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

van Haastert, Petrus; Vries, Martinus J. de; Penning, Louis C.; Roovers, Edwin; Kaay, Jeroen van der; Erneux, Christophe; Lookeren Campagne, Michiel M. van

Published in:
Default journal

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

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

Publication date:
1989

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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

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

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chemoattractant and guanosine 5'-[γ-thio]triphosphate induce the accumulation of inositol 1,4,5-trisphosphate in Dictyostelium cells that are labelled with [3H]inositol by electroporation

Peter J. M. VAN HAASTERT,* Martinus J. DE VRIES,* Louis C. PENNING,* Edwin ROOVERS,* Jeroen VAN DER KAAY,* Christophe ERNEUX† and Michiel M. VAN LOOKEREN CAMPAGNE*‡
*Cell Biology and Genetics Unit, Zoological Laboratory, University of Leiden, P.O. Box 9316, NL-2300 Leiden, The Netherlands, and †Institute of Interdisciplinary Research (IRIBHN), School of Medicine, Free University of Brussels (U.L.B.), Campus Erasme (Bât. C), Route de Lennik 808, B-1070 Brussels, Belgium.

The analysis of the inositol cycle in Dictyostelium discoideum cells is complicated by the limited uptake of [3H]inositol (0.2% of the applied radioactivity in 6 h), and by the conversion of [3H]inositol into water-soluble inositol metabolites that are eluted near the position of inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] on anion-exchange h.p.l.c. columns. The uptake was improved to 2.5% by electroporation of cells in the presence of [3H]inositol; electroporation was optimal at two 210 μs pulses of 7 kV. Cells remained viable and responsive to chemotactic signals after electroporation. The intracellular [3H]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)P3 did not co-elute with authentic [32P]Ins(1,4,5)P3 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 [3H]Ins(1,4,5)P3 was identified by (a) co-elution with authentic [32P]Ins(1,4,5)P3 and (b) degradation by a partially purified Ins(1,4,5)P3 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)P3; we did not detect radioactivity in inositol 1,3,4trisphosphate or inositol 1,3,4,5-tetakisphosphate [Ins(1,3,4,5)P3]. In vitro, Ins(1,4,5)P3 was metabolized to inositol 1,4- and 4,5-bisphosphate, but not to Ins(1,3,4,5)P4 or another tetakisphosphate 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)P3 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)P3 induces the accumulation of cGMP in saponin-permeabilized Dictyostelium cells (Europe-Finner & Newell, 1985). Furthermore, Ins(1,4,5)P3 releases Ca2+ from non-mitochondrial pools, and Ins(1,4,5)P3 and Ca2+ 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)P3 in Ca2+ and cGMP metabolism in Dictyostelium.

In mammalian cells, Ins(1,4,5)P3 is formed by the agonist and G-protein stimulation of a specific phospholipase C that hydrolyses PtdIns(4,5)P2 (reviewed by Berridge, 1987). Ins(1,4,5)P3 is dephosphorylated to Ins(1,4)P2 or phosphorylated to Ins(1,3,4,5)P4, which is then dephosphorylated to Ins(1,3,4)P2. The dephosphorylation of Ins(1,4,5)P3 in Dictyostelium in vitro follows at least two routes. A 2,3-bisphosphoglycerate-sensitive enzyme yields Ins(1,4)P2, and a Li+-sensitive enzyme yields Ins(4,5)P2; both InsP2 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)P3 in Dictyostelium revealed an increase in radioactivity that was eluted from Dowex columns in the InsP2 and InsP3 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 PtdInsP2 and 'Dowex InsP3', a doubling of radioactivity in 'Dowex InsP3' after stimulation of cells with cAMP.

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; PtdIns, phosphatidylinositol phosphate; PtdInsP2, phosphatidylinositol bisphosphate; Ins, InsP2, InsP3, InsP4, etc., myo-inositol mono-, bis-, tris- (etc.) phosphates with isomeric numbering (all -) as appropriates; GroPIns, glycerophospho-myo-inositol; GTP[S], guanosine 5'-[γ-thio]triphosphate; PB, 10 mm-KH2PO4/Na2HPO4, pH 6.5.

† Present address: Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY10032, U.S.A.
but no detectable decrease in radioactivity in PtdInsP₂.
Furthermore, analysis of the radioactivity in the ‘Dowex
InsP₃’ fraction by h.p.l.c. revealed that only about 10% of
it co-eluted with authentic Ins(1,4,5)P₃. 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), PtdInsP-kinase (M. M. Van
Lookeren Campagne, unpublished work) and (poly)-
phosphoinositide phosphatases (Van Lookeren Campagne
et al., 1988).

We have re-evaluated the receptor- and G-protein-
stimulated formation of Ins(1,4,5)P₃ in Dictyostelium.
The uptake of [³H]inositol was improved from 0.2% to
2.5% by electroporation of Dictyostelium cells. The
metabolism of [³H]inositol to PtdIns, PtdInsP and
PtdInsP₂ was identified by t.l.c., and that to [³H]Ins-
(1,4,5)P₃ by ion-pair reversed-phase h.p.l.c. We show
that the chemoattractant cAMP and the non-
hydrolysable GTP analogue GDP[S] induce a small
transient increase in the radioactivity in Ins(1,4,5)P₃. On
the contrary, we did not detect radioactivity being eluted as
Ins(1,3,4)P₃ or Ins(1,3,4,5)P₄. In addition, no detectable
Ins(1,4,5)P₃ 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 Ins(1,4,5)P₃ may be different.

MATERIALS AND METHODS

Materials

L-myo-[1,2-³H]inositol (1500 GBq/mmoll), [³H]Ins₁P
(200 GBq/mmoll), [³H]Ins₄P (56 GBq/mmoll), [³H]-
Ins(1,4)P₂ (93 GBq/mmoll), [³H]Ins(1,4,5)P₃ (740 GBq/
mmoll), [⁴,⁵-³²P]Ins(1,4,5)P₃ (6030 GBq/mmoll) and
[³H]Ins(1,3,4,5)P₄ (185 GBq/mmoll) were from New
England Nuclear. [³H]Ins(4,5)P₂ was synthesized from
[³H]Ins(1,4,5)P₃ with an Ins(1,4,5)P₃ 1-phosphatase from
Dictyostelium (Van Lookeren Campagne et al., 1988).
[³H]Ins(1,3,4)P₃ was synthesized from [³H]Ins-
(1,3,4,5)P₄ with human erythrocyte ghosts.

Ins(1,4,5)P₃ 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[³H]-
Ins(1,4,5)P₃ (C. Erneux, unpublished work). The
hydrolysat of InsP₃ (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-KH₂PO₄/Na₂HPO₄, 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 centri-
fugation for 2 min at 100 g and resuspended in PB at
a density of 2 × 10⁶ cells/ml. Cells (1.5 ml) were mixed
with 185 kBq (5 μCi) of [³H]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-MgCl₂ and 0.2 mm-CaCl₂. 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 [³H]inositol was 11.1 MBq/ml
(300 μCi/ml). After electroporation cells were incubated
for 10 min on ice in PB with Ca²⁺/Mg²⁺, centrifuged,
twice washed with PB and shaken for an additional
45 min in PB at a density of 10⁶ cells/ml. Cells were then
washed twice and resuspended in PB at a density of
1.5 × 10⁶ cells/ml.

Extraction and analysis of phospholipids

Phospholipids were analysed from [³H]inositol-labelled
cells, which were obtained as described above, or from
[³²P]P₃-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 [³²P]P₃/ml at a density of 10⁶ cells/ml. At the times
indicated, 2 × 10⁷ cells were washed with ice-cold BSS
and resuspended in BSS to a density of 2 × 10⁶ cells/ml.
The extraction of phospholipids from [³H]- and [³²P]-
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 N₂. 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 [³H]inositol-labelled phospholipids was used
usually performed in chloroform/methanol/NH₄ (40 \%/water
(90:90:5:22, by vol.). [³²P]-labelled phospholipids were
usually analysed with chloroform/methanol/methyl-
amine (40 %)/water (90:36:5:5, by vol). In some
experiments, where cells were labelled with [³H]inositol
and [³²P]P₃, thin-layer plates were also chromato-
graphed in chloroform/methanol/acetic acid/acetone

Extraction and analysis of water-soluble inositol
phosphates

A 100 μl portion of [³H]inositol-labelled cells was
added to 100 μl of 3.5% (w/v) HClO₄ or 18% (w/v)
trichloroacetic acid, both in the presence of 10 mm-
EDTA and InsP₃ hydrolysate (100 μg/ml). About 10 Bq
of [³²P]Ins(1,4,5)P₃ was added, and after 10 min on ice
samples were centrifuged for 2 min at 10000 g. The
HClO₄ supernatant was neutralized with 50 μl of
KHCO₃ (50 % saturated at 20 °C) and the trichloroacetic
acid supernatant was extracted with 3 × 500 μl 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-Na₂B₄O₇ (GroPln5), 150 mM-ammonium formate in 5 mM-Na₂B₄O₇ (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 then washed 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 (Partisol 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-K₂PO₄/10% 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 mM-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 lystate, 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 [³²H]inositol by shaking them in [³²H]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 [³²H]inositol.

Electroporation conditions

The uptake of [³²H]inositol was considerably improved by electroporation of cells in the presence of [³²H]inositol (Fig. 1). Optimal conditions are two 210 µs pulses at

![Fig. 1. Conditions for optimal electroporation](image-url)
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 x 10^6 cells/ml). After the electroshocks, 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. 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 mM-2'-deoxy-cAMP and 10 mM-dithiothreitol (a) or with 0.1 mM-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 (×).

P. J. M. Van Haastert and others
The inositol cycle of Dictyostelium

The kinetics of incorporation of \(^{3}\text{H}\) and \(^{32}\text{P}\) radioactivity in these spots is shown in Fig. 5. After electro- portrayal with \(^{3}\text{H}\)inositol, radioactivity was rapidly incorporated into PtdIns and more slowly into PtdIns\(_{2}\), PtdIns\(_{4}\) and lyso-PtdIns; optimal labelling of PtdIns\(_{4}\) was obtained at about 45 min after electro- portrayal (Fig. 5). The kinetics of \(^{3}\text{H}\) labelling was different; PtdIns\(_{2}\) and PtdIns\(_{4}\) were rapidly labelled, and a steady state was reached within about 90 min. Incorporation of \(^{32}\text{P}\) 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; \(^{32}\text{P}\)phosphate in PtdIns (as well as in phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and lyso-PtdIns) is derived from the slowly labelled \(\beta\)-phosphate of CTP. PtdIns is then translocated to the plasma membrane, where it is phosphorylated to PtdIns\(_{2}\) and PtdIns\(_{4}\); these additional \(^{32}\text{P}\)phosphates are derived from the rapidly labelled \(\gamma\)-phosphate of ATP. The kinetics of labelling of phosphatidylinositol phosphates with \(^{3}\text{H}\)inositol and \(^{32}\text{P}\)P 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. 4. T.l.c. of phospholipids

Phospholipids were extracted from Dictyostelium cells that were labelled with \(^{3}\text{H}\)inositol or \(^{32}\text{P}\)P, and separated on silica-gel 60 plates with chloroform/methanol/N\(_{2}\)/water (90:90:5:22, by vol.). The plates were autoradiographed for the localization of \(^{3}\text{H}\)P and scraped in 0.5 or 0.25 cm fractions for the localization of \(^{3}\text{H}\)-labelled compounds. Cells were labelled for 30 min with \(^{3}\text{H}\)inositol or for 30 min with \(^{32}\text{P}\)P. Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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

Phospholipids were extracted at the times indicated and separated by t.l.c. on silica-gel 60 plates developed in chloroform/methanol/N\(_{2}\)/water (90:90:5:22, by vol.) (a) or in chloroform/methanol/methylamine/water (90:36:5:5) (b). Symbols: \(\bullet\), PtdIns; \(\bigcirc\), PtdIns\(_{2}\); \(\Delta\), PtdIns\(_{4}\); \(\times\), 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 Ins\(_{3}\) 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 Ins\(_{3}\), the efficiency being over 95%.

The trichloroacetic acid extract of \(^{3}\text{H}\)inositol-labelled cells was chromatographed on Dowex columns, which were eluted with increasing concentrations of ammonium formate to separate Ins, GroPIns, Ins\(_{2}\), Ins\(_{3}\), Ins\(_{4}\) and Ins\(_{5}\) (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 Ins\(_{3}\) and Ins\(_{4}\) 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...
The inositol cycle of Dictyostelium

Table 1. Uptake and metabolism of \([H]i\)nositol in Dictyostelium

Cells were labelled with \([H]i\)nositol 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 \([4,5-\textit{32P}]\text{Ins}(1,4,5)\text{P}_3\) and analysed by Dowex columns as described in the Materials and methods section. The ‘Dowex InsP\(_3\)’ and ‘Dowex InsP\(_4\)’ fractions were concentrated and desalted by chromatography on a Sephadex G10 column (50 cm x 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).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied inositol</td>
<td>10000000</td>
</tr>
<tr>
<td>Taken up by the cells</td>
<td>243715</td>
</tr>
<tr>
<td>In organic phase</td>
<td></td>
</tr>
<tr>
<td>In PtdIns</td>
<td>108800</td>
</tr>
<tr>
<td>In lyso-PtdIns</td>
<td>8674</td>
</tr>
<tr>
<td>In PtdIns(_P)</td>
<td>3470</td>
</tr>
<tr>
<td>In PtdIns(_P)</td>
<td>2970</td>
</tr>
<tr>
<td>In protein interphase</td>
<td></td>
</tr>
<tr>
<td>In Dowex InsP(_3)</td>
<td>47385</td>
</tr>
<tr>
<td>In Dowex InsP(_3)</td>
<td>71734</td>
</tr>
<tr>
<td>In Dowex InsP(_3)</td>
<td>49241</td>
</tr>
<tr>
<td>In Dowex GroPIns</td>
<td>741</td>
</tr>
<tr>
<td>In Dowex InsP(_4)</td>
<td>1152</td>
</tr>
<tr>
<td>In Dowex InsP(_4)</td>
<td>2870</td>
</tr>
<tr>
<td>In h.p.l.c. Ins(1,4,5)(_P)</td>
<td>257</td>
</tr>
<tr>
<td>In Dowex InsP(_4)</td>
<td>11495</td>
</tr>
<tr>
<td>In h.p.l.c. Ins(1,3,4,5)(_P)</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

Table 2. Cross-analysis of InsP\(_3\) on three separation systems

Equal amounts of radioactivity (70000 c.p.m.) in the trichloroacetic acid extract from \([H]i\)nositol-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 \([\textit{32P}]\text{Ins}(1,4,5)\text{P}_3\). The fractions containing \(\textit{32P}\) 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 \([\textit{32P}]\text{Ins}(1,4,5)\text{P}_3\).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Purified Radioactivity (c.p.m.)</th>
<th>Dowex Radioactivity (c.p.m.)</th>
<th>Partisil SAX Radioactivity (c.p.m.)</th>
<th>LiChrosorb RP18 Radioactivity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dowex InsP(_3)</td>
<td>2585</td>
<td>–</td>
<td>521 c.p.m. ‘InsP(_3)’ (20%)</td>
<td>119 c.p.m. fraction 6 (5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80% not eluted</td>
<td>301 c.p.m. ‘InsP(_3)’ (12%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1907 c.p.m. fractions 70–80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(74%)</td>
</tr>
<tr>
<td>SAX InsP(_3)</td>
<td>759</td>
<td>–</td>
<td></td>
<td>95 c.p.m. fraction 6 (13%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250 c.p.m. ‘InsP(_3)’ (33%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54% not eluted</td>
</tr>
<tr>
<td>RP10 InsP(_3)</td>
<td>276</td>
<td>–</td>
<td>257 c.p.m. ‘InsP(_3)’ (93%)</td>
<td>–</td>
</tr>
<tr>
<td>RP18 fraction 63</td>
<td>11500</td>
<td>1271 c.p.m. ‘InsP(_3)’ (11%)</td>
<td>251 c.p.m. fraction 7 (2%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9453 c.p.m. ‘InsP(_3)’ (82%)</td>
<td>930 c.p.m. fractions 23–28</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>601 c.p.m. fraction 65 (5%)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>85% not eluted</td>
<td>–</td>
</tr>
</tbody>
</table>

bottom\footnote{containing \([\textit{32P}]\text{Ins}(1,4,5)\text{P}_3\) were collected from four separations on this system, and each was concentrated and incubated at 37 °C in a total volume of 100 \(\mu\)l with 40 mm-Hepes/NaOH, 20 mm-MgCl\(_2\) 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 InsP\(_3\) fraction was determined and expressed as a percentage of that from an identical incubation from which the 5-phosphatase was omitted.}
with (Fig. 6b) was analysed by experiments and observed that the water-soluble Chromatography of inositol of the Dowex columns and the amount of InsP₄ was determined. The negative result with *Dictyostelium* extract was reproduced at least 10 times, also under many other incubation conditions (see the Results section).

![Fig. 7. cAMP- and GTP[S]-mediated accumulation of Ins(1,4,5)P₃](image)

(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 show in Fig. 4. Symbols: ●, Ins(1,4,5)P₃; ○, InsP₂; ▲, PtdInsP₂. (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 ± S.D. of triplicate determinations.

*Dictyostelium* 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 PtdInsP or PtdInsP after stimulation of cells with cAMP (s.d. ± 5%), which was unexpected, since the radioactivity in PtdInsP was only 2970 c.p.m., whereas that of the ‘Dowex InsP₃’ 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 InsP₃ from Dowex columns was analysed more carefully, with an internal standard of authentic [³²P]Ins(1,4,5)P₃. It appears that the [³H] radioactivity in the Dowex InsP₃ fraction was not eluted as a defined peak, and did not co-elute with [³²P]Ins(1,4,5)P₃ (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 [³²P]Ins(1,4,5)P₃ was analysed by anion exchange h.p.l.c. on a Partisol SAX column (Fig. 6b) and by reversed-phase ion-pair h.p.l.c. on a LiChrosorb RP18 column (Fig. 6c). Chromatography on the SAX column revealed a peak of radioactivity at fraction 32 that co-eluted with [³²P]Ins(1,4,5)P₃. 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 [³²P]Ins(1,4,5)P₃ 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 [³²P] radioactivity were desalted, concentrated and re-analysed on the two other columns. The elution of [³H] radioactivity was corrected for the recovery of [³²P]Ins(1,4,5)P₃, and the results of the experiments are presented in Table 2. About 20% and 12% of the [³H] radioactivity from the Dowex InsP₃ fraction co-eluted with [³²P]Ins(1,4,5)P₃ 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 InsP₃ fraction was re-analysed on the RP18 column: only 33% co-eluted with [³²P]Ins(1,4,5)P₃; 13% was found in fraction 6, and 54% was not eluted from the column. Finally, the radioactivity in the InsP₃ fraction from the RP18 column was re-analysed on the SAX column: essentially all radioactivity was recovered and co-eluted with [³²P]Ins(1,4,5)P₃.

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)P₃; analysis by Dowex and SAX columns overestimates InsP₃ 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 InsP₃ fraction (11%) and InsP₃ fraction (82%); however, most of the radioactivity (85%) was not eluted from the SAX column, and only small amounts

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Degradation (%)</th>
<th>Ins(1,4,5)P₃ (%)</th>
<th>InsP₄ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>4</td>
<td>28</td>
<td>68</td>
</tr>
<tr>
<td><em>Dictyostelium</em></td>
<td>6</td>
<td>94</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Rat brain +</td>
<td>8</td>
<td>34</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 3. Phosphorylation of Ins(1,4,5)P₃

Extracts of rat brain or *Dictyostelium* were incubated with [³H]Ins(1,4,5)P₃ 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 InsP₄ was determined. The negative result with *Dictyostelium* extract was reproduced at least 10 times, also under many other incubation conditions (see the Results section).
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)P3 in *Dictyostelium*. To prove that the 3H radioactivity in this peak is present in Ins(1,4,5)P3 we have purified the 3H-labelled compound with an internal standard of [32P]Ins(1,4,5)P3 and incubated the mixture with a partially purified Ins(1,4,5)P3 5-phosphatase from rat brain. The results (Fig. 6c, inset) demonstrate that the compounds containing 3H and 32P radioactivity were degraded at approximately the same rate, which identifies the 3H-labelled compound as Ins(1,4,5)P3.

The chromatographic conditions for the RP18 column were optimized for the separation of Ins(1,3,4)P3, Ins(1,4,5)P3 and Ins(1,3,4,5)P3 (Fig. 6c). During the analysis we have never found a significant amount of radioactivity in Ins(1,3,4)P3, Ins(1,3,4,5)P3 or another isomer of InsP3 (see also Fig. 6b). The peak of radioactivity that was eluted in fraction 63 of the SAX column has the chromatographic behaviour of InsP3, but was not analysed further.

**cAMP- and GTP[S]-mediated accumulation of Ins(1,4,5)P3**

The kinetics of Ins(1,4,5)P3 accumulation after stimulation of sensitive cells with cAMP is shown in Fig. 7(a). Ins(1,4,5)P3 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)P3 (s.d. 6%). The amounts of PtdInsP2, InsP2 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)P3 or at or close to the position of Ins(1,3,4,5)P3 in any of the samples of Fig. 7(a) (results not shown).

The accumulation of Ins(1,4,5)P3 after stimulation with GTP[S] was investigated in saponin-permeabilized cells. The results show a small increase in Ins(1,4,5)P3 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)P3 kinase in vitro**

The phosphorylation of Ins(1,4,5)P3 by a putative kinase was investigated in a lysate of *D. discoideum* under conditions in which the Ins(1,4,5)P3 3-kinase from rat brain shows optimal activity (Table 3). About 70% of Ins(1,4,5)P3 was converted into Ins(1,3,4,5)P3 by the rat brain extract, but no InsP3 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)P3 3-kinase from rat brain (Table 3). We have not found Ins(1,4,5)P3 kinase activity in *D. discoideum* under various conditions (results not shown), including variation of the ATP concentration between 0.1 and 10 mm, the Mg2+ 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-Ca2+, 10 mm-Li+, 0.25 mm-2,3-bisphosphoglycerate, or an ATP-regeneration system, did not yield detectable amounts of InsP3. It is possible that under these conditions the InsP3 formed is immediately dephosphorylated to an isomer of InsP3. This was investigated in the two experiments. First, a mixture of [3H]Ins(1,4,5)P3 and [4,5-32P]Ins(1,4,5)P3 was incubated under conditions for kinase activity, and the ratio of 3H/32P radioactivity in the InsP3 fraction was monitored. This 3H/32P ratio did not alter during the incubation (results not shown). Second, [4,5-32P]-Ins(1,4,5)P3 was incubated under conditions for kinase activity, mixed with [3H]Ins(1,4,5)P3 and [3H]Ins(1,3,4,5)P3 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 32P label in InsP3 exactly co-migrated with the internal standard [3H]Ins(1,4,5)P3 and no 32P label was eluted at or near the position of [3H]Ins(1,3,4,5)P3 (results not shown). Both experiments suggest that the InsP3 isomer after the kinase reaction is still Ins(1,4,5)P3. Finally, we have analysed the phosphorylation of Ins, InsP, Ins4P, Ins(4,5)P2 and Ins(4,5)P3, 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)P3 to an isomer of InsP3 in *D. discoideum*.

**CONCLUSIONS**

Although Ins(1,4,5)P3 induces Ca2+ release from non-mitochondrial pools and the activation of guanylate cyclase in *Dictyostelium* (Europe-Finnér & Newell, 1985, 1986a), experiments designed to measure a receptor-stimulated accumulation of Ins(1,4,5)P3 have not provided evidence for the identification of this compound (Europe-Finnér & 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)P3 or Ins(1,3,4,5)P3. We have used several techniques to improve labelling with [3H]inositol and to identify and analyse several inositol metabolites, notably Ins(1,4,5)P3.

At the end of this analysis we have shown that cAMP induces a transient increase in Ins(1,4,5)P3, 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)P3. Since we have not been able to demonstrate that cAMP or GTP[S] induces a decrease in PtdInsP3, we have no formal evidence that the increase in Ins(1,4,5)P3 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 polyphosphates from *Dictyostelium* cells we have never detected radioactivity in Ins(1,3,4,5)P3 or Ins(1,3,4,5)P3; we have also been unable to demonstrate InsP3 kinase activity in *Dictyostelium* extracts. This could imply that *Dictyostelium* does not have the phosphorylation/dephosphorylation pathway of Ins(1,4,5)P3 that is present in most vertebrate cells (Majerus et al., 1988). It is possible that Ins(1,4,5)P3 follows a different metabolic route in *Dictyostelium* from that in mammalian cells, since we have observed that in vitro Ins(1,4,5)P3 is dephosphorylated mainly to Ins(4,5)P2 by a specific 1-phosphatase and partly to Ins(1,4)P2 by a specific 5-phosphatase (Van Looikeren Campagne et al., 1988). The metabolism of Ins(1,4,5)P3 in vivo has to be confirmed by the analysis of the InsP3.
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)P₃ after stimulation of cells with the chemoattractant cAMP and the non-hydrolysable GTP analogue GDP[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).

REFERENCES


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