Evidence for phospholipases from *Trypanosoma cruzi* active on phosphatidylinositol and inositolphosphoceramide

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The lipid moiety in the glycosylphosphatidylinositol anchors of glycoproteins of *Trypanosoma cruzi* consists of an alkylacylglycerol, a lysoalkylglycerol or a ceramide. Previously, we showed that the inositolphosphoceramides (IPCs) are the major components in the precursor inositolphospholipids of epimastigote and trypomastigote forms. Using ^{3}H-labelled subfractions of IPC, phosphatidylinositol (PI) and glycoinositolphospholipids (GIPLs) as substrates with a cell-free system, we now demonstrate the association of at least five enzyme activities with the trypanosomal membranous particulate material. These include: phospholipase A_1 and phospholipase A_2, enzymes that release free fatty acid from the PI and GIPLs; an acyltransferase responsible for the acylation of the generated monoacyl or monoalkylglycerolipids with endogenous unlabelled fatty acid; two activities of phospholipase C, one releasing ceramide from IPC and the other alkylacylglycerol, alkylglycerol or diacylglycerol from PI. The neutral lipids were also generated on incubation of the GIPLs. The phospholipase C activities were inhibited by *p*-chloromercuriphenylsulphonic acid, as reported for other PI phospholipases C. An IPC-fatty-acid hydrolase, releasing fatty acid from the labelled IPC, was also observed. The enzyme activities reported in the present study may be acting in remodelling reactions leading to the anchor of the mature glycoproteins of *T. cruzi*.

Key words: ceramide, glycoinositolphospholipids, inositolphosphoceramide, phospholipases A, phospholipase C.

INTRODUCTION

Trypanosoma cruzi, the agent of Chagas disease, has three main stages in its life cycle: epimastigotes, the non-infective form found in the gut of the insect vector; the infective trypomastigotes, which have the ability of entering many different vertebrate cells; amastigotes, the intracellular replicating form that originates by differentiation of trypomastigotes after invasion [1].

Phospholipases play important roles in generating lipid second messengers [2,3]. One of the best characterized is phosphatidylinositol (PI)-specific phospholipase (PL) C (PI-PLC) [4,5]. Susceptibility to the bacterial PI-PLC is usually taken as indicative of the presence of an acyl or alkylglycerolipid anchor in a protein [6]. We have previously shown that ceramide is also released by bacterial PI-PLC from the ceramide-containing glycoinositolphospholipids (GIPLs) from *Trypanosoma cruzi* [7]. Most membrane proteins in *T. cruzi* are linked via glycosylphosphatidylinositol (GPI) to the C-terminus of the protein [8–10]. The lipid moieties can be quite variable, and a summary of the major structures found in *T. cruzi* GPI anchors has been published [11]. In contrast with the African trypanosomes, no diacylglycerol (DAG) was found. Hexadecylglycerol has been previously found in the Tc85 glycoprotein, which is specifically from the trypomastigote forms of *T. cruzi* [12]. An acylated alkylglycerol component is present in the 1G7 anchor of metacyclic forms [13]; interestingly a small proportion of a putative inositolphosphoceramide (IPC) was reported [14]. In mucins of epimastigote forms the 1-*O*-hexadecyl-2-*O*-palmitoylglycerol is replaced by ceramide when the parasites differentiate to metacyclic forms [15]. Also, in the Ssp-4 glycoprotein of amastigote forms, a ceramide was identified as the anchor lipid. Moreover, it was shown that extracellular differentiation of trypomastigotes to amastigotes is accompanied by an increase in the level of free ceramide in the parasite [16] concomitant with the release of Ssp-4, suggesting cleavage of the GPI anchor by an endogenous PI-PLC activity [17]. In trans-sialidase, a shed antigen of the trypomastigote stage, lyso-1-*O*-hexadecylglycerol and ceramide are present in a 1:3 ratio [11]. In free GIPLs of *T. cruzi*, both 1-*O*-hexadecyl-2-*O*-palmitoylglycerol and ceramide are present in epimastigotes collected in the logarithmic phase of growth [18], whereas only ceramide was found in the related lipopeptidophosphoglycan (LPPG) from epimastigotes collected at the stationary phase [7]. All these variations suggest that remodelling mechanisms, as reported for *Saccharomyces cerevisiae* [19–21], may be working in *T. cruzi*.

The biosynthetic pathway for the introduction of ceramide in the anchor of glycoproteins of *T. cruzi* is not known. Apparently, ceramide was not described as an anchor of mammalian glycoproteins. This fact suggests that inhibition of ceramide incorporation into the anchor could be a good target for chemotherapy of Chagas disease.

In contrast with *T. brucei*, for which the important role of PLA_1 in remodelling the lipid anchor has been extensively studied [22,23], as far as we know there are no reports on PLA_1 in *T. cruzi*.

In a first approach to identify the lipases that could be responsible for remodelling reactions in *T. cruzi*, we used, as exogenous substrates, the radioactive inositolphospholipids (IPLs) and GIPLs from *T. cruzi* for incubation with membranes of epimastigote cells. IPCs account for 80–85% of the total IPLs

Abbreviations used: AAG, alkylacylglycerol; DAG, diacylglycerol; GIPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; IPC, inositolphosphoceramide; IPL, inositolphospholipid; LPPG, lipopeptidophosphoglycan; MAG, monoacylglycerol; *p*CMPSA, *p*-chloromercuriphenylsulphonic acid; PI, phosphatidylinositol; PL, phospholipase (e.g. PLA or PLC); PI-PLC, PI-specific phospholipase C; RPTLC, reverse-phase TLC; Tos-Lys-CH_2Cl, N^{α}-*p*-tosyl-L-lysylchloromethane.

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in epimastigotes [24], and they contribute to 58 % of the IPLs in infective trypomastigotes [25]. In the present work evidence has been found for the existence of PLA_1, PLA_2 and PLC in membranous particulate material from *T. cruzi* epimastigotes. The PLC activity was demonstrated by cleavage of a ceramide from IPC and cleavage of a glycerolipid from PI. The results also suggest the presence of an acyltransferase and an IPC-fatty-acid-hydrolase activity.

EXPERIMENTAL

Preparation of *T. cruzi* membranes

Epimastigote cells of *T. cruzi*, Y strain, were hypotonically lysed by rapidly vortexing in 10 mM Hepes, pH 7.9, 0.1 mM N^{α}-*p*-tosyl-L-lysylchloromethane (Tos-Lys-CH_2Cl, 'TLCK'), 0.5 mM dithiothreitol, 1mM EGTA, 0.5mM $NaHCO_3$ for 15 min at 0 °C. Further disruption was achieved by sonicating six times for 10 s at intervals of 30 s. Lysis was confirmed by microscopic examination, and the homogenate was clarified by centrifugation at 1000 ***g*** for 15 min. The supernatant was then centrifuged at 80000 ***g*** for 1 h, and the pellet was resuspended and stored in aliquots at −70 °C in buffer A [50 mM Hepes, pH 7.4, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 25 mM KCl, 0.5 mM dithiothreitol, 0.1 mM Tos-Lys-CH_2Cl, 1 μg/ml leupeptin and 20 % (v/v) glycerol].

Preparation of [9,10-^{3}H]palmitic-acid-labelled IPLs and GIPLs from *T. cruzi*

After two days of culture growth, epimastigotes (5×10^9 cells) were labelled by incubation with [^{3}H] palmitic acid as previously reported [24] and were extracted with chloroform/methanol (2:1, v/v) and (1:1, v/v). The extracts were pooled and dried, and the IPLs were purified by DEAE-Sephadex A-25 (acetate form). Fractions containing IPC or PI were separated by column chromatography [24].

For isolation of GIPLs the pellet remaining after exhaustive extraction of the cells with chloroform/methanol was extracted twice with 10 ml chloroform/methanol/water (10:10:3, by vol.). The radiolabelled glycolipids present in this fraction were susceptible to PI-PLC and HNO_2 treatments, characteristic of GPI structures.

Preparation of [^{3}H]DAG/alkylacylglycerol and [^{3}H]ceramide

For the preparation of the ^{3}H-labelled neutral lipids, PI and IPC were treated with PI-PLC from *Bacillus thuringiensis* as described below. The lipids were extracted with butanol, and the purity of the glycerolipids and ceramide was tested by TLC. The glycerolipids were further purified by silica column chromatography. The fraction containing the DAG and alkylacylglycerol (AAG) was eluted from the column with hexane/ethyl acetate (6:1, v/v).

Assay of phospholipase activities

The radioactive PI, IPC and GIPLs isolated as described above were used as substrates for the following experiments. Aliquots of lysate were thawed and washed twice with 8 ml of buffer B (50 mM Tris/HCl, pH 7.4, 0.1 mg/ml PMSF, 0.1 mg/ml Tos-Lys-CH_2Cl and 2.5 μg/ml leupeptin) by centrifugation for 1 h at 100000 ***g*** at 4 °C. The standard incubation mixture consisted of the radioactive substrate (600000 c.p.m.) and 5×10^7 cell equivalents of *Trypanosoma* membranes in 100 μl of buffer B containing 0.1 % Triton X-100. The substrate, dissolved in chloroform/methanol (1:1, v/v), was dried in the incubation tubes under a N_2 stream, and then the membranes, suspended in the buffer, were added. After incubation at 30 °C the reaction was stopped at different times (15, 60, 120 min and 18 h) by addition of 660 μl of chloroform/methanol (1:1, v/v). For the 18 h incubation of PI and IPC several reagents [*p*-chloromercuriphenylsulphonic acid (*p*CMPSA), EDTA, *o*-phenanthroline, $CaCl_2$ and $MgCl_2$] were tested at a concentration of 10 mM. Also, an 18 h incubation of the substrates in the buffer without membranes was done as a control.

Assays for DAG/AAG and ceramide lipase activities

The radioactive DAG/AAG (200000 c.p.m.) or ceramide (500000 c.p.m.) was incubated for 60 min and 18 h with the cell-free system under the same conditions as described for the phospholipases assay. An 18 h incubation of the substrates in the buffer without membranes was used as a control.

Lipid extraction

After incubation, the membranes were extracted twice with chloroform/methanol/water (10:10:3, by vol.). The organic extracts were dried in a Speed-vac evaporator and then subjected to butanol/water partition. The radiolabelled lipids were recovered in the butanol phases, which were washed with water, dried and analysed by TLC.

Analytical methods

TLC was performed using silica-gel 60 plates (Merck), developed with the following solvent systems: A, chloroform/methanol/2.5 M NH_4OH (15:10:2, by vol.); B, chloroform/methanol (38:3, v/v); C, chloroform/methanol/2.5 M NH_4OH (40:10:1, by vol.); D, hexane/ethyl acetate (4:1, v/v); E, chloroform/methanol/(conc.) NH_4OH (100:10:1, by vol.); F, chloroform/methanol/(conc.) NH_4OH (300:25:2, by vol.); G, chloroform/methanol/water (10:10:3, by vol.). When necessary, the lipids were eluted from the silica gel with chloroform/methanol/water (5:5:1, by vol.).

Reverse-phase TLC (RPTLC) was performed on RP-18 F_{254s} (Merck) using the following solvents: H, chloroform/methanol/water (40:100:3, by vol.) and I, acetic acid/acetonitrile (1:1, v/v).

Radiolabelled lipids were detected by fluorography. The TLC plates were sprayed with EN^{3}HANCE (New England Nuclear, Le Blanc Mesnil, France) and were exposed to Kodak X-Omat A-R5 films (Rochester, NY, U.S.A) at −70 °C. Quantification was performed with a densitometer, or alternatively spots were scraped from the plates, extracted as described above and counted directly.

The non-radioactive standards were detected by spraying the plates with a solution containing 10 % (v/v) conc. H_2SO_4, 0.04 M $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 3 mM $Ce(SO_4)_2$ and heating to 200 °C.

AAG standards were prepared as previously described [26].

Enzyme treatments

Samples of the IPLs were resuspended in 100 μl of 50 mM Tris/HCl, pH 7.2, containing 0.1 % deoxycholate and incubated with 0.35 unit of PI-PLC from *Bacillus thuringiensis* (Oxford GlycoSciences, Abingdon, Oxon., U.K), for 90 min at 37 °C. The lipids were extracted with butanol (3×0.5 ml) and analysed by TLC (solvents B and D). The PLA_2 reaction was performed in the same buffer with 2.5 mM $CaCl_2$ added. The samples were incubated with 25 units of bee venom PLA_2 from *Apis mellifera*

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(Sigma, 1200 units/mg of solid, 1350 units/mg of protein) for 2–4 h at 37 °C. After the reaction the lipids were extracted with butanol and analysed by TLC.

Acid methanolysis

Ceramides were hydrolysed for 18 h at 70–78 °C with HCl (conc.)/methanol/water (3:29:4, by vol.). After several evaporations with methanol the samples were treated with 14% (w/v) BF_3 in methanol, at 80 °C for 45 min to complete methylation of the fatty acids. The lipids were extracted with toluene and analysed by TLC in solvent C.

Alkaline hydrolysis

The original lipids or the lipids isolated after PI-PLC treatment, were hydrolysed with 0.1M NaOH in 90% methanol for 1 h at 37 °C. After neutralization with 10% (v/v) glacial acetic acid, the lipids were extracted with butanol and analysed by TLC.

Hydrogenation

Fatty acid methyl esters were subjected to hydrogenation with palladium on activated carbon [palladium content 10% (w/w), Aldrich], using a hydrogen pressure of 3 atmospheres. The reaction was performed for 4–5 h with shaking at room temperature. As a control, a sample of linolenic acid methyl ester ($C_{18:2}$) was treated under the same conditions.

RESULTS

PLC activities in *T. cruzi* membranes

Radioactive IPLs from epimastigote forms of *T. cruzi* were obtained by metabolic labelling with [^{3}H]palmitic acid and separated into PI and IPC subfractions [24]. The latter could not be freed from some PI and lysoPI.

To investigate an PI-PLC activity in membranes of *T. cruzi*, the radioactive PI (Figure 1A) and IPC (Figure 1B) were incubated with the *T. cruzi* membranes for different times. Also the action of various reagents was studied in the 18 h incubations. After the indicated times the lipids were extracted and analysed by TLC (solvent A). The formation of less polar lipids can be observed in all the lanes, except in the controls incubated without membranes (Figures 1A and 1B, lane 10). The compounds of higher mobility, I, II and VI, were eluted from the silica and rechromatographed in solvent B (Figure 2A). Lipid I, which has been cleaved from PI, has the mobility of an AAG or a DAG, as these are not differentiated from each other with this TLC solvent (Figure 2A, lane 1). Lipid II was identified as alkylglycerol (Figure 2A, lane 2). On the other hand, lipid VI, generated from IPC, was shown to be a ceramide (Figure 2A, lane 3). To further confirm the identity of the glycerolipid (Figure 2A, lane 1) it was chromatographed in solvent D, which discriminates AAG from DAG (Figure 2B). As can be seen, most of the lipid corresponds to AAG with DAG as a minor component, although in the PI substrate both glycerolipids were present in similar amounts. The fact that the 1,3 isomer is more abundant is due to intramolecular

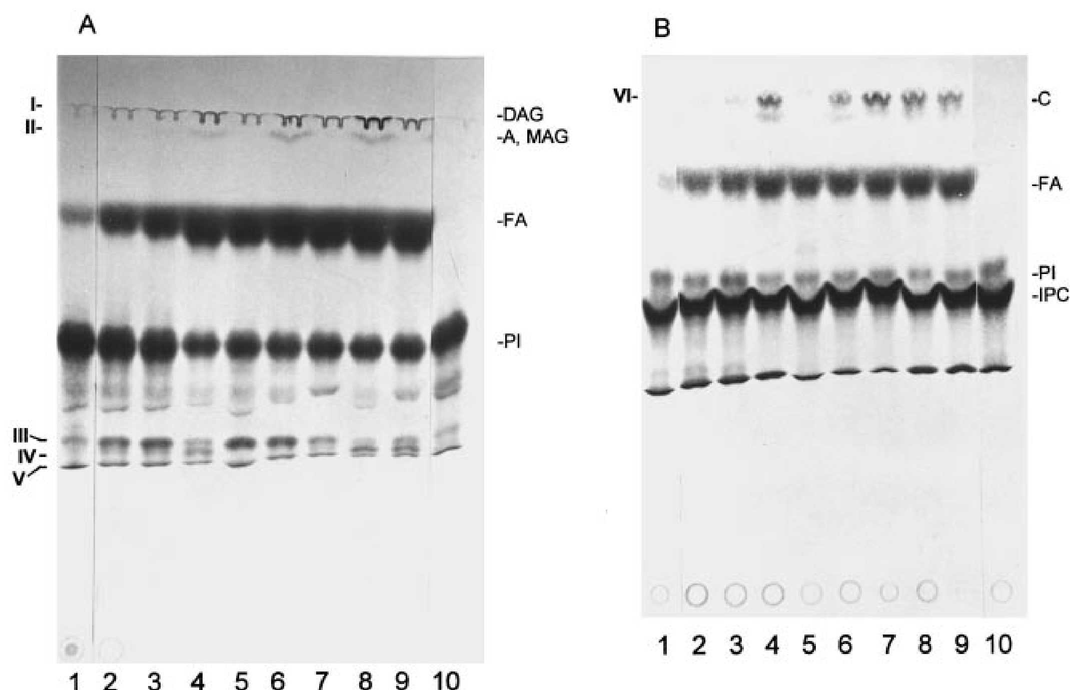

Figure 1 PLA$_1$, PLA$_2$, IPC-fatty-acid hydrolase and PI-PLC activities in *T. cruzi* membranes

[^{3}H]palmitic-acid-labelled PI **(A)** and IPC **(B)** (600000 c.p.m.) purified from *T. cruzi* epimastigotes were incubated for different times (15 min, lane 1; 1 h, lane 2; 2 h, lane 3; 18 h, lane 4) at 30 °C with a membrane fraction from *T. cruzi* (5×10^7 cells) in 50 mM Tris/HCl, pH 7.4, containing 0.1% Triton X-100. The effect of different reagents (all at 10 mM) was studied in the 18 h incubation (*p*CMPSA, lane 5; EDTA, lane 6; *o*-phenanthroline, lane 7; $CaCl_2$, lane 8; $MgCl_2$, lane 9). A control incubated with the buffer without membranes is shown in lane 10. After the incubation the radiolabelled lipids were extracted with chloroform/methanol/water (10:10:3, by vol.), the solvent was dried, the lipids were subjected to water/butanol partition and the butanol phases were analysed by TLC using solvent system A as described in the Experimental section. The positions of standards are indicated on the right: DAG, 1,2-di-*O*-palmitoyl-glycerol; A, alkylglycerol (1-*O*-hexadecylglycerol); FA, fatty acid; MAG, 1-*O*-palmitoylglycerol; C, ceramide (palmitoyldihydrosphingosine).

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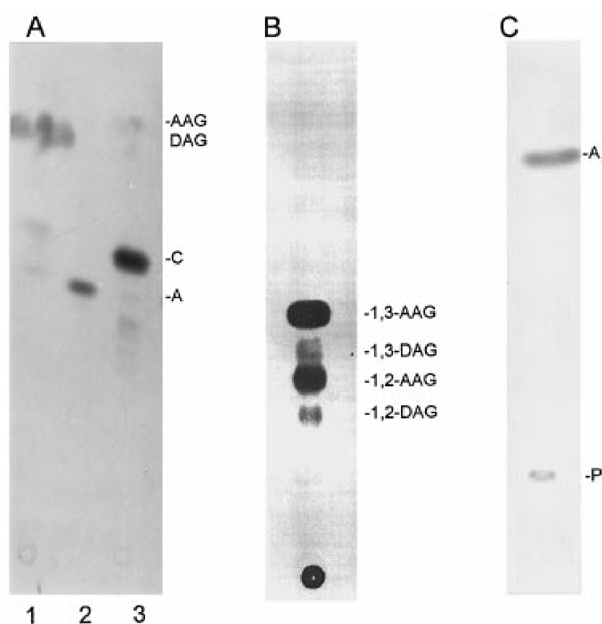

Figure 2 Analysis of the lipids released by a PLC activity during the incubation of PI and IPC with the *T. cruzi* membranes

(**A**) Compounds I, II and VI from Figure 1 were eluted from the plates and rechromatographed in solvent B (lanes 1, 2 and 3, respectively). (**B**) The glycerolipid from lane 1, in (**A**), was eluted and analysed in solvent D. (**C**) The compounds with the mobility of the two AAGs in (**B**) were eluted, mild base-treated and analysed by TLC using solvent system E. The positions of standards are indicated on the right of each panel: AAG, alkylacylglycerol (1-*O*-hexadecyl-2-*O*-palmitoylglycerol); DAG, diacylglycerol (1,2-di-*O*-palmitoyl-glycerol); C, ceramide (palmitoyl-dihydrosphingosine); A, alkylglycerol (1-*O*-hexadecylglycerol); P, palmitic acid.

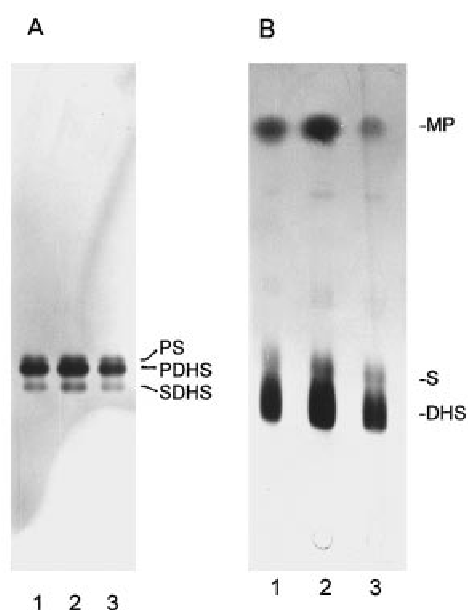

Figure 3 The ceramide released from the IPC by the endogenous PLC has the same composition as the ceramide of the original IPC, but radioactive fatty acid is partly exchanged by unlabelled fatty acid

(**A**) The IPCs incubated with the *T. cruzi* membranes for 18 h (Figure 1B, lane 4) and the control (Figure 1B, lane 10) were treated with PI-PLC of *B. thuringiensis*. The lipids were extracted with butanol and an aliquot was analysed by RPTLC in solvent H (lanes 1 and 2, respectively). The ceramide released by the endogenous PLC is shown in lane 3. (**B**) The ceramides analysed in (**A**) lanes 1, 2 and 3, were subjected to acid methanolysis, and the products were analysed by TLC in solvent system C (lanes 1–3, respectively). Standards: PS, palmitoylsphingosine; PDHS, palmitoyldihydrosphingosine; SDHS, stearoyldihydrosphingosine; MP, palmitic acid methyl ester; S, sphingosine; DHS, dihydrosphingosine.

acyl migration from position 2 during manipulation of the samples, a phenomenon usually observed [27]. As expected saponification of the AAG afforded alkylglycerol and fatty acid (Figure 2C).

The nature of the ceramide released by incubation of the IPC with the membranes was investigated by reverse phase thin layer chromatography (RPTLC) with solvent H. The main component is palmitoyldihydrosphingosine with minor amounts of stearoyl-dihydrosphingosine and palmitoylsphingosine (Figure 3A, lane 3). Ceramides from the remaining IPC after 18 h incubation (eluted from the plates shown in Figure 1B, lane 4) and from the control IPC (eluted from lane 10 of the plate shown in Figure 1B), were obtained by treatment with bacterial PI-PLC and analysed in the same TLC (Figure 3A, lanes 1 and 2 respectively). No difference in ceramide composition was observed. The structure of the ceramides released by the endogenous IPC-PLC was confirmed by acid methanolysis and TLC analysis in solvent C (Figure 3B). Dihydrosphingosine and traces of sphingosine were detected together with the methyl ester of the fatty acids (Figure 3B, lane 3). The same pattern was obtained by methanolysis of the ceramides released from control IPC (Figure 3B, lane 2) and from the IPC remaining after incubation with membranes (Figure 3B, lane 1).

We further showed that the PI-PLC activity generates ceramide and AAG from the [^{3}H]GIPLs (Figure 4). After overnight incubation with the cell-free system a less polar lipid was detected with the concomitant decrease of the GIPLs (Figure 4A, lane 3). The upper lipid was also visualized after 1 h incubation upon longer exposure (results not shown). To determine the composition of the generated lipid, it was eluted (Figure 4A, lane 3) and further analysed with solvent system F (Figure 4B), showing the presence of ceramides and AAG (Figure 4B, lane 1). A control, showing the lipids released from the GIPLs using a commercial PI-PLC, was performed (Figure 4B, lane 2).

The results conclusively demonstrate a PLC activity, cleaving lipids from PI, IPC and GIPLs, in membranes of *T. cruzi* epimastigotes. The fact that these substrates are only partially degraded may be attributed to the dilution effect of significant amounts of endogenous substrates in the preparations.

Effect of inhibitors on PI-PLC activity

The effect of different reagents on the PLC activity with PI as substrate was investigated (Figure 1A). PI-PLC activity was inhibited (40 %) with 10 mM *p*CMPSA (lane 5). *p*CMPSA is a reagent reported to inhibit GPI-PLC from *T. brucei* [28,29]. In a reinvestigation of the substrate specificity of GPI-PLC it was found that the enzyme was also active for PI under appropriate conditions, and hydrolysis of the latter was also inhibited by *p*CMPSA [30]. Maximal activity was obtained with the addition of $CaCl_2$ (lane 8). However, EDTA (lane 6) did not cause inhibition of the PLC activity for PI, and no effect was observed with $MgCl_2$ (lane 9).

Effect of inhibitors on IPC-PLC activity

Phospholipase C activity for IPC was completely inhibited by millimolar concentrations of *p*CMPSA (Figure 1B, lane 5) and partially inhibited with EDTA (45 %) (lane 6). We have observed

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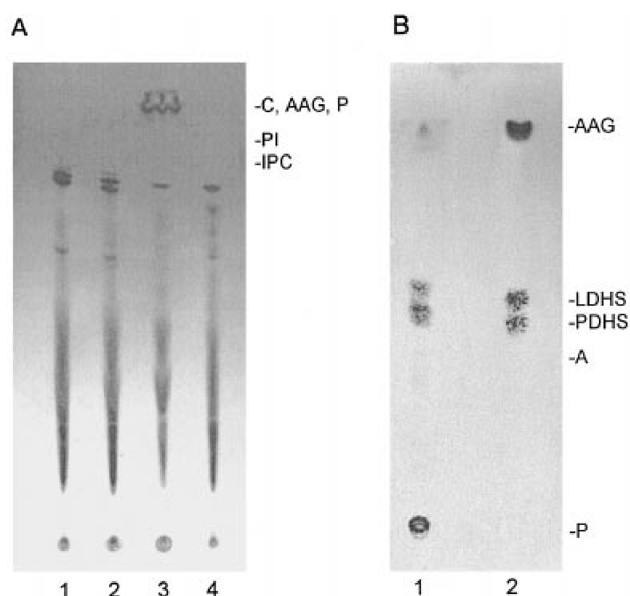

Figure 4 Analysis of the lipids released during incubation of GIPLs with the *T. cruzi* membranes

(**A**) [^{3}H]palmitic-acid-labelled GIPLs (500000 c.p.m.) purified from *T. cruzi* were incubated for different times (15 min, lane 1; 1 h, lane 2; 18 h, lane 3) and the lipids extracted as described in the legend to Figure 1. A control incubated with the buffer without membranes is shown in lane 4. The TLC was developed in solvent system G. (**B**) The compound of highest mobility in (**A**), lane 3, was eluted from the plate and rechromatographed (lane 1). A control of the lipids released from GIPLs by bacterial PI-PLC is shown in lane 2. The TLC was developed with solvent system F. Standards: A, alkylglycerol; AAG, alkylacylglycerol; C, ceramide; LDHS, lignoceroyldihydrosphingosine; PDHS, palmitoyldihydrosphingosine; P, palmitic acid.

a slight activation with $CaCl_2$ (lane 8), whereas $MgCl_2$ (lane 9) caused 60 % inhibition of the IPC-PLC activity. As reported for other PI-PLC activities [31,32], *o*-phenanthroline did not inhibit ceramide release (Figure 1B, lane 7).

Activities of PLA_1 and PLA_2

The main degradation observed by incubation of radioactive PI with membranes of *T. cruzi* was the release of fatty acid in a time-dependent reaction (Figure 1A). The activity was not significantly influenced by any of the added reagents.

The fatty acids of Figure 1(A) were eluted from the plate, methylated, and the methyl esters were analysed by RPTLC (solvent I) before (Figure 5, lane 1) and after (lane 2) catalytic hydrogenation.

The different profiles suggested the presence of unsaturated fatty acids. Thus compounds 'a' and 'b' from lane 1, Figure 5 were eluted, hydrogenated and analysed (lanes 5 and 6, respectively of Figure 5). Compound 'a' gave spots corresponding to $C_{16:0}$ and $C_{18:0}$, indicating the original presence of traces of $C_{16:1}$ and $C_{18:2}$. Compound 'b' was partly hydrogenated to $C_{18:0}$ indicating the original presence of $C_{18:1}$. In conclusion, the main fatty acid released from the PIs corresponds to stearic acid. Also palmitic acid, oleic acid and traces of palmitoleic and linoleic acids were found. Although in the unlabelled PIs the main fatty acids found were oleic acid and linoleic acid, in the radioactive PIs palmitic acid was mainly incorporated as stearic acid with minor incorporation as palmitic acid.

Endogenous PLA activity generated lysoPIs. For their identification, lipid V from lanes 1–3 (Figure 1A) and lipid III from

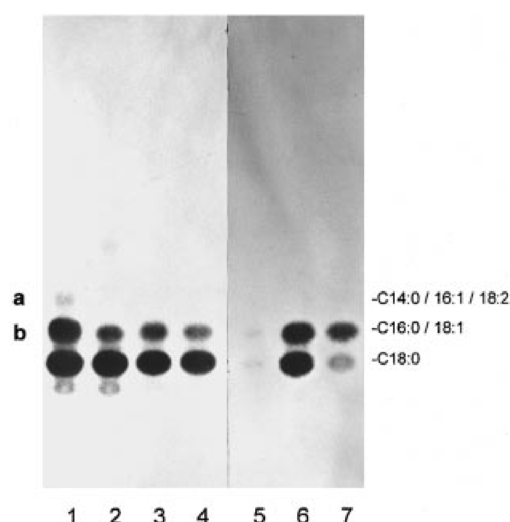

Figure 5 Analysis of the fatty acids released by PLA_1 and PLA_2 activities during the incubation of IPLs with the membranes of *T. cruzi*

Fatty acids (FA) from Figures 1(A) and 1(B) were eluted from the plate, methylated and analysed by RPTLC before (lanes 1 and 3, respectively) and after catalytic hydrogenation (lanes 2 and 4, respectively). Compound **a** from lane 1 and compound **b** from lanes 1 and 3 were eluted separately, hydrogenated and re-analysed (lanes 5, 6 and 7, respectively). The RPTLC was developed in solvent system I. Standards: $C_{14:0}$, myristic acid methyl ester; $C_{16:0}$, palmitic acid methyl ester; $C_{16:1}$, palmitoleic acid methyl ester; $C_{18:0}$, stearic acid methyl ester; $C_{18:1}$, oleic acid methyl ester; $C_{18:2}$, linoleic acid methyl ester.

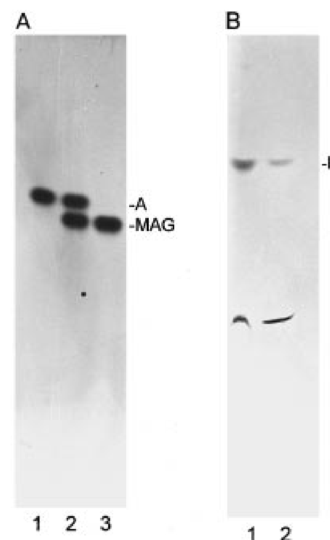

Figure 6 Analysis of the lysoPIs obtained during incubation of the PI with *T. cruzi* membranes

(**A**) Compounds III, IV and V from Figure 1(**A**) were treated with bacterial PI-PLC; the radiolabelled lipids were extracted with butanol and analysed by TLC in solvent E. Lane 1, lipid released from compound V; lane 2, lipids released from compounds III–V; lane 3, lipid released from compound III. (**B**) Compounds III–V from Figure 1(**A**) were treated with mild base (lane 1) or with PLA_2 (lane 2), then the lipids were extracted with butanol and analysed by TLC in solvent system A. Standards: A, alkylglycerol (1-*O*-hexadecylglycerol); MAG, 1-*O*-palmitoylglycerol; P, palmitic acid.

lanes 2, 3, 5 and 6 (Figure 1A) were eluted and treated with PI-PLC from *B. thuringiensis*. Lipid V generated a compound with the mobility of alkylglycerol in solvent E (Figure 6A, lane 1),

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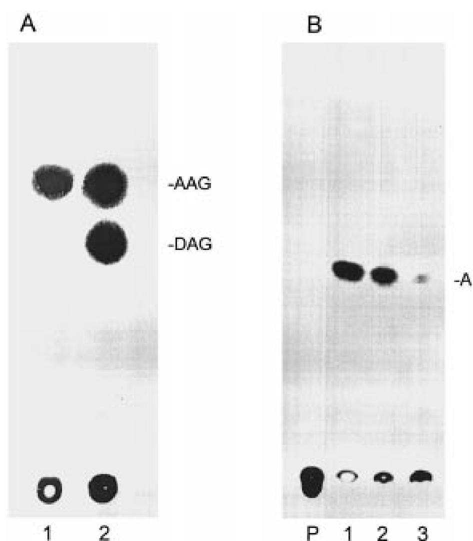

Figure 7 The radioactive fatty acid in the glycerolipid is exchanged by unlabelled fatty acid upon incubation of PI with membranes of *T. cruzi*

(**A**) The PIs remaining after incubation for 18 h with membranes, eluted from the TLC of Figure 1(**A**), lane 4, were treated with PI-PLC from *B. thuringiensis* (lane 1). A control PI was eluted from lane 10, of Figure 1(**A**) and treated in the same way (lane 2). The lipids were extracted with butanol and analysed in solvent D. (**B**) The glycerolipids in (**A**) were eluted, subjected to mild base treatment, and analysed in solvent F. Lane 1, products obtained from the AAG of lane 1, panel (**A**); lane 2, products obtained from the AAG of lane 2, panel (**A**); and lane 3, products from DAG of lane 2, panel (**A**). Standards: AAG, alkylacylglycerol; DAG, diacylglycerol; A, alkylglycerol; P, palmitic acid.

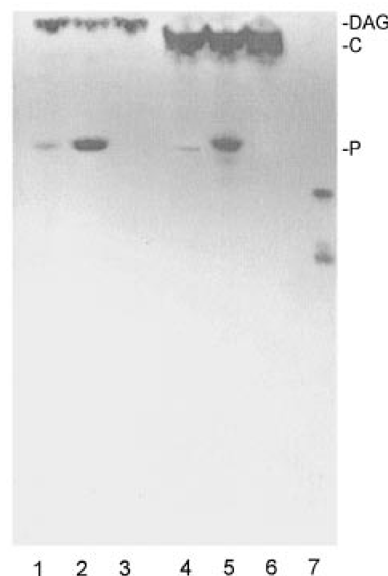

Figure 8 DAG/AAG and ceramide lipase activities

[^{3}Hpalmitic-acid-labelled DAG/AAG (200000 c.p.m.) or ceramide (500000 c.p.m.) obtained from the IPLs were incubated with the *T. cruzi* membranes for 1 h or 18 h (lanes 1 and 2 for DAG/AAG; lanes 4 and 5 for the ceramide). The lipids were extracted as described in the legend of Figure 1. Controls incubated for 18 h with the buffer are: DAG/AAG, lane 3, and ceramide, lane 6. The TLC was developed in solvent system A. Radioactive IPLs of *T. cruzi* are shown in lane 7. Standards: C, ceramide; DAG, diacylglycerol; P, palmitic acid.

whereas lipid III gave monoacylglycerol (MAG) (Figure 6A, lane 3). PI-PLC treatment of the triple band from lanes 4 and 9 (Figure 1A) generated both alkylglycerol and MAG (Figure 6A, lane 2), indicating that the middle band, (compound IV, Figure 1A) corresponds to the isomeric MAG, as only 1-*O*-alkylglycerol should be present.

For further proof of the presence of two MAGs two aliquots of the mixture of lipids III–V were saponified or subjected to PLA_2 treatments (Figure 6B). Release of fatty acid by the PLA_2 treatment (Figure 6B, lane 2) confirms the action of an endogenous PLA_1 on the original radioactive PI affording the 2-*O*-acylglycerophosphoinositol. The fact that saponification (Figure 6B, lane 1) gave a larger amount of fatty acid indicates that the 1-*O*-acyl derivative was also present. Lipid III is the 2-*O*-acyl derivative since it was degraded by PLA_2 treatment (results not shown).

To investigate the composition of the PI lipids remaining after incubation with the membranes, the PI was eluted from lane 4, Figure 1(A), treated with PI-PLC, and the lipid analysed together with the neutral lipid released from a PI control obtained from lane 10, Figure 1(A). It can be seen in Figure 7(A) that only radioactive AAG was present in PI incubated with membranes, whereas AAG and DAG were shown in the control. Furthermore, after saponification of the AAG (Figure 7A, lane 1) most of the label was present in the alkyl group (Figure 7B, lane 1). The recovered alkylglycerol/fatty acid radioactivity ratio was 12.4, whereas a 1.3 ratio was found for the AAG from the control (Figure 7B, lane 2). The low radioactivity found in the acyl group of the AAG after incubation indicates an acyltransferase activity which will introduce nonradioactive fatty acid after the action of a PLA_2. This agrees with the result obtained after saponification of the AAG produced by the endogenous PI-PLC which showed most of the radioactivity in the alkylglycerol (Figure 2C), in contrast with results previously reported when analysing precursor glycolipids [33].

The facts that no radioactive DAG was present in the PI after incubation with the membranes, and that the amount of lysoPIs does not correlate with the radioactive fatty acid released, are in agreement with the presence of PLA_1, PLA_2 and acyltransferase activities.

Analysis of the lipids generated from the [^{3}H]GIPLs showed a spot close to the origin (Figure 4B, lane 1), which when rechromatographed in solvent A had the mobility of a fatty acid (results not shown). The radioactivity in the AAG (Figure 4B, lane 1) was significantly lower than in the glycerolipid obtained from the substrate using a commercial PI-PLC (Figure 4B, lane 2). No alkylglycerol was detected, suggesting that an acyltransferase which introduces non radioactive fatty acid could be also working on the GIPLs.

IPC-fatty-acid hydrolase and acyltransferase activities

Incubation of the radioactive IPC with the membranes also released fatty acid in a time-dependent reaction (Figure 1B). Although the substrate could not be cleared of all traces of PI, the radioactivity in the latter could not account for all of the labelled free fatty acid found. The fatty acids (Figure 1B) were methylated and analysed by RPTLC in solvent I (Figure 5, lane 3). The pattern changed slightly by hydrogenation (Figure 5, lane 4). When the upper spot of lane 3 was separately hydrogenated a small amount of stearic acid was detected (lane 7) indicating the presence of some oleic acid. Ceramides of palmitic and stearic acid with dihydrosphingosine as base were previously found as components of the IPCs [24]. The fact that the ratio of radioactive oleic to palmitic acid released from the PIs is

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1.25 and from the IPC fraction the ratio is 0.35 confirms that the fatty acid (Figure 1B) originates from an IPC-fatty-acid hydrolase activity.

On the other hand, the radioactivity ratio, long chain base/fatty acid, in the original IPC was 1.4, whereas this ratio found in the ceramide released by the endogenous PLC was 4.8. The lower amount of radioactivity found in the fatty acid of the free ceramide suggests an acyltransferase activity introducing unlabelled fatty acid. Further experiments are necessary to demonstrate if this enzyme acts before and/or after ceramide release.

DAG/AAG and ceramide lipase activities

To evaluate the presence of deacylating enzyme activities releasing fatty acid from the neutral lipids, we performed two independent experiments, incubating [^{3}H]DAG/AAG and [^{3}H]ceramide with the membranes. A trace of fatty acid is released after 1 h incubation (Figure 8, lanes 1 and 4), and a larger amount is generated in an overnight incubation (Figure 8, lanes 2 and 6). The solvent system used in the TLC of Figure 8 does not discriminate disubstituted from monosubstituted glycerol. When the upper compound was eluted and analysed in solvent system B, no MAG or alkylglycerol was detected (results not shown), again indicating that an acyltransferase is acting on the neutral lipids and introducing nonradioactive fatty acids.

DISCUSSION

This is the first report presenting evidence of PLs in *T. cruzi* that are active on IPLs and GIPLs, in particular of a ceramide-releasing PLC activity. Enzymic activities which solubilize the membrane form of the variant surface glycoprotein ('mfVSG') were detected in *T. cruzi* epimastigote and metacyclic lysates, and these activities were membrane bound in trypomastigotes [34,35]. We have not investigated the soluble fractions for the enzymic activities now reported.

Ceramide is a constituent of the anchor in glycoproteins shed from the parasite. Thus the Ssp-4 glycoprotein characteristic of amastigote forms of *T. cruzi* is anchored by a ceramide [16]. Also, the trans-sialidase of trypomastigote forms, actively shed during the acute period of infection [36], is anchored by a ceramide [11,37]. The ceramide could be released from the isolated anchor-bearing glycoproteins by exogenous bacterial PI-PLC. The membrane-bound IPC-PLC activity reported in the present study could be responsible for the shedding of the antigens, with the concomitant release of ceramide. The enzyme levels in amastigote and trypomastigote forms could be higher than in epimastigotes. Further experiments will be required to verify this hypothesis.

The IPC-PLC as well as the PI-PLC activities determined in the particulate fraction were inhibited by *p*CMPSA, which was reported as an inhibitor of bacterial [38] and *T. brucei* PI-PLC [30] as well as of the GPI-PLC from *T. brucei* [28,29]. Also, it was shown that the amount of AAG was increased by the presence of Ca^{2+}, although the enzyme was also effective in the absence of added Ca^{2+}. In the case of the IPC hydrolysing activity, although inhibition by EDTA was observed, the activity was slightly increased by Ca^{2+}. Both PLC activities were not affected by *o*-phenanthroline. The same *T. cruzi* enzyme could be acting on PI and IPC, with the slight differences found with the inhibitors due to different interactions with the substrates; however, the possibility that two different enzymes are operating cannot be ruled out.

We have previously found that in GIPLs purified from the early stages of growth of *T. cruzi*, AAG or ceramide are linked to the same glycan [18]. Ceramide was not found in glycolipid precursors [33] nor in the IPL-GlcN formed by incubation of a cell-free system of *T. cruzi* with UDP-[^{3}H]GlcNAc (L. E. Bertello, M. J. M. Alves, W. Colli and R. M. de Lederkremer, unpublished work). Remodelling reactions, as reported in *S. cerevisiae* [19–21], could take place. Whether the IPC is the source of the ceramide introduced in the LPPG and in the protein anchors in *T. cruzi* is unknown at present. It was recently described that in *S. cerevisiae* the ceramide used for remodelling GPI anchors is newly synthesized and is not taken from IPC [39].

Two major ceramides in the LPPG are palmitoylsphinganine and lignoceroylsphinganine. However, no lignoceric acid was present in the radioactive precursor IPCs nor in the unlabelled samples previously obtained from *T. cruzi* [24]. The $C_{24:0}$ is probably acquired from the medium and either used for the synthesis of a new ceramide substrate for the LPPG or participates directly in a remodelling reaction leading to one of the species of LPPG. In this respect, $C_{24:0}$ was found free and in triacylgycerol, phosphatidylcholine and phosphatidylethanolamine, only when unlabelled parasites were analysed but not in metabolically labelled trypomastigotes [40], indicating incorporation from the medium.

In the present study, we have demonstrated for the first time that PLA_1 and PLA_2 enzymes are present in membranes of *T. cruzi*. PLA_1 activity is important in other trypanosomatids [41]. In particular, in *T. brucei* the enzyme is mainly cytoplasmic and was unable to cleave the ester linkage in the membrane form of the variant surface glycoprotein [42]. A membrane-bound PLA_1 activity, generating free fatty acid from lysophospholipids, and an acyltransferase were also reported in *T. brucei* [22].

The fact that two lysoIPLs containing MAG are formed by the enzymic action is consistent with the presence of membrane-bound PLA_1 and PLA_2 activities. Probably, the lysolipid containing alkylglycerol is generated by the same PLA_2.

An acyltransferase acting on the lysolipids and introducing unlabelled endogenous fatty acid would account for the larger amount of radioactive fatty acid compared with the lysolipids. This hypothesis was confirmed by analysing the PI remaining after 18 hncubation; only AAG was observed (Figure 7A) and the label was found mainly in the alkylglycerol (Figure 7B). Slow generation of fatty acid from DAG/AAG or ceramide was also shown. However, fatty acid was released from PI or IPC before a significant amount of neutral lipids were detected. On the other hand the presence of PLC inhibitors during the incubations (Figures 1A and 1B, lane 5) did not significantly affect the release of fatty acid. These data confirm that PLA activities are involved in the generation of fatty acid, although a small contribution from the neutral lipids cannot be excluded.

AAG but not DAG was found in free GIPLs and in the anchors of proteins from *T. cruzi*. The AAG was always identified as hexadecyl-*O*-palmitoylglycerol; however, in the putative precursor PI obtained from [^{3}H]palmitic-acid-labelled cells, hexadecylglycerol is mainly esterified with stearic acid in both epimastigotes [24] and trypomastigotes [25]. Also we usually found a lower amount of radioactivity in the 2-acyl group as compared to the alkyl group when *in vivo* incorporations were performed. These results, together with the fact that in the unlabelled samples the unsaturated fatty acids $C_{18:2}$ and $C_{18:1}$ were found [24], confirm that fatty acid exchange is taking place. Although fatty acid exchange has been extensively studied in *T. brucei* [23,43–45], there are no publications on the mechanisms of remodelling taking place in *T. cruzi*.

Further experiments will be necessary to define the role of the different PL activities in the biosynthesis and final structure of the GIPLs, either free or protein-bound, in *T. cruzi*.

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