Phospholipase C in Dictyostelium discoideum
Cyclic AMP surface receptor and G-protein-regulated activity in vitro

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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 activity in Dictyostelium cells, because it is hydrolysed mainly to glycerophosphoinositol instead of Ins(1,4,5)P_3. Enzyme activity was determined with endogenous unlabelled PtdInsP_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 μM, cell lysates were prepared in EGTA and the enzyme reaction was started by adding 10 μM 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 vitro. Addition of 10 μM guanosine 5’-[γ-thio]triphosphate during lysis activated the enzyme to the same extent, and this effect was antagonized by guanosine 5’-[β-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 vacuolated 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, β and γ 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 PtdInsP_2 cannot be used for the characterization of phospholipase C in Dictyostelium, because it is rapidly degraded to glycerophosphoinositol (GroPIns). In this paper we describe a method for the analysis of phospholipase C activity in vitro, using endogenous unlabelled PtdInsP_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’-[γ-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

[^3H]Ins(1,4,5)P_3 (20–60 Ci/mmol) [γ-32P]ATP (> 3000 Ci/mmole) were obtained from Amersham International. All other radiolabelled inositol phosphates were from New England Nuclear. EGTA, Heps, Ins(1,4,5)P_3 and phytase were from Sigma;
GTP, GTP[S] and guanosine 5'-[β-thio]diphosphate GDP[S] were from Boehringer Mannheim. All other reagents 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 KH₂PO₄, 3.0 g of Na₂HPO₄ 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 10⁶ 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 x 10⁶ cells/cm²; 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 x 10⁶ 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 PtdInsP₂ as a substrate for phospholipase C

[³H]PtdInsP₂ 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 μm. At 10 s after lysis, either samples were quenched by adding an equal volume of 3.5% HClO₄, or CaCl₂ 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% HClO₄. For Ins(1,4,5)P₃ determination, samples were neutralized with 50% saturated KHCO₃ and 20 &mu;l samples were assayed for Ins(1,4,5)P₃ by using the Ins(1,4,5)P₃-binding protein from bovine liver as described [30]. Phospholipase C activity is defined as the amount of Ins(1,4,5)P₃ produced during the 20 s incubation with CaCl₂.

Identification of produced Ins(1,4,5)P₃

To identify the compound that showed cross-reactivity with the Ins(1,4,5)P₃-binding protein as authentic Ins(1,4,5)P₃, 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 [³H]Ins(1,4,5)P₃ [34]. The amount of enzyme was chosen such that 50–75% [³H]Ins(1,4,5)P₃ 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 μl of water and centrifuging at 14000 g for 2 min, and inositol phosphates in the aqueous phase were separated by using Dowex A×1 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)P₃. Samples from the phospholipase C incubation were mixed with 1000 c.p.m. each of [³H]Ins(1,4)P₁, [³²P]Ins(1,4,5)P₃ and [³²P]Ins(1,3,4,5)P₄. Tributylammonium phosphate (TBAP; 500 μl 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 PtdInsP₂

Total lipid was extracted from 2.5 x 10⁶ cells in 50 μl by addition of 0.5 ml of chloroform/methanol/HCl (20:40:1, by vol.); phase separation was induced by adding 200 μl of water and centrifuging for 1 min at 14000 g. The organic phase was removed, and the aqueous phase was washed once with 100 μl 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 μl 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 HCIO₄. Insoluble KClO₄ was sedimented, and Ins(1,4,5)P₃ levels were determined by the isotope-dilution assay. Recovery of lipids and the efficiency of hydrolysis were determined by using [³H]PtdInsP₂ as an internal standard.

Assay for PtdIns and PtdIns₃ kinase

Cells were lysed through Nuclepore polycarbonate filters (3 μm 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 x 10⁶ cells/ml. Kinase reactions were performed by adding 35 μl of membranes to a reaction mixture containing 5 mM MgCl₂, 0.25 μCi of [³²P]ATP, 10 μM ATP with or without 1 μM cAMP in a total volume of 15 μl of lysis buffer. Reactions were carried out for 2 min at 22 °C and quenched by adding 500 μl of chloroform/methanol/HCl (20:40:1, by vol.). Phase separation was induced by addition of 200 μl 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 μl of chloroform/methanol (9:1, v/v) and applied to silica 60 TLC plates pre-activated for 1 h at 120 °C. Plates were developed in chloroform/methanol/NH₄OH/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 [3H]labelled phospholipids as standards, which were detected by using En3Hance (NEN DuPont).

RESULTS

Use of exogenous PtdInsP₂ 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 [3H]PtdInsP₂ 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)P₂ proved unsuitable, since GroPIns was the major water-soluble product (Table 1). Different mixtures of phospholipids and different concentrations of Mg²⁺ and Ca²⁺ were used, as well as inhibitors of inositol-phosphate phosphatases, but significant [3H]Ins(1,4,5)P₃ production remained undetectable. Comparable data were obtained with PtdInsP 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 PtdInsP₂

Two assays may allow the detection of phospholipase activity in vitro when endogenous substrate is used; either to measure the production of [3H]Ins(1,4,5)P₃ in membranes derived from [3H]inositol-labelled cells, as described by Cubitt and Firtel [32], or to use the Ins(1,4,5)P₃ mass assay to determine the amount of Ins(1,4,5)P₃ produced form unlabelled PtdInsP₂. 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 Ca²⁺, a large production of Ins(1,4,5)P₃ was observed (results not shown). If lysis took place in the presence of the Ca²⁺-chelator EGTA, no activity could be detected over a period of at least 1 min (Figure 1a). Re-adding Ca²⁺ to the lysate led to the re-activation of the enzyme (Figure 1a). Thus, by using EGTA and Ca²⁺ the enzyme can be turned off and on; the lysate if made in the presence of EGTA and Ins(1,4,5)P₃ formation is allowed to proceed for a fixed period of time by starting the reaction with Ca²⁺ and terminating it with HClO₄.

The time course of Ins(1,4,5)P₃ production after Ca²⁺ addition (Figure 1a) shows that the rate of Ins(1,4,5)P₃ production was constant up to 30 s and then slowly declined; 20 s was chosen as a standard incubation time. Addition of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺.

Identification of the reaction product as Ins(1,4,5)P₃

Two experiments were performed to demonstrate that the compound cross-reacting with the Ins(1,4,5)P₃-binding protein is authentic Ins(1,4,5)P₃. In the first experiment a sample from the phospholipase C assay was mixed with authentic [3H]Ins(1,4,5)P₃ and incubated with a purified rat brain Ins(1,4,5)P₃ 5-phosphatase or a partially purified Dictyostelium Ins(1,4,5)P₃ 1-phosphatase. If the cross-reactivity was due to Ins(1,4,5)P₃, both cross-reactivity and [3H]Ins(1,4,5)P₃ 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 [3H]PtdInsP₂ and [3H]PtdInsP₃ in Dictyostelium discoideum**

<table>
<thead>
<tr>
<th>Amount (% of soluble radioactivity)</th>
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<tbody>
<tr>
<td>Product</td>
</tr>
<tr>
<td>Ins</td>
</tr>
<tr>
<td>GroPIns</td>
</tr>
<tr>
<td>InsP</td>
</tr>
<tr>
<td>GroPInsP</td>
</tr>
<tr>
<td>InsP₂</td>
</tr>
<tr>
<td>InsP₃</td>
</tr>
</tbody>
</table>

**Figure 1** Ins(1,4,5)P₃ production in lysates

(a) Time course of Ins(1,4,5)P₃ production after lysis in the presence of 5.9 mM EGTA with (●) or without (○) 5.9 mM CaCl₂ re-added to the lysate. CaCl₂ was added at 10 s after lysis, which is at t = 0 in the Figure. (b) Time course of re-addition of CaCl₂ to the lysate on Ins(1,4,5)P₃ production. The enzyme reactions were conducted for 20 s; t = 0 is the moment of cell lysis. The free Ca²⁺ concentration for both panels was 10 μM. Ins(1,4,5)P₃ levels before lysis were subtracted. Data are expressed as means ± S.E.M. of 3 independent experiments in triplicate.
chromatographed with an authentic Ins(1,4,5)P₃ 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)P₃-binding protein from bovine liver, (ii) is degraded to approximately the same extent as authentic Ins(1,4,5)P₃ by Ins(1,4,5)P₃ 5-phosphatase, and (iii) co-chromatographs with authentic Ins(1,4,5)P₃ on a RP-18 h.p.l.c. system.

In order to establish whether the amount of substrate (i.e. PtdInsP₂) present in the lysate was sufficient and not a limiting factor in the phospholipase C assay, the total mass of PtdInsP₂ was determined (Table 2). Cells contain about 300 pmol of PtdInsP₂/mg of protein. PtdInsP₂ levels of a lysate prepared in EGTA were decreased to approx. 110 pmol/mg. Re-addition of Ca²⁺ to the lysate induced the production of 47 pmol of Ins(1,4,5)P₃/mg; the level of PtdInsP₂ was still 94 pmol/mg, indicating that sufficient substrate remained.

Table 2. PtdInsP₂ and Ins(1,4,5)P₃ contents of lysates after phospholipase C reaction

<table>
<thead>
<tr>
<th></th>
<th>PtdInsP₂ (pmol/mg)</th>
<th>Ins(1,4,5)P₃ (pmol/mg)</th>
<th>PLC activity (pmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>302</td>
<td>15.4</td>
<td>100</td>
</tr>
<tr>
<td>Lysate</td>
<td>111</td>
<td>15.6</td>
<td>0</td>
</tr>
<tr>
<td>Lysate + Ca²⁺</td>
<td>94</td>
<td>62.3</td>
<td>140</td>
</tr>
</tbody>
</table>

Therefore, the presence of the phospholipase C activity was measured in stimulated and unstimulated cells, demonstrating that less than 10% of Ins(1,4,5)P₃ was degraded under phospholipase C assay conditions in the absence or presence of Ca²⁺. To address these questions, phosphatase activity was measured in stimulated and unstimulated cells, showing that Ca²⁺ had no effect on the stimulation of PtdInsP₂ by PtdInsP₁. Inhibition of phosphatase activity or an increased turnover of PtdInsP₁ to PtdInsP₂ could also explain the observed enhanced accumulation of Ins(1,4,5)P₃. To address these questions, phosphatase activity was measured in stimulated and unstimulated cells, demonstrating that less than 10% of Ins(1,4,5)P₃ was degraded under phospholipase C assay conditions in the absence or presence of Ca²⁺ (Table 3). In addition, the incorporation of radioactivity from [γ-³²P]ATP into PtdInsP and PtdInsP₁ was measured, showing that Ca²⁺ had no effect on PtdIns kinase and PtdInsP kinase activities in vitro. These results indicate that the increase in Ins(1,4,5)P₃ production in a cell lysate prepared from cAMP-stimulated cells is due to an increased phospholipase C activity.

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

Main Figure: h.p.l.c. analysis. Lysates in GSTA from 2 × 10⁷ cells were incubated without (○) or with 10 μM free Ca²⁺ (●), 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)P₃ assay buffer and assayed for cross-reactivity with the Ins(1,4,5)P₃-binding protein. Arrows indicate the fractions in which authentic Ins(1,4,5)P₃ (A), Ins(1,4,5)P₃ (B) and Ins(3,4,5)P₂ (C) were eluted. Inset: enzymic degradation of cross-reactivity. A sample from the phospholipase C reaction was mixed with [³²P]Ins(1,4,5)P₃, and incubated with purified rat brain 5-phosphatase (5) and partially purified Dictyostelium Ins(1,4,5)P₃ 1-phosphatase (1). Then each sample was divided: one part was analysed for the degradation of radioactive Ins(1,4,5)P₃ (B) and the other part for the degradation of cross-reactivity in the Ins(1,4,5)P₃-binding protein assay (22).

**Figure 3** Ca²⁺-dependency of phospholipase C

Phospholipase C activity was determined at 5.9 mM EGTA and different CaCl₂ concentrations to yield the indicated free Ca²⁺ concentrations. E, activity in the presence of 5.9 mM EGTA with added CaCl₂. Data shown are means ± S.E.M. of 3 independent experiments in triplicate.

**Ca²⁺-dependency of phospholipase C**

Since most phospholipase C enzymes described so far are dependent on Ca²⁺, the Ca²⁺-sensitivity of Dictyostelium phospholipase C was characterized further by using Ca²⁺/EGTA buffers to generate fixed Ca²⁺ concentrations. Figure 3 shows a dose-response curve for Ca²⁺ which is bell-shaped. Half-maximal activity was observed at 0.1 μM, activity was maximal between 1 and 100 μM, and only at concentrations above 100 μM 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)P₃ levels in vitro [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 μM cAMP.

Although activation of phospholipase C is the most likely cause for the observed increase in Ins(1,4,5)P₃, inhibition of phosphatase activity or an increased turnover of PtdInsP₁ to PtdInsP₂ could also explain the observed enhanced accumulation of Ins(1,4,5)P₃ in vitro. To address these questions, phosphatase activity was measured in stimulated and unstimulated cells, demonstrating that less than 10% of Ins(1,4,5)P₃ was degraded under phospholipase C assay conditions in the absence or presence of cAMP (Table 3). In addition, the incorporation of radioactivity from [γ-³²P]ATP into PtdInsP and PtdInsP₁ was measured, showing that cAMP had no effect on PtdIns kinase and PtdInsP kinase activities in vitro. These results indicate that the increase in Ins(1,4,5)P₃ 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,
Table 3  Ins(1,4,5)P_3 phosphatase and Ptdlns/PtdInsP 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 [3H]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 [γ-32P]ATP in the absence or presence of 1 μM cAMP. The phospholipids were isolated by t.l.c. and quantified.

<table>
<thead>
<tr>
<th>Phospholipase C</th>
<th>Phosphatase</th>
<th>Kinase with product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pmol/min)</td>
<td>[% degradation of Ins(1,4,5)P_3]</td>
<td>[% incorporation of [γ-32P]ATP]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PtdInsP</td>
</tr>
<tr>
<td>Control</td>
<td>156</td>
<td>13</td>
</tr>
<tr>
<td>cAMP</td>
<td>294</td>
<td>10</td>
</tr>
</tbody>
</table>

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.

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 μM cAMP; ○, phospholipase C activity after lysis in the presence of 10 μM GTP[S]. Data shown are means of a typical experiment performed in triplicate.

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 μM cAMP, 10 μM GTP[S] and 100 μM GDP[S]. Data are means ± S.E.M. of 3 independent experiments in triplicate.* significantly above control (P < 0.05).

<table>
<thead>
<tr>
<th>Addition</th>
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<tbody>
<tr>
<td></td>
<td>Phospholipase C activity (pmol/min. per mg)</td>
</tr>
<tr>
<td>−</td>
<td>122 ± 41</td>
</tr>
<tr>
<td>cAMP</td>
<td>254 ± 51*</td>
</tr>
<tr>
<td>GDP[S]</td>
<td>235 ± 65*</td>
</tr>
<tr>
<td>GTP[S] + GDP[S]</td>
<td>142 ± 46</td>
</tr>
<tr>
<td>GDP[S]</td>
<td>115 ± 50</td>
</tr>
<tr>
<td>cAMP + GTP[S]</td>
<td>256 ± 69*</td>
</tr>
</tbody>
</table>

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 μM 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 μM 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...
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)P₂ and the strict Ca²⁺-dependency of the enzyme. During lysis, Ca²⁺ is removed to decrease enzyme activity. Subsequently, Ca²⁺ is re-added to the lysate for a fixed period of time, allowing accurate determination of phospholipase C activity. The InsP₃ produced is shown to be Ins(1,4,5)P₃. Several basic properties of the enzyme are as follows. (i) The enzyme is Ca²⁺-dependent, with half-maximal activity at 0.1 μM Ca²⁺ and optimal activity at 10 μM Ca²⁺. (ii) The EGTA-inactivated enzyme is stable for up to 30 s. (iii) The Ca²⁺-activated enzyme is stable for at least 1 min, and Ins(1,4,5)P₃ production is linear with time for at least 30 s. (iv) GTP[S] 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 Ca²⁺-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)P₂ resulted in the production of GroPLins instead of Ins(1,4,5)P₃. Apparently, phospholipase A and the phosphatases are more active than phospholipase C. The fatty acid composition of the PtdIns(4,5)P₂ used may explain this result; commercially available PtdInsP₂ is an arachidonyl-stearoyl species, yet Dictyostelium discoideum has been shown to lack C₄₀₀₄ 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 PtdInsP₂ in small unilamellar vesicles exposes PtdInsP₂ 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 [H]PtdInsP₂, a phospholipase C was described in partially purified membranes. More recently Cubitt and Firtel [32] showed the presence of a membrane-bound activity, using endogenously [H]inositol-labelled PtdInsP₂ as a substrate. Enzyme kinetics, Ca²⁺-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)P₃ 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 Ca²⁺-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-δ class. Expression of phospholipase C-δ 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-δ 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)P₃ 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)P₃, we have shown that a 2-fold increase in phospholipase C activity is sufficient to generate the observed increase in Ins(1,4,5)P₃ (J. Van der Kaay, A. A. Bominara and P. J. M. Van Haastert, unpublished work).

Unexpectedly, cAMP not only activates phospholipase C; at concentrations above 10 μM 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 role of the enzyme in 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.

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