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Nucleus-associated phosphorylation of Ins(1,4,5)P_3 to InsP_6 in *Dictyostelium*

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Although many cells contain large amounts of InsP_6, its metabolism and function is still largely unknown. In *Dictyostelium* lysates, the formation of InsP_6 by sequential phosphorylation of inositol via Ins(3,4,6)P_3 has been described [Stevens and Irvine (1990) Nature (London) **346**, 580–583]; the second messenger Ins(1,4,5)P_3 was excluded as a potential substrate or intermediate for InsP_6 formation. However, we observed that mutant cells labelled *in vivo* with [^{3}H]inositol showed altered labelling of both [^{3}H]Ins(1,4,5)P_3 and [^{3}H]InsP_6. In this report we demonstrate that Ins(1,4,5)P_3 is converted into InsP_6 *in vitro* by nucleus-associated enzymes, in addition to the previously described stepwise phosphorylation of inositol to InsP_6 that occurs in the cytosol. HPLC analysis indicates that Ins(1,4,5)P_3 is converted into InsP_6 via sequential phosphorylation at the 3-, 6- and 2-positions. Ins[^{32}P]P_6, isolated from cells briefly labelled with [^{32}P]P_i, was analysed using *Paramecium* phytase, which removes the phosphates of InsP_6 in a specific sequence. The 6-position contained significantly more ^{32}P radioactivity than the 4- or 5-positions, indicating that the 6-position is phosphorylated after the other two positions. The results from these *in vivo* and *in vitro* experiments demonstrate a metabolic route involving the phosphorylation of Ins(1,4,5)P_3 via Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 to InsP_6 in a nucleus-associated fraction of *Dictyostelium* cells.

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)P_2 producing two second messengers: diacylglycerol and Ins(1,4,5)P_3 [1]. Ins(1,4,5)P_3 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)P_3 and Ins(1,3,4,5)P_4 are involved in Ca^{2+} regulation in many systems [9–11]. Ins(1,3,4,5,6)P_5 is involved in the regulation of the affinity of oxygen for avian haemoglobin [12], and InsP_6 is thought to serve as a phosphate buffer in plants [13]. Ins(1,3,4,5,6)P_5 and InsP_6 may also serve as neurotransmitters [14]. Recently an InsP_6-binding protein has been isolated and recognized as the AP2 clathrin-assembly protein [15].

The recent characterization of a new class of inositol phosphates, the inositol polyphosphate pyrophosphates, shows that InsP_6 is not a metabolic end point [16]. Evidence for the existence of InsP_7 and InsP_8 arose from the detection of ^{3}H-labelled compounds more polar that InsP_6, which were formed in *Dictyostelium* cells labelled with [^{3}H]inositol *in vivo* [17]. After structural analysis, these compounds were identified as D/L-1-diphosphoinositol pentakisphosphate and D/L-bis-(1,4)-diphosphoinositol tetrakisphosphate [16]. 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* InsP_6 is present at high concentration (about 0.6 mM [21]), no function has been attributed to it yet. A route for InsP_6 formation via stepwise phosphorylation of *myo*-inositol in *Dictyostelium* has been described by Stephens and Irvine [22]. They excluded Ins(1,4,5)P_3 as a direct precursor in the InsP_6 synthesis. We have also reported experiments suggesting the absence of Ins(1,4,5)P_3 kinase activity in *Dictyostelium* lysates [23]. However, when *Dictyostelium* cells expressing an oncogenic *ras* gene are labelled with [^{3}H]inositol, they show increased conversion of [^{3}H]PtdIns into [^{3}H]PtdIns4P and as a consequence elevated levels of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]Ins(1,4,5)P_3 [24]. The observations that the levels of [^{3}H]InsP_6 were also increased [24,25] suggest a link between [^{3}H]InsP_6 formation and [^{3}H]Ins(1,4,5)P_3 levels. In this study we have further investigated the formation of InsP_6 in *Dictyostelium* cells, and indeed observed that Ins(1,4,5)P_3 can be phosphorylated to InsP_6 *in vitro*. The intermediates were identified as Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5. This enzymic conversion was observed in a preparation of broken nuclei, whereas the formation of InsP_6 from inositol was detected only in the soluble fraction of a cell lysate.

We conclude that *Dictyostelium* cells have two metabolic routes to InsP_6: 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 [$(NH_4)_2SO_4$ 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 Nuclepore. [^{14}C]Ins3P, [^{3}H]Ins(1,4,5)P_3, [^{3}H]inositol (20 Ci/mmol), Ins[4-^{32}P](1,4,5)P_3 (200 Ci/mmol), Ins(1,4,5)[5-^{32}P]P_3 (200 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham. [^{3}H]Ins1P, [^{3}H]Ins4P, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]InsP_6 (23 Ci/mmol) were from NEN-Dupont. [^{3}H]Ins(1,3,4,6)P_4 and purified Ins(1,4,5)P_3 3-kinase were gifts from C. Erneux (IRIBHN, Université Libre de Bruxelles); [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,3,4,5,6)P_5 were kindly provided by B. Hoiting (University of Groningen).

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Preparation of [^{3}H]Ins(4,5)P_2, Ins[5-^{32}P](1,3,4,5)P_4 and Ins[3-^{32}P](1,3,4,5)P_4

[^{3}H]Ins(4,5)P_2 was prepared by incubation of [^{3}H]Ins(1,4,5)P_3 with a partially purified *Dictyostelium* Ins(1,4,5)P_3 1-phosphatase in the presence of 0.25 mM 2,3-diphosphoglyceric acid and 5 mM $MgCl_2$ as described [26].

Ins[3-^{32}P](1,3,4,5)P_4 was prepared by phosphorylation of Ins(1,4,5)P_3 with a purified Ins(1,4,5)P_3 3-kinase in the presence of [γ-^{32}P]ATP [27]. Ins[5-^{32}P](1,3,4,5)P_4 was prepared by phosphorylation of Ins[5-^{32}P](1,4,5)P_3 with the 3-kinase in the presence of ATP [27]. Incubations with the Ins(1,4,5)P_3 3-kinase were in 25 μl 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 μM ATP, 6.25 μM Ins(1,4,5)P_3 and 50 mM Hepes, pH 7.5 (final concentrations). To obtain Ins[3-^{32}P](1,3,4,5)P_4 and Ins[5-^{32}P](1,3,4,5)P_4, 1.5 μCi of [γ-^{32}P]ATP or 0.125 μCi of Ins[5-^{32}P](1,4,5)P_3 was added respectively. Reactions were terminated by boiling for 2 min. The InsP_4 produced was isolated by HPLC on a Zorbax SAX column eluted with gradient A (see below). Salt was removed by dialysis for 3 × 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 10^7 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 μm 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 μm 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.

InsP_6 formation *in vitro*

Assay mixtures (100 μl) contained 50 μl of enzyme preparation (5 × 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 [^{32}P]P_i incorporation into ATP and InsP_6

Dictyostelium cells were harvested and starved for 2 h in 20 mM Hepes, pH 6.5 (HB buffer), at 10^7 cells/ml. Cells were again harvested and resuspended in HB buffer at a density of 2 × 10^7 cells/ml and incubated with 0.25 mCi of [^{32}P]P_i/ml. After t = 5, 15, 30, 60, 120, 150 and 180 min a 1 ml sample was centrifuged for 60 s at 1500 ***g***; the cells were washed twice with 1 ml of HB buffer, resuspended in 100 μl of HB and lysed by addition of 100 μl 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 μl of 2 M acetic acid and 70 μl 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 × 90 min against 500 vol. of 10 mM Hepes, pH 7.1.

The radioactivity at the γ-position of [^{32}P]ATP was determined by converting glucose and [^{32}P]ATP into [^{32}P]glucose 6-phosphate and [^{32}P]ADP with hexokinase. The [^{32}P]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 μl of hexokinase (100 units/ml) in 100 μl 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 Ins[^{32}P]P_6 isolated from *Dictyostelium* cells labelled with [^{32}P]P_i

Cells were labelled with [^{32}P]P_i, extracted with $HClO_4$, neutralized with $KHCO_3$ as described above and subsequently extracted with charcoal to remove nucleotides: 20 μl 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 [^{3}H]InsP_6, dialysed overnight against 3 × 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 InsP_6 were pooled and diaysed overnight against 3 × 500 vol. of 10 mM Hepes, pH 7.1, to remove the ammonium phosphate.

This purified [^{3}H]Ins[^{32}P]P_6 mixture was dephosphorylated stepwise at the 6-, 5- and 4-positions using 20 μl of *Paramecium* phytase for t = 0, 10 and 120 min in a total volume of 100 μl containing 50 mM Tris/HCl, pH 7.0, about 5000 d.p.m. Ins[^{32}P]P_6 and 5000 d.p.m. [^{3}H]InsP_6 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 s were collected and 4 ml of emulsifier 299 was added. Radioactivity was determined with a dual-label counting program and using 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 s 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

InsP_6 formation *in vitro* from inositol and Ins(1,4,5)P_3 in the cytosol and nucleus-associated fraction

Dictyostelium cells were lysed by passage through a Nuclepore filter of pore size 3 μm (which is smaller than cells but larger than the nucleus). The lysate was centrifuged at low speed to

Table 1 Subcellular localization of InsP_6 formation from inositol and Ins(1,4,5)P_3

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

	InsP_6 formation (% of substrate)	
Fraction	[^{3}H]Inositol	[^{3}H]Ins(1,4,5)P_3
Cytosol	10.8 ± 2.2	1.36 ± 2.71*
Microsomes	< 0.1	< 0.1
Nuclei	< 0.1	< 0.1
Broken nuclei	< 0.1	12.8 ± 1.2

* In three out of five experiments no phosphorylation of Ins(1,4,5)P_3 was detected; in two experiments 0.62% and 6.2% of Ins(1,4,5)P_3 respectively was converted into InsP_6; we assume that in these experiments a small portion of the nuclei were broken.

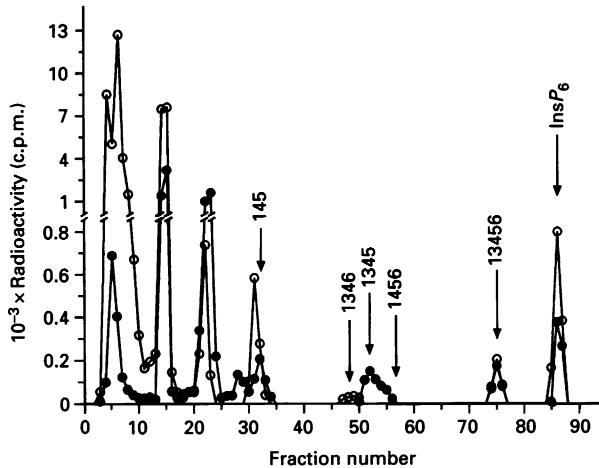


Figure 1 Combined HPLC separation after *in vitro* phosphorylation of [^{3}H]Ins in a cytosolic fraction and Ins[^{32}P](1,4,5)P_3 in a nuclear extract

A *Dictyostelium* cytosolic fraction and a nuclear extract were incubated with [^{3}H]inositol and Ins[^{32}P](1,4,5)P_3 respectively. The combined reaction products were separated by HPLC; ○, ^{3}H-labelled compounds, ●, ^{32}P-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 [^{3}H]inositol or [^{3}H]Ins(1,4,5)P_3 and formation of [^{3}H]InsP_6 was analysed by HPLC (Table 1). Using the cytosolic fraction, [^{3}H]inositol was successfully phosphorylated to [^{3}H]InsP_6, but reproducible phosphorylation of [^{3}H]Ins(1,4,5)P_3 to [^{3}H]InsP_6 was not observed (Table 1). In the microsomal and nuclear fractions neither [^{3}H]inositol nor [^{3}H]Ins(1,4,5)P_3 was phosphorylated to [^{3}H]InsP_6. However, significant and reproducible conversion of [^{3}H]Ins(1,4,5)P_3 into [^{3}H]InsP_6 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, [^{3}H]inositol was not converted into [^{3}H]InsP_6 using this nuclear extract.

In conclusion, phosphorylation of Ins(1,4,5)P_3 to InsP_6 is mediated by soluble nucleus-associated enzyme(s), whereas the enzyme(s) involved in conversion of inositol into InsP_6 are cytosolic.

Table 2 InsP_6 formation in the cytosol and nuclear extract using different substrates

The data shown are means ± S.D. of three experiments, or the means of two experiments. ND, not determined. —, Below the limit of detection.

	InsP_6 formation (% of substrate)	
Substrate	Nuclear extract	Cytosol
Ins	—	11.98 ± 3.25
Ins1P	—	—
Ins3P	—	9.33 ± 0.82
Ins4P	—	—
Ins(1,4)P_2	—	—
Ins(3,4)P_2	—	—
Ins(4,5)P_2	0.53 ± 0.15*	0.53 ± 0.15*
[^{3}H]Ins(1,4,5)P_3	13.68 ± 1.78	†
Ins[4-^{32}P](1,4,5)P_3	14.06 ± 1.94	ND
Ins[5-^{32}P](1,4,5)P_3	14.14 ± 2.47	ND
[^{3}H]Ins(1,3,4,5)P_4	8.26 ± 1.21	†
Ins[3-^{32}P](1,3,4,5)P_4	5.86	ND
Ins[5-^{32}P](1,3,4,5)P_4	7.27	ND

* The subcellular localization of the phosphorylation of Ins(4,5)P_2 was not investigated.
† No reproducible phosphorylation; see Table 1.

Routes of InsP_6 formation *in vitro*

Figure 1 shows a combined HPLC profile of *in vitro* phosphorylation of [^{3}H]inositol in the cytosol and [^{32}P]Ins(1,4,5)P_3 in the nuclear extract. The [^{3}H]InsP_3 isomer and the [^{3}H]InsP_4 isomer derived from [^{3}H]inositol did not co-migrate with standard Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 respectively. The [^{3}H]InsP_5 isomer co-migrated with Ins(1,3,4,5,6)P_5. To establish further the routes of InsP_6 formation in the cytosol, several inositol phosphates were tested as possible precursors of InsP_6 (Table 2). Ins3P is the only inositol monophosphate that is converted in the cytosol into InsP_6. In the cytosolic fraction the inositol polyphosphates tested are poor precursors of InsP_6 formation, including Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Stephens and Irvine [22] have identified the intermediates of the sequential phosphorylation in the cytosolic fraction of [^{3}H]inositol to InsP_6 as Ins3P, Ins(3,6)P_2, Ins(3,4,6)P_3, Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5. The elution profile of the [^{3}H]inositol phosphate intermediates (Figure 1) and the substrate specificity for the formation of InsP_6 in the cytosol (Table 2) are fully consistent with this route of InsP_6 formation.

The phosphorylation of Ins(1,4,5)P_3 in the nuclear extract was investigated in more detail. Assuming that during InsP_6 formation no dephosphorylation occurs (for which proof will be given below), only three InsP_4 isomers can be formed from Ins(1,4,5)P_3, namely Ins(1,2,4,5)P_4, Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4. The Ins[^{32}P]P_4 isomer that was formed from Ins[^{32}P](1,4,5)P_3 in the nuclear extract co-migrated with [^{3}H]Ins(1,3,4,5)P_4 and not with [^{3}H]Ins(1,4,5,6)P_4 (Figure 1). {When [^{3}H]Ins(1,4,5)P_3 was incubated with ATP and *Dictyostelium* enzymes for a prolonged period, a second InsP_4 isomer was formed besides Ins(1,3,4,5)P_4. This product was neither Ins(1,3,4,6)P_4 nor Ins(3,4,5,6)P_4 [or Ins(1,4,5,6)P_4] which have different retention times in our chromatographic system. After a relatively short incubation

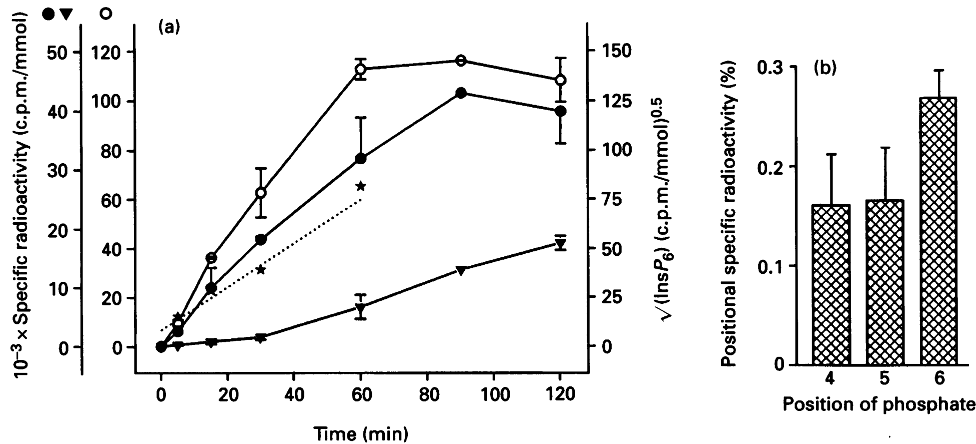


Figure 2 ***In vivo*** **Ins[^{32}P]P_6 formation after labelling of cells with [^{32}P]P$_i$; (a) kinetics of labelling [γ-^{32}P]ATP and InsP_6; (b) distribution of label over three positions**

(**a**) *Dictyostelium* cells were incubated with [^{32}P]P$_i$ 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 γ-position. The data shown are presented as c.p.m./mmol using a concentration of 2.6 mM P$_i$ (▼), 1 mM ATP (○) or 0.6 mM InsP_6 (●) (for InsP_6 the data were divided by 6 to account for the six phosphates). The data for InsP_6 are also presented as the square root of specific activity versus time (★), 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 γ-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[ATP]. The observed labelling of each position of InsP_6 has a mean rate of 181 c.p.m./mmol per min, yielding a rate constant of $a = 0.0047$ min^{-1} ($t_{1/2} = 146$ min). Pre-steady 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 γ-position of ATP is approximately linear with time showing a rate of 619 c.p.m./mmol per min (i.e. [ATP] = 619t). During this phase the rate of InsP_6 phosphorylation is given by d[InsP_6]/dt = a[ATP] = 619at. Integration yields InsP_6 = 619/2 at^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.$^{0.5}$/mmol$^{0.5}$ 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 (from 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)$^{0.5}$ per min, $r = 0.993$. (**b**) Cells were labelled with [^{32}P]P$_i$ for 15 min, lysed and the water-soluble fraction was extracted with charcoal to remove nucleotides; [^{3}H]InsP_6 was added and [^{3}H]Ins[^{32}P]P_6 was isolated by HPLC. This mixture was incubated with *Paramecium* phytase as described in Figure 3. The fraction of ^{32}P radioactivity at positions 6, 5 and 4 was calculated. The data shown are means ± S.D. of three (**a**) or five (**b**) determinations.

period (1 h), considerable amounts of Ins(1,3,4,5)P_4 and low levels of InsP_6 were formed, whereas the unknown InsP_4 product was not detectable. On prolonged incubation (more than 4 h) a decrease in Ins(1,3,4,5)P_4 was accompanied by an increase in InsP_6 and the appearance of the unidentified product. This InsP_4 isomer was assumed to be a degradation product of InsP_6 and not further identified.} As an authentic standard of Ins(1,2,4,5)P_4 is not available, the possible co-migration with this isomer cannot be examined. If Ins(1,2,4,5)P_4 were the InsP_4 intermediate, the InsP_5 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 Ins[^{32}P]P_5 isomer co-migrated with Ins(1,3,4,5,6)P_5, and not Ins(1,2,3,4,5)P_5 or Ins(1,2,4,5,6)P_5, the InsP_4 intermediate cannot have been Ins(1,2,4,5)P_4 and is therefore identified as Ins(1,3,4,5)P_4. This isomer can give rise to only two InsP_5 isomers, Ins(1,2,3,4,5)P_5 and Ins(1,3,4,5,6)P_5. Co-migration of the Ins[^{32}P]P_5 produced with Ins(1,3,4,5,6)P_5 and not with Ins(1,2,3,4,5)P_5 identifies it as Ins[^{32}P](1,3,4,5,6)P_5. Thus, in the nuclear extract, Ins(1,4,5)P_3 is phosphorylated via Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 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_3 and Ins(1,3,4,5)P_4 (i.e. no dephosphorylation reactions) is provided by six series of experiments. (i) [^{3}H]Ins(1,4,5)P_3 in the presence of 1 mM inositol was converted to the same extent into [^{3}H]InsP_6 as in the absence of inositol; conversion of [^{3}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_3 to inositol does not precede InsP_6 formation. (ii) Degradation of Ins(1,4,5)P_3 to Ins1P, Ins4P or Ins(1,4)P_2 followed by phosphorylation to InsP_6 is excluded, as these isomers are not substrates for InsP_6 formation (Table 2). (iii) Ins[4-^{32}P](1,4,5)P_3 and Ins[5-^{32}P](1,4,5)P_3 were converted into Ins[^{32}P]P_6 at the same rate as the [^{3}H]Ins(1,4,5)P_3 internal control (Table 2), indicating that the phosphates at the 4- and 5-positions of Ins(1,4,5)P_3 are retained during the phosphorylation reactions to P_6. (iv) Although Ins(4,5)P_2 shows some conversion into InsP_6, degradation of Ins(1,4,5)P_3 to this isomer with subsequent phosphorylation is not a likely route, because the simultaneous incubation of [^{3}H]Ins(1,4,5)P_3 and Ins[^{32}P](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_3 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_4 by using Ins[5-^{32}P](1,3,4,5)P_4 and Ins[3-^{32}P](1,3,4,5)P_4, which were converted into InsP_6 at about the same rate as ^{3}H-labelled Ins(1,3,4,5)P_4 (Table 2), indicating that the phosphates at positions 3 and 5 were retained. (vi) The InsP_5 isomer detected after phosphorylation of Ins(1,3,4,5)P_4 *in vitro* was co-eluted with Ins(1,3,4,5,6)P_5, and not with Ins(1,2,3,4,5)P_5/Ins(1,2,3,5,6)P_5 or Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5. These combined data reveal that all phosphates of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are retained during phosphorylation via Ins(1,3,4,5,6)P_5 to InsP_6.

Summarizing, 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_3 into InsP_6 via Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5; interestingly, the InsP_5 isomers of the two routes are identical.

In the subsequent experiments we investigated the possible route(s) of InsP_6 formation *in vivo*. Cells were labelled with [^{32}P]P_i, which is incorporated into the γ-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 Ins[^{32}P]P_6 *in vivo* on labelling with [^{32}P]P_i

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_i via [γ-^{32}P]ATP into InsP_6.

Cells were labelled with [^{32}P]P_i 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 γ-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_i relatively slowly with a half-time to equilibrium of about 30 min (Figure 2a). The rate of ^{32}P incorporation at the γ-position of ATP follows the same kinetics. Together with the observation that 38 % of the label in ATP is at the γ-position, irrespective of the labelling period, this indicates that intracellular P_i 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_i 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 γ-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 [γ-^{32}P]ATP and 2035 c.p.m./mmol for InsP_6. {The specific radioactivities of P_i, 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 [γ-^{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 γ-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 [γ-^{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.

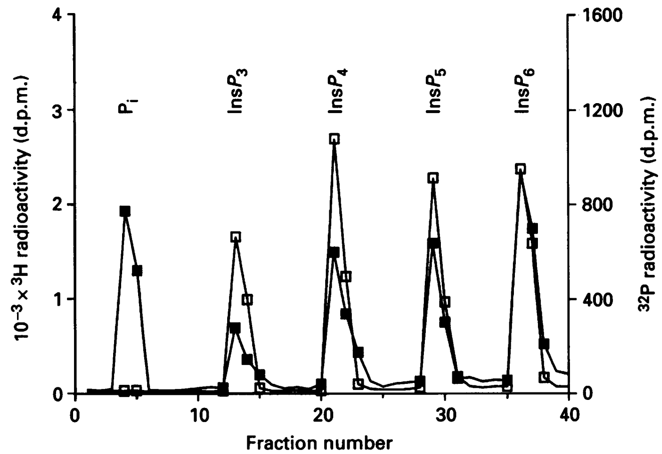


Figure 3 HPLC separation after degradation by *Paramecium* phytase of a mixture of authentic [^{3}H]InsP_6 and Ins[^{32}P]P_6 isolated from *Dictyostelium* cells

A mixture of authentic [^{3}H]InsP_6 and Ins[^{32}P]P_6 isolated from *Dictyostelium* cells after labelling for 15 min with [^{32}P]P_i was incubated with *Paramecium* phytase for 10 min and the reaction products were separated by HPLC. The ordinates are adjusted such that [^{3}H]InsP_6 and Ins[^{32}P]P_6 peaks are of equal size. The reduced level of ^{32}P radioactivity (■) relative to ^{3}H radioactivity (□) at a specific dephosphorylation step is a measure of the positional specific radioactivity at the position of dephosphorylation. *Paramecium* phytase dephosphorylates InsP_6 in the strict sequence 6, 5, 4. Data from five experiments are combined in Figure 2(b).

Route of Ins[^{32}P]P_6 formation *in vivo* on labelling cells with [^{32}P]P_i

When cells are labelled with [^{32}P]P_i for a very short period, the six phosphates of Ins[^{32}P]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 Ins(1,4,5)P_3. Thus, after a very brief labelling period of cells with [^{32}P]P_i, 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 Ins(1,4,5)P_3.

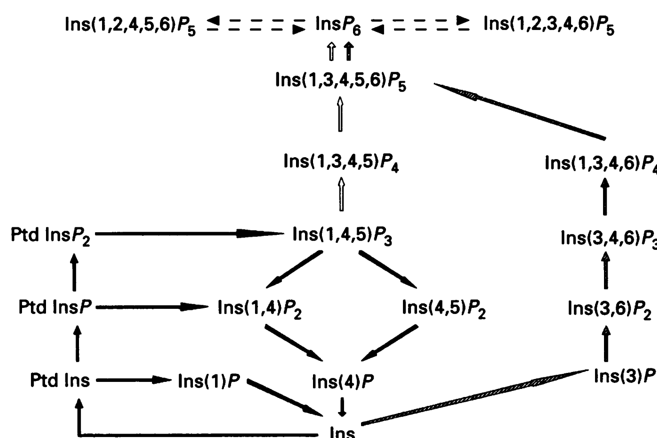
Cells were labelled for 15 min with [^{32}P]P_i and quenched with $HClO_4$. Authentic [^{3}H]InsP_6 was added to the extract and the [^{3}H]Ins[^{32}P]P_6 mixture was isolated by HPLC. The mixture was incubated with *Paramecium* phytase, and the reaction products were separated by HPLC (Figure 3). The ^{32}P/^{3}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 Ins[^{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 InsP_6 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*, InsP_6 can be formed by sequential phosphorylation of inositol [22]. We have investigated the formation of InsP_6 in some detail, because we observed that [^{3}H]inositol-labelled mutants with altered formation of [^{3}H]Ins(1,4,5)P_3 also showed altered labelling of [^{3}H]InsP_6, suggesting that at least part of [^{3}H]InsP_6 is formed from [^{3}H]Ins(1,4,5)P_3 [24,25]. The present results indeed show that *Dictyostelium* cells contain a newly identified pathway, which involves the phosphorylation of Ins(1,4,5)P_3 via Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 to InsP_6; this route is only detected in nuclear extracts and not in the cytosol, microsomes or intact nuclei.

The pathway of Ins(1,4,5)P_3 metabolism to InsP_6 was unravelled by identifying the intermediates. The observation that all the phosphates of Ins(1,4,5)P_3 were retained in InsP_6 indicated that Ins(1,4,5)P_3 was not (even partly) degraded before it was phosphorylated. The InsP_5 isomer produced co-migrated with Ins(1,3,4,5,6)P_5 and not with any of the other five InsP_5 isomers. Only three InsP_4 isomers can be formed from Ins(1,4,5)P_3: Ins(1,2,4,5)P_4, Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4. The observed product co-migrated with Ins(1,3,4,5)P_4 and not with Ins(1,4,5,6)P_4. Since the InsP_5 produced does not contain phosphate at the 2-position, Ins(1,2,4,5)P_4 cannot be an intermediate. Thus InsP_6 formation from Ins(1,4,5)P_3 proceeds via Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5. In the green alga *Chlamydomonas* and in turkey erythrocytes, similar enzyme activities have been observed phosphorylating Ins(1,4,5)P_3 via Ins(1,3,4,5)P_4 to Ins(1,3,4,5,6)P_5; however, in these systems no InsP_6 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)P_3; this part is present in both cytosolic and nucleus-associated compartments. The open arrows refer to phosphorylation of Ins(1,4,5)P_3 to InsP_6 in the nucleus. The hatched arrows demonstrate the phosphorylation of inositol to InsP_6 in the cytosol, and the broken arrows indicate the two futile InsP_5/InsP_6 cycles.

Stephens and Irvine [22] characterized three InsP_5 isomers after labelling of *Dictyostelium* cells *in vivo* with [^{3}H]inositol: Ins(1,3,4,5,6)P_5, Ins(1,2,3,4,6)P_5 and Ins(1,2,4,5,6)P_5. The reported half-times of conversion into InsP_6 were 25, 6.4 and 0.8 s respectively. The Ins(1,3,4,5,6)P_5 isomer was shown to be the precursor of InsP_6 *in vivo*, and the other two isomers were degradation products of InsP_6 and were rephosphorylated to InsP_6 in futile cycles.

The three different routes of InsP_6 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 InsP_6 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 InsP_6. 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)P_3 into InsP_6. This involves PtdIns turnover; therefore inositol and the six phosphates of InsP_6 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. Sipma, A. A. Bominaar 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)P_3 kinase activity either *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_2O and Ins[^{32}P](1,4,5)P_3 revealed that the ^{32}P label was excluded from the nuclei relative to the ^{3}H label, indicating that the nucleus was closed to Ins(1,4,5)P_3 (J. Van der Kaay, H. Sipma, A. A. Bominaar 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 lysate was used.

Once several routes for the formation of InsP_6 had been detected *in vitro*, experiments were designed to obtain an indication of the importance of each route *in vivo*. The kinetics of [^{32}P]P_i incorporation into ATP and InsP_6 demonstrated that phosphate in no position in InsP_6 was in rapid (minutes) equilibrium with ATP; the phosphates of InsP_6 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 InsP_6, whenever present *in vivo*, involve only a small portion of the InsP_6 pool. In a second experiment, *Paramecium* phytase, which dephosphorylates InsP_6 in a strict sequence, was used to determine the distribution of ^{32}P over the different positions of InsP_6. The radioactivity in Ins[^{32}P]P_6 isolated from cells after a brief labelling period with [^{32}P]P_i 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 InsP_6 is labelled after the 4- and 5-positions. This sequence of phosphorylation reactions is compatible with the route of InsP_6 formation from Ins(1,4,5)P_3, but not with the route from inositol or the futile cycle at the 5-position (Scheme 1). These conclusions on the formation of InsP_6 *in vivo* after labelling with phosphate

are supported by experiments in which Dd-RAS-THR[12] mutant cells were labelled with [^{3}H]inositol, which showed a close correlation between the rate of label incorporation into [^{3}H]Ins(1,4,5)P_3 and InsP_6 [24,25]. These combined experiments strongly suggest that, in cells, at least part of InsP_6 is formed from Ins(1,4,5)P_3.

Dictyostelium nuclei contain several enzymes that are involved in inositol phosphate metabolism, as well as many inositol phosphates. The spectrum of [^{3}H]inositol phosphates isolated from the nucleus or the cytosol of [^{3}H]inositol-labelled cells is very similar. Moreover, inositol phospholipid turnover and degradation of Ins(1,4,5)P_3 to inositol do occur in the nuclei (J. Van der Kaay, H. Sipma, A. A. Bominaar 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]. Maliviya et al. [37] have reported Ins(1,4,5)P_3-mediated Ca^{2+} release from isolated purified rat liver nuclei, which have specific high-affinity binding sites for Ins(1,4,5)P_3. Besides Ca^{2+} 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 InsP_6 formation, the nucleus of *Dictyostelium* may have a specialized function in inositol phosphate metabolism.

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