Membrane-associated phosphoinositidase C activity in *Dictyostelium discoideum*

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Membrane-associated phosphoinositidase C activity has been identified in *Dictyostelium discoideum* using phosphatidylinositol 4,5-bisphosphate as exogenous substrate. Maximal activity was observed with 0.4 mM phosphatidylinositol 4,5-bisphosphate at pH 7.0. The enzyme was stimulated by micromolar concentrations of free calcium with maximal activity at 100 μM.

Phosphoinositidase C; Phosphatidylinositol 4,5-bisphosphate; Membrane; Signal transduction; *Dictyostelium discoideum*

1. INTRODUCTION

Cell signalling in the cellular slime mould *Dictyostelium discoideum* involves two distinct signal transduction systems: one involving adenylate cyclase for relay of the cyclic AMP signal and the other involving inositol 1,4,5-trisphosphate [1,2]. Evidence for the inositol phosphate pathway has come from studies using intact and saponin-permeabilized amoebae [2–4]. The presence of plasma membrane-associated phosphoinositidase C catalysing the hydrolysis of PIP_2 to diacylglycerol and inositol 1,4,5-trisphosphate can be postulated from these data. However, this is so far the first report that describes a membrane-associated PIP_2 phosphoinositidase C in *D. discoideum*.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]PIP_2 was purchased from Amersham and the non-radiolabeled PIP_2 (bovine brain) was from Sigma. AG 1-x8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad. All other chemicals were from Sigma.

2.2. *Harvesting of amoebae*

D. discoideum cells (strain NC4, wild-type cells) were grown in association with *Klebsiella aerogenes* (strain OXF1) on SM nutrient agar as described earlier [3,5]. The cells were harvested from the bacterial plates in cold 0.5 mM $MgCl_2$, 5 mM glycine, pH 8.5, and freed from the bacteria by repeated centrifugation at 190 × *g* for 2 min in the buffer. The *D. discoideum* cells were finally resuspended at $5 \cdot 10^7$ cells/ml in this buffer also containing 30 μg leupeptin/ml, 30 μg antipain/ml and 30 μg PMSF/ml (lysis buffer).

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetracetic acid

2.3. *Cell lysis and membrane preparation*

$1 \cdot 10^9$ cells in lysis buffer were allowed to warm to room temperature. The cells were lysed by pressing them through 5 μm pores of two Nucleopore filters (Nucleopore Corp.) according to the method by Das and Henderson [6]. The following steps were performed at 0–4°C. The cell lysate was centrifuged at 200 × *g* for 5 min to remove whole cells (more than 99% lysis) and then centrifuged at 5800 × *g* for 20 min. The pellet was hand-homogenized by 3 strokes with a teflon pestle in the same volume of 10 mM Tricine-NaOH, pH 7.5, containing 30 μg leupeptin/ml, 30 μg antipain/ml and 30 μg PMSF/ml and centrifuged, 38 000 × *g* for 30 min. The membranes were resuspended to approx. 10 mg protein/ml in 10 mM Tricine-NaOH at pH 7.5, 30 μg leupeptin/ml, 30 μg antipain/ml and the phosphoinositidase C activity towards exogenous PIP_2 was assayed immediately. Protein was determined according to Lowry et al. [7] in the presence of 1% sodium dodecylsulphate using bovine serum albumin as standard.

2.4. *Determination of PIP_2 phosphoinositidase C activity*

The standard incubation mixture contained 50 mM Tris-maleate, pH 7.0, 100 μM $CaCl_2$, 0.4 mM PIP_2 (5000 dpm/nmol) and 150 μg of membrane protein in a final volume of 50 μl. For experiments in which calcium sensitivity of PIP_2 phosphoinositidase C was measured, EGTA was included in the assay according to [8]. The reaction was started by the addition of PIP_2 which was prepared by evaporating the lipid in solvent to dryness under a stream of N_2 followed by sonication in the Tris-maleate buffer for 45 s. Control samples were boiled for 5 min and cooled to incubation temperature prior to the addition of radiolabeled inositol phospholipid substrate. Incubations in duplicate were for 5 min at 37°C and they were stopped by addition of 50 μl ice-cold 100 mM CDTA, 1 mM mannitol [9] followed by 1 ml cold chloroform/methanol 2:1 (v/v). After addition of 250 μl of 1 M HCl to each sample and vigorous vortexing, they were kept on ice for 30–60 min. The samples were then centrifuged for 45 s in a Beckman Microfuge [10]. The upper phase was neutralized and used for analysis of inositol trisphosphate by anion exchange chromatography on AG 1-x8 formate columns as described earlier [11] before liquid scintillation counting. The data presented are representative of 3–4 independent membrane isolations. Duplicate analyses within an experiment deviated between 1 and 5% from the mean value.

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In some experiments, lipids of the organic phase were analyzed by thin layer chromatography for determination of phosphomonoesterase products [10] on Silica Gel plates (Merck AG) impregnated with potassium oxalate according to [12]. The solvent system used to separate the inositol phospholipids was as described earlier [13] and appropriate bands were scraped off before counting the radioactivity.

3. RESULTS AND DISCUSSION

Phosphoinositidase C (inositol phospholipid-specific phospholipase C) has proven to be a ubiquitous activity in eukaryotic cells. The phosphoinositidase C isozymes, which cleave inositol phospholipids to diacylglycerol and inositol phosphates, are present in most mammalian cells as well as in plants [14]. The present work was aimed at identifying such a membrane-associated phosphoinositidase C activity with PIP_2 in the lower eukaryote *D. discoideum*. Such activity could be postulated from earlier studies using intact and saponin-permeabilized amoebae in which stimulation of cell-surface cyclic AMP receptors induced transient elevation of inositol 1,4,5-trisphosphate levels [2–4]. Phosphoinositidase C activity in a crude membrane fraction from *D. discoideum* cells was tested using added PIP_2 as substrate. As the PIP_2 phosphoinositidase C in these cells had not previously been demonstrated some of its properties were studied in more detail.

The phosphoinositidase C activity increased linearly with substrate concentration reaching a maximal level at 15 to 20 nmol added PIP_2 (Fig. 1). Fig. 2 shows that the reaction was linear with time for approx. 5 min with 20 nmol of added PIP_2.

Variation of pH from 5.5 to 8.0 was performed under standard incubation conditions. The phosphoinositidase C activity against PIP_2 showed activity optimum in the pH range 6.5–7.0; in this pH range the activity was almost 9- and 2-fold higher than at pH 5.5 and 8.0, respectively (Fig. 3).

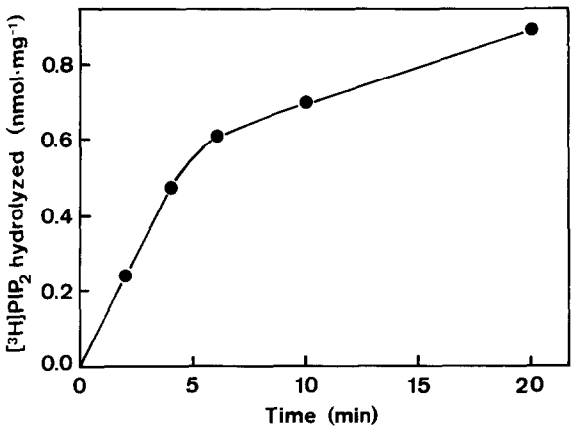

Fig. 2. PIP_2 phosphoinositidase C activity as a function of incubation time. See section 2 for further details.

When the water-soluble reaction products formed were analyzed by chromatography on Ag 1-x8, 68% was found to be inositol trisphosphate but a relatively large amount of the radioactivity, i.e. 24 and 8% was recovered as inositol bisphosphate and inositol phosphate, respectively. The formation of some inositol bisphosphate and inositol phosphate might have been explained by the observation of phosphomonoesterase products of PIP_2 in the organic phase analyzed by thin layer chromatography. It was conceivable that in the membrane fraction the PIP_2 was being hydrolyzed first by phosphomonoesterase(s) to PIP and PI, which were then in turn being hydrolyzed by phosphodiesterase activity. However, under standard incubation conditions, only 2.5% and 2.0% of the added PIP_2 was recovered as PIP and PI, respectively. The high level of inositol bisphosphate may be better explained by the particulate inositol phosphatase activity in *D. discoideum* cells earlier reported by Van

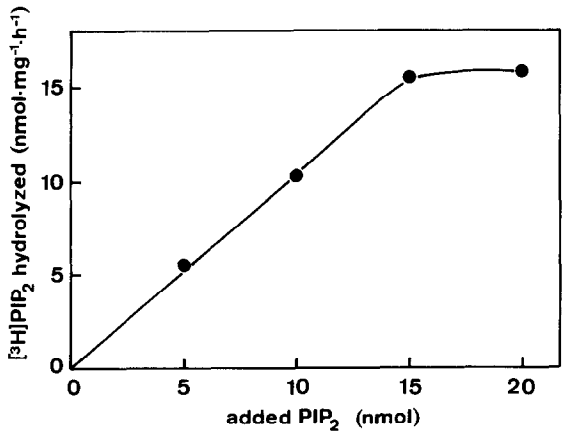

Fig. 1. PIP_2 phosphoinositidase C as a function of substrate concentration. Incubations in duplicate were performed as described in section 2 and are representative of 3 similar experiments.

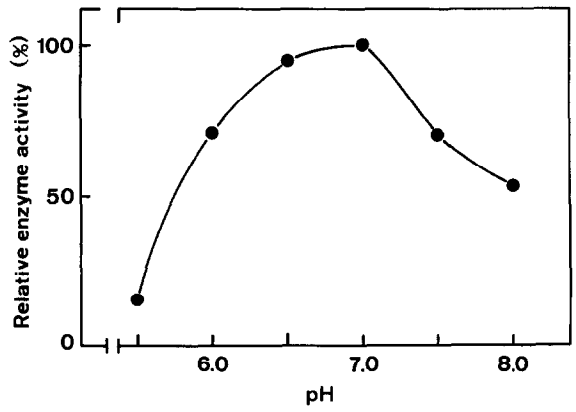

Fig. 3. PIP_2 phosphoinositidase C activity as a function of pH. Incubations in duplicate were performed as in section 2 with 50 mM (final concentration) Tris-maleate buffer. The specific activity observed at pH 7.0 was 9.3 nmol [^{3}H]PIP_2 hydrolyzed per mg protein per hour.

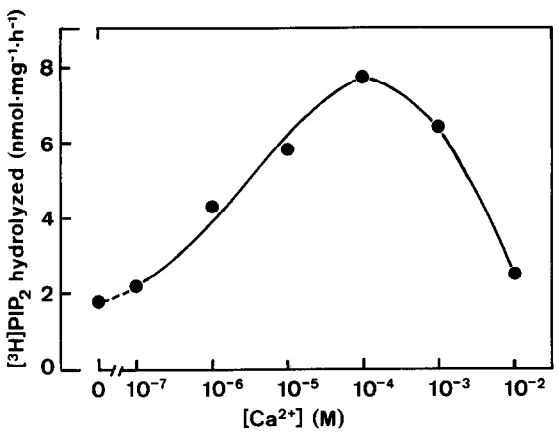

Fig. 4. PIP_2 phosphoinositidase C activity as a function of Ca^{2+} concentration. The concentration of free Ca^{2+} was varied by different EGTA-$CaCl_2$ mixtures [8]. Incubations in duplicate were performed as described in section 2.

Lookeren Campagne et al. [15] which converts inositol 1,4,5-trisphosphate to inositol 1,4-bisphosphate and inositol 4,5-bisphosphate. Phospholipase D activity hydrolysing PIP_2 to phosphatidic acid and inositol bisphosphate cannot be excluded.

In order to determine the effects of varying the Ca^{2+} concentration, the PIP_2 phosphoinositidase C activity was assayed over a range of free Ca^{2+} concentrations using EGTA-$CaCl_2$ buffers [8] at pH 7.0 (Fig. 4). Basal activity was observed without addition of Ca^{2+} (just the EGTA present) or with those submicromolar concentrations of free Ca^{2+} that would be expected to occur in vivo. The hydrolysis of PIP_2 increased in the range of micromolar Ca^{2+} concentrations reaching a maximum at 100 μM. Ca^{2+} in the millimolar range rather inhibited the PIP_2 phosphoinositidase C activity. In summary, the PIP_2 phosphoinositidase C in *D. discoideum* cells is Ca^{2+}-dependent, but no definite conclusion can be drawn about whether it is Ca^{2+}-controlled.

This study is the first to establish the presence of membrane-associated phosphoinositidase C activity against PIP_2 in *Dictyostelium*. These results support the involvement of the inositol phospholipid cycle in signal transduction in the amoebae. As a logical progression toward biochemically characterizing inositol phospholipid signal transduction in *Dictyostelium* it will be of particular interest to purify the enzyme and study interactions with the putative guanine nucleotide-binding protein [16].

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