Diacylglycerol pyrophosphate: a novel metabolite in the *Trypanosoma cruzi* phosphatidic acid metabolism

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Abstract This work provides evidence that phosphatidic acid (PA) is metabolized to diacylglycerol pyrophosphate (DGPP) in *Trypanosoma cruzi*. Also the presence of the enzymatic activities involved in its regulation, phosphatidate kinase (PA-k) and phosphatidate phosphatase, is demonstrated. The increase of DGPP levels in *T. cruzi* epimastigotes or in its membrane fraction after exogenous PA addition or phospholipase (PLD) pre-treatment suggests that PA-k may be involved in the regulation of PA levels after its stimulation.

Key words: Diacylglycerol pyrophosphate; Diacylglycerol kinase; Phosphatidate kinase; Phosphatidic acid; Phospholipid; *Trypanosoma cruzi*

1. Introduction

In previous studies [1–3], we demonstrated that different stimuli are able to modify PI and PA metabolism in *Trypanosoma cruzi*, a protozoan parasite of man and causative agent of Chagas' disease. These results were consistent with the activation of the inositol phosphate/diacylglycerol pathway in the parasite as a response to external signals. Thus, they showed the existence of phosphoinositide PLC activation and of a possible cross-talk mechanism with PKC and c-AMP dependent protein kinase A [3]. Phosphatidic acid, intermediate of lipid metabolism, has been suggested to be an important second messenger in higher eukaryotic cells. It is rapidly produced in receptor-stimulated cells by phospholipase D and/or the combined action of phospholipase C/DG kinase activities [4]. In general, PA is converted to DG by PA phosphatase or to lyso-PA by PLA2 [5]. Therefore, these enzymes may regulate the signaling functions of phosphatidate.

Recently, a new mechanism of PA attenuation was reported in which this phospholipid is converted into diacylglycerol pyrophosphate (DGPP) by a phosphatidic acid kinase. This enzyme was first found in the plant kingdom [6,7]; recently, it has also been detected in *Sacharomyces cerevisiae* [8]. At present, it is not clear what role DGPP plays in the lipid metabolism and cell signaling, but it has been suggested that it may function as a signaling molecule itself [9]. The knowledge of the molecular mechanism in the interconvertion of PA and DG is essential to understand the regulatory mechanism of signal transduction linked to phospholipid turnover.

In this paper we investigated the regulation of *T. cruzi* PA levels, one of the products following phosphoinositide PLC stimulation, through its convertion in DGPP. The results reported here describe, for the first time, the PA kinase activity in *T. cruzi* membrane fraction and suggest possible physiological consequences of this new pathway.

2. Materials and methods

2.1. Organisms and growth condition

The *T. cruzi* Tulahuen strain was used in this study. The epimastigote forms were grown during 6 days at 28°C in a modified Warren medium [10], as was described by Racagni et al. [11].

2.2. Preparation of epimastigote membranes

The washed cells were suspended in 5 volumes of 50 mM HEPES (pH 7.4) containing: 0.25 M sucrose, 5 mM KCl, 1 mM EDTA and 4μg/ml leupeptin. This suspension was frozen at −180°C and thawed three times, homogenized and centrifuged at $100\times g$ for 15 min to remove unbroken cells and cell debris. The supernatant was then centrifuged at $105\,000\times g$ for 60 min to obtain the membrane fraction. The membranes were washed and resuspended with 50 mM HEPES (pH 7.4) (1.5 mg protein/ml buffer). These membranes were used as a source of PA kinase and DG kinase activities.

2.3. PLD pre-treatment

In other experiments, whole washed cells or $105\,000\times g$ membrane fraction were incubated with exogenous phospholipase D (PLD) from *Streptomyces cromofocus* (Sigma P8023, 100 U/ml) in KRT buffer (pH 8), for 30 min at 28°C. PLD pre-treated membranes were inactivated at 100°C for 30 min and then used as a source of exogenous substrate.

2.4. Determination of lipid kinase activities

PA kinase and the other lipid kinases were simultaneously assayed using endogenous lipids as substrates, unless otherwise stated. The membrane fraction isolated (30–80 μg of protein), unless otherwise indicated, was added to thermally equilibrated buffer 50 mM HEPES (pH 7.4) containing (mM): EDTA (0.1), DTE (0.5), $MgCl_2$ (10), sodium *o*-vanadate (0.1), Na_2ATP (1.0), specific activity 250 cpm/pmol. Endogenous lipid phosphorylation was allowed to proceed for 5 min at 30°C in a final volume of 100 μl. The incubation mixture was subsequently quenched with 1.5 of chloroform/methanol (1:2, v/v). In some experiments, phosphatidic acid, dioleoyl (C18:1, [cis]-9) (Sigma, P2767) and 1,2-dihexanoyl-*sn*-glycerol (Sigma, D8531) were used as an exogenous substrate. The chloroform stock solutions were prepared into a glass test tube and then evaporated under a stream of N_2, resuspended in 50 mM HEPES (pH 7.4) and then sonified 6-fold for 30 s at 0°C. When the effect of polyamines was

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Abbreviations: DG, diacylglycerol; DG-k, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; PAP, PA phosphatase; PI, phosphatidylinositol phosphate; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D

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studied the membrane fractions were incubated in ice for 15 min with (μM): spermine (300), spermidine (500) or putrescine (500). In these cases the assay mix contained 0.2 mM sodium *o*-vanadate and 10 mM Mg ion.

2.5. Lipid extraction and separation

The lipids were extracted from membranes according to Schacht [12]. Chromatograms were developed with solvent systems: (I) chloroform/methanol/ammonium hydroxide/water (90:70:4:16), (II) chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v), (III) chloroform/pyridine/formic acid (35:30:7, v/v). The positions of labeled phospholipids on the chromatograms were determined by autoradiography. The amount of labeled phospholipids was determined by scintillation counting.

2.6. Pre-incubation of epimastigotes with radioactive precursor

The cells harvested in the logarithmic phase were pre-incubated and gently agitated in a shaking bath at 28°C for 12 h in KRT buffer [8], containing 0,1% bovine serum albumin (BSA), 10% calf serum and 5 μCi/25 mg cells of [2-^{3}H]glycerol. Incorporation of radioactivity in the cells ranged between 20–30%.

2.7. Protein determination

Protein content of membrane samples was determined according to Bradford [13] with bovine albumin as standard.

2.8. Calculations

The values of the tests were calculated as means and standard deviations for three experiments. The values presented without standard deviations are values of single representative tests. Statistical significance was not determined.

2.9. Materials

All chemicals were reagent grade. Solvents and TLC plates (Art. 5721) were purchased from Merck (Darmstadt, Germany). Phospholipid standards and phosphatidic acid were obtained from Sigma Chemical Co. (USA). [γ^{32}P]ATP and [2-^{3}H]glycerol Net 022 from NEN Life Science Products (Dupont, USA).

3. Results

3.1. DGPP as a lipid component of T. cruzi

The phosphorylation of membrane fraction by [γ^{32}P]ATP, isolated from *T. cruzi* epimastigote forms, resulted in rapid labeling of chloroform-soluble products. They were identified as PA, PIP, DGPP and PIP2 based on their chromatographic mobilities on solvent mixture I [9]. The chromatographic properties of DGPP on solvent mixture II and III, indicated that it was a pyrophosphorylated derivate from PA [6,7] (Fig. 1).

3.2. PA kinase activity

DGPP formation was dependent on Mg ion concentration, MgATP, pH and protein concentration. The phosphorylation of endogenous PA under standard conditions was linear for 5 min with 30 μg of protein, 5 pmol/min/mg protein. PA kinase showed an optimum pH between pH 7 and 7.5, with a rapid decrease of activity below pH 6.6 (Fig. 2).

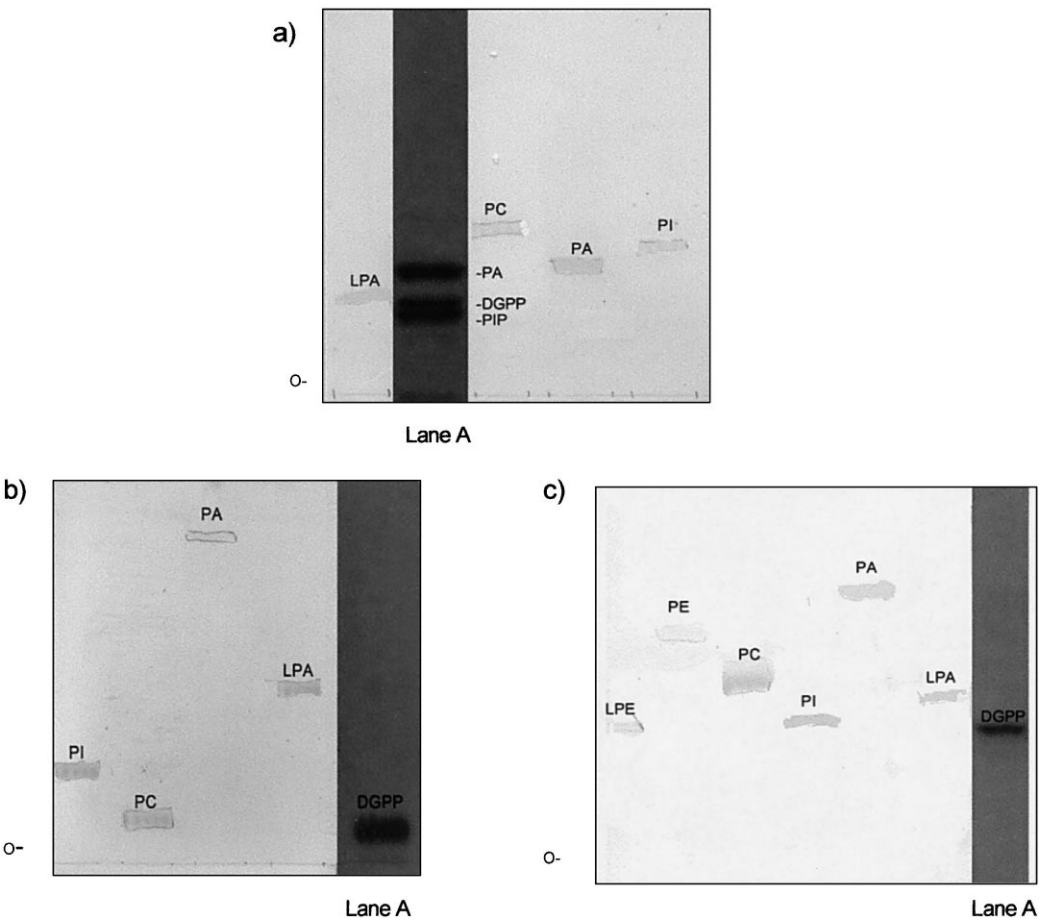

Fig. 1. Autoradiography of lipid kinase products and reference compounds separated by thin layer chromatography. *T. cruzi* membrane lipids were phosphorylated in the presence of exogenous [γ^{32}P]ATP and endogenous lipid substrates (lane A), extracted and separated with solvent mixture I (a), II (b) or III (c) (Section 2). Standard phospholipids were stained with iodine and their spots were mounted according to their Rf-values: PA, phosphatidic acid; LPA, lyso-PA; PC, phosphatidylcholine; PI, phosphatidylinositol; LPE, lyso-phosphatidylethanolamine.

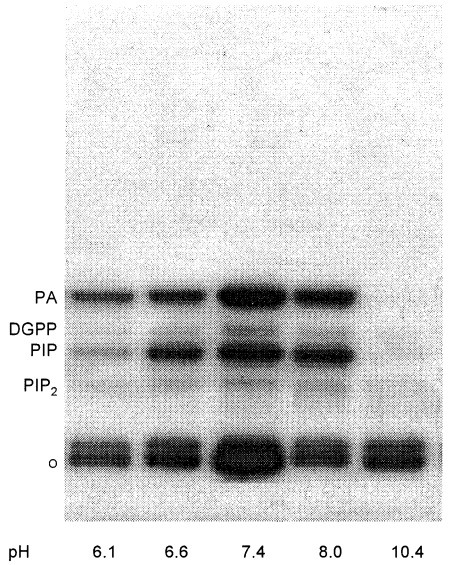

Fig. 2. Dependence of PA kinase activity on pH. PA kinase activity was assayed under standard conditions at the indicated pH values. *T. cruzi* membrane lipids were phosphorylated in the presence of exogenous [γ^{32}P]ATP and endogenous lipid substrates, extracted and separated with solvent mixture I (Section 2).

Since *S. cerevisiae* DGPP phosphatase exhibits a broad optimum pH between 6.0 and 8.5 [8], in this study *T. cruzi* PA kinase activity was routinely measured in the presence of a variety of phosphatase effectors (mM): sodium vanadate (0.2), p-nitrophenyl phosphate (pNPP) (0.1) and NaF (10).

PA kinase followed Michaelis-Menten kinetics with respect to MgATP. The values of K_m and V_{max}, determined under the conditions mentioned and in the presence of 2.5 mM Mg ions, were 80 μM and 20 pmol/min/mg, respectively. Mg ions were inhibitory up to 4 mM; the activity obtained with 10 mM Mg ions was approximately 30% lower than 2.5 mM (14–16 pmol/min/mg). In addition, Mn ions inhibited the activity at concentrations above 1.5 mM in the presence of 10 mM Mg^{2+}. In the absence of Mg ions, Mn ions were only slightly stimulating.

The analysis of phospholipids from the cells incubated for 12 h with [2-^{3}H]glycerol showed that the DGPP cellular concentration accounted for 4% of the total phospholipids (5×10^{-5} pmol/mg of wet weight), as in the case of PA [11]. In spite of this, PA-k specific activity was 5-fold lower than DG-k (75 pmol/min/mg) in the presence of 10 mM Mg ions.

3.3. Effectors of PA kinase activity

The inhibitory or stimulatory effects of DGPP phosphatase effectors on PA-k activity were studied using endogenous and exogenous PA as substrate.

In Fig. 3 we showed that PA-k activity increased with respect to the control, 36 ± 10% and 91 ± 42%, in the absence and presence of exogenous PA respectively when NaF was added to the assay system. In contrast, the additions of Mn ions (0.1 mM) to the assay system, known as an inhibitor of DGPP phosphatase activity, did not result in a significant change of PA-k [8,14]. PA-k activity decreased 51 ± 11% after the pre-incubation of membrane with sodium deoxycholate (DOC), a liver PAP activator [15]; however, it was followed by an increase of 62 ± 29% with the addition of exogenous

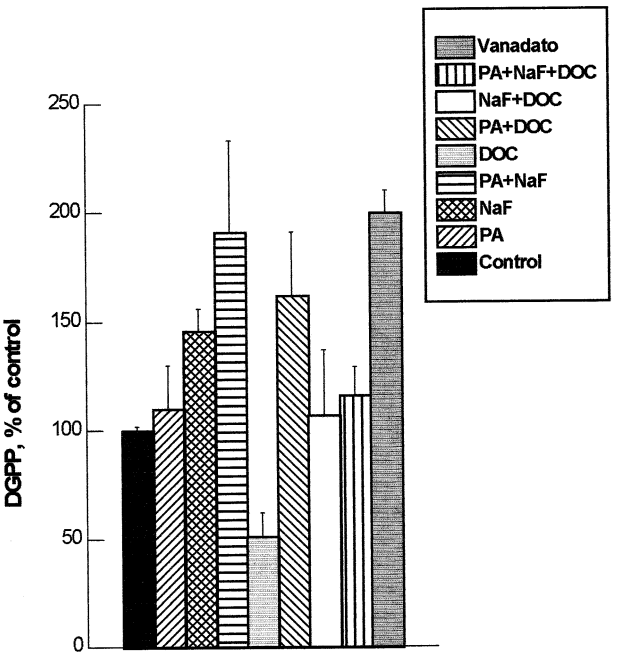

Fig. 3. Effectors of PA-k activity. PA kinase activity was measured under standard assayed conditions as described in Section 2 in the presence of the following effectors (mM): 0.08 PA, 10 NaF, 1 sodium deoxycholate (DOC), 0.2 sodium *o*-vanadate. The results shown are representatives of four separated experiments ± S.D.

PA. The PA-k activity remained constant when NaF and DOC were present in the assay system independently of exogenous PA presence.

When the ATPase and phosphatase inhibitor vanadate was added to the medium, PA-k activity increased 100% with re-

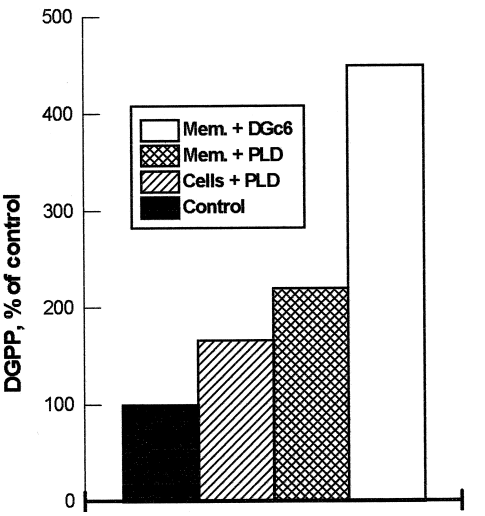

Fig. 4. Effect of the addition of exogenous PLD and dihexanoylglycerol on DGPP formation. Cells or membrane fractions of epimastigote forms were incubated for 30 min at 30°C with exogenous phospholipase D (PLD) of *Streptomyces chromofuscus* (100 U/ml). 105 000×*g* membranes were isolated from whole cells and phosphorylated as described in Section 2 ($n=2$ experiments). In the case indicated, the membrane control was phosphorylated in the presence of 0.5 mM 1,2-dihexanoyl-*sn*-glycerol (DG C6:0) resulting in an activity of 450 ± 30% of control ($n=3$ experiments).

spect to the control. This fact may indicate the presence of phosphatase and ATPase activity in our system [16].

3.4. PA-k activity after PLD treatment or the addition of exogenous DG

The effects of endogenous PA formation in the *T. cruzi* membranes were studied by treating the cells or membrane fractions with exogenous phospholipase D or by addition of exogenous DG. Fig. 4 shows that PLD treatment increased the DGPP formation in the cells and membranes about 60% and 120% respectively. The new amount of DGPP formed seemed related to the PA formed. This fact and a 350% increase of PA-k activity after the addition of exogenous dihexanoylglycerol led us to infer that DGPP was a metabolic product of PA.

3.5. Effect of polyamines

Positively charged compounds such as polyamines were reported to affect membrane associated lipid kinases in different types of cells [17]. Thus, we investigated the effect of spermine, putrescine and spermidine on the PA-k activity of *T. cruzi*. All of them increased the enzymatic activity; 300 μM spermine, in the presence of 0.2 mM sodium *o*-vanadate, had the greatest stimulatory effect, so the specific activity observed increased 2-fold with respect to the control (10–12 pmol/min/mg protein) without polyamines, whereas 500 μM putrescine and spermidine increased the enzymatic activity only 1- and 0.8-fold, respectively.

4. Discussion

In previous studies, we showed that *T. cruzi* was able to phosphorylate PI, PIP and DG sequentially under stimulation conditions [1]; now in this paper, we demonstrated, for the first time, the presence of DGPP, the pyrophosphorylated derivate of PA in this parasite. Our findings evidenced that *T. cruzi* metabolized PA as DGPP after treating the cells with PLD or the addition of dihexanoylglycerol, a substrate of DG-k. These results showed that the increase of endogenous PA levels in the membrane was converted 'in vitro' to its derivated DGPP. Indeed, the addition of exogenous PA evidenced that this enzyme may also use exogenous PA as substrate.

Under our standard assay conditions PA-k activity was similar to that reported for the crude extract from *S. cerevisiae* (7 pmol/min/mg), another lower eukaryotic microorganism [8]. The activity PA-k in *T. cruzi* was improved in the presence of inhibitors of phosphatases, vanadate and NaF. This indicated that the pyrophosphorylated product may be rapidly hydrolyzated by a higher phosphatase activity. In spite of this, the phosphatase activity was apparently insensitive to inhibition by Mn ions, a characteristic property of the DGPP phosphatase isolated from *S. cerevisiae* [8] and from *E. coli* [14].

In agreement with other *T. cruzi* lipid kinases, PA-k measured in the presence of phosphatases inhibitors was dependent on MgATP and Mg ion was an activator of the reaction. A requirement for a second enzyme-bound divalent metal cation is not unusual in kinases. However, Mn ions at concentrations above 1.5 mM were inhibitory to the Mg^{2+}-stimulated activity. This result may indicate competition between Mn and Mg ions by ATP.

Polyamines are present in both mammalian and plant cells and are required for optimal growth; they are also involved in plant stress responses [18]. The DGPP level increase due to the addition of polyamines to the membrane fractions may imply an additional physiological role for the polyamines. Although spermidine and putrescine do not have much effect on PA-k, spermine can significantly affect the kinase activity within physiological concentrations. Many of the known cellular effects of the polyamines are due to their polycationic nature; acidic phospholipid precursors of second messengers as PIP, PIP2 and PA, are the primary polyamine-binding sites in membrane [19]. Results of our laboratory (Hernandez et al., paper in preparation) indicated that in *T. cruzi* the activity of membrane associated PIP-k was stimulated only by spermine and, as a consequence, PIP2 levels also increased. In this way, it may be thought that the DGPP increased formation can be due to the activation of PA-k or inhibition of PAP activity as was reported by Wu and Carman [20].

On the other hand, in the green alga *Chlamydomonas*, mastoparan – another positively charged compound and activator of G protein and PLD – stimulated the DGPP formation in parallel to PA level variation [21]. Similarly, Marchesini [22] showed that mastoparan induced an increase in the PA and DGPP levels in *T. cruzi* epimastigote forms. In addition, as in this parasite different stimuli are able to modify PI and PA metabolism [1–3], the results presented here suggest the interesting possibility that PA-k has important implications for the regulation of cellular responses by agonist-induced phosphoinositide and phosphatidic acid turnover. However, additional studies are needed to determine the role of DGPP in *T. cruzi* cellular signaling.

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