Chemoattractant and guanosine 5'-[γ -thio]triphosphate induce the accumulation of inositol 1,4,5-trisphosphate in *Dictyostelium* cells that are labelled with [3 H]inositol by electroporation

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The analysis of the inositol cycle in *Dictyostelium discoideum* cells is complicated by the limited uptake of [3 H]inositol (0.2% of the applied radioactivity in 6 h), and by the conversion of [3 H]inositol into water-soluble inositol metabolites that are eluted near the position of inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] on anion-exchange h.p.l.c. columns. The uptake was improved to 2.5% by electroporation of cells in the presence of [3 H]inositol; electroporation was optimal at two 210 μ s pulses of 7 kV. Cells remained viable and responsive to chemotactic signals after electroporation. The intracellular [3 H]inositol was rapidly metabolized to phosphatidylinositol and more slowly to phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. More than 85% of the radioactivity in the water-soluble extract that was eluted on Dowex columns as Ins(1,4,5) P_3 did not co-elute with authentic [32 P]Ins(1,4,5) P_3 on h.p.l.c. columns. Chromatography of the extract by ion-pair reversed-phase h.p.l.c. provided a good separation of the polar inositol polyphosphates. Cellular [3 H]Ins(1,4,5) P_3 was identified by (a) co-elution with authentic [32 P]Ins(1,4,5) P_3 and (b) degradation by a partially purified Ins(1,4,5) P_3 5-phosphatase from rat brain. The chemoattractant cyclic AMP and the non-hydrolysable analogue guanosine 5'-[γ -thio]triphosphate induced a transient accumulation of radioactivity in Ins(1,4,5) P_3 ; we did not detect radioactivity in inositol 1,3,4-trisphosphate or inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5) P_4]. *In vitro*, Ins(1,4,5) P_3 was metabolized to inositol 1,4- and 4,5-bisphosphate, but not to Ins(1,3,4,5) P_4 or another tetrakisphosphate isomer. We conclude that *Dictyostelium* has a receptor- and G-protein-stimulated inositol cycle which is basically identical with that in mammalian cells, but the metabolism of Ins(1,4,5) P_3 is probably different.

INTRODUCTION

Dictyostelium discoideum lives in the soil, where it feeds on bacteria. Exhaustion of the food supply induces cell aggregation which is mediated by chemotaxis to extracellular cAMP. The binding of cAMP to cell-surface receptors induces the activation of many processes, including the G-protein-mediated activation of adenylate cyclase and the activation of guanylate cyclase. The cAMP produced is secreted and may activate other cells; thus the chemotactic signal is relayed through the population of cells (for reviews see Gerisch, 1987; Janssens & Van Haastert, 1987).

It has been observed that Ins(1,4,5) P_3 induces the accumulation of cGMP in saponin-permeabilized *Dictyostelium* cells (Europe-Finner & Newell, 1985). Furthermore, Ins(1,4,5) P_3 releases Ca^{2+} from non-mitochondrial pools, and Ins(1,4,5) P_3 and Ca^{2+} induce the association of actin with the Triton-insoluble cytoskeleton (Europe-Finner & Newell, 1986a,b). These results suggest a direct or indirect role of Ins(1,4,5) P_3 in Ca^{2+} and cGMP metabolism in *Dictyostelium*.

In mammalian cells, Ins(1,4,5) P_3 is formed by the agonist and G-protein stimulation of a specific phospholipase C that hydrolyses PtdIns(4,5) P_2 (reviewed by Berridge, 1987). Ins(1,4,5) P_3 is dephosphorylated to Ins(1,4) P_2 or phosphorylated to Ins(1,3,4,5) P_4 , which is then dephosphorylated to Ins(1,3,4) P_3 . The dephosphorylation of Ins(1,4,5) P_3 in *Dictyostelium* *in vitro* follows at least two routes. A 2,3-bisphosphoglycerate-sensitive enzyme yields Ins(1,4) P_2 , and a Li^+ -sensitive enzyme yields Ins(4,5) P_2 ; both Ins P_2 isomers are dephosphorylated to Ins4P and then to Ins (Van Lookeren Campagne *et al.*, 1988).

Experiments designed to detect receptor- and G-protein-stimulated formation of Ins(1,4,5) P_3 in *Dictyostelium* revealed an increase in radioactivity that was eluted from Dowex columns in the Ins P_3 and Ins P_4 fractions (Europe-Finner & Newell, 1987a,b). We have repeated these experiments and simultaneously analysed the phosphatidylinositol (poly)phosphates. Our experiments indicated similar amounts of radioactivity in PtdIns P_2 and 'Dowex Ins P_3 ', a doubling of radioactivity in 'Dowex Ins P_3 ' after stimulation of cells with cAMP,

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; PtdIns, phosphatidylinositol phosphate; PtdIns P_2 , phosphatidylinositol bisphosphate; Ins, InsP, Ins P_2 , Ins P_3 etc., myo-inositol mono-, bis-, tris- (etc.) phosphates with isomeric numbering (all D-) as appropriate; GroPIns, glycerophospho-myoinositol; GTP[S], guanosine 5'-[γ -thio]triphosphate; PB, 10 mM-KH $_2$ PO $_4$ /Na $_2$ HPO $_4$, pH 6.5.

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but no detectable decrease in radioactivity in $\text{PtdIns}P_2$. Furthermore, analysis of the radioactivity in the 'Dowex $\text{Ins}P_3$ ' fraction by h.p.l.c. revealed that only about 10% of it co-eluted with authentic $\text{Ins}(1,4,5)P_3$. Finally, it should be mentioned that a phospholipase C activity has not yet been demonstrated in *Dictyostelium* (Irvine *et al.*, 1980), although many enzymes that are thought to be part of the inositol cycle have been identified in *Dictyostelium*. These enzymes are PtdIns kinase (Varela *et al.*, 1987), diacylglycerol kinase (Jimenez *et al.*, 1988), CDP-diacylglycerol:inositol phosphatidyltransferase (Machon *et al.*, 1980), $\text{PtdIns}P$ -kinase (M. M. Van Lookeren Campagne, unpublished work) and (poly)-phosphoinositol phosphatases (Van Lookeren Campagne *et al.*, 1988).

We have re-evaluated the receptor- and G-protein-stimulated formation of $\text{Ins}(1,4,5)P_3$ in *Dictyostelium*. The uptake of $[^3\text{H}]$ inositol was improved from 0.2% to 2.5% by electroporation of *Dictyostelium* cells. The metabolism of $[^3\text{H}]$ inositol to PtdIns , $\text{PtdIns}P$ and $\text{PtdIns}P_2$ was identified by t.l.c., and that to $[^3\text{H}]\text{Ins}(1,4,5)P_3$ by ion-pair reversed-phase h.p.l.c. We show that the chemoattractant cAMP and the non-hydrolysable GTP analogue GTP[S] induce a small transient increase in the radioactivity in $\text{Ins}(1,4,5)P_3$. On the contrary, we did not detect radioactivity being eluted as $\text{Ins}(1,3,4)P_3$ or $\text{Ins}(1,3,4,5)P_4$. In addition, no detectable $\text{Ins}(1,4,5)P_3$ kinase activity could be shown *in vitro*. These results suggest that *Dictyostelium* has a receptor- and G-protein-stimulated inositol cycle which could be similar to that in vertebrate cells, but that the metabolism of $\text{Ins}(1,4,5)P_3$ may be different.

MATERIALS AND METHODS

Materials

L-*myo*-[1,2- ^3H]Inositol (1500 GBq/mmol), $[^3\text{H}]\text{Ins}1P$ (200 GBq/mmol), $[^3\text{H}]\text{Ins}4P$ (56 GBq/mmol), $[^3\text{H}]\text{Ins}(1,4)P_2$ (93 GBq/mmol), $[^3\text{H}]\text{Ins}(1,4,5)P_3$ (740 GBq/mmol), $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ (6030 GBq/mmol) and $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ (185 GBq/mmol) were from New England Nuclear. $[^3\text{H}]\text{Ins}(4,5)P_2$ was synthesized from $[^3\text{H}]\text{Ins}(1,4,5)P_3$ with an $\text{Ins}(1,4,5)P_3$ 1-phosphatase from *Dictyostelium* (Van Lookeren Campagne *et al.*, 1988). $[^3\text{H}]\text{Ins}(1,3,4)P_3$ was synthesized from $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ with human erythrocyte ghosts.

$\text{Ins}(1,4,5)P_3$ 5-phosphatase was purified 50-fold from rat brain particulate fraction; the activity was 50 nmol/min per mg of protein at 37 °C and at 1 μM - $[^3\text{H}]\text{Ins}(1,4,5)P_3$ (C. Erneux, unpublished work). The hydrolysate of $\text{Ins}P_6$ (phytic acid; Sigma) was prepared as described by Wreggett & Irvine (1987).

Culture conditions

Dictyostelium cells were grown in association with *Escherichia coli* on buffered glucose-peptone agar, harvested in 10 mM- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.5 (PB), and starved for 3 h in this buffer.

Optimization of electroporation conditions

The standard conditions for electroporation were as follows. *Dictyostelium* cells were washed by centrifugation for 2 min at 100 g and resuspended in PB at a density of 2×10^8 cells/ml. Cells (1.5 ml) were mixed with 185 kBq (5 μCi) of $[^3\text{H}]$ inositol and placed in the electroporation chamber. Electroporation was performed

in a workshop-made apparatus which has 1 cm between the electrodes. Unless indicated otherwise, two pulses of 210 μs duration were given at 7 kV and at 5 s interval. Cells were immediately taken out of the electroporation chamber and incubated on ice for 10 min in PB with 2 mM- MgCl_2 and 0.2 mM- CaCl_2 . Cells were centrifuged (2 min, 100 g), and washed twice with PB; radioactivity was determined after lysis of the cells in 1% (w/v) SDS.

Electroporation for labelling inositol metabolites

Electroporation was done as described above, except that the concentration of $[^3\text{H}]$ inositol was 11.1 MBq/ml (300 μCi /ml). After electroporation cells were incubated for 10 min on ice in PB with $\text{Ca}^{2+}/\text{Mg}^{2+}$, centrifuged, washed twice with PB and shaken for an additional 45 min in PB at a density of 10^7 cells/ml. Cells were then washed twice and resuspended in PB at a density of 1.5×10^8 cells/ml.

Extraction and analysis of phospholipids

Phospholipids were analysed from $[^3\text{H}]$ inositol-labelled cells, which were obtained as described above, or from $[^{32}\text{P}]\text{P}_i$ -labelled cells. The latter cells were starved for 4 h in Bonner's salt solution (BSS) (Bonner, 1947), washed three times in BSS and incubated with 1.85 MBq (50 μCi) of $[^{32}\text{P}]\text{P}_i$ /ml at a density of 10^8 cells/ml. At the times indicated, 2×10^7 cells were washed with ice-cold BSS and resuspended in BSS to a density of 2×10^8 cells/ml. The extraction of phospholipids from ^3H - and ^{32}P -labelled cells was identical; 100 μl of the cell suspension was added to 500 μl of chloroform/methanol/conc. HCl (20:40:1, by vol.). After vigorous shaking, 200 μl of water was added, and samples were shaken for a further 10 min. Then samples were centrifuged for 2 min at 10000 g, and the upper water phase was removed. The organic phase was washed with 200 μl of water. The combined water phases were used for the analysis of water-soluble inositol phosphates (see below). The organic phase was taken from underneath the protein interphase, and this interphase was washed once with chloroform. The combined organic phases were dried completely under a stream of N_2 . Phospholipids were dissolved in 50 μl of chloroform/methanol (9:1, v/v) and applied to a heat-activated silica-gel 60 plate (Merck). T.l.c. of $[^3\text{H}]$ inositol-labelled phospholipids was usually performed in chloroform/methanol/ NH_3 (40%) /water (90:90:5:22, by vol.). ^{32}P -labelled phospholipids were usually analysed with chloroform/methanol/methylamine (40%) /water (90:36:5:5, by vol.). In some experiments, where cells were labelled with $[^3\text{H}]$ inositol and $[^{32}\text{P}]\text{P}_i$, thin-layer plates were also chromatographed in chloroform/methanol/acetic acid/acetone/water (40:13:12:15:8, by vol.).

Extraction and analysis of water-soluble inositol phosphates

A 100 μl portion of $[^3\text{H}]$ inositol-labelled cells was added to 100 μl of 3.5% (w/v) HClO_4 or 18% (w/v) trichloroacetic acid, both in the presence of 10 mM-EDTA and $\text{Ins}P_6$ hydrolysate (100 μg /ml). About 10 Bq of $[^{32}\text{P}]\text{Ins}(1,4,5)P_3$ was added, and after 10 min on ice samples were centrifuged for 2 min at 10000 g. The HClO_4 supernatant was neutralized with 50 μl of KHCO_3 (50% saturated at 20 °C) and the trichloroacetic acid supernatant was extracted with $3 \times 500 \mu\text{l}$ of water-saturated ether.

The extracts were chromatographed by several methods. The anion-exchange Dowex AG1X2 columns (0.5 ml) were eluted (1 ml fractions) with 10 ml each of water (for inositol), 60 mM-ammonium formate in 5 mM- $\text{Na}_2\text{B}_4\text{O}_7$ (GroPIIns), 150 mM-ammonium formate in 5 mM- $\text{Na}_2\text{B}_4\text{O}_7$ (InsP), 300 mM-ammonium formate in 100 mM-formic acid (InsP₂), 750 mM-ammonium formate in 100 mM-formic acid (InsP₃), and 1200 mM-ammonium formate in 100 mM-formic acid (InsP₄). The eluate was mixed with 4 ml of Instagel (Packard), and the radioactivity was determined by liquid-scintillation counting by using a dual-label program. The h.p.l.c. anion-exchange column (Partisil SAX; Whatman) was eluted at a flow rate of 1.0 ml/min with a gradient of ammonium formate up to 2.0 M, pH 3.4, as described in Fig. 6(b). The h.p.l.c. reversed-phase column LiChrosorb RP18 was eluted isocratically with 5 mM-tributylammonium phosphate/100 mM- KH_2PO_4 /10 % methanol, pH 4.0, at a flow rate of 1.2 ml/min. The eluate from the h.p.l.c. columns was divided in 34 s fractions and the radioactivity was determined with 4 ml of Instagel. It is important for ion-pair reversed-phase h.p.l.c. that the sample is diluted to 300 μl in 50 mM-tributylammonium phosphate in order to bind inositol phosphates to the reversed-phase column (P. J. M. Van Haastert, A. Vink & J. Van der Kaay, unpublished work).

Ins(1,4,5)P₃ kinase

Cells were lysed by pressing them through a Nuclepore filter (Das & Henderson, 1983) in 20 mM-Tris/HCl (pH 7.5)/400 μM -phenylmethanesulphonyl fluoride/5 μM -leupeptin/250 mM-sucrose. The extract was used immediately, or centrifuged for 5 min at 10000 g; the pellet was washed once in the lysis buffer, and the supernatant was re-centrifuged for 5 min at 160000 g. The incubation contained 700 Bq of Ins(1,4,5)P₃ or [4,5-³²P]Ins(1,4,5)P₃, 50 mM-Hepes/NaOH, pH 7.5, 0.1–10 mM-ATP, 2–

20 mM-MgCl₂, 5 mM-2-mercaptoethanol and 5 μl of enzyme in a total volume of 20 μl . The enzyme used was *Dictyostelium* lysate, 10000 g pellet or 160000 g supernatant, and/or rat brain 180000 g supernatant (Takazawa *et al.*, 1988). The reaction was terminated after 2–15 min by addition of 500 μl of chloroform/methanol/conc. HCl (20:40:1, by vol.); samples were vigorously shaken, and phase separation was induced by addition of 200 μl of water. The water phase was chromatographed on 0.5 ml Dowex (AG1X2) columns that were eluted with 10 ml of 300 mM-ammonium formate in 100 mM-formic acid (Ins + InsP + InsP₂), then with 10 ml of 750 mM-ammonium formate in 100 mM-formic acid [Ins(1,4,5)P₃] and finally with 10 ml of 1200 mM-ammonium formate in 100 mM-formic acid (InsP₄). Under these conditions, over 90 % of the radioactivity of authentic standards was eluted in the appropriate fraction.

RESULTS AND DISCUSSION

Dictyostelium cells have previously been labelled with [³H]inositol by shaking them in [³H]inositol for 6 h (Europe-Finner & Newell, 1987a,b). By this method cells take up about 0.2 % of the radioactivity. In early experiments, when we analysed the inositol phosphates by h.p.l.c. instead of on Dowex columns, we observed that only 0.1 % of the radioactivity taken up by the cells co-eluted with Ins(1,4,5)P₃. Obviously, economic reasons prevented a detailed analysis of the inositol cycle in *Dictyostelium* when each sample required the input of 3.7 MBq (0.1 mCi) of [³H]inositol.

Electroporation conditions

The uptake of [³H]inositol was considerably improved by electroporation of cells in the presence of [³H]inositol (Fig. 1). Optimal conditions are two 210 μs pulses at

Fig. 1. Conditions for optimal electroporation

Dictyostelium cells were treated with different numbers of pulses (a), at different voltages (b), and at different cell densities (c). The standard conditions were two pulses at 7 kV and 2×10^8 cells/ml (7 % cell density). After 10 min cells were washed twice and cell-associated radioactivity was determined.

Fig. 2. Kinetics of electroporation

(a) Cells were electroporized in the presence of [³H]-inositol and washed at the times indicated. (b) Cells were electroporized in the absence of [³H]inositol; then [³H]inositol was added at the times indicated, and cells were washed 10 min later. For the zero-time point, [³H]-inositol was present during electroporation.

7 kV. The uptake was linear with cell density and rather efficient, since about 2% of the radioactivity was taken up when the cells occupy about 7% of the volume of the electroporation chamber (at 2×10^8 cells/ml). After the electroschocks, cells had to be incubated with [³H]inositol for an additional 10 min (Fig. 2a). When [³H]inositol was added after electroporation, the radioactivity was rapidly excluded from the cells (Fig. 2b). These data suggest that cells seal after electroporation with a half-time of about 2 min.

Viability of cells after electroporation

The viability of the cells after electroporation was tested by plating them on agar supports. Cell aggregation was not affected by electroporation (results not shown). At 30 min after electroporation, cells reacted to the chemotactic signal cAMP with the activation of adenylate cyclase and guanylate cyclase (Fig. 3). Guanylate cyclase, but not adenylate cyclase, could even be activated within 30 s after electroporation, while cells were not yet sealed.

Metabolism of inositol to phospholipids

Dictyostelium cells were labelled with [³H]inositol by electroporation, or with [³²P]P_i. At regular time intervals phospholipids were extracted and separated by t.l.c. An example of such a separation is demonstrated in Fig. 4, showing that the ³H radioactivity was eluted as one major spot (90%) and three minor spots (each containing about 3%). These spots also contained ³²P radioactivity, and co-eluted in three different chromatographic systems (see Fig. 4 and the Materials and methods section). The phospholipids were tentatively identified by their chromatographic behaviour and kinetics of labelling (see below).

Fig. 3. Chemoattractant-induced accumulation of cGMP and cAMP

Cells were stimulated with 10 μ M-2'-deoxy-cAMP and 10 mM-dithiothreitol (a) or with 0.1 μ M-cAMP (b). Cells were lysed at the times indicated, and cAMP (a) or cGMP (b) was measured. The symbols indicate the response before electroporation (●), at 30 s after electroporation (○) and at 30 min after electroporation (×).

Fig. 4. T.l.c. of phospholipids

Phospholipids were extracted from *Dictyostelium* cells that were labelled with [^3H]inositol or [^{32}P]P_i, and separated on silica-gel 60 plates with chloroform/methanol/NH₃/water (90:90:5:22, by vol.). The plates were autoradiographed for the localization of ^{32}P and scraped in 0.5 or 0.25 cm fractions for the localization of ^3H -labelled compounds. Cells were labelled for 30 min with [^3H]inositol or for 30 min with [^{32}P]P_i. Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

The kinetics of incorporation of ^3H and ^{32}P radioactivity in these spots is shown in Fig. 5. After electroporation with [^3H]inositol, radioactivity was rapidly incorporated into PtdIns and more slowly into PtdInsP, PtdInsP₂ and lyso-PtdIns; optimal labelling of PtdInsP₂ was obtained at about 45 min after electroporation (Fig. 5). The kinetics of ^{32}P labelling was different; PtdInsP and PtdInsP₂ were rapidly labelled, and a steady state was reached within about 90 min. Incorporation of ^{32}P radioactivity into PtdIns (Fig. 5b), lyso-PtdIns, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (results not shown) was more slowly, and a steady state was not obtained within the time period of the experiment.

The metabolic route of phosphatidylinositol phosphates in mammalian cells has been described in detail by Downes & Michell (1985). PtdIns is formed in the endoplasmic reticulum from CDP-diacylglycerol and inositol; [^{32}P]phosphate in PtdIns (as well as in phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and lyso-PtdIns) is derived from the slowly labelled β -phosphate of CTP. PtdIns is then translocated to the plasma membrane, where it is phosphorylated to PtdInsP and PtdInsP₂; these additional [^{32}P]phosphates are derived from the rapidly labelled γ -phosphate of ATP. The kinetics of labelling of phosphatidylinositol phosphates with [^3H]inositol and [^{32}P]P_i in *Dictyostelium* is similar to the kinetics of labelling in mammalian cells, and may proceed via a similar metabolic pathway, since most of the required enzymes have been identified in *Dictyostelium* (Machon *et al.*, 1980; Varela *et al.*, 1987; Jimenez *et al.*, 1988; Van Lookeren Campagne *et al.*, 1988).

Fig. 5. Kinetics of phosphatidylinositol phosphate labelling with [^3H]inositol (a) or [^{32}P]P_i (b)

Phospholipids were extracted at the times indicated and separated by t.l.c. on silica-gel 60 plates developed in chloroform/methanol/NH₃/water (90:90:5:22, by vol.) (a) or in chloroform/methanol/methylamine/water (90:36:5:5) (b). Symbols: ●, PtdIns; ○, PtdInsP; ▲, PtdInsP₂; ×, lyso-PtdIns. For abbreviations in Fig. 5(b), see Fig. 4 legend; also PS, phosphatidylserine.

Extraction of water-soluble inositol phosphates and chromatography on Dowex columns

Three methods for the extraction of the water-soluble inositol phosphates were compared (see the Materials and methods section). In addition, the effect of EDTA and a hydrolysate of InsP₆ on the extraction efficiency was investigated, since these compounds may prevent the precipitation of inositol polyphosphates (Wreggett & Irvine, 1987). The results (not shown) reveal that the extraction of inositol polyphosphates was optimal with trichloroacetic acid in the presence of EDTA and a hydrolysate of InsP₆, the efficiency being over 95%.

The trichloroacetic acid extract of [^3H]inositol-labelled cells was chromatographed on Dowex columns, which were eluted with increasing concentrations of ammonium formate to separate Ins, GroPIns, InsP, InsP₂, InsP₃ and InsP₄ (Table 1). The results are similar to those reported by Europe-Finner & Newell (1987a,b), who found approx. 2-fold increases in the radioactivity eluted in the InsP₃ and InsP₄ fractions after stimulation of *Dictyo-*

Fig. 6. Separation of water-soluble inositol phosphates

An equal amount of a trichloroacetic acid extract from [³H]inositol-labelled cells with an internal standard of [³²P]Ins(1,4,5)P₃ was chromatographed on an open-bed Dowex anion-exchange column (a), a h.p.l.c. Partisil SAX anion-exchange column (b) or a h.p.l.c. LiChrosorb 10RP18 reversed-phase ion-pair column (c). The columns were eluted as described in the Materials and methods section, and the radioactivity in the eluate was determined. Symbols: Δ, ³H radioactivity, presented on two scales; ×, [³²P]Ins(1,4,5)P₃ (shaded peak). The arrows indicate the elution of authentic standards. For the inset in (c), the fractions

Table 1. Uptake and metabolism of [³H]inositol in *Dictyostelium*

Cells were labelled with [³H]inositol under standard conditions, and extracted with chloroform/methanol/HCl as described in the Materials and methods section. The organic phase was analysed by t.l.c. as described in the legend of Fig. 4. The water phase was spiked with 350 c.p.m. of [4,5-³²P]Ins(1,4,5)*P*₃ and analysed by Dowex columns as described in the Materials and methods section. The 'Dowex Ins*P*₃' and 'Dowex Ins*P*₄' fractions were concentrated and desalted by chromatography on a Sephadex G10 column (50 cm × 1.6 cm) that was eluted with 7% (v/v) propan-1-ol. The fractions containing radioactivity were combined, concentrated and analysed by h.p.l.c. on the anion exchange column Partisil SAX and the reversed-phase column LiChrosorb RP18 (see the Materials and methods and the Results sections).

Fraction	Radioactivity (c.p.m.)
Applied inositol	10 000 000
Taken up by the cells	2 437 15
In organic phase	123 918
In PtdIns	108 800
In lyso-PtdIns	8 674
In Ptd-Ins <i>P</i>	3 470
In PtdIns <i>P</i> ₂	2 970
In protein interphase	47 385
In water phase	71 734
In Dowex Inositol	49 241
In Dowex Gro <i>P</i> Ins	741
In Dowex Ins <i>P</i>	1 887
In Dowex Ins <i>P</i> ₂	1 152
In Dowex Ins <i>P</i> ₃	2 870
In h.p.l.c. Ins(1,4,5) <i>P</i> ₃	257
In Dowex Ins <i>P</i> ₄	11 495
In h.p.l.c. Ins(1,3,4,5) <i>P</i> ₄	< 20

Table 2. Cross-analysis of Ins*P*₃ on three separation systems

Equal amounts of radioactivity (70 000 c.p.m.) in the trichloroacetic acid extract from [³H]inositol-labelled cells were chromatographed on three columns, Dowex, Partisil SAX and LiChrosorb RP18; the samples also contained an internal standard of 350 c.p.m. of [³²P]Ins(1,4,5)*P*₃. The fractions containing ³²P radioactivity were isolated and reanalysed on the three columns as indicated. The fractions refer to the elution profiles of the columns as presented in Fig. 6. All data were corrected for the recovery of [³²P]Ins(1,4,5)*P*₃.

Purified		Re-analysed on		
Fraction	Radioactivity (c.p.m.)	Dowex	Partisil SAX	LiChrosorb RP18
Dowex Ins <i>P</i> ₃	2585	—	521 c.p.m. 'Ins <i>P</i> ₃ ' (20 %) 80 % not eluted	119 c.p.m. fraction 6 (5 %) 301 c.p.m. 'Ins <i>P</i> ₃ ' (12 %) 1907 c.p.m. fractions 70–80 (74 %)
SAX Ins <i>P</i> ₃	759	—	—	95 c.p.m. fraction 6 (13 %) 250 c.p.m. 'Ins <i>P</i> ₃ ' (33 %) 54 % not eluted
RP10 Ins <i>P</i> ₃	276	—	257 c.p.m. 'Ins <i>P</i> ₃ ' (93 %)	—
RP18 fraction 63	11 500	1271 c.p.m. 'Ins <i>P</i> ₃ ' (11 %) 9453 c.p.m. 'Ins <i>P</i> ₄ ' (82 %)	251 c.p.m. fraction 7 (2 %) 930 c.p.m. fractions 23–28 (8 %) 601 c.p.m. fraction 65 (5 %) 85 % not eluted	—

containing [³²P]Ins(1,4,5)*P*₃ were collected from four separations on this system, and each was concentrated and incubated at 37 °C in a total volume of 100 μl with 40 mM-Hepes/NaOH, 20 mM-MgCl₂ and 50 ng of partially purified Ins(1,4,5)*P*₃ 5-phosphatase from rat brain. The reactions were terminated by trichloroacetic acid, and the lysates were re-chromatographed on this system. The radioactivity in the Ins*P*₃ fraction was determined and expressed as a percentage of that from an identical incubation from which the 5-phosphatase was omitted.

Fig. 7. cAMP- and GTP[S]-mediated accumulation of Ins(1,4,5) P_3 .

(a) [^3H]inositol-labelled cells were stimulated with 0.1 μM -cAMP and lysed at the times indicated. The water-soluble and water-insoluble extracts were analysed respectively by h.p.l.c. as shown in Fig. 6(c) and by t.l.c. as shown in Fig. 4. Symbols: ●, Ins(1,4,5) P_3 ; ○, Ins P_2 ; ▲, PtdIns P_2 . (b) Cells were permeabilized with saponin (1 mg/ml) and 5 mM-ATP during the last 30 min period of the labelling with [^3H]inositol, then washed and stimulated with 0.1 μM -cAMP (●) or 100 μM -GTP[S] (▲). The results shown are means $\pm$ S.D. of triplicate determinations.

stelium cells with cAMP. We have reproduced their experiments with similar results (not shown). In these experiments we also analysed the inositol phospholipids, and observed no decrease in PtdIns P or PtdIns P_2 after stimulation of cells with cAMP (S.D. $\pm$ 5%), which was unexpected, since the radioactivity in PtdIns P_2 was only 2970 c.p.m., whereas that of the 'Dowex Ins P_3 ' fraction was 2870 c.p.m. (Table 2), and increased to about 4000 c.p.m. after stimulation of cells with cAMP. Therefore the elution of Ins P_3 from Dowex columns was analysed more carefully, with an internal standard of authentic [^{32}P]Ins(1,4,5) P_3 . It appears that the ^3H radioactivity in the Dowex Ins P_3 fraction was not eluted as a defined peak, and did not co-elute with [^{32}P]Ins(1,4,5) P_3 (Fig. 6a).

Chromatography of inositol polyphosphates by h.p.l.c.

The water-soluble fraction of [^3H]inositol-labelled cells with an internal standard of [^{32}P]Ins(1,4,5) P_3 was analysed by anion exchange h.p.l.c. on a Partisil SAX column (Fig. 6b) and by reversed-phase ion-pair h.p.l.c.

Table 3. Phosphorylation of Ins(1,4,5) P_3

Extracts of rat brain or *Dictyostelium* were incubated with [^3H]Ins(1,4,5) P_3 and ATP as described in the Materials and methods section. After the reaction, the incubation mixture was chromatographed on Dowex columns and the amount of Ins P_4 was determined. The negative result with *Dictyostelium* extract was reproduced at least 10 times, also under many other incubation conditions (see the Results section).

Enzyme preparation	Degradation (%)	Ins(1,4,5) P_3 (%)	Ins P_4 (%)
Rat brain	4	28	68
<i>Dictyostelium</i>	6	94	< 1
Rat brain + <i>Dictyostelium</i>	8	34	58

on a LiChrosorb RP18 column (Fig. 6c). Chromatography on the SAX column revealed a peak of radioactivity at fraction 32 that co-eluted with [^{32}P]Ins(1,4,5) P_3 . On the RP18 column, this peak of radioactivity was eluted in fraction 23 (Fig. 6c). However, the peak contained 750 c.p.m. on the SAX column and only 250 c.p.m. on the RP18 column, but the recovery of the internal standard [^{32}P]Ins(1,4,5) P_3 was about 80% in both cases.

In cross-analysis experiments, the water-soluble fraction of [^3H]inositol-labelled cells was chromatographed on the Dowex, the SAX or the RP18 column, and the fractions containing ^{32}P radioactivity were desalted, concentrated and re-analysed on the two other columns. The elution of ^3H radioactivity was corrected for the recovery of [^{32}P]Ins(1,4,5) P_3 , and the results of the experiments are presented in Table 2. About 20% and 12% of the ^3H radioactivity from the Dowex Ins P_3 fraction co-eluted with [^{32}P]Ins(1,4,5) P_3 on the SAX and RP18 column, respectively; the remaining radioactivity was not eluted from the SAX column, or was eluted partly from the RP18 column in fraction 6 (5%) and mainly in fractions 70–80 (74%). The radioactivity that was eluted from the SAX column in the Ins P_3 fraction was re-analysed on the RP18 column: only 33% co-eluted with [^{32}P]Ins(1,4,5) P_3 ; 13% was found in fraction 6, and 54% was not eluted from the column. Finally, the radioactivity in the Ins P_3 fraction from the RP18 column was re-analysed on the SAX column: essentially all radioactivity was recovered and co-eluted with [^{32}P]Ins(1,4,5) P_3 .

This cross-analysis demonstrates that, from the 70000 c.p.m. in the water-soluble extract of [^3H]inositol-labelled cells, no more than about 250 c.p.m. can possibly be Ins(1,4,5) P_3 ; analysis by Dowex and SAX columns overestimates Ins P_3 by about 2200 and 500 c.p.m. respectively. Since part of these contaminations is apparently eluted from the RP18 column at the end of the chromatogram (in fractions 68–75), we have also purified this radioactivity and re-analysed its chromatography on Dowex and SAX columns. The results (Table 2) demonstrate that the radioactivity was eluted from the Dowex column in the Ins P_3 fraction (11%) and Ins P_4 fraction (82%); however, most of the radioactivity (85%) was not eluted from the SAX column, and only small amounts

were eluted in fraction 7 (2%), fractions 23–28 (8%) and fraction 65 (5%).

The results of Table 2 suggest that the RP18 column may be the only separation system that can be used for the quantification of [^3H]Ins(1,4,5) P_3 in *Dictyostelium*. To prove that the ^3H radioactivity in this peak is present in Ins(1,4,5) P_3 we have purified the ^3H -labelled compound with an internal standard of [^{32}P]Ins(1,4,5) P_3 and incubated the mixture with a partially purified Ins(1,4,5) P_3 5-phosphatase from rat brain. The results (Fig. 6c, inset) demonstrate that the compounds containing ^3H and ^{32}P radioactivity were degraded at approximately the same rate, which identifies the ^3H -labelled compound as Ins(1,4,5) P_3 .

The chromatographic conditions for the RP18 column were optimized for the separation of Ins(1,3,4) P_3 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 (Fig. 6c). During the analysis we have never found a significant amount of radioactivity in Ins(1,3,4) P_3 , Ins(1,3,4,5) P_4 or another isomer of Ins P_4 (see also Fig. 6b). The peak of radioactivity that was eluted in fraction 63 of the SAX column has the chromatographic behaviour of Ins P_6 , but was not analysed further.

cAMP- and GTP[S]-mediated accumulation of Ins(1,4,5) P_3

The kinetics of Ins(1,4,5) P_3 accumulation after stimulation of sensitive cells with cAMP is shown in Fig. 7(a). Ins(1,4,5) P_3 increased by about 40–50%, with a maximum at about 9 s after stimulation. Although the increase is relatively small, statistical analysis shows that it is very significant, owing to the accurate method for detection of Ins(1,4,5) P_3 (S.D. 6%). The amounts of PtdIns P_2 , Ins P_2 and the large peak at the end of the reversed-phase h.p.l.c. chromatogram did not significantly change after chemotactic stimulation. Furthermore, no radioactivity was eluted at the position of Ins(1,3,4) P_3 or at or close to the position of Ins(1,3,4,5) P_4 in any of the samples of Fig. 7(a) (results not shown).

The accumulation of Ins(1,4,5) P_3 after stimulation with GTP[S] was investigated in saponin-permeabilized cells. The results show a small increase in Ins(1,4,5) P_3 after stimulation of saponin-permeabilized cells with cAMP or GTP[S] similar to that in non-permeabilized cells with cAMP (Fig. 7b).

Ins(1,4,5) P_3 kinase *in vitro*

The phosphorylation of Ins(1,4,5) P_3 by a putative kinase was investigated in a lysate of *D. discoideum* under conditions in which the Ins(1,4,5) P_3 3-kinase from rat brain shows optimal activity (Table 3). About 70% of Ins(1,4,5) P_3 was converted into Ins(1,3,4,5) P_4 by the rat brain extract, but no Ins P_4 was formed in the extract of *D. discoideum*. Furthermore, a mixture of *D. discoideum* and rat brain extracts shows approximately the activity of the rat brain extract, indicating that *D. discoideum* has no inhibitory or degradative influence on the Ins(1,4,5) P_3 3-kinase from rat brain (Table 3). We have not found Ins(1,4,5) P_3 kinase activity in *D. discoideum* under various conditions (results not shown), including variation of the ATP concentration between 0.1 and 10 mM, the Mg^{2+} concentration between 2 and 20 mM, and analysis in the pellet or the high-speed supernatant of the extract. Also the inclusion of 0.1–100 μM - Ca^{2+} , 10 mM- Li^+ , 0.25 mM-2,3-bisphosphoglycerate, or an ATP-regeneration system, did not yield detectable amounts of Ins P_4 . It is

possible that under these conditions the Ins P_4 formed is immediately dephosphorylated to an isomer of Ins P_3 . This was investigated in the two experiments. First, a mixture of [^3H]Ins(1,4,5) P_3 and [4,5- ^{32}P]Ins(1,4,5) P_3 was incubated under conditions for kinase activity, and the ratio of $^3\text{H}/^{32}\text{P}$ radioactivity in the Ins P_3 fraction was monitored. This $^3\text{H}/^{32}\text{P}$ ratio did not alter during the incubation (results not shown). Second, [4,5- ^{32}P]Ins(1,4,5) P_3 was incubated under conditions for kinase activity, mixed with [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4,5) P_4 after the reaction was terminated, and analysed by h.p.l.c. in the reversed-phase system as described in Fig. 6(c). We observed that all the ^{32}P label in Ins P_3 exactly co-migrated with the internal standard [^3H]Ins(1,4,5) P_3 and no ^{32}P label was eluted at or near the position of [^3H]Ins(1,3,4,5) P_4 (results not shown). Both experiments suggest that the Ins P_3 isomer after the kinase reaction is still Ins(1,4,5) P_3 . Finally, we have analysed the phosphorylation of Ins, Ins1 P , Ins4 P , Ins(1,4) P_2 and Ins(4,5) P_2 , but did not detect a phosphorylated product under the conditions used (results not shown). Although these negative results are not conclusive, none of the experiments provide evidence for the phosphorylation of Ins(1,4,5) P_3 to an isomer of Ins P_4 in *D. discoideum*.

CONCLUSIONS

Although Ins(1,4,5) P_3 induces Ca^{2+} release from non-mitochondrial pools and the activation of guanylate cyclase in *Dictyostelium* (Europe-Finner & Newell, 1985, 1986a), experiments designed to measure a receptor-stimulated accumulation of Ins(1,4,5) P_3 have not provided evidence for the identification of this compound (Europe-Finner & Newell, 1987a,b). The results reported here in fact demonstrate that analysis of inositol polyphosphates by Dowex (and probably also Partisil SAX) columns did not detect Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4 . We have used several techniques to improve labelling with [^3H]inositol and to identify and analyse several inositol metabolites, notably Ins(1,4,5) P_3 .

At the end of this analysis we have shown that cAMP induces a transient increase in Ins(1,4,5) P_3 , with a maximum at about 9 s after stimulation. In saponin-permeabilized cells cAMP or GTP[S] induces a similar accumulation of Ins(1,4,5) P_3 . Since we have not been able to demonstrate that cAMP or GTP[S] induces a decrease in PtdIns P_2 , we have no formal evidence that the increase in Ins(1,4,5) P_3 is produced by the receptor- and G-protein-mediated activation of a phospholipase C. Conclusive evidence for this reaction probably has to come from the detection *in vitro* of this enzymic conversion.

During the analysis of inositol phosphates from *Dictyostelium* cells we have never detected radioactivity in Ins(1,3,4,5) P_4 or Ins(1,3,4) P_3 ; we have also been unable to demonstrate Ins P_3 kinase activity in *Dictyostelium* extracts. This could imply that *Dictyostelium* does not have the phosphorylation/dephosphorylation pathway of Ins(1,4,5) P_3 that is present in most vertebrate cells (Majerus *et al.*, 1988). It is possible that Ins(1,4,5) P_3 follows a different metabolic route in *Dictyostelium* from that in mammalian cells, since we have observed that *in vitro* Ins(1,4,5) P_3 is dephosphorylated mainly to Ins(4,5) P_2 by a specific 1-phosphatase and partly to Ins(1,4) P_2 by a specific 5-phosphatase (Van Lookeren Campagne *et al.*, 1988). The metabolism of Ins(1,4,5) P_3 *in vitro* has to be confirmed by the analysis of the Ins P_2

and InsP isomers that are formed *in vivo* before and after stimulation of cells with cAMP.

In summary, we have explored the inositol cycle of the eukaryotic micro-organism *Dictyostelium*, and present conditions for the quantification of Ins(1,4,5) P_3 after stimulation of cells with the chemoattractant cAMP and the non-hydrolysable GTP analogue GTP[S]. These conditions can now be used for experiments in which *Dictyostelium* can be advantageous: the analysis of signal-transducing mutants and transformants with modified genes of signal-transducing components.

We thank Bert Van Duijn and Kees Donkersloot for the design and construction of the electroporation instrument. This study was supported by a grant to P.J.M.V.H. from the C. and C. Huygens Fund, which is subsidized by the Netherlands Organization for Scientific Research, and to C.E. under contract of the Ministère de la Politique Scientifique (Belgium).

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Received 28 July 1988/20 October 1988; accepted 4 November 1988