by phosphorylation of \(\text{Ins}(1,4,5)P_2\) with a purified \(\text{Ins}(1,4,5)P_2\) 3-kinase in the presence of \([\gamma-^{32P}]\text{ATP}\) [27]. \([^{5-32P}]\text{(1,3,4,5)P}_4\) was prepared by phosphorylation of \([^{5-32P}]\text{(1,4,5)P}_2\) with the 3-kinase in the presence of ATP [27]. Incubations with the \(\text{Ins}(1,4,5)P_2\) 3-kinase were in 25 \(\mu\)l at 37°C for 1 h and the mixtures contained: 12.5 mM MgCl_2, 1 mM EGTA, 1.1 mM CaCl_2, 6.25 \(\mu\)M ATP, 6.25 \(\mu\)M \(\text{Ins}(1,4,5)P_2\) and 50 mM Hepes, pH 7.5 (final concentrations). To obtain \([^{3-32P}]\text{(1,3,4,5)P}_4\) and \([^{5-32P}]\text{(1,3,4,5)P}_4\), 1.5 \(\mu\)Ci of \([\gamma-^{32P}]\text{ATP}\) or 0.125 \(\mu\)Ci of \([^{5-32P}]\text{(1,4,5)P}_2\) was added respectively. Reactions were terminated by boiling for 2 min. The \(\text{Ins}_2P_2\) produced was isolated by HPLC on a Zorbax SAX column eluted with gradient A (see below). Salt was removed by dialysis for 3 \(\times\) 2 h against 500 vol. of 10 mM Hepes, pH 7.1 [28].

Preparation of cytosolic and nuclear extracts of Dictyostelium

Wild-type AX3 cells were grown in modified HL5 medium containing 10 g/l d-glucose as described [29] and starved in 10 mM sodium/potassium phosphate buffer for 2 h at 10°C cells/ml. Cells were harvested and washed once in 40 mM Hepes/0.5 mM EDTA, pH 6.5. All subsequent steps were performed at 4°C. Cells were lysed through polycarbonate filters of 3 \(\mu\)m pore size and the lysate was centrifuged for 2 min at 1500 \(g\). The pellet, which contained the nuclei, was washed twice with washing buffer and checked for the absence of unlysed cells. The pellet was resuspended and after a second passage through a double 3 \(\mu\)m polycarbonate filter, the extract was centrifuged for 5 min at 10000 \(g\) and the supernatant for 35 min at 100000 \(g\). The resulting high-speed supernatant is called nuclear extract. The 1500 \(g\) supernatant of the original lysate was centrifuged for 35 min at 100000 \(g\); the supernatant is called cytosolic extract. The pellet obtained in this last centrifugation was resuspended and is the microsomal fraction.

\(\text{Ins}_2P_2\) formation in vitro

Assay mixtures (100 \(\mu\)l) contained 50 \(\mu\)l of enzyme preparation (5 \(\times\) 10^6 cell equivalents), labelled substrates as indicated in the Figure legends, 20 mM MgCl_2, 10 mM ATP, 1 mM EGTA, 1 mM CaCl_2, 10 mM LiCl, 0.25 mM 2,3-diphosphoglyceric acid and 50 mM Tris/HCl, pH 8.0. The mixtures were incubated at room temperature and the reactions were quenched by boiling for 2 min; the mixtures were analysed by HPLC analysis using gradient A (see under ‘HPLC analysis’).

In vivo kinetics of \([^{32P}]\text{P}\), incorporation into ATP and \(\text{Ins}_2P_2\)

Dictyostelium cells were harvested and starved for 2 h in 20 mM Hepes, pH 6.5 (HB buffer), at 10^6 cells/ml. Cells were again harvested and resuspended in HB buffer at a density of 2 x 10^6 cells/ml with 1 ml of HB buffer, resuspended in 100 \(\mu\)l of HB and lysed by addition of 100 \(\mu\)l of 3.5% HClO_4 containing 10 mM EDTA and 10 mM EGTA. The period between the first centrifugation of the labelled cells and the addition of HClO_4 was about 9 min. Subsequently, 10 \(\mu\)l of 2 M acetic acid and 70 \(\mu\)l of 1.12 M KHCO_3 were added. After centrifugation for 1 min at 14000 \(g\), the supernatant was removed and applied to a Zorbax anion-exchange HPLC column which was eluted with gradient B (see under ‘HPLC analysis’). The Čerenkov radiation of the fractions was determined and those that contained ATP were pooled; salts were removed by dialysis for 2 x 90 min against 500 vol. of 10 mM Hepes, pH 7.1.

The radioactivity at the \(\gamma\)-position of \([^{32P}]\text{ATP}\) was determined by converting glucose and \([^{32P}]\text{ATP}\) into \([^{32P}]\text{Glucose 6-phosphate}\) and \([^{32P}]\text{ADP}\) with hexokinase. The \([^{32P}]\text{Glucose 6-phosphate}\) produced was quantified by HPLC analysis using a Zorbax column eluted with gradient C (see under ‘HPLC analysis’). Reaction mixtures contained 20 \(\mu\)l of hexokinase (100 units/ml) in 100 \(\mu\)l of 50 mM Hepes, pH 7.5, containing 0.2 mM glucose and 2 mM MgCl_2. Incubations were at room temperature for 35 min and terminated by the addition of 0.5 ml of 10 mM EDTA and 2 min boiling.

Determination of positional specific radioactivities of \([^{32P}]\text{P}\) isolated from Dictyostelium cells labelled with \([^{32P}]\text{P}\)

Cells were labelled with \([^{32P}]\text{P}\), extracted with HClO_4, neutralized with KHCO_3 as described above and subsequently extracted with charcoal to remove nucleotides: 20 \(\mu\)l of a suspension of charcoal [20% (w/v) in 0.1 M NaCl] was added to the supernatants which were incubated for 15 min on ice. Samples were centrifuged for 1 min at 14000 \(g\) and the supernatants were extracted once with charcoal. The extracted supernatants were mixed with \([^{32P}]\text{Ins}_{2}P_{2}\), dialysed overnight against 3 x 500 vol. of 10 mM Hepes, pH 7.1, to remove excess \(^{32}P\), and applied to a Zorbax HPLC column eluted with gradient B (see under ‘HPLC analysis’). The fractions containing \(\text{Ins}_{2}P_{2}\) were pooled and dialysed overnight against 3 x 500 vol. of 10 mM Hepes, pH 7.1, to remove the ammonium phosphate.

This purified \([^{32P}]\text{Ins}_{2}P_{2}\) mixture was dephosphorylated stepwise at the 6-, 5- and 4-positions using 20 \(\mu\)l of Paramecium phytase for 

\(t=0\), 10 and 120 min in a total volume of 100 \(\mu\)l containing 50 mM Tris/HCl, pH 7.0, about 5000 d.p.m. \([^{32P}]\text{P}\), and 5000 d.p.m. \([^{32P}]\text{P}\) as described in the preceding paper [30]. The samples were analysed using a Zorbax HPLC column eluted with gradient B (see under ‘HPLC analysis’). Fractions of 20 \(\mu\)l were collected and 4 ml of emulsifier 299 was added. Radioactivity was determined with a dual-label counting program and used a quench-correction curve.

HPLC analysis

The Zorbax HPLC column was eluted with gradients consisting of water in pump A and 1.2 M ammonium phosphate, pH 3.7, in pump B at a flow rate of 1.5 ml/min; fractions of 20 \(\mu\)l were collected. Linear gradients were generated between the following break points: gradient A: 0 min 0% B; 5 min 37% B; 20 min 45% B; 25 min 100% B; 30 min 100% B; 31 min 0% B; 40 min 0% B; gradient B: 0 min 0% B; 1 min 5% B; 4 min 10% B; 20 min 100% B; 24 min 100% B; 25 min 0% B; 35 min 0% B; gradient C: 0 min 0% B; 1 min 30% B; 7 min 75% B; 8 min 100% B; 12 min 100% B; 13 min 0% B; 20 min 0% B.

RESULTS

\(\text{Ins}_2P_2\) formation in vitro from Insitol and \(\text{Ins}(1,4,5)P_2\) in the cytosol and nucleus-associated fraction

Dictyostelium cells were lysed by passage through a Nucleapore filter of pore size 3 \(\mu\)m (which is smaller than cells but larger than the nucleus). The lysate was centrifuged at low speed to
Table 1 Subcellular localization of InsP₄ formation from inositol and Ins(1,4,5)P₃

The data (means ± S.D.) are from three to five independent experiments. < 0.1 indicates below the limit of detection.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[3H]inositol</th>
<th>[3H]Ins(1,4,5)P₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>10.8±2.2</td>
<td>1.36±2.71*</td>
</tr>
<tr>
<td>Microsomes</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Nuclei</td>
<td>&lt; 0.1</td>
<td>12.8±1.2</td>
</tr>
<tr>
<td>Broken nuclei</td>
<td>&lt; 0.1</td>
<td></td>
</tr>
</tbody>
</table>

* In three out of five experiments no phosphorylation of Ins(1,4,5)P₃ was detected; in two experiments 0.62% and 6.2% of Ins(1,4,5)P₃ respectively was converted into InsP₄; we assume that in these experiments a small portion of the nuclei were broken.

Figure 1 Combined HPLC separation after in vitro phosphorylation of [3H]ins in a cytosolic fraction and Ins[32P](1,4,5)P₃ in a nuclear extract

A Dicystostelium cytosolic fraction and a nuclear extract were incubated with [3H]inositol and Ins[32P](1,4,5)P₃ respectively. The combined reaction products were separated by HPLC: ○, [3H]-labelled compounds, ●, 32P-labelled compounds. The elution of standard compounds is indicated.

remove the nuclei; high-speed centrifugation provided a soluble cytosolic fraction and a microsomal fraction. These subcellular fractions were incubated with ATP and [3H]inositol or [3H]Ins(1,4,5)P₃ and formation of [3H]InsP₄ was analysed by HPLC (Table 1). Using the cytosol fraction, [3H]inositol was successfully phosphorylated to [3H]InsP₄, but reproducible phosphorylation of [3H]Ins(1,4,5)P₃ to [3H]InsP₄ was not observed (Table 1). In the microsomal and nuclear fractions neither [3H]inositol nor [3H]Ins(1,4,5)P₃ was phosphorylated to [3H]InsP₄. However, significant and reproducible conversion of [3H]Ins(1,4,5)P₃ into [3H]InsP₄ was detected after the nuclei were broken by a second passage through a double 3 μm Nuclepore filter (Table 1); on centrifugation of these broken nuclei, enzyme activity was detected in the soluble extract of the nuclei (results not shown). In contrast, [3H]inositol was not converted into [3H]InsP₄ using this nuclear extract.

In conclusion, phosphorylation of Ins(1,4,5)P₃ to InsP₄ is mediated by soluble nucleus-associated enzyme(s), whereas the enzyme(s) involved in conversion of inositol into InsP₄ are cytosolic.

Routes of InsP₄ formation in vitro

Figure 1 shows a combined HPLC profile of in vitro phosphorylation of [3H]inositol in the cytosol and [3H]Ins(1,4,5)P₃ in the nuclear extract. The [3H]InsP₄ isomer and the [3H]Ins(1,4,5)P₃ isomer derived from [3H]inositol did not co-migrate with standard Ins(1,4,5)P₃ and Ins(1,3,4,5,6)P₅ respectively. The [3H]InsP₄ isomer co-migrated with Ins(1,3,4,5,6)P₅. To establish further the routes of InsP₄ formation in the cytosol, several inositol phosphates were tested as possible precursors of InsP₄ (Table 2). Ins3P is the only inositol monophosphate that is converted in the cytosol into InsP₄. In the cytosolic fraction the inositol polyphosphates tested are poor precursors of InsP₄ formation, including Ins(1,4,5)P₃ and Ins(1,3,4,5,6)P₅. Stephens and Irvine [22] have identified the intermediates of the sequential phosphorylation in the cytosolic fraction of [3H]inositol to InsP₄ as Ins3P, Ins(3,6)P₂, Ins(3,4,6)P₃, Ins(1,3,4,6)P₄, and Ins(1,3,4,5,6)P₅. The elution profile of the [3H]inositol phosphate intermediates (Figure 1) and the substrate specificity for the formation of InsP₄ in the cytosol (Table 2) are fully consistent with this route of InsP₄ formation.

The phosphorylation of Ins(1,4,5)P₃ in the nuclear extract was investigated in more detail. Assuming that during InsP₄ formation no dephosphorylation occurs (for which proof will be given below), only three InsP₄ isomers can be formed from Ins(1,4,5)P₃, namely Ins(1,2,4,5)P₄, Ins(1,3,4,5)P₄ and Ins(1,3,4,5,6)P₅. The Ins[32P]P₄ isomer that was formed from Ins[32P](1,4,5)P₃ in the nuclear extract co-migrated with [3H]Ins(1,3,4,5)P₄ and not with [3H]Ins(1,4,5,6)P₅ (Figure 1). When [3H]Ins(1,4,5)P₃ was incubated with ATP and Dicystostelium enzymes for a prolonged period, a second InsP₄ isomer was formed besides Ins(1,3,4,5)P₄. This product was neither Ins(1,3,4,6)P₄ nor Ins(3,4,5,6)P₅ [or Ins(1,4,5,6)P₅] which have different retention times in our chromatographic system. After a relatively short incubation
period (1 h), considerable amounts of Ins(1,3,4,5)P$_2$ and low levels of InsP$_6$ were formed, whereas the unknown InsP$_6$ product was not detectable. On prolonged incubation (more than 4 h) a decrease in Ins(1,3,4,5)P$_2$ was accompanied by an increase in InsP$_6$ and the appearance of the unidentified product. This InsP$_6$ isomer was assumed to be a degradation product of InsP$_6$ and not further identified. As an authentic standard of Ins(1,4,5)P$_3$ is not available, the possible co-migration with this isomer cannot be examined. If Ins(1,2,4,5,6)P$_5$ were the InsP$_6$ intermediate, the InsP$_6$ intermediate would have to have a phosphate at the 2-position, i.e. it would have to be either Ins(1,2,3,4,5)P$_5$ or Ins(1,2,4,5,6)P$_5$. Since the InsP$_6$ isomer co-migrated with Ins(1,3,4,5,6)P$_2$ and not Ins(1,2,3,4,5)P$_2$ or Ins(1,2,4,5,6)P$_2$, the InsP$_6$ intermediate cannot have been Ins(1,2,4,5)P$_2$ and is therefore identified as Ins(1,3,4,5)P$_2$. This isomer can give rise to only two InsP$_6$ isomers, Ins(1,3,4,5,6)P$_2$ and Ins(1,3,4,5,6)P$_2$. Co-migration of the InsP$_6$ isomer produced with Ins(1,3,4,5,6)P$_2$ and not with Ins(1,2,3,4,5)P$_2$ identifies it as Ins(1,3,4,5,6)P$_2$. Thus, in the nuclear extract, Ins(1,4,5)P$_3$ is phosphorylated via Ins(1,3,4,5)P$_2$ and Ins(1,3,4,5,6)P$_2$ to InsP$_6$. This deduction is based on the assumption that no dephosphorylation steps have to be taken into account.

Evidence for direct phosphorylation of Ins(1,4,5)P$_2$ and Ins(1,3,4,5)P$_2$ (i.e. no dephosphorylation reactions) is provided by six series of experiments. (i) [H]$^32$P[Ins(1,4,5)P$_2$] in the presence of 1 mM inositol was converted to the same extent into [H]InsP$_6$ as in the absence of inositol; conversion of [H]inositol into InsP$_6$ in the cytosol was completely inhibited by 1 mM inositol (results not shown). This experiment indicates that degradation of Ins(1,4,5)P$_2$ to inositol does not precede InsP$_6$ formation. (ii) Degradation of Ins(1,4,5)P$_2$ to InsP$_4$, Ins4P or Ins(1,4)P$_2$ followed by phosphorylation to InsP$_6$ was excluded, as these isomers are not substrates for InsP$_6$ formation (Table 2). (iii) Ins[H]Ins(1,4,5)P$_2$ and Ins[H]Ins(4,5)P$_2$ were converted into InsP$_6$ at the same rate as the [H]Ins(1,4,5)P$_2$ internal control (Table 2), indicating that the phosphates at the 4- and 5-positions of Ins(1,4,5)P$_2$ are retained during the phosphorylation reactions to InsP$_6$. (iv) Although Ins(4,5)P$_2$ shows some conversion into InsP$_6$, degradation of Ins(1,4,5)P$_2$ to this isomer with subsequent phosphorylation is a not a likely route, because the simultaneous incubation of [H]Ins(1,4,5)P$_2$ and [H]Ins(4,5)P$_2$ revealed that the latter was not as efficiently phosphorylated to InsP$_6$ as the former (results not shown). Thus InsP$_6$ production from Ins(1,4,5)P$_2$ occurred with retention of the phosphates at the 1-, 4- and 5-positions. (v) Similar experiments were performed for Ins(1,3,4,5)P$_2$ by using Ins(3,4,P)[1,3,4,5]P$_2$ and Ins(3,4,P)[1,3,4,5]P$_2$, which were converted into InsP$_6$ at about the same rate as 1$^H$-labelled Ins(1,3,4,5)P$_2$ (Table 2), indicating that the phosphates at positions 3 and 5 were retained. (vi) The InsP$_6$ isomer detected after phosphorylation of Ins(1,3,4,5)P$_2$ in vivo was co-eluted with Ins(1,3,4,5,6)P$_2$ and not with Ins(1,2,3,4,5)P$_2$ or Ins(2,3,4,5,6)P$_2$. These combined data reveal that all phosphates of Ins(1,4,5)P$_2$ and Ins(1,4,5)P$_2$ are retained during phosphorylation via Ins(1,3,4,5,6)P$_2$ to InsP$_6$.

Summary, in addition to the stepwise phosphorylation of inositol to InsP$_6$ by cytosolic enzymes as described by Stephens and Irvine [22]. Dictyostelium possesses nucleus-associated enzymes that convert Ins(1,4,5)P$_2$ to InsP$_6$ via Ins(1,3,4,5)P$_2$ and Ins(1,3,4,5,6)P$_2$; interestingly, the InsP$_6$ isomers of the two routes are identical.

Figure 2 In vivo [H]$^3$P]P$_6$ formation after labelling of cells with [H]$^3$P]P$_6$; (a) kinetics of labelling [H]$^3$P]ATP and InsP$_6$; (b) distribution of label over three positions

(a) Dictyostelium cells were incubated with [H]$^3$P]P$_6$ for the times indicated. Cells were lysed and the water-soluble extract was analysed by HPLC. The fractions containing ATP were isolated to determine the fraction of label at the $\gamma$-position. The data shown are presented as c.p.m./mmol using a concentration of 2.6 mM P$_6$, 1 mM ATP (C) or 0.6 mM InsP$_6$ (C) (for InsP$_6$ the data were divided by 5 to account for the six phosphates). The data for InsP$_6$ are also presented as the square root of specific activity versus time (A), to calculate the rate constant of phosphate turnover at short incubation times. Two quantitative approaches were used to calculate the mean phosphate turnover time of InsP$_6$. First, between 60 and 120 min after the onset of labelling, the $\gamma$-position of ATP has reached an equilibrium value of 38190 c.p.m./mmol. During this period the incorporation of label into InsP$_6$ is approximated to d[InsP$_6$]/dt = a(3P)]. The observed labelling of each position of InsP$_6$ has a mean rate of 1 c.p.m./mmol per min, yielding a rate constant of a = 0.0047 min$^{-1}$ (t$_{1/2}$ = 146 min). Pre-steady state data are used for the second calculation of phosphate incorporation into InsP$_6$. During the first 30 min of the labelling period the incorporation of label at the $\gamma$-position of ATP is approximately linear with time showing a rate of 619 c.p.m./mmol per min (i.e. [ATP] = 619). During this phase the rate of InsP$_6$ phosphorylation is given by d[InsP$_6$]/dt = a(3P)]. Integration yields InsP$_6$ = 691/2 a$^2$. Presentation of the radioactivity in InsP$_6$ as the square root of the specific activity versus time provides a linear line with a slope of 1.2 c.p.m./min$^{-1}$ per min, indicating a rate constant of InsP$_6$ phosphorylation equal to a = 0.0046 min$^{-1}$ (t$_{1/2}$ = 149 min). Linear regression analysis: ATP formation (up to 60 min) yields intercept abscissa = 0.1 min, slope = 619 c.p.m./mmol per min, r = 0.998; InsP$_6$ formation (30 to 120 min) yields intercept abscissa = 21.9 min, slope = 181 c.p.m./mmol per min, r = 0.998; square root of InsP$_6$ formation (up to 60 min) yields intercept abscissa = -6.7 min, slope = 1.2 c.p.m./mmol$^{-1/2}$ per min, r = 0.993. (b) Cells were labelled with [H]$^3$P]P$_6$ for 15 min, lysed and the water-soluble fraction was extracted with charcoal to remove nucleotides. [H]$^3$P]InsP$_6$ was added and [H]$^3$P]InsP$_6$ was isolated by HPLC. This mixture was incubated with Phenomenex phytase as described in Figure 3. The fraction of [H]$^3$P] radioactivity at positions 6, 5 and 4 was calculated. The data shown are means ± S.D. of three (A) or five (B) determinations.
In the subsequent experiments we investigated the possible route(s) of Insp$_6$ formation in vivo. Cells were labelled with $[^{32}P]P_6$, which is incorporated into the $\gamma$-position of ATP and subsequently into Insp$_6$. The kinetics of labelling of specific positions of Insp$_6$ with $^{32}P$ may be used to address specific questions on how Insp$_6$ is formed in vivo.

**Kinetics of formation of Insp$[^{32}P]_6$ in vivo on labelling with $[^{32}P]P_6$.**

Stephens and Irvine [22] have observed that in vitro Insp$_6$ is rapidly dephosphorylated and rephosphorylated at the 3- and 5-positions. They calculated a phosphate turnover time at these positions of less than 1 min if these futile cycles are present in vivo. On the other hand, labelling of cells with $[^{3}H]$inositol suggests a turnover time of total Insp$_6$ of the order of several hours [23,31]. Thus the difference between de novo Insp$_6$ synthesis (hours) and futile dephosphorylation/phosphorylation cycles (minutes, when present) should be easily detectable by analysing the initial rate of incorporation of $[^{32}P]P_6$ via $[^{\gamma-32}P]ATP$ into Insp$_6$.

Cells were labelled with $[^{32}P]P_6$, for different periods, washed and lysed. The uptake of $^{32}P$ by the cells and its subsequent incorporation into ATP and Insp$_6$ was determined by HPLC analysis of the extract. ATP was isolated to determine the fraction of radioactivity at the $\gamma$-position, which is the presumed phosphate donor of Insp$_6$ (this fraction was 37.7 ± 2.6 % of the total radioactivity in ATP at all labelling times). Dictyostelium cells take up $[^{32}P]P_6$, relatively slowly with a half-time to equilibrium of about 30 min (Figure 2a). The rate of $^{32}P$ incorporation at the $\gamma$-position of ATP follows the same kinetics. Together with the observation that 38 % of the label in ATP is at the $\gamma$-position, irrespective of the labelling period, this indicates that intracellular P$_\gamma$ is in rapid equilibrium with ATP. This notion is consistent with the reported turnover time of ATP of only a few seconds in Dictyostelium [32].

In contrast with the rapid equilibrium between P$_\gamma$ and ATP, the labelling of Insp$_6$ shows a substantial lag phase. At 15 min after the onset of labelling, ATP contains 10157 ± 348 c.p.m. at the $\gamma$-position, whereas Insp$_6$ contains only 1221 ± 71 c.p.m. The concentrations of ATP and Insp$_6$ have been determined by several methods, yielding a specific radioactivity of 10157 c.p.m./mmol for $[^{\gamma-32}P]ATP$ and 2035 c.p.m./mmol for Insp$_6$. (The specific radioactivities of P$_\gamma$, ATP and Insp$_6$ were calculated using concentrations of 2.6, 1 and 0.6 mM respectively, as determined simultaneously by NMR [21]; other methods yield 0.5 mM Insp$_6$ (metal dye detection [4]), and 0.9 mM ATP (enzyme assay [32]).) Even if all $^{32}P$ label in Insp$_6$ is located on only one position, the specific radioactivity of that position is still much lower than that in $[^{\gamma-32}P]ATP$, indicating that no position in Insp$_6$ can be in rapid equilibrium with ATP. We conclude that there is no evidence for futile dephosphorylation/phosphorylation cycles in vivo on a minute time scale.

To obtain an estimate of the rate of phosphate incorporation into Insp$_6$, the labelling of Insp$_6$ is presented as the specific radioactivity averaged over the six phosphate positions (Figure 2a). After 60 min ATP labelling has reached equilibrium, whereas incorporation of label into Insp$_6$ is still increasing. After 120 min of labelling, the mean specific radioactivity of Insp$_6$ is about 40 % of the specific radioactivity at the $\gamma$-position of ATP, suggesting a phosphate turnover time in Insp$_6$ of more than 1 h. At 4 and 6 h of labelling the specific radioactivity of Insp$_6$ and $[^{\gamma-32}P]ATP$ have similar values, indicating that equilibrium is reached (results not shown). Quantitative analysis of the data (see the legend to Figure 2) reveals that $^{32}P$-labelled Insp$_6$ is formed with a half-time of about 2.4 h.

**Route of Insp$[^{32}P]_6$ formation in vivo on labelling cells with $[^{32}P]P_6$.**

When cells are labelled with $[^{32}P]P_6$, for a very short period, the six phosphates of Insp$[^{32}P]_6$ do not have an equal amount of radioactivity: positions that are phosphorylated at the end of the pathway will have a higher $^{32}P$ content than positions that are phosphorylated at the beginning. Paramecium phytase dephosphorylates Insp$_6$ in a specific order at the 6-, 5- and 4-positions, which allows this enzyme to be used to determine the distribution of $^{32}P$ over these positions [30,33]. For Insp$_6$ formation from inositol these three positions are phosphorylated in the sequence 6, 4, 5, whereas the sequence is 4, 5, 6 for Insp$_6$ formation via Insp$_4(1,4,5)P_3$. Thus, after a very brief labelling period of cells with $[^{32}P]P_6$, the $^{32}P$ content at the 5- and 4-positions will be higher than at the 6-position when Insp$_6$ is derived from sequential phosphorylation of inositol, but lower than at the 6-position when Insp$_6$ is formed via Insp$_4(1,4,5)P_3$.

Cells were labelled for 15 min with $[^{32}P]P_6$, and quenched with HClO$_4$. Authentic $[^{3}H]$Insp$_5$ was added to the extract and the $[^{3}H]$Insp$[^{32}P]P_6$ mixture was isolated by HPLC. The mixture was incubated with Paramecium phytase, and the reaction products were separated by HPLC (Figure 3). The $^{32}P$/H ratios of the inositol phosphates were calculated and divided by that of Insp$_6$. The decrease in this ratio from 1.00 in Insp$_6$ to 0.73 ± 0.03 in Insp$_5$ represents the fraction of $^{32}P$ label at the 6-position (Figure 2b). Thus the $[^{32}P]$phosphate content of position 6 was 27 ± 3 % of the total of Insp$[^{32}P]P_6$ after 15 min of labelling. In contrast, both positions 5 and 4 contained about only 16 ± 5 % of the total $[^{32}P]$phosphate content of Insp$_6$. The short labelling period shows...
that position 6 is labelled to a larger extent than positions 4 and 5, indicating that position 6 is labelled after positions 4 and 5.

**DISCUSSION**

Inositol polyphosphates with more than four phosphates attain very high concentrations in some cells. In *Dictyostelium* a concentration of 0.6 mM InsP6 has been reported. This compound could be used to store phosphate, but may also be a source of other inositol phosphates. The metabolism of these higher inositol phosphates is only partly understood. In *Dictyostelium*, InsP6 can be formed by sequential phosphorylation of inositol [22]. We have investigated the formation of InsP6 in some detail, because we observed that [3H]inositol-labelled mutants with altered formation of [3H]Ins(1,4,5)P3 also showed altered labelling of [3H]InsP6, suggesting that at least part of [3H]InsP6 is formed from [3H]Ins(1,4,5)P3 [24,25]. The present results indeed show that *Dictyostelium* cells contain a newly identified pathway, which involves the phosphorylation of Ins(1,4,5)P3 via Ins(1,3,4,5)P2 and Ins(1,3,4,5,6)P5 to InsP6; this route is only detected in nuclear extracts and not in the cytosol, microsomes or intact nuclei.

The pathway of Ins(1,4,5)P3 metabolism to InsP6 was unravelled by identifying the intermediates. The observation that all the phosphates of Ins(1,4,5)P3 were retained in InsP6 indicated that Ins(1,4,5)P3 was not (even partly) degraded before it was phosphorylated. The InsP6 isomer produced co-migrated with Ins(1,3,4,5,6)P5 and not with any of the other five InsP6 isomers. Only three InsP6 isomers can be formed from Ins(1,4,5)P3: Ins(1,2,4,5)P4, Ins(1,3,4,5)P5 and Ins(1,3,4,5,6)P6. The observed product co-migrated with Ins(1,3,4,5,6)P6 and not with Ins(1,4,5)P3. Since the InsP6 produced does not contain phosphate at the 2-position, Ins(1,2,4,5)P4 cannot be an intermediate. Thus InsP6 formation from Ins(1,4,5)P3 proceeds via Ins(1,3,4,5)P3 and Ins(1,3,4,5,6)P5. In the green alga *Chlamydomonas* and in turkey erythrocytes, similar enzyme activities have been observed phosphorylating Ins(1,5)P3 via Ins(1,3,4,5)P3 to Ins(1,3,4,5,6)P5; however, in these systems no InsP6 formation was detected [5].

**Scheme 1**  
**Inositol phosphate metabolism in Dictyostelium**

The closed arrows refer to phospholipid turnover and degradation of Ins(1,4,5)P3; this part is present in both cytosolic and nucleus-associated compartments. The open arrows refer to phosphorylation of Ins(1,4,5)P3 to InsP6 in the nucleus. The hatched arrows demonstrate the phosphorylation of inositol to InsP6 in the cytosol, and the broken arrows indicate the two futile InsP6/InsP5 cycles.

Stephens and Irvine [22] characterized three InsP6 isomers after labelling of *Dictyostelium* cells *in vivo* with [3H]inositol: Ins(1,3,4,5,6)P6, Ins(1,2,3,4,6)P6 and Ins(1,2,4,5,6)P6. The reported half-times of conversion into InsP6 were 25, 6.4 and 0.8 s respectively. The Ins(1,3,4,5,6)P6 isomer was shown to be the precursor of InsP6 *in vivo*, and the other two isomers were degradation products of InsP6 and were rephosphorylated to InsP5 in futile cycles.

The three different routes of InsP6 formation in *Dictyostelium* are summarized in Scheme 1. The first route is the sequential phosphorylation of inositol in the cytosol. In this pathway inositol is incorporated into InsP6 at approximately the same rate as the six phosphates. The second route comprises the futile dephosphorylation/phosphorylation cycles at the 3- and 5-positions of InsP6. In this route, phosphates are rapidly exchanged at these positions, whereas the inositol moiety is not renewed. The third route has been identified in this study as the nucleus-associated conversion of Ins(1,4,5)P3 into InsP6. This involves PtdIns turnover; therefore inositol and the six phosphates of InsP6 are combined via different metabolic pathways, perhaps in different compartments. Inositol and the phosphate at the 1-position are derived from the condensation of CDP-diacylglycerol with inositol to give PtdIns; we have not investigated the presence of this reaction in the nucleus. The phosphates at the 4- and 5-positions are produced by phospholipid kinases and have a relatively high turnover. Phospholipid kinases have been detected in the nucleus of *Dictyostelium* (J. Van der Kaay, H. Simpa, A. A. Bommena and P. J. M. Van Haastert, unpublished work). Finally, the phosphates at the 2-, 3- and 6-positions are derived from inositol phosphate kinase(s) present in the nucleus.

Previously we [23] and others [22] were unable to detect Ins(1,4,5)P3 kinase activity *in vitro* or *in vivo*. The present data reveal that this enzyme activity is detected only in broken nuclei. Incubation of nuclei with a mixture of [3H]H2O and InsP[32P](1,4,5)P3 revealed that the 32P label was excluded from the nuclei relative to the 3H label, indicating that the nucleus was closed to Ins(1,4,5)P3 (J. Van der Kaay, H. Simpa, A. A. Bommena and P. J. M. Van Haastert, unpublished work). In retrospect, in previous experiments either the nuclei were lost in the preparation of high-speed supernatants or they remained intact when a complete lyase was used.

Once several routes for the formation of InsP6 had been detected in *vitro*, experiments were designed to obtain an indication of the importance of each route in *in vivo*. The kinetics of [33P]P, incorporation into ATP and InsP6 demonstrated that phosphate in no position in InsP6 was in rapid (minutes) equilibrium with ATP; the phosphates of InsP6 were exchanged with an average half-time of about 2.5 h. Thus no evidence was obtained for futile dephosphorylation/phosphorylation cycles with a phosphate turnover of a few minutes. This observation suggests that the proposed futile cycles at positions 3 and 5 of InsP6, whenever present in *in vivo*, involve only a small portion of the InsP6 pool. In a second experiment, *Paramecium* phytase, which dephosphorylates InsP5 in a strict sequence, was used to determine the distribution of 32P over the different positions of InsP6. The radioactivity in InsP[32P]P6 isolated from cells after a brief labelling period with [33P]P was distributed over the 6-, 5- and 4-positions as 27±3, 16±5 and 16±5 % of the total radioactivity respectively. These data imply that the 6-position of InsP6 is labelled after the 4- and 5-positions. This sequence of phosphorylation reactions is compatible with the route of InsP6 formation from Ins(1,4,5)P3, but not with the route from inositol or the futile cycle at the 5-position (Scheme 1). These conclusions on the formation of InsP6 *in vivo* after labelling with phosphate...
are supported by experiments in which Dd-RAS-THR14 mutant cells were labelled with [3H]inositol, which showed a close correlation between the rate of label incorporation into [3H]Ins(1,4,5)P3 and InsP6 [24,25]. These combined experiments strongly suggest that, in cells, at least part of InsP6 is formed from Ins(1,4,5)P3.

Dictyostelium nuclei contain several enzymes that are involved in inositol phosphate metabolism, as well as many inositol phosphates. The spectrum of [3H]inositol phosphates isolated from the nucleus or the cytosol of [3H]inositol-labelled cells is very similar. Moreover, inositol phospholipid turnover and degradation of Ins(1,4,5)P3 to inositol do occur in the nuclei (J. Van der Kaay, H. Sipma, A. A. Boininaar and P. J. M. Van Haastert, unpublished work), suggesting that nuclei may have a complete inositol cycle. Several recent reports of enzymes and enzyme activities in preparations of nuclei suggest the existence of a nuclear inositol cycle in different organisms. Phospholipase C, PtdIns kinases, protein kinase C isoforms and diacylglycerol kinase have been shown to be present in nuclei of rat liver cells and mouse NIH 3T3 fibroblasts [34–36]. Malviya et al. [37] have reported Ins(1,4,5)P3-mediated Ca2+ release from isolated purified rat liver nuclei, which have specific high-affinity binding sites for Ins(1,4,5)P3. Besides Ca2+ regulation, a nuclear inositol cycle might also contribute via the diacylglycerol/protein kinase C pathway to nuclear processes such as phosphorylation of transcription factors [38]. Together with InsP6 formation, the nucleus of Dictyostelium may have a specialized function in inositol phosphate metabolism.

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