In *Dictyostelium discoideum* inositol 1,3,4,5-tetrakisphosphate is dephosphorylated by a 3-phosphatase and a 1-phosphatase

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The degradation of Ins(1,3,4,5)P_4 in *Dictyostelium* was investigated using a mixture of [^{3}H]Ins(1,3,4,5)P_4 and [3-^{32}P]Ins-(1,3,4,5)P_4. After incubation of this mixture with a *Dictyostelium* homogenate the ^{32}P/^{3}H ratio found in the InsP_3 product was reduced to 24% of the ratio in the substrate. ^{32}P-labelled inorganic phosphate was found as well, whereas hardly any InsP_2 was detected. This indicates that Ins(1,3,4,5)P_4 is mainly degraded by a 3-phosphatase. The other enzyme was characterized by identification of the ^{32}P-labelled InsP_3 isomer. This isomer did not co-elute with Ins(1,3,4)P_3, indicating that no 5-phosphatase was present in *Dictyostelium*. The ^{32}P-labelled InsP_3 could be oxidized using $NaIO_4$. The only InsP_3 isomer that has these characteristics is Ins(3,4,5)P_3, indicating 1-phosphatase activity. The 1-phosphatase appeared to be dependent on $MgCl_2$, whereas the 3-phosphatase was still active in the absence of $MgCl_2$. An analogue of Ins(1,3,4,5)P_4 with a thiophosphate substitution at the 1-position was found to be almost completely resistant to hydrolysis by the 1-phosphatase, but was degraded by the 3-phosphatase.

INTRODUCTION

It is generally accepted that the second messenger Ins(1,4,5)P_3, generated by the action of phospholipase C, is involved in calcium mobilization from non-mitochondrial stores [1]. In mammalian cells Ins(1,4,5)P_3 can be phosphorylated by a 3-kinase, resulting in the formation of Ins(1,3,4,5)P_4 [2]. Both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are dephosphorylated by a 5-phosphatase yielding Ins(1,4)P_2 and Ins(1,3,4)P_3 respectively [3,4]. For rat liver and human platelets it has been shown that Ins(1,3,4,5)P_4 can also be dephosphorylated by a 3-phosphatase [5–7]. It was suggested that this activity provides a way to sustain Ins(1,4,5)P_3 signals, but when the hepatic 3-phosphatase was purified its substrate specificity indicated that the preferred substrates *in vivo* probably are InsP_6 and Ins(1,3,4,5,6)P_5 [8]. Furthermore, it is shown that the 3-phosphatase is located inside the endoplasmic reticulum, probably without access to its substrates [9]. Therefore, the cellular function of the 3-phosphatase remains unclear.

In the cellular slime mould *Dictyostelium discoideum* there are indications for the function of Ins(1,4,5)P_3 in calcium mobilization [10,11]. Furthermore Ins(1,4,5)P_3 has been shown to be generated by phospholipase C-mediated hydrolysis of PtdIns-(4,5)P_2 [12–14]. The metabolism of Ins(1,4,5)P_3 in *Dictyostelium* is different from that in mammalian cells: in vegetative and aggregation competent cells Ins(1,4,5)P_3 is degraded by a 5-phosphatase and a 1-phosphatase to yield Ins(1,4)P_2 and Ins-(4,5,)P_2 respectively [15]. In *Dictyostelium* slug cells, however, the Ins(1,4,5)P_3 is solely dephosphorylated by the 1-phosphatase [16].

Recently a *Dictyostelium* cell line lacking the gene for phospholipase C was constructed [17]. This cell line has levels of Ins(1,4,5)P_3 that are only slightly significantly lower than those in wild-type cells [18]. This indicates that there should be at least one additional route for Ins(1,4,5)P_3 formation. Among several other possible routes, the dephosphorylation of higher inositol polyphosphates could provide Ins(1,4,5)P_3. Ins(1,3,4,5)P_4, which is present in *Dictyostelium*, could be the immediate precursor of Ins(1,4,5)P_3. The level of Ins(1,3,4,5)P_4 is 10 pmol/10^7 cells, whereas the level of Ins(1,4,5)P_3 is about 5 pmol/10^7 cells [18].

In this study the degradation of Ins(1,3,4,5)P_4 by *Dictyostelium* homogenates was investigated. We found that Ins(1,3,4,5)P_4 is degraded by a 3-phosphatase to yield Ins(1,4,5)P_3, as in mammalian cells. Unlike in mammalian cells, Ins(1,3,4,5)P_4 is not degraded by a 5-phosphatase, but an additional enzyme hydrolysing Ins(1,3,4,5)P_4 at the 1-position was identified.

MATERIALS AND METHODS

Materials

[2-^{3}H]Ins(1,3,4,5)P_4 (21 Ci/mmol) and [2-^{3}H]Ins(1,4,5)P_3 (40 Ci/mmol) were from Du Pont–New England Nuclear. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham International. The Zorbax HPLC column (6.2 mm × 8 cm) was from Du Pont. Emulsifier 299 scintillation cocktail was from Packard. The Visking dialysis membrane (12–14 kDa cutoff) and PMSF were from Serva. Hexokinase (2 mg/ml), adenosine 5′-[γ-thio]triphosphate (ATP[S]) and leupeptin were purchased from Boehringer. BSA and Ins(1,4,5)P_3 were from Sigma. All other chemicals were from Merck. DL-*myo*-Inositol 4,5-bisphosphate 1-phosphorothioate [Ins(1)PS(4,5)P_2] was a generous gift from Dr. B. V. L. Potter. A cDNA clone encoding the C-terminal part of the Ins(1,4,5)P_3 3-kinase (clone C5 in [19]) was a generous gift from Dr. C. Erneux.

Organism and culture conditions

Dictyostelium discoideum strains AX3, HD10 and HD11 were grown axenically in HL5 medium [20] containing 10 g/l glucose instead of 16 g/l glucose. Cells were harvested in PB (10 mM sodium/potassium phosphate buffer, pH 6.5), washed by repeated centrifugation (3 min, 300 *g*), and starved for 5 h in PB at

Abbreviations used: Ins(1)PS(4,5)P_2, DL-*myo*-inositol 4,5-bisphosphate 1-phosphorothioate; Ins(1)PS(3,4,5)P_3, DL-*myo*-inositol 3,4,5-trisphosphate 1-phosphorothioate.

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a density of 1×10^7 cells/ml. Cells were washed in 40 mM Hepes, pH 6.5, containing 0.5 mM EDTA. After resuspending in 40 mM Hepes, pH 6.5, 0.5 mM EDTA and 200 mM sucrose, cells were homogenized at a density of 1×10^8/ml by pressing them through a Nuclepore filter (pore size 3 μm).

Preparation of recombinant Ins(1,4,5)P_3 3-kinase

LB medium (10 g/l NaCl, 10 g/l bactotryptone, 5 g/l bactoyeast extract) containing 50 μg of ampicillin/ml was inoculated with a single colony of the Bluescript plasmid harbouring the cDNA insert encoding rat brain Ins(1,4,5)P_3 3-kinase (clone C5; [19]). Cultures (50 ml) were incubated overnight at 37 °C. The Ins(1,4,5)P_3 3-kinase was induced by the addition of isopropyl β-thiogalactoside (IPTG) to a final concentration of 1 mM. After 4 h cells were harvested and resuspended in 0.5 ml of cold lysis buffer (50 mM Tris/HCl, pH 8, 1 mM EDTA, 12 mM 2-mercaptoethanol, 0.2 mM PMSF, 2.5 μM leupeptin, 1% Triton X-100, 10% sucrose). The suspension was shaken for 20 min at 4 °C followed by centrifugation (15000 *g*, 5 min). The supernatant showed Ins(1,4,5)P_3 3-kinase activity and was immediately stored at −80 °C.

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

Ins(1,4,5)P_3 3-kinase activity was used in a 50 μl reaction volume containing 84 mM Hepes, pH 7.5, 1 mg/ml BSA, 20 mM $MgCl_2$, 10 μM ATP, 10 μM Ins(1,4,5)P_3, [γ-^{32}P]ATP and enzyme [enzyme activity in the mixture: 94 pmol/min per ml at 10 μM Ins(1,4,5)P_3 and 37 °C]. The mixture was incubated for 8 min at 37 °C. This was followed by boiling for 2 min and centrifugation for 5 min at 15 000 *g*. The supernatants were incubated for 35 min with 5 μl of hexokinase (1.1 units) in the presence of 100 μM glucose followed by boiling for 2 min to remove residual [γ-^{32}P]ATP. The reaction mixture was centrifuged (2 min, 14000 *g*) and analysed by HPLC as described below. The fractions containing [3-^{32}P]Ins(1,3,4,5)P_4 were desalted by dialysis against 10 mM Hepes, pH 7.0, for 4 h.

Ins(1,3,4,5)P_4 phosphatase assay

Ins(1,3,4,5)P_4 phosphatase activity was determined in a mixture containing 2000 d.p.m. [^{3}H]Ins(1,3,4,5)P_4 and 2000 d.p.m. [3-^{32}P]Ins(1,3,4,5)P_4. After incubation for 30–60 min at room temperature, $(NH_4)_2HPO_4$ was added to a final concentration of 120 mM and the sample was boiled for 2 min. The sample was centrifuged for 2 min at 10000 *g*, and the supernatant was applied to an HPLC column.

HPLC analysis of labelled inositol phosphates

Samples were analysed using a Zorbax HPLC column equipped with a guard column. The column was eluted with a gradient of water (buffer A) and 1.2 M $(NH_4)_2HPO_4$ (buffer B) at 1.5 ml/min. Linear gradients of %B were formed at the following time points.

Gradient 1: 0% at 0 min, 30% at 5 min, 39% at 11 min, 100% at 13 min, 100% at 16 min. Gradient 2: 0% at 0 min, 50% at 0.1 min, 100% at 5 min, 100% at 7.5 min. Gradient 3: 0% at 0 min, 5% at 3 min, 15% at 8 min, 20% at 9 min, 25% at 14 min, 30% at 15 min, 39% at 21 min, 100% at 21.1 min, 100% at 27 min. Gradient 4: 0% at 0 min, 30% at 1 min, 70% at 10 min, 100% at 11 min, 100% at 16 min. Fractions of 0.5 ml were collected to which 4 ml of scintillation cocktail was added. Radioactivity in the fractions was determined in a Beckmann liquid-scintillation counter.

Oxidation of Ins(1,3,4,5)P_4 degradation product

[3-^{32}P]Ins(1,3,4,5)P_4 was incubated with a *Dictyostelium* homogenate. The sample was split in two. One of the samples was used as a control. To the other sample $NaIO_4$ (pH 2) was added to a final concentration of 175 mM. The reaction was carried out for 36 h at room temperature in the dark. Afterwards both samples were analysed by HPLC as described above.

RESULTS AND DISCUSSION

For the investigation of Ins(1,3,4,5)P_4 degradation by wild-type (AX3) *Dictyostelium* homogenates we made use of [3-^{32}P]Ins(1,3,4,5)P_4. This compound was prepared from unlabelled Ins(1,4,5)P_3 and [γ-^{32}P]ATP using Ins(1,4,5)P_3 3-kinase. As this compound yields unlabelled Ins(1,4,5)P_3 and ^{32}P-labelled inorganic phosphate when degraded by a 3-phosphatase, it provides a simple assay for Ins(1,3,4,5)P_3 3-phosphatase activity. On the other hand, degradation of Ins(1,3,4,5)P_4 by a 5-phosphatase results in the formation of ^{32}P-labelled Ins(1,3,4)P_3, a compound that elutes before Ins(1,4,5)P_3 in the HPLC system used.

A mixture of [3-^{32}P]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,3,4,5)P_4 was incubated with *Dictyostelium discoideum* homogenate in the presence of 10 mM EDTA. In Figure 1 a typical HPLC profile of the reaction products is shown; the relative ^{32}P/^{3}H ratios of the different peaks are indicated in the Figure, with the ratio in Ins(1,3,4,5)P_4 set at 1.0. These data reveal that the ^{32}P/^{3}H ratio in the InsP_3 fraction (0.24) is markedly reduced relatively to the ^{32}P/^{3}H ratio of the Ins(1,3,4,5)P_4 substrate. In addition, hardly any InsP_2 is formed in the reaction. Furthermore the ^{32}P label that is lost in the InsP_3 fraction is found back as ^{32}P-labelled inorganic phosphate. The significant loss of ^{32}P in the InsP_3 fraction on the one hand, and the formation of ^{32}P-labelled inorganic phosphate in the absence of significant InsP_2 formation on the other hand, indicates that 76% of Ins(1,3,4,5)P_4

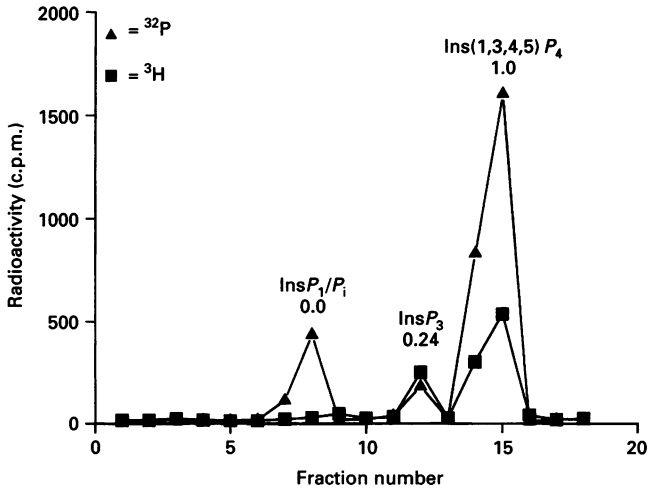

Figure 1 [3-^{32}P]Ins(1,3,4,5)P_4 degradation in the presence of EDTA

HPLC profile of the degradation of a mixture of [^{3}H]Ins(1,3,4,5)P_4 and [3-^{32}P]Ins(1,3,4,5)P_4 by a wild-type *Dictyostelium* homogenate in the presence of 10 mM EDTA. Gradient 4 was used to elute the column. The ^{32}P/^{3}H ratio in the InsP_3 has dropped to 0.24, indicating 76% Ins(1,3,4,5)P_4 3-phosphatase activity. The removed ^{32}P is found back as inorganic phosphate (Ins$P$/P_i fraction).

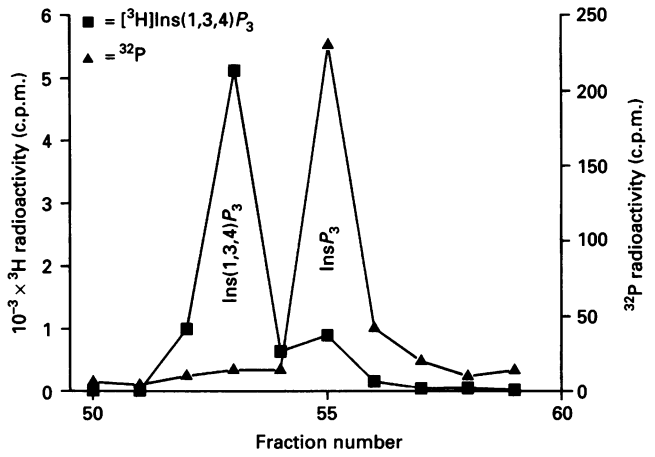

Figure 2 Absence of co-chromatography of the [^{32}P]InsP_3 and [^{3}H]Ins-(1,3,4)P_3

HPLC profile of [^{32}P]InsP_3 product formed from [3-^{32}P]Ins(1,3,4,5)P_4 after incubation with a wild-type *Dictyostelium* homogenate. The [^{32}P]InsP_3 did not co-elute with authentic [^{3}H]Ins-(1,3,4)P_3 which was included as an internal standard. The HPLC column was eluted with gradient 3.

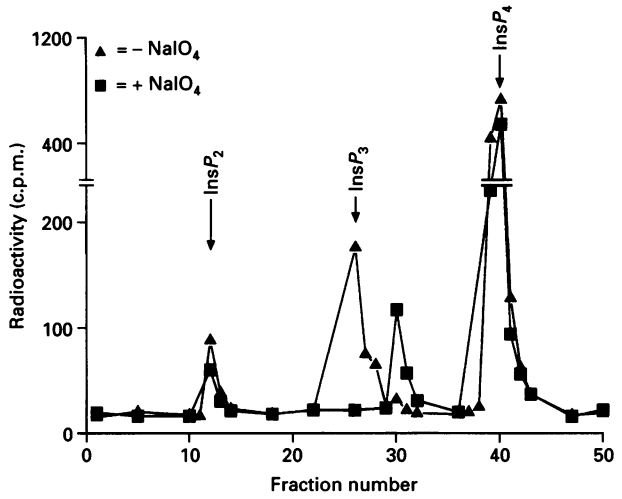

Figure 3 Effect of incubation with $NaIO_4$ on the [3-^{32}P]Ins(1,3,4,5)P_4 degradation products

[3-^{32}P]Ins(1,3,4,5)P_4 was incubated with a wild-type *Dictyostelium* homogenate in the presence of 10 mM EDTA. Half of the sample was incubated with $NaIO_4$, and the other half was used as a control. The position of the InsP_3 in the control sample is indicated in the Figure. In the $NaIO_4$-treated sample the retention time of this compound is altered, indicating that it was oxidized and cannot be Ins(1,3,5)P_3. Gradient 1 was used to elute the column. The experiment was done twice with identical results.

degradation is caused by a 3-phosphatase. Figure 1 also shows that 24% of the produced InsP_3 is ^{32}P-labelled. Exclusion of EDTA and addition of 5 mM $MgCl_2$ in the reaction mixture resulted in about a 4-fold increase of total Ins(1,3,4,5)P_4 phosphatase activity and in the enhanced formation of a ^{32}P-labelled InsP_3 isomer as the ^{32}P/^{3}H ratio in the InsP_3 fraction has increased from 0.24 in the presence of EDTA to 0.84 in the presence of $MgCl_2$ (data not shown). It should be noted that in the presence of $MgCl_2$ the ^{32}P/^{3}H ratio in the InsP_3 fraction is still lower than the ^{32}P/^{3}H ratio in Ins(1,3,4,5)P_4, indicating that part of the Ins(1,3,4,5)P_4 is still degraded by the 3-phosphatase.

In mammalian cells Ins(1,3,4,5)P_4 is degraded by a 3-phosphatase and a 5-phosphatase. The latter would yield [3-^{32}P]Ins-(1,3,4)P_3 from [3-^{32}P]Ins(1,3,4,5)P_4. Figure 2 reveals that the [3-^{32}P]InsP_3 isomer produced by *Dictyostelium* homogenates in the presence of Mg^{2+} does not co-elute with authentic [^{3}H]Ins(1,3,4)P_3, indicating that no 5-phosphatase is present.

In order to identify the second phosphatase activity we identified the nature of the formed [^{32}P]InsP_3, which could be either Ins(3,4,5)P_3 or Ins(1,3,5)P_3 (assuming that phosphate migration does not occur). From these two compounds Ins-(3,4,5)P_3 can be oxidized in a Malaprade reaction, whereas Ins(1,3,5)P_3 can not be oxidized because it has no vicinal hydroxyl groups, which are the requirements for the Malaprade reaction [21]. Incubation of the [^{32}P]InsP_3 with $NaIO_4$ altered the retention time of this compound (Figure 3). We therefore conclude that it was oxidized, indicating that it must have been Ins(3,4,5)P_3 and that the phosphatase is an Ins(1,3,4,5)P_4 1-phosphatase. *Dictyostelium* cells contain less than 1 pmol of D/L-Ins(3,4,5)P_3 per 1×10^7 cells [18]. Therefore either the formation of Ins(3,4,5)P_3 has little significance *in vivo* or Ins(3,4,5)P_3 is rapidly metabolized further.

The *Dictyostelium* Ins(1,3,4,5)P_4 phosphatases were investigated using a labelled Ins(1,3,4,5)P_4 analogue with thiophosphate substitution at the 1-position. It was prepared from Ins(1)PS-(4,5)P_2 using recombinant 3-kinase and [γ-^{32}P]ATP as described previously [22]. The compound was purified using HPLC and desalted by dialysis. It eluted several fractions after authentic Ins(1,3,4,5)P_4 (Figure 4). This compound was degraded in the presence of 5 mM $MgCl_2$ by an homogenate with [^{3}H]Ins-(1,3,4,5)P_4 as internal standard. Under these conditions Ins-(1,3,4,5)P_4 is degraded mainly (84%) by the 1-phosphatase. From Figure 4 it can be seen that hardly any [^{32}P]InsP_3 is formed from [3-^{32}P]DL-*myo*-inositol 3,4,5-trisphosphate 1-phosphorothioate [Ins(1)PS(3,4,5)P_3], but that instead most of the ^{32}P radioactivity

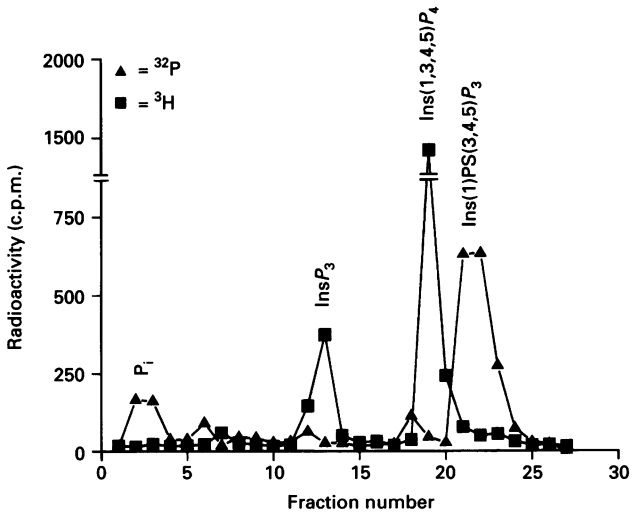

Figure 4 Degradation of [3-^{32}P]Ins(1)PS(3,4,5)P_3

HPLC profile of the degradation of a mixture of [^{3}H]Ins(1,3,4,5)P_4 and [3-^{32}P]Ins(1)PS(3,4,5)P_3 by a wild-type *Dictyostelium* homogenate in the presence of 5 mM $MgCl_2$. Ins(1)PS(3,4,5)P_3 is not degraded to an InsP_3 compound, indicating that no degradation by the 1-phosphatase has occurred. ^{32}P-labelled inorganic phosphate is formed which indicates that the analogue is degraded by the 3-phosphatase. Gradient 2 was used to elute the column.

is found back as inorganic phosphate. This indicates that the analogue is hardly degraded by the 1-phosphatase, but mainly by the 3-phosphatase. Using Ins(1,4,5)P_4 5-phosphatase, it has been shown before that thiophosphate-substituted inositol phosphates are resistant to hydrolysis of the thiophosphate group [23]. Now we can extend this observation to a phosphatase that hydrolyses a different phosphate group.

The magnesium dependence of both phosphatase activities was determined by carrying out reactions in the presence of 5 mM $MgCl_2$ or 10 mM EDTA. The relative enzyme activities were determined with the activity of the 1-phosphatase in the presence of $MgCl_2$ set at 100%. Under this condition 3-phosphatase activity was 25.9 ± 3.0%. Replacing $MgCl_2$ with EDTA resulted in a decrease of 1-phosphatase activity to 9.3 ± 0.6%. The 3-phosphatase on the other hand, is hardly dependent on the presence of $MgCl_2$ as its activity in the presence of EDTA was still 21.9 ± 0.6%.

The Ins(1,3,4,5)P_4 3-phosphatase activity, as a percentage of total Ins(1,3,4,5)P_4 phosphatase activity in the presence of $MgCl_2$, was not significantly different in various *Dictyostelium* strains. In wild-type (AX3) cells, 3-phosphatase was 20.6 ± 3.0% of total Ins(1,3,4,5)P_4 phosphatase activity. In a phospholipase C-lacking mutant (HD10) and in a control cell line for HD10 (HD11) these values were 18.5 ± 3.3% and 15.0 ± 3.2% respectively. This indicates that this route of Ins(1,4,5)P_3 formation is not caused by knocking out phospholipase C, but is a normally existing route in *Dictyostelium*.

The degradation of Ins(1,3,4,5)P_4 in *Dictyostelium* is in some aspects similar to that in mammalian cells, whereas other aspects are different. In mammalian cells two enzymes for Ins(1,3,4,5)P_4 degradation have also been demonstrated. One of them is a Mg^{2+}-independent 3-phosphatase [5]. However, in mammalian cells the second enzyme is not a 1-phosphatase but a 5-phosphatase [4]. In our experiments we could not demonstrate any Ins(1,3,4,5)P_4 5-phosphatase activity as we could not detect the formation of Ins(1,3,4)P_3 (Figure 2). In mammalian cells three different types of 5-phosphatases have been identified: types I and III degrade both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 whereas type II only hydrolyses Ins(1,4,5)P_3 [4]. In *Dictyostelium* Ins(1,4,5)P_3 5-phosphatase activity has been identified [15]. The absence of Ins(1,3,4,5)P_4 5-phosphatase activity suggests that the enzyme is a type-II 5-phosphatase.

The 3-phosphatase could be similar to the 3-phosphatase in mammalian cells. It shares the characteristic that it is $MgCl_2$-independent. In mammalian cells it has been shown that the 3-phosphatase is localized in the endoplasmic reticulum without apparent access to its substrate [9]. We are currently investigating the localization of the 3-phosphatase in order to see whether it could provide a way to form Ins(1,4,5)P_3 *in vivo*.

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