# Phospholipase C in Dictyostelium discoideum

Bominaar, Anthony A.; Kesbeke, Fanja; Haastert, Peter J.M. van

*Published in:*
Biochemical Journal



*Publication date:*
1994

# Phospholipase C in *Dictyostelium discoideum*

## Cyclic AMP surface receptor and G-protein-regulated activity *in vitro*

Anthony A. BOMINAAR,*‡ Fanja KESBEKE† and Peter J. M. VAN HAASTERT*

*Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands,
and †Cell Biology and Genetics, State University of Leiden, Zoological Laboratory, Kaiserstraat 63, 2311 GP Leiden, The Netherlands

The cellular slime mould *Dictyostelium discoideum* shows several responses after stimulation with the chemoattractant cAMP, including a transient rise in cyclic AMP (cAMP), cGMP and Ins(1,4,5)$P_3$. In this paper the regulation of phospholipase C *in vitro* is described. Under our experimental conditions commercial PtdIns(4,5)$P_2$ cannot be used to analyse phospholipase C activity in *Dictyostelium* lysates, because it is hydrolysed mainly to glycerophosphoinositol instead of Ins(1,4,5)$P_3$. Enzyme activity was determined with endogenous unlabelled PtdIns$P_2$ as a substrate. The product was measured by isotope-dilution assay and identified as authentic Ins(1,4,5)$P_3$. Since phospholipase C is strictly $Ca^{2+}$-dependent, with an optimal concentration range of 1–100 $\mu$M, cell lysates were prepared in EGTA and the enzyme reaction was started by adding 10 $\mu$M free $Ca^{2+}$. Phospholipase C activity increased 2-fold during *Dictyostelium* development up to 8 h of starvation, after which the activity declined to less than 10 % of the vegetative level. Enzyme activity *in vitro* increased up to 2-fold after stimulation of cells with the agonist cAMP *in vivo*. Addition of 10 $\mu$M guanosine 5′-[$\gamma$-thio]triphosphate during lysis activated the enzyme to the same extent, and this effect was antagonized by guanosine 5′-[$\beta$-thio]diphosphate. These results strongly suggest that surface cAMP receptors and G-proteins regulate phospholipase C during *Dictyostelium* development.

## INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is a eukaryotic micro-organism. Living in soil and feeding on bacteria, it has developed a mechanism to overcome unfavourable environmental conditions. Upon depletion of its food source a developmental program is initiated, resulting in aggregation of single cells into a multicellular structure which will eventually differentiate into a sorocarp. This sorocarp consists of two different cell types: highly vacuolized dead cells, providing the stalk on which a small droplet containing spore cells is positioned (for reviews see [1–4]).

Aggregation of cells is effected via chemotactic movement of the individual cells in a gradient of the chemoattractant cyclic AMP (cAMP). Chemotactic movement is the final result of cAMP-mediated transmembrane signal transduction (see [3]). Signal transduction in the cellular slime mould *Dictyostelium discoideum* has many characteristics in common with the mechanisms observed in higher eukaryotes. Following stimulation of cells with cAMP, a number of responses can be observed. These responses include a transient increase in the levels of the second messengers cAMP, cyclic GMP and Ins(1,4,5)$P_3$ (see [4]).

In the past few years inositol phosphates have received particular interest as second messengers in signal transduction. The commonly accepted inositol phosphate in signalling is Ins(1,4,5)$P_3$ [5,6], which is generated from PtdIns(4,5)$P_2$ by the action of the enzyme phospholipase C [7]. In the same reaction the second messenger *sn*-1,2-diacylglycerol is formed. Ins(1,4,5)$P_3$ has been shown to release $Ca^{2+}$ from internal stores [8,9], and both diacylglycerol and $Ca^{2+}$ are involved in the activation of protein kinase C [10,11]. For many higher eukaryotes the activity of phospholipase C has been shown *in vitro* [12–17], and evidence has been obtained that the activity is regulated via G-proteins [18–23]. Interaction of phospholipase C with receptor tyrosine kinases has been observed also [24–26], indicating that phospholipase C activity can be associated with different types of signal transduction. G-proteins and receptor tyrosine kinases appear to interact with different phospholipase C isoenzymes, $\beta$ and $\gamma$ respectively [18,26].

Since the cellular slime mould *Dictyostelium discoideum* shows a small but significant increase in Ins(1,4,5)$P_3$ levels in response to the chemoattractant cAMP [27–30], a receptor-coupled phospholipase C was to be expected. Basal phospholipase C activity was detected recently [31,32], but this enzyme activity was not shown to be regulated by surface receptors or G-proteins. We observed that exogenous PtdIns$P_2$ cannot be used for the characterization of phospholipase C in *Dictyostelium*, because it is rapidly degraded to glycerophosphoinositol (Gro*P*Ins). In this paper we describe a method for the analysis of phospholipase C activity *in vitro*, using endogenous unlabelled PtdIns$P_2$. The product was detected by using a specific Ins(1,4,5)$P_3$-binding protein from bovine liver. *Dictyostelium* phospholipase C is characterized and shown to be regulated by the receptor and G-protein agonists cAMP and guanosine 5′-[$\gamma$-thio]triphosphate (GTP[S]), respectively. In the accompanying paper [33] we demonstrate that multiple receptors and G-proteins regulate phospholipase C, identify the individual components and elucidate the complex pathways of interactions.

## MATERIALS AND METHODS

### Chemicals

[$^3$H]Ins(1,4,5)$P_3$ (20–60 Ci/mmol) [$\gamma$-$^{32}$P]ATP (> 3000 Ci/mmol) were obtained from Amersham International. All other radiolabelled inositol phosphates were from New England Nuclear. EGTA, Hepes, Ins(1,4,5)$P_3$ and phytase were from Sigma;

Abbreviations used: cAMP, cyclic AMP; Gro*P*Ins, glycerophosphoinositol; GTP[S], guanosine 5′[$\gamma$-thio]triphosphate; GDP[S], guanosine 5′-[$\beta$-thio]diphosphate.

‡ To whom reprint requests should be addressed.

GTP, GTP[S] and guanosine 5'-[$\beta$-thio]diphosphate GDP[S] were from Boehringer Mannheim. All other reagents used were of at least analytical grade.

### Cell culture

*Dictyostelium discoideum* strain NC-4 was grown in co-culture with *Klebsiella aerogenes* on agar plates containing (per litre) 3 g of glucose, 3 g of peptone (Difco), 4.5 g of $KH_2PO_4$, 3.0 g of $Na_2HPO_4$ and 15 g of agar at pH 6.5. Cells were harvested just before clearing of the bacterial lawn in 10 mM Na/K phosphate buffer, pH 6.5 (PB), and washed free of bacteria by repeated centrifugation at 300 *g*. Cells were resuspended at $10^7$ cells/ml in PB and shaken for 4 h at 150 rev./min. to obtain aggregation competence. For the developmental time series, cells were starved on PB-buffered agar plates at a density of $5 \times 10^6$ cells/cm$^2$; after starvation, cells were harvested, and multicellular structures, when present, were dissociated by incubation with 5 mg/ml cellulase in 10 mM Na/K phosphate/1 mM EDTA, pH 7.0, followed by an extra wash with PB to remove EDTA and cellulase. All cell preparations were then washed once in 40 mM Hepes/NaOH, pH 6.5, and resuspended in this buffer at $5 \times 10^7$ cells/ml. Before the experiment, cells were aerated for 10 min to obtain a homogeneous suspension and to provide oxygen; aeration was continued during the experiments.

### Exogenous PtdIns$P_2$ as a substrate for phospholipase C

[$^3$H]PtdIns$P_2$ was incorporated into mixed lipid micelles and incubated with a low-speed supernatant (2 min at 10000 *g*) from a *Dictyostelium* lysate as described by Taylor and Exton [21]. Reactions were quenched with 20% trichloroacetic acid. The trichloroacetic acid was removed from the supernatant by ether extraction, and the supernatant was applied to a Partisil SAX anion-exchange h.p.l.c. column, equilibrated in water. Elution was performed with a linear gradient of ammonium acetate, pH 3.4, from 0 to 1.3 M. Fractions were mixed with 3 ml of Scintillator 299, and radioactivity was determined. Compounds were identified by comparison with the elution positions of authentic radiolabelled inositol-containing substances.

### Phospholipase C assay

Samples of the cell suspension were brought to 5.9 mM EGTA, and cells were lysed by rapid elution through Nuclepore polycarbonate filters, pore size 3 $\mu$m. At 10 s after lysis, either samples were quenched by adding an equal volume of 3.5% $HClO_4$, or $CaCl_2$ was added to a final concentration of 5.9 mM. In the latter case the lysate was left for 20 s, after which the reaction was terminated by addition of an equal volume of 3.5% $HClO_4$. For Ins(1,4,5)$P_3$ determination, samples were neutralized with 50%-satd. $KHCO_3$ and 20 $\mu$l samples were assayed for Ins(1,4,5)$P_3$ by using the Ins(1,4,5)$P_3$-binding protein from bovine liver as described [30]. Phospholipase C activity is defined as the amount of Ins(1,4,5)$P_3$ produced during the 20 s incubation with $CaCl_2$.

### Identification of produced Ins(1,4,5)$P_3$

To identify the compound that showed cross-reactivity with the Ins(1,4,5)$P_3$-binding protein as authentic Ins(1,4,5)$P_3$, samples were incubated with purified rat brain 5-phosphatase (kindly provided by Dr. C. Erneux, Brussels) or *Dictyostelium* 1-phosphatase for 15 min at 20 °C, with or without 1000 c.p.m. of [$^3$H]Ins(1,4,5)$P_3$ [34]. The amount of enzyme was chosen such that 50–75% [$^3$H]Ins(1,4,5)$P_3$ was degraded. The samples with the radioactive tracer were treated further as phosphatase samples: reactions were quenched by addition of 0.5 ml of chloroform/methanol/HCl (20:40:1, by vol.), phase separation was induced by adding 200 $\mu$l of water and centrifuging at 14000 *g* for 2 min, and inositol phosphates in the aqueous phase were separated by using Dowex Ax1 as described [35]. The samples without tracer were quenched by heating to 100 °C for 2 min and assayed by the isotope-dilution assay as described above. Degradation of radiolabel and cross-reactivity were compared.

Ion-pair h.p.l.c. was used as the second method to identify the cross-reacting compound as Ins(1,4,5)$P_3$. Samples from the phospholipase C incubation were mixed with 1000 c.p.m. each of [$^3$H]Ins(1,4)$P_2$, [$^{32}$P]Ins(1,4,5)$P_3$ and [$^{32}$P]Ins(1,3,4,5)$P_4$. Tributylammonium phosphate (TBAP; 500 $\mu$l of 0.1 M) was added and the sample was loaded on a Lichrosorb RP-18 h.p.l.c. column, pre-equilibrated with 10 mM TBAP and 25% methanol. Elution was performed isocratically in equilibration buffer. Fractions (0.5 ml) were collected, of which 0.25 ml was mixed with 3 ml of Scintillator 299 and quantified using a liquid-scintillation counter (Beckmann), and 0.25 ml was freeze-dried, resuspended in 100 mM Tris, pH 9.0, and assayed for cross-reactivity by the isotope-dilution assay.

### Mass determination of PtdIns$P_2$

Total lipid was extracted from $2.5 \times 10^6$ cells in 50 $\mu$l by addition of 0.5 ml of chloroform/methanol/HCl (20:40:1, by vol.); phase separation was induced by adding 200 $\mu$l of water and centrifuging for 1 min at 14000 *g*. The organic phase was removed, and the aqueous phase was washed once with 100 $\mu$l of chloroform. The combined samples of the organic phase were dried under a stream of nitrogen gas. Hydrolysis of lipids was performed by adding 250 $\mu$l of 1 M KOH and boiling the samples for 30 min. Subsequently the samples were cooled on ice and adjusted to neutral pH by adding $HClO_4$. Insoluble $KClO_4$ was sedimented, and Ins(1,4,5)$P_3$ levels were determined by the isotope-dilution assay. Recovery of lipids and the efficiency of hydrolysis were determined by using [$^3$H]PtdIns$P_2$ as an internal standard.

### Assay for PtdIns and PtdIns*P* kinase

Cells were lysed through Nuclepore polycarbonate filters (3 $\mu$m pore size) and membranes were isolated by centrifuging for 2 min at 14000 *g*; the supernatant was removed and the pellet was washed and resuspended in lysis buffer (40 mM Hepes/NaOH, pH 6.5) to the equivalent of $4 \times 10^8$ cells/ml. Kinase reactions were performed by adding 35 $\mu$l of membranes to a reaction mixture containing 5 mM $MgCl_2$, 0.25 $\mu$Ci of [$^{32}$P]ATP, 10 $\mu$M ATP with or without 1 $\mu$M cAMP in a total volume of 15 $\mu$l of lysis buffer. Reactions were carried out for 2 min at 22 °C and quenched by adding 500 $\mu$l of chloroform/methanol/HCl (20:40:1, by vol.). Phase separation was induced by addition of 200 $\mu$l of water and centrifuging at 14000 *g* for 2 min. The organic phase was removed and dried under a stream of nitrogen gas. Samples were dissolved in 10 $\mu$l of chloroform/methanol (9:1, v/v) and applied to silica 60 t.l.c. plates pre-activated for 1 h at 120 °C. Plates were developed in chloroform/methanol/$NH_4OH$/water (90:90:5:22, by vol.) and autoradiographed on Kodak X-ray film for 16 h. Radioactive spots were quantified

with an LKB Laserscan densitometer. Spots were identified by using [$^3$H]labelled phospholipids as standards, which were detected by using En$^3$Hance (NEN DuPont).

## RESULTS

### Use of exogenous PtdIns$P_2$ as a substrate for phospholipase C

The first attempt to demonstrate phospholipase C made use of previously described methods for assaying the enzyme *in vitro*. Exogenous [$^3$H]PtdIns$P_2$ in mixed phospholipid micelles was incubated with cell lysates, and the water-soluble products were analysed by h.p.l.c. Unfortunately, exogenously supplied PtdIns(4,5)$P_2$ proved unsuitable, since Gro*P*Ins was the major water-soluble product (Table 1). Different mixtures of phospholipids and different concentrations of $Mg^{2+}$ and $Ca^{2+}$ were used, as well as inhibitors of inositol-phosphate phosphatases, but significant [$^3$H]Ins(1,4,5)$P_3$ production remained undetectable. Comparable data were obtained with PtdIns*P* as a substrate (Table 1). Apparently, exogenous phosphoinositides are a better substrate for phospholipase A and phosphatases than for phospholipase C.

### Assay for phospholipase C activity using unlabelled endogenous PtdIns$P_2$

Two assays may allow the detection of phospholipase activity *in vitro* when endogenous substrate is used; either to measure the production of [$^3$H]Ins(1,4,5)$P_3$ in membranes derived from [$^3$H]inositol-labelled cells, as described by Cubitt and Firtel [32], or to use the Ins(1,4,5)$P_3$ mass assay to determine the amount of Ins(1,4,5)$P_3$ produced form unlabelled PtdIns$P_2$. The latter method was chosen, because it is fast, convenient, inexpensive, and multiple samples can be processed simultaneously.

After lysis of cells in the presence of $Ca^{2+}$, a large production of Ins(1,4,5)$P_3$ was observed (results not shown). If lysis took place in the presence of the $Ca^{2+}$-chelator EGTA, no activity could be detected over a period of at least 1 min (Figure 1a). Re-adding $Ca^{2+}$ to the lysate led to the re-activation of the enzyme (Figure 1a). Thus, by using EGTA and $Ca^{2+}$ the enzyme can be turned off and on; the lysate if made in the presence of EGTA and Ins(1,4,5)$P_3$ formation is allowed to proceed for a fixed period of time by starting the reaction with $Ca^{2+}$ and terminating it with $HClO_4$.

The time course of Ins(1,4,5)$P_3$ production after $Ca^{2+}$ addition (Figure 1a) shows that the rate of Ins(1,4,5)$P_3$ production was constant up to 30 s and then slowly declined; 20 s was chosen as a standard incubation time. Addition of $Ca^{2+}$ to the lysate at different times after lysis demonstrates (Figure 1b) that the enzyme can be re-activated to the same extent up to 15 s after lysis; addition of $Ca^{2+}$ at longer times after cell lysis did not lead to full recovery of enzyme activity. In order to obtain the highest reproducibility, 10 s after lysis was chosen as the standard time for readdition of $Ca^{2+}$.

### Identification of the reaction product as Ins(1,4,5)$P_3$

Two experiments were performed to demonstrate that the compound cross-reacting with the Ins(1,4,5)$P_3$-binding protein is authentic Ins(1,4,5)$P_3$. In the first experiment a sample from the phospholipase C assay was mixed with authentic [$^3$H]Ins(1,4,5)$P_3$ and incubated with a purified rat brain Ins(1,4,5)$P_3$ 5-phosphatase or a partially purified *Dictyostelium* Ins(1,4,5)$P_3$ 1-phosphatase. If the cross-reactivity was due to Ins(1,4,5)$P_3$, both cross-reactivity and [$^3$H]Ins(1,4,5)$P_3$ should be degraded at identical rates by these enzymes. The results show that this was indeed the case (Figure 2 inset). In the second experiment, co-chromatography on an h.p.l.c. ion-pair system was used as a criterion for identity. Figure 2 reveals that the major cross-reacting compound in the binding-protein assay co-

**Table 1 Water-soluble products formed from exogenous [$^3$H]PtdIns$P_2$ and [$^3$H]PtdIns*P* in *Dictyostelium discoideum***

A *Dictyostelium* low-speed supernatant was incubated with [$^3$H]PtdIns$P_2$ or [$^3$H]PtdIns*P* for 10 min. The reaction was quenched with chloroform/methanol/HCl, and the [$^3$H]inositol-containing compounds in the water phase were analysed by anion-exchange h.p.l.c. ND, not detectable.

| | | Amount (% of soluble radioactivity) | |
|---|---|---|---|
| Product | Substrate... | PtdIns$P_2$ | PtdIns*P* |
| Ins | | 6.3 | 21 |
| Gro*P*Ins | | 52.2 | 30 |
| Ins*P* | | 30.8 | 35 |
| Gro*P*Ins*P* | | 3.5 | 10 |
| Ins$P_2$ | | 6.0 | 4 |
| Ins$P_3$ | | 1.3 | ND |

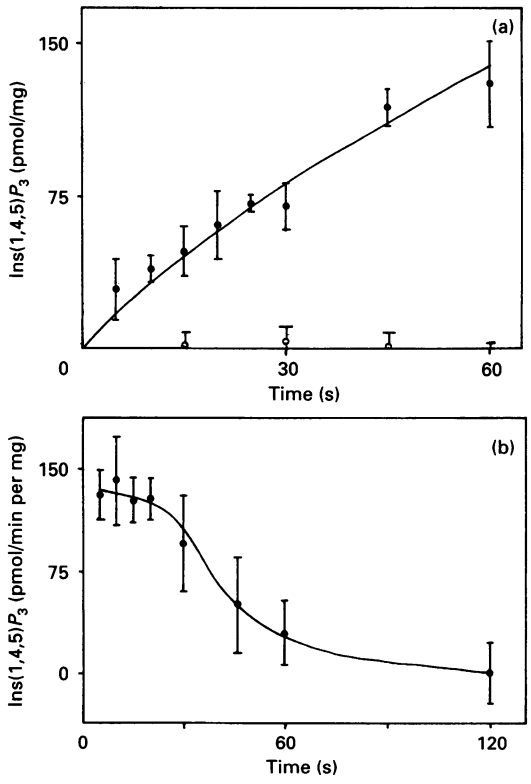


**Figure 1 Ins(1,4,5)$P_3$ production in lysates**

(**a**) Time course of Ins(1,4,5)$P_3$ production after lysis in the presence of 5.9 mM EGTA with (●) or without (○) 5.9 mM $CaCl_2$ re-added to the lysate. $CaCl_2$ was added at 10 s after lysis, which is at $t = 0$ in the Figure. (**b**) Time course of re-addition of $CaCl_2$ to the lysate on Ins(1,4,5)$P_3$ production. The enzyme reactions were conducted for 20 s; $t = 0$ is the moment of cell lysis. The free $Ca^{2+}$ concentration for both panels was 10 $\mu$M; Ins(1,4,5)$P_3$ levels before lysis were subtracted. Data are expressed as means ± S.E.M. of 3 independent experiments in triplicate.

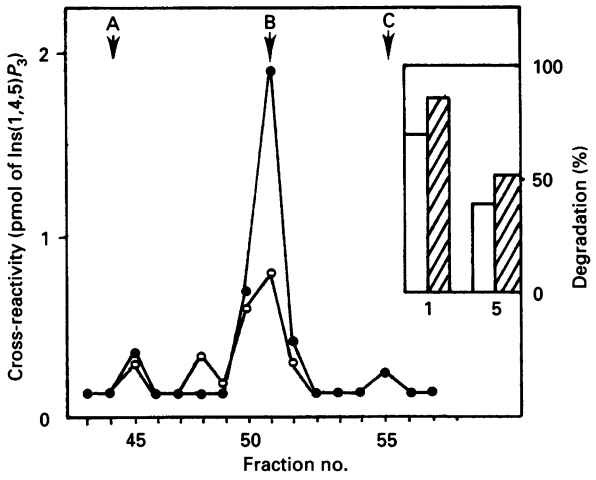


**Figure 2 H.p.l.c. and enzymic identification of the reaction product**

Main Figure: h.p.l.c. analysis. Lysates in EGTA from $2 \times 10^7$ cells were incubated without (○) or with 10 $\mu$M free $Ca^{2+}$ (●), quenched, mixed with radioactive standards, and chromatographed by h.p.l.c. Fractions of the eluate were freeze-dried, reconstituted in Ins(1,4,5)$P_3$ assay buffer and assayed for cross-reactivity with the Ins(1,4,5)$P_3$-binding protein. Arrows indicate the fractions in which authentic Ins(1,4)$P_2$ (A), Ins(1,4,5)$P_3$ (B) and Ins(1,3,4,5)$P_4$ (C) were eluted. Inset: enzymic degradation of cross-reactivity. A sample from the phospholipase C reaction was mixed with [$^3$H]Ins(1,4,5)$P_3$, and incubated with purified rat brain Ins(1,4,5)$P_3$ 5-phosphatase (5) and partially purified *Dictyostelium* Ins(1,4,5)$P_3$ 1-phosphatase (1). Then each sample was divided: one part was analysed for the degradation of radioactive Ins(1,4,5)$P_3$ (□) and the other part for the degradation of cross-reactivity in the Ins(1,4,5)$P_3$-binding protein assay (▨).

**Table 2 PtdIns$P_2$ and Ins(1,4,5)$P_3$ contents of lysates after phospholipase C reaction**

| | PtdIns$P_2$ (pmol/mg) | Ins(1,4,5)$P_3$ (pmol/mg) | PLC activity (pmol/min per mg) |
|---|---|---|---|
| Cells | 302 | 15.4 | – |
| Lysate | 111 | 15.6 | 0 |
| Lysate + $Ca^{2+}$ added | 94 | 62.3 | 140 |

chromatographed with an authentic Ins(1,4,5)$P_3$ standard on h.p.l.c. Thus the compound produced in the phospholipase C assay (i) cross-reacts with the highly specific Ins(1,4,5)$P_3$-binding protein from bovine liver, (ii) is degraded to approximately the same extent as authentic Ins(1,4,5)$P_3$ by Ins(1,4,5)$P_3$ 5- and 1-phosphatase, and (iii) co-chromatographs with authentic Ins(1,4,5)$P_3$ on a RP-18 h.p.l.c. system.

In order to establish whether the amount of substrate (i.e. PtdIns$P_2$) present in the lysate was sufficient and not a limiting factor in the phospholipase C assay, the total mass of PtdIns$P_2$ was determined (Table 2). Cells contain about 300 pmol of PtdIns$P_2$/mg of protein. PtdIns$P_2$ levels of a lysate prepared in EGTA were decreased to approx. 110 pmol/mg. Re-addition of $Ca^{2+}$ to the lysate induced the production of 47 pmol of Ins(1,4,5)$P_3$/mg; the level of PtdIns$P_2$ was still 94 pmol/mg, indicating that sufficient substrate remained.

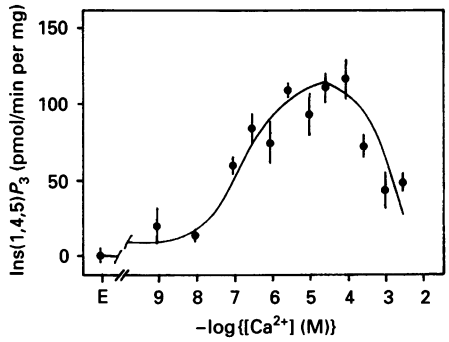


**Figure 3 $Ca^{2+}$-dependency of phospholipase C**

Phospholipase C activity was determined at 5.9 mM EGTA and different $CaCl_2$ concentrations to yield the indicated free $Ca^{2+}$ concentrations. E, activity in the presence of 5.9 mM EGTA without added $CaCl_2$. Data shown are means ± S.E.M. of 3 independent experiments in triplicate.

## $Ca^{2+}$-dependence of phospholipase C

Since most phospholipase C enzymes described so far are dependent on $Ca^{2+}$, the $Ca^{2+}$-sensitivity of *Dictyostelium* phospholipase C was characterized further by using $Ca^{2+}$/EGTA buffers to generate fixed $Ca^{2+}$ concentrations. Figure 3 shows a dose–response curve for $Ca^{2+}$ which is bell-shaped. Half-maximal activity was observed at 0.1 $\mu$M, activity was maximal between 1 and 100 $\mu$M, and only at concentrations above 100 $\mu$M did the activity decrease again.

## Regulation of phospholipase C by the agonist cAMP

Extracellular cAMP binds to surface receptors and induces a small increase in Ins(1,4,5)$P_3$ levels *in vivo* [27–30]. Therefore the regulation of phospholipase C by cAMP and guanine nucleotides was investigated. Stimulation of lysates with cAMP had no effect on phospholipase C activity (results now shown). Similar observations have been made for the regulation of adenylate and guanylate cyclases by cAMP in lysates (see [4]). Activation of these enzymes can be measured *in vitro* by stimulating cells before lysis. Thus cells were stimulated with different concentrations of cAMP, lysed and assayed for phospholipase C activity. Phospholipase C activity was stimulated up to 2-fold by cAMP (Figure 4); half-maximal stimulation was observed at 0.1 $\mu$M cAMP.

Although activation of phospholipase C is the most likely cause for the observed increase in Ins(1,4,5)$P_3$, inhibition of phosphatase activity or an increased turnover of PtdIns$P$ to PtdIns$P_2$ could also explain the observed enhanced accumulation of Ins(1,4,5)$P_3$. To address these questions, phosphatase activity was measured in stimulated and unstimulated cells, demonstrating that less than 10% of Ins(1,4,5)$P_3$ was degraded under phospholipase C assay conditions in the absence or presence of cAMP (Table 3). In addition, the incorporation of radioactivity from [$\gamma$-$^{32}$P]ATP into PtdIns$P$ and PtdIns$P_2$ was measured, showing that cAMP had no effect on PtdIns kinase and PtdIns$P$ kinase activities *in vitro*. These results indicate that the increase in Ins(1,4,5)$P_3$ production in a cell lysate prepared from cAMP-stimulated cells is due to an increased phospholipase C activity.

## Regulation of phospholipase C by guanine nucleotides

All four surface cAMP receptors in *Dictyostelium* known to date have a putative structure with seven transmembrane domains,

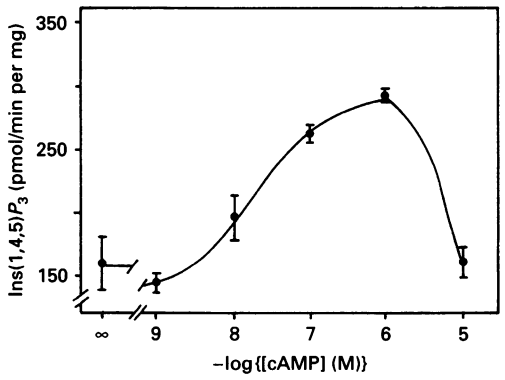


**Figure 4 Dose–response curve of cAMP-stimulated phospholipase C**

Cells were stimulated with different concentrations of cAMP. After 20 s, cells were lysed and phospholipase C activity was determined. Data shown are means ± S.E.M. of 3 independent experiments in triplicate.

**Table 3 Ins(1,4,5)$P_3$ phosphatase and PtdIns/PtdIns$P$ kinase activities after stimulation with cAMP**

Phosphatase activity was measured in lysates prepared from control and cAMP-stimulated cells under phospholipase C assay conditions; the lysate was incubated for 20 s with 1000 c.p.m. of [$^3$H]Ins(1,4,5)$P_3$, and the degradation was measured by ion-exchange chromatography. Kinase activity was measured in membranes which were incubated with [$\gamma$-$^{32}$P]ATP in the absence or presence of 1 $\mu$M cAMP. The phospholipids were isolated by t.l.c. and quantified.

| | Phospholipase C (pmol/min per mg) | Phosphatase [% degradation of Ins(1,4,5)$P_3$] | Kinase with product (% incorporation from [$\gamma$-$^{32}$P]ATP) | |
|---|---|---|---|---|
| | | | PtdIns$P$ | PtdIns$P_2$ |
| Control | 156 | 13 | 0.38 | 0.11 |
| cAMP | 294 | 10 | 0.36 | 0.10 |

**Table 4 Effect of guanine nucleotides on phospholipase C activity**

Cells were lysed in the presence of guanine nucleotides and assayed for phospholipase C activity. Phospholipase C activity in lysates from cAMP-stimulated cells is shown for comparison. The concentrations used were 1 $\mu$M cAMP, 10 $\mu$M GTP[S] and 100 $\mu$M GDP[S]. Data are means ± S.E.M. of 3 independent experiments in triplicate: *significantly above control ($P < 0.05$).

| Addition | Phospholipase C activity (pmol/min. per mg) |
|---|---|
| – | 122 ± 41 |
| cAMP | 254 ± 51* |
| GTP[S] | 235 ± 65* |
| GTP[S] + GDP[S] | 142 ± 46 |
| GDP[S] | 115 ± 50 |
| cAMP + GTP[S] | 256 ± 69* |

typical of G-protein-coupled receptors [36,37]. To test the hypothesis that G-proteins regulate *Dictyostelium* phospholipase C activity, cells were lysed in the presence of 10 $\mu$M GTP[S] and phospholipase C activity was measured. Table 4 shows that GTP[S] leads to a 2-fold stimulation of phospholipase C activity. This stimulation could be antagonized by a 10-fold excess of GDP[S], whereas GDP[S] on its own had no effect. Stimulation of cells with a saturating cAMP concentration, followed by lysing in the presence of 10 $\mu$M GTP[S], showed no enhancement of phospholipase C activity compared with lysing in the absence of GTP[S], indicating that stimulation of phospholipase C by surface receptors and G-proteins is not additive (Table 4).

## Developmental regulation of phospholipase C

Cells were allowed to starve for different periods of time, thereby going through the various stages of development. At regular intervals samples were taken and analysed for basal phospholipase C activity. As shown in Figure 5 the basal activity increased

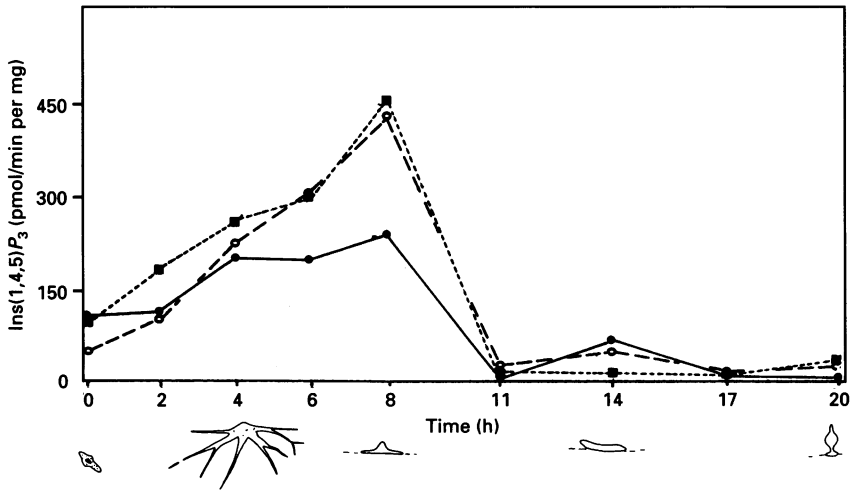


**Figure 5 Developmental regulation of phospholipase C activity**

Cells were starved for different periods on agar, and phospholipase C activity was determined. Drawings below the time axis show the stages of development at the time indicated. ●, Basal phospholipase C activity; ■, phospholipase activity after stimulation of cells before lysis with 1 $\mu$M cAMP; ○, phospholipase C activity after lysis in the presence of 10 $\mu$M GTP[S]. Data shown are means of a typical experiment performed in triplicate.

about 2-fold from vegetative amoeba to the tipped aggregate at 8 h of development. During the transition from the tipped aggregate to the first slug-like structure the activity declined rapidly, not to return again throughout the rest of the differentiation program. The status of the enzyme in spores is as yet unknown, since spores cannot be lysed by the methods described here.

At the different developmental stages, cells were stimulated with cAMP before lysis or lysed in the presence of GTP[S], and phospholipase C was measured. In the vegetative stage cAMP did not alter enzyme activity; activation started at 2–4 h of development and was maximal at 6–8 h. This time course coincides with the expression of the major cAMP surface receptor [36,37]. The effect of lysis in the presence of GTP[S] on phospholipase C activity was more complex. In vegetative cells GTP[S] inhibited phospholipase C by about 50%, whereas a 2-fold stimulation was observed at later stages of development (Figure 5). These data suggest multiple pathways for the regulation of phospholipase C by G-proteins. In the accompanying manuscript [33] this regulation will be examined in more detail.

## DISCUSSION

In this paper we describe a phospholipase C in *Dictyostelium discoideum* which is stimulated by the receptor agonist cAMP and can be activated by the non-hydrolysable GTP analogue GTP[S]. The assay is based on the use of endogenous PtdIns(4,5)$P_2$ and the strict $Ca^{2+}$-dependency of the enzyme. During lysis, $Ca^{2+}$ is removed to decrease enzyme activity. Subsequently, $Ca^{2+}$ is re-added to the lysate for a fixed period of time, allowing accurate determination of phospholipase C activity. The Ins$P_3$ produced is shown to be Ins(1,4,5)$P_3$. The basic properties of the enzyme are as follows. (i) The enzyme is $Ca^{2+}$-dependent, with half-maximal activity at 0.1 $\mu$M $Ca^{2+}$ and optimal activity at 10 $\mu$M $Ca^{2+}$. (ii) The EGTA-inactivated enzyme is stable for up to 30 s. (iii) The $Ca^{2+}$-activated enzyme is stable for at least 1 min, and Ins(1,4,5)$P_3$ production is linear with time for at least 30 s. (iv) cAMP increases the activity 2-fold. (v) GTP[S] activates the enzyme to the same extent as cAMP. (vi) The effects of cAMP and GTP[S] are not additive. The $Ca^{2+}$-dose-dependence of the described phospholipase C is in good accordance with what has been shown for phospholipase C from other organisms, although the complete absence of activity in EGTA is uncommon [7].

As described in the Results section, the use of commercial exogenous PtdIns(4,5)$P_2$ resulted in the production of Gro*P*Ins instead of Ins(1,4,5)$P_3$. Apparently, phospholipase A and the phosphatases are more active than phospholipase C. The fatty acid composition of the PtdIns(4,5)$P_2$ used may explain this result; commercially available PtdIns$P_2$ is an arachidonyl-stearoyl species, yet *Dictyostelium discoideum* has been shown to lack $C_{20:4}$ arachidonyl chains [38]. Recent work suggests that *Dictyostelium discoideum* phospholipase C may recognize specific phospholipids (A. D. Tepper, J. Van der Kaay, and P. J. M. Van Haastert, unpublished work). Another possibility is that application of PtdIns$P_2$ in small unilamellar vesicles exposes PtdIns$P_2$ in a conformation favoured by phosphatases and phospholipase A.

Recently, phospholipase C activity was demonstrated in *Dictyostelium discoideum* by using two different methods. In a paper by Lundberg and Newell [31], using exogenous [$^3$H]PtdIns$P_2$, a phospholipase C was described in partially purified membranes. More recently Cubitt and Firtal [32] showed the presence of a membrane-bound activity, using endogenously [$^3$H]inositol-labelled PtdIns$P_2$ as a substrate. Enzyme kinetics, $Ca^{2+}$-sensitivity and cellular localization suggest that both groups have studied the same phospholipase C. The enzyme activity observed by Lundberg and Newell [31] is less than 1% of the activity that we report here. An estimation of the activity found by Cubitt and Firtel [32], based on the ratio of radioactivity in the phospholipid precursor and the Ins(1,4,5)$P_3$ produced, indicates a similar low activity. Neither Lundberg and Newell [31] nor Cubitt and Firtel [32] have demonstrated that phospholipase C is regulated by either cAMP or GTP[S]. The absence of stimulation by cAMP or GTP[S] in these preparations is consistent with our finding that addition of cAMP or GTP[S] after lysis has no effect on phospholipase C activity. The long period for preparation of membranes could be another possible cause for the lack of stimulation as well as for the low activity of phospholipase C, because we observed (Figure 1b) that phospholipase C is very unstable. Although there are several differences between the phospholipase C shown by Cubitt and Firtel [32] and Lundberg and Newell [31] and the one that we describe here, the $Ca^{2+}$-dependency and the developmental regulation are comparable.

Recently a cDNA coding for phospholipase C in *Dictyostelium discoideum* has been cloned. Expression of the cDNA in *Dictyostelium* resulted in an increased basal phospholipase C activity, measured by the methods described here [39]. Structural analysis of the primary sequence shows that *Dictyostelium* phospholipase C belongs to the phospholipase C-$\delta$ class. Expression of phospholipase C-$\delta$ mRNA as determined by Northern-blot analysis [39] coincides with the expression of enzyme activity throughout development (Figure 5). This would mean that this is the first phospholipase C-$\delta$ known to date that is shown to be G-protein-coupled. Currently a cell line with a disrupted phospholipase C gene is under construction in order to obtain direct evidence for the relationship between the cloned phospholipase C gene and the observed phospholipase C activity.

After stimulation of cells with cAMP, only a relatively small increase in phospholipase C activity was observed. This is in accordance with the observed small increase in Ins(1,4,5)$P_3$ levels *in vivo* [27–30]. Using a computer-based model for the inositol cycle, including all known parameters for synthesis and degradation of Ins(1,4,5)$P_3$, we have shown that a 2-fold increase in phospholipase C activity is sufficient to generate the observed increase in Ins(1,4,5)$P_3$ (J. Van der Kaay, A. A. Bominaar and P. J. M. Van Haastert, unpublished work).

Unexpectedly, cAMP not only activates phospholipase C; at concentrations above 10 $\mu$M cAMP a decrease in activity can be observed. A comparable effect is seen in the vegetative stage after lysis in the presence of GTP[S]. These observations suggest that in *Dictyostelium* phospholipase C activity is modulated in a complex manner by cAMP and G-proteins. The methods described here are used in the accompanying paper [33] to investigate the regulate of the enzyme by the chemoattractant cAMP and the role of G-proteins in cell lines with defined deletions in receptor and G-protein genes, which allowed the identification of stimulatory and inhibitory receptors and G-proteins and their complex interactions.

We thank Peter Braun for analysing Ins(1,4,5)$P_3$ cross-reactivity on the RP-18 h.p.l.c. system, and Christophe Erneux for the kind gift of rat brain 5-phosphatase. Part of this work was supported by grants from the NWO Council for Medical Research and the Pieter Langerhuizen Fund.

## REFERENCES

1 Janssens, P. M. W. and Van Haastert, P. J. M. (1987) Microbiol. Rev. **51**, 396–418
2 Schaap, P. (1986) Differentiation **33**, 1–16
3 Caterina, M. J. and Devreotes, P. N. (1991) FASEB J. **5**, 3078–3085

4 Van Haastert, P. J. M., Janssens, P. M. W. and Erneux, C. (1991) Eur. J. Biochem. **195**, 289–303
5 Berridge, M. J. and Irvine, R. F. (1984) Nature (London) **317**, 315–321
6 Irvine, R. F. (1989) in Inositol Lipids in Cell Signalling (Michell, R. H., Drummond, A. H. and Downes, C. P., eds.), pp. 135–161, Academic Press, London
7 Rhee, S. G., Suh, P. G., Ryu, S. H. and Lee, S. Y. (1989) Science **244**, 546–550
8 Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–205
9 Europe-Finner, G. N. and Newell, P. C,. (1986) Biochim. Biophys. Acta **887**, 335–340
10 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. **255**, 2273–2276
11 Ludérus, M. E. E., Van der Most, R. G., Otte, A. P. and Van Driel, R. (1989) FEBS Lett. **353**, 71–75
12 Wilson, D. B., Bross, T. E., Hofman, S. L. and Majerus, P. W. (1984) J. Biol. Chem. **259**, 11718–11724
13 Melin, P. M., Sundler, R. and Jergil, B. (1986) FEBS Lett. **198**, 85–88
14 Fukui, T., Lutz, R. J. and Lowenstein, J. M. (1988) J. Biol. Chem. **263**, 17730–17737
15 Banno, Y., Yu, A., Nakashima, T., Homma, Y., Takenawa, T. & Nozawa, Y. (1990) Biochem. Biophys. Res. Commun. **167**, 396–401
16 Meldrum, E., Katan, M. and Parker, P. (1989) Eur. J. Biochem. **182**, 673–677
17 Baldassare, J. J., Henderson, P. A. and Fisher, G. J. (1989) Biochemistry **28**, 6010–6016
18 Smrcka, A. V., Hepler, J. R., Brown, K. O. and Sternweis, P. C. (1991) Science **251**, 804–807
19 Gierschik, P. and Jakobs, K. H. (1987) FEBS Lett. **224**, 219–223
20 Nakashima, S., Hattori, H., Shirato, L., Takenaka, A. and Nozawa, Y. (1987) Biochem. Biophys. Res. Commun. **148**, 971
21 Taylor, S. J. and Exton, J. H. (1987) Biochem. J. **248**, 791–799
22 Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. and Capon, D. J. (1989) Cell **56**, 487–493
23 Deckmyn, H., Tu, S.-M. and Majerus, P. W. (1986) J. Biol. Chem. **261**, 16553–16558
24 Moolenaar, W. H., Bierman, A. J., Tilly, B. C., Verlaan, I., Defize, L. H., Honegger, A. M., Ullrich, A. and Sclessinger, J. (1988) EMBO J. **3**, 707–710
25 Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K. and Takenawa, T. (1989) J. Biol. Chem. **264**, 21885–21890
26 Kin, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J. and Rhee, S. G. (1990) Cell **65**, 435–441
27 Europe-Finner, G. N. and Newell, P. C. (1987) J. Cell Sci. **87**, 513–518
28 Europe-Finner, G. N., Gammon, B., Wood, C. A. and Newell, P. (1989) J. Cell Sci. **93**, 585–592
29 Van Haastert, P. J. M., De Vries, M. J., Penning, L. C., Roovers, E., Van Der Kaay, J., Erneux, C. and Van Lookeren Campagne, M. M. (1989) Biochem. J. **258**, 577–586
30 Van Haastert, P. J. M. (1989) Anal. Biochem. **177**, 115–119
31 Lundberg, G. A. and Newell, P. C. (1990) FEBS Lett. **270**, 181–183
32 Cubitt, A. B. and Firtel, R. A. (1992) Biochem. J. **283**, 371–378
33 Bominaar, A. A. and Van Haastert, P. J. M. (1994) Biochem. J. **297**, 189–193
34 Erneux, C., Lemos, M., Verjans, B., Vanderhaeghen, P., Delvaux, A. and Dumont, J. E. (1989) Eur. J. Biochem. **181**, 319–324
35 Van Lookeren Campagne, M. M., Erneux, C., Van Eijk, R. and Van Haastert, P. J. M. (1988) Biochem. J. **254**, 343–350
36 Klein, P., Sun, T. J., Saxe, C. L., Kimmel, A. R., Johnson, R. and Devreotes, P. N. (1988) Science **241**, 1467–1472
37 Saxe, C. L., Klein, P., Sun, T. J., Kimmel, A. R. and Devreotes, P. N. (1988) Dev. Genet. (N.Y.) **9**, 227–235
38 Weeks, G. (1976) Biochim. Biophys. Acta **450**, 21–32
39 Drayer, A. L. and Van Haastert, P. J. M. (1992) J. Biol. Chem. **261**, 18387–18392


Received 7 June 1993/27 August 1993; accepted 1 September 1993