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 PIP2 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 PIP2 phosphoinositidase C in D. discoideum.

2. MATERIALS AND METHODS

2.1. Materials

[3H]PIP2 was purchased from Amersham and the non-radio-labeled PIP2 (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 MgCl2, 5 mM glycine, pH 8.5, and freed from the bacteria by repeated centrifugation at 190 x g for 2 min in the buffer. The D. discoideum cells were finally resuspended at 5 x 10^7 cells/ml in this buffer also containing 30 μg leupeptin/ml, 30 μg antipain/ml and 30 μg PMSF/ml (lysis buffer).

2.3. Cell lysis and membrane preparation

1 x 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 x g for 5 min to remove whole cells (more than 99% lysis) and then centrifuged at 3800 x 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 x 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 PIP2 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 PIP2 phosphoinositidase C activity

The standard incubation mixture contained 50 mM Tris-maleate, pH 7.0, 100 mM CaCl2, 0.4 mM PIP2 (5000 dpm/nmol) and 150 μg of membrane protein in a final volume of 50 μl. For experiments in which calcium sensitivity of PIP2 phosphoinositidase C was measured, EGTA was included in the assay according to [8]. The reaction was started by the addition of PIP2 which was prepared by evaporating the lipid in solvent to dryness under a stream of N2 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 PIP2 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 PIP2 as substrate. As the PIP2 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 PIP2 (Fig. 1). Fig. 2 shows that the reaction was linear with time for approx. 5 min with 20 nmol of added PIP2.

Variation of pH from 5.5 to 8.0 was performed under standard incubation conditions. The phosphoinositidase C activity against PIP2 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).

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 PIP2 in the organic phase analyzed by thin layer chromatography. It was conceivable that in the membrane fraction the PIP2 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 PIP2 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. 4. PIP2 phosphoinositidase C activity as a function of Ca\(^{2+}\) concentration. The concentration of free Ca\(^{2+}\) was varied by different EGTA-Ca\(^{2+}\) 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 PIP2 to phosphatidic acid and inositol bisphosphate cannot be excluded.

In order to determine the effects of varying the Ca\(^{2+}\) concentration, the PIP2 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 PIP2 increased in the range of micromolar Ca\(^{2+}\) concentrations reaching a maximum at 100 \(\mu\)M. Ca\(^{2+}\) in the millimolar range rather inhibited the PIP2 phosphoinositidase C activity. In summary, the PIP2 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 PIP2 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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