Structural analysis of inositol phospholipids from *Trypanosoma cruzi* epimastigote forms

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Inositol phospholipids (IPL) from epimastigote forms of *Trypanosoma cruzi* have been investigated by metabolic labelling with [³H]palmitic acid and by GLC–MS analysis of the lipids obtained from non-labelled parasites. The IPL fraction was separated into phosphatidylinositol (PI) and inositol-phosphoceramide subfractions, the latter accounting for 80–85% of the total IPL. The neutral lipids released from the IPLs by PI-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* were analysed by silica-gel and reverse-phase TLC for the radioactive lipids and by GLC–MS for the non-radioactive samples. Ceramides containing dihydrosphingosine and sphingosine with C_{18:0}

and C_{18:0} fatty acids were identified. The main component in the [³H]palmitic acid-labelled ceramides was palmitoyldihydrosphingosine, while in the non-labelled sample the ceramides contained mainly sphingosine. This could reflect partial uptake of phospholipid from the medium. The PI contain both alkylacyl- and diacyl-glycerol lipids, with the ether lipid being more abundant. The latter was identified as 1-*O*-hexadecylglycerol esterified by C_{18:2} and C_{18:1} fatty acids. Interestingly, the same lipid had been identified in the anchor of the 1G7 glycoprotein of *T. cruzi* metacyclic forms.

INTRODUCTION

Phospholipids are essential components of plasma membranes of eukaryotes. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (PI) are the major classes found in *T. cruzi* epimastigote forms [1]. In particular, inositol phospholipids (IPL) are the precursors of the glycosyl-phosphatidylinositol (GPI) anchors of membrane glycoproteins. This type of anchorage is particularly frequent in parasitic protozoa [2]. It is believed that some of these glycoproteins in *T. cruzi* are involved in the infectious process [3,4].

The biosynthetic pathway has been well elucidated for the GPI of the variant surface glycoprotein of *T. brucei*. A glycerol-

phospholipid containing stearic acid in the *sn*-1 position and a mixture of fatty acids at *sn*-2 [5] is the acceptor for the construction of ethanolamine phosphate Man α 1-2Man α 1-6Man1-4GlcN α 1-6-PI ('EtNPM₃-GlcN-PI'). Interestingly, this glycolipid undergoes a series of fatty acid remodelling to give dimyristoylglycerol as the lipid moiety [6].

Free glyco-inositol phospholipids (GIPLs) with GPI anchor-like structures have been also characterized in other parasites. The best known are the lipopeptidophosphoglycan (LPPG) of *T. cruzi* [7,8] and the GIPLs of *Leishmania* spp. [2]. While the lipid in *Leishmania* is an alkylacylphosphatidylinositol, a ceramide phosphoinositol has been characterized in LPPG [9]. However, the structure of the lipid component is developmentally regulated, as we have concomitantly found hexadecylpalmitoylglycerol or ceramide linked to the same glycan structure in cells collected at the exponential phase of growth (2 days) [10]. Also, ceramide-containing GIPLs have been found in the trypanosomatids, *Leptomonas samueli* [11], *Endotrypanum* [12] and *Herpetomonas samuelpeesoai* [13]. In yeast the GPI anchor of glycoproteins is heterogeneous, and ceramide or a glycerolipid can be found. However, most mature proteins are anchored by a ceramide-containing GPI [14,15].

In *Trypanosoma cruzi*, hexadecyl-2-*O*-acylglycerol is present in the anchor of the 1G7 glycoprotein of metacyclic forms [16]. Also, the mucin-like glycoproteins, which are the major acceptors of sialic acid in *T. cruzi* metacyclic forms, are anchored by an alkylacylglycerolipid [17].

Hexadecylpalmitoylglycerol was found in the mucins of epimastigotes (C. Lima and R. M. de Lederkremer, unpublished work). In trypomastigote forms, 1-*O*-hexadecylglycerol is the lipid moiety in the anchor of the Tc85 glycoprotein [3] involved in host-cell invasion [18]. Unlike Tc85 isolated from the parasite lysate, shed Tc85 has an acylinositol-modified GPI anchor (G. Abuin, A. S. Couto, R. M. de Lederkremer, O. L. Casal, C. Galli, W. Colli and M. J. M. Alves, unpublished work). The

Figure 1 Schematic representation of GPI anchors or free GIPLs in different stages of *T. cruzi*

Epimastigote: GIPLs [10], LPPG [7–9]. Metacyclic: 1G7 [32,16], mucins [17]. Copy numbers per cell are indicated when they were reported. The shaded area represents an oligosaccharide.

Abbreviations used: PI-PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; IPL, inositol phospholipid; GPI, glycosyl-phosphatidylinositol; GIPL, glyco-inositol phospholipid; IPC, inositol phosphoceramide; LPPG, lipopeptidophosphoglycan; AAG, alkylacylglycerol; DAG, diacylglycerol; LIT medium, liver-infusion/tryptose medium; RPTLC, reverse-phase TLC; Fr, fraction.

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shed acute-phase antigen ('SAPA'), a member of the *trans*-sialidase family [4,19] could be released by PI-PLC from the surface of trypomastigotes, thus suggesting the presence of a GPI anchor. In the amastigote stage, the major surface glycoprotein Ssp-4 is anchored by a GIPL [20], although its structure was not described. To our knowledge a ceramide has not yet been reported to be a constituent of glycoprotein anchors in *T. cruzi*. A schematic representation showing the presence of IPL in the anchor of glycoproteins or in free GIPLs of different stages of *T. cruzi* is shown in Figure 1.

Inhibition of membrane anchorage in the parasite is an attractive approach for chemotherapy. Contribution in this direction is the knowledge of the structure and biosynthesis of the IPL.

In the present work we analyse the molecular species of IPL that are available for the construction of the GIPLs. Metabolic labelling of the IPL of the *T. cruzi* epimastigotes by incorporation of [^3H]palmitic acid was performed.

MATERIALS AND METHODS

Parasites

Epimastigote forms of *T. cruzi*, Y strain, were cultivated in liver-infusion/tryptose medium (LIT medium) as described in [21]. Cells were harvested at the exponential phase of growth (2 days) by centrifugation at 4400 *g* unless otherwise stated. The pellet was washed three times with 0.15 M NaCl/0.01 M sodium phosphate, pH 7.2.

Metabolic labelling

Cells were cultured for 2 days in LIT medium, transferred to TAU-3AG [22] medium containing 0.2% fetal-calf serum at 8×10^7 – 2×10^8 cells/ml and were adapted for 6 h at 28 °C. Labelling was performed with [9,10- ^3H]palmitic acid (54 Ci/mmol; 40–60 $\mu\text{Ci/ml}$) for 18 h at 28 °C. Microscopic observation after incorporation showed that all of the parasites remained viable.

Extraction and purification of inositolphospholipids

Labelled cells (5×10^9) of *T. cruzi* were freeze-dried and extracted with 2:1 (v/v; 20 ml) and 1:1 (v/v; 2×20 ml) of chloroform/methanol. The extract was dried, the residue washed with hexane to remove excess [^3H]palmitic, and the inositol phosphate-containing lipids were separated from the neutral lipids by DEAE-Sephadex A25 (acetate form) column chromatography [23]. Neutral lipids were eluted with 50 ml of methanol/chloroform/water (30:15:4, by vol.) and the acidic lipids with 50 ml of methanol/chloroform/0.8 M sodium acetate (30:15:4). The latter fraction was evaporated and salts were removed by passing through a C_{18} clean-up cartridge (Worldwide Monitoring, Horsham, PA, U.S.A.). Salts were removed with water, and IPL were eluted with methanol. Further purification was performed on a column of Unisil (200–325 mesh; Clarkson Chemical Co., South Williamsport, PA, U.S.A.) equilibrated with chloroform, eluting with 4:1 (v/v) and then 1:1 (v/v) chloroform/methanol. Non-radioactive lipids were extracted from 10^{12} epimastigotes and purified by the same procedure.

Analytical methods

TLC was performed using silica-gel 60 (Merck) 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.); and

(D) hexane/ethyl acetate (4:1, v/v). When necessary, IPL 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: (E) chloroform/methanol/water (10:25:1, by vol.); (F) chloroform/methanol/water (20:55:3, by vol.); and (G) acetic acid/acetonitrile (1:1, v/v).

Radiolabelled lipids were detected by fluorography. The TLC plates were sprayed with EN³HANCE (New England Nuclear) and were exposed to Kodak X-Omat A-R5 films at –70 °C. The non-radioactive compounds were detected by spraying the plates with 10% H_2SO_4 /0.04 M $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ /3 mM $\text{Ce}(\text{SO}_4)_2$ and heating to 200 °C.

Capillary GLC was carried out with a Hewlett–Packard 5890 gas chromatograph with nitrogen as the carrier gas. The following conditions were used: (a) SP 2330 column (0.25 mm $\times$ 30 m; Supelco) at 230 °C; (b) HP-1 column (0.20 mm $\times$ 12 m). The temperature was programmed from 250 to 320 °C at 6 °C/min.

GLC–MS was performed on a TRIO-2VG MASSLAB at 70 eV. The columns and conditions used were: (c) DB-5 column (0.25 mm $\times$ 30 m). The oven temperature program was 180 °C (5 min) to 230 °C at 2 °C/min and to 250 °C at 5 °C/min; (d) HP-1 column (0.20 mm $\times$ 12 m) at 140 °C (2 min) to 310 °C at a rate of 20 °C/min. Trimethylsilylation of the lipids was performed with 20–50 μl of Sylon HTP (Supelco) by heating at 70 °C for 10 min.

Treatment with PI-PLC

Samples of the IPL were resuspended in 50 μl of 50 mM Tris/HCl, pH 7.2, containing 0.1% deoxycholate and incubated with 0.35 unit of PLC from *Bacillus thuringiensis* (a gift from Dr. M. G. Low, Department of Physiology and Cellular Biophysics, Columbia University, New York, NY, U.S.A.), for 90 min at 37 °C. The lipid was extracted with diethyl ether (4 $\times$ 0.5 ml) and analysed by TLC (solvents B and D). The non-radioactive lipids were analysed as trimethylsilyl derivatives by capillary GLC (condition b) and by GLC–MS (condition d).

The aqueous phase that remained after treatment of the IPL with PI-PLC and diethyl ether extraction was hydrolysed with 4 M HCl for 18 h at 105 °C and, after several evaporations with the addition of water, it was desalted on Amberlite MB3 resin. After acetylation the sample was analysed by GLC using condition (a).

Acid methanolysis

Radioactive IPL were hydrolysed for 18 h at 70–78 °C with conc. HCl/methanol/water (3:29:4, by vol.), and the hydrolysate was extracted with diethyl ether. The long-chain bases or the alkyl-glycerol were analysed by TLC in solvent C, and the fatty acid methyl esters were analysed by RPTLC using solvent G. In order to complete methylation, samples were treated with 14% BF_3 in methanol, at 80 °C for 45 min before analysis.

Alkaline hydrolysis

The original lipids or the lipids isolated after PI-PLC treatment, were hydrolysed with 0.1 M NaOH in 90% ethanol for 1 h at 37 °C. After neutralization, the lipids were extracted with diethyl ether and analysed by GLC–MS (condition c) as their trimethylsilyl derivatives. The product from the radioactive samples was analysed by TLC (solvent A).

Preparation of alkylacylglycerol standards

1-*O*-hexadecyl-2-*O*-palmitoylglycerol, 1-*O*-hexadecyl-2-*O*-stearoylglycerol, 1-*O*-hexadecyl-2-*O*-myristoylglycerol and 1-*O*-hexadecyl-2-*O*-oleoylglycerol were prepared by lipase-catalysed esterification of 1-*O*-hexadecylglycerol. A typical reaction mixture consisted of 0.1 mmol of 1-*O*-hexadecyl-*rac*-glycerol (Sigma), 0.05 mmol of fatty acid and 1 mg of lipase from *Candida cylindracea*, type VII (Sigma), suspended in 2 ml of hexane. The reactions were carried out at 30 °C with shaking at 200 rev./min in a New Brunswick Science Innova 4000 incubator. The progress of the reaction was monitored by TLC using solvent D. The products were purified by column chromatography on silica-gel 60 (Merck) with hexane/ethyl acetate (23:2, v/v). Hexadecyl-2-*O*-acylglycerol (R_F 0.50 for $C_{16:0}$ acyl), was thus separated from the 3-*O*-acylglycerol (R_F 0.63) and from starting material (R_F 0.07).

The products were characterized by their $^1\text{H-NMR}$ spectra using a Bruker AC 200 spectrometer. Details of their preparation and properties are available from R. M. de L. on request.

RESULTS

Isolation and purification of IPL

IPLs were isolated from epimastigote cells in the exponential phase of growth (day 2) or when they reached the stationary phase after 4 days. In typical experiments, 5×10^9 [^3H]palmitic acid-labelled epimastigotes or 10^{12} epimastigotes were extracted with chloroform/methanol and the acid lipids were separated by chromatography on DEAE-Sephadex. This purification step separates the IPL from the amino-containing phospholipids, phosphatidylethanolamine and phosphatidylcholine, which amount to 75–80% of the total phospholipids in *T. cruzi* epimastigotes [1]. Analysis by TLC of the radioactive lipids (Figure 2a) showed one strong double band and a minor one, the latter with the same mobility as the PI standard. The relative proportions (4:1) were maintained in cells collected after 4 days.

Figure 2 TLC followed by fluorography of the [^3H]palmitic acid-labelled IPL from *T. cruzi* epimastigotes

(a) The acid lipids were extracted from cells of 2 days (lane 1) or 4 days of culture (lane 2) and purified on DEAE-Sephadex. (b) The acid lipids of lane (a) 1 were separated in UNISIL. Fr 1 was eluted with chloroform/methanol (4:1, v/v) and Fr 2 with chloroform/methanol (1:1, v/v). The solvent used was chloroform/methanol/2.5 M NH_4OH (15:10:2, by vol.). The standard was PI.

Figure 3 TLC and fluorography of the [^3H]palmitic acid-labelled lipids released by PI-PLC from IPL

(a) Fr 1 and Fr 2 (Figure 2b) were eluted from the plate and treated with 0.35 unit of PI-PLC from *B. thuringiensis*. The lipids (Fr 2, lane 1, and Fr 1, lane 2) were extracted with diethyl ether and analysed with chloroform/methanol (38:3, v/v) as solvent. (b) Lipids released from Fr 1 analysed with hexane/ethyl acetate (4:1, v/v). Standards were: H, 1-*O*-hexadecylglycerol; C, ceramide (palmitoyldihydrospingosine); DAG (1,2-di-*O*-palmitoylglycerol); AAG (1-*O*-hexadecyl-2-*O*-palmitoylglycerol).

Figure 4 Analysis of the products obtained by acid methanolysis of labelled Fr 2

(a) TLC in silica gel with chloroform/methanol/2.5 M NH_4OH (40:10:1, by vol.). (b) RPTLC with acetic acid/acetonitrile (1:1, v/v). Standards were: S, sphingosine; DHS, dihydro-sphingosine; C_{12} , C_{14} , C_{16} , C_{18} , C_{20} , methyl esters of dodecanoic acid, myristic acid, palmitic acid, stearic acid and arachidic acid respectively.

All the following experiments were performed with the cells of 2 days.

A partial purification in fractions 1 and 2 (Fr 1 and Fr 2) was obtained by silicic acid column chromatography (Figure 2b). The general structure of IPL was confirmed by hydrolysis with PI-PLC. Practically all the radioactivity was extracted with diethyl ether after enzymic digestion. It was previously shown [9] that the enzyme from *B. thuringiensis* degrades ceramide-containing GIPLs as well as GIPLs with acyl- or alkyl-glycerol. Analysis by TLC with solvent B (Figure 3a) showed that Fr 2 gave a lipid with the R_F of a ceramide standard, while a component

Figure 5 Reverse-phase TLC of the labelled ceramides released from Fr 2 (Figure 2) by PI-PLC

(a) The ceramides released from the entire Fr 2 were analysed by RPTLC. (b) Fr 2 was separated in an upper band (s) and a lower one (i) which were individually treated with PI-PLC. The solvent was chloroform/methanol/water (10:25:1, by vol.). The standards were: PS, palmitoylsphingosine; PDHS, palmitoyldihydrosphingosine, SDHS, stearoyldihydrosphingosine; LDHS, lignoceroyldihydrosphingosine.

running as diacylglycerol (DAG) or alkylacylglycerol (AAG) was obtained from Fr 1. Solvent B does not discriminate between the glycerolipids. However, we could separate DAG from AAG with solvent D (Figure 3b). Accordingly, Fr 2 was not altered under saponification conditions. In contrast, Fr 1 gave a component with a lower R_f and the fatty acid in solvent A (results not shown).

Identification of inositol phosphoceramides (IPCs)

To confirm that the lipid of Fr 2 was a ceramide, acid methanolysis was performed and the products were analysed by TLC (Figure 4a) and RPTLC (Figure 4b). Dihydrosphingosine was the main base, detected together with a minor amount of sphingosine. The methyl ester of the fatty acid was also detected. The RPTLC showed the $C_{16:0}$ fatty acid with a trace of the $C_{18:0}$ fatty acid. Moreover, the ceramides released by PI-PLC were resolved into four bands by RPTLC in solvent E (Figure 5). The main components are the ceramides of the saturated long-chain base dihydrosphingosine with palmitic acid and stearic acid respectively. Minor spots corresponding to palmitoylsphingosine and probably stearoylsphingosine were detected (Figure 5a). When the two bands of Fr 2 (Figure 2b) were separately eluted from the plate and respectively hydrolysed with PI-PLC, the upper band gave the dihydrosphingosine ceramides and the lower band afforded mainly the sphingosine ceramides (Figure 5b).

The IPL were also obtained from non-labelled epimastigotes by separation on DEAE-Sephadex followed by silicic acid column chromatography. The ceramides were released from the IPC fraction by PI-PLC digestion and extraction with diethyl ether. They could be analysed as the trimethylsilyl derivatives by GLC using a short capillary column (12 m) and condition b (Figure 6). The same ceramides, palmitoylsphingosine (retention time 15.51 min), palmitoyldihydrosphingosine (16.09 min), stearoylsphingosine (19.39 min) and stearoyldihydrosphingosine (20.21 min) were found in a 5.2:1.4:1 ratio. A peak at 9.68 min corresponded to deoxycholic acid from the buffer used with the

Figure 6 GLC analysis of the ceramides released from the IPC fraction by treatment with PI-PLC

Ceramides were analysed as the trimethylsilyl derivatives on a HP-1 capillary column (0.20 mm $\times$ 12 m). The temperature program was from 250 to 320 $^{\circ}$ C at 6 $^{\circ}$ C/min. The retention times correspond to the trimethylsilyl derivatives of standards of palmitoylsphingosine ($C_{16/18:1}$, 15.511 min), palmitoyldihydrosphingosine ($C_{16/18:0}$, 16.095 min) and stearoyldihydrosphingosine ($C_{18/18:0}$, 20.217 min). The peak at 19.394 min ($C_{18/18:1}$) was determined by GLC-MS as stearoylsphingosine. The peak at 9.68 min (DOC) was due to deoxycholic acid.

PI-PLC. The retention times were coincident with those for commercial standards; only stearoylsphingosine was not available.

The structures were confirmed by GLC-MS. The mass fragments were compared with reported values [24]. Peaks at 15.51 min and 19.39 min showed significant ions at m/z 243, 311, 336, 426 present in all sphingosine ceramides, while peaks at 16.09 min and 20.21 min gave the fragments characteristic of the dihydrosphingosine base at m/z 313 and 217 [9,24]. The fatty acid fragments for the first two peaks appeared at m/z 370 and 443 corresponding to the $C_{16:0}$ fatty acid. The fragments obtained for the last peaks at 398 and 471, that is, shifted 28 units, corresponded to the $C_{18:0}$ fatty acid constituent of the ceramides.

Thus the IPC obtained from the non-labelled parasites have the same ceramides as the radioactive lipids, although the sphingosine ceramides are the major components in the non-radioactive samples.

Identification of the AAG and DAG species

For the identification of the alkylglycerol, the upper component of Fr 1 (Figure 2b), labelled with [3 H]palmitic acid, was subjected to acid methanolysis. After separation of the lipids by TLC (solvent C) the component with the mobility of an alkylglycerol was eluted from the plate and analysed by RPTLC in solvent F

Figure 7 RPTLC of the [^3H]palmitic acid-labelled alkylglycerol obtained from the glycerophosphoinositol

Labelled Fr 1 was hydrolysed with HCl (c)/methanol/water (3:29:4, by vol.) at 70–78 °C for 18 h. The lipids were separated by preparative TLC (solvent C) and the component with the mobility of alkylglycerol was eluted from the plate and analysed by RPTLC. The solvent was chloroform/methanol/water (20:55:3, by vol.). Standards were: D, 1-*O*-dodecylglycerol; H, 1-*O*-hexadecylglycerol; O, 1-*O*-octadecylglycerol.

(Figure 7), showing the same R_F as 1-*O*-hexadecylglycerol. On the other hand, when Fr 1 was treated with PI-PLC, the lipid extracted into diethyl ether and separated by TLC (solvent D) revealed an AAG and a DAG (Figure 3b). The radioactive lipids were recovered in a 3:2 ratio. The non-labelled glycerolipids obtained after PI-PLC treatment were analysed by GLC (condition b) showing the AAG (retention time 20.01 min) and the DAG (R_t 28.92 min) in a 2.4:1 ratio. The different species of each type could not be separated, although standards of 1-*O*-hexadecyl-2-*O*-acylglycerol with $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids were well resolved.

The components of the AAG and DAG were respectively analysed after saponification, by GLC–MS (condition c) of the trimethylsilyl ethers. Only one alkylglycerol, namely 1-*O*-hexadecylglycerol, with a retention time of 27.3 min and two fatty acids, linoleic acid, (16.9 min) and oleic acid (17.2 min) were shown (Figure 8). Peak 1 corresponds to the trimethylsilyl ether of linoleic acid, the MS results were identical with those obtained from an authentic sample, showing a very low molecular ion at m/z 352 and the $M-15$ ion (20%). Accordingly peak 2 showed the corresponding mass fragments shifted 2 units, thus appearing at 354 and 339 (30%). The MS of peak 3, showed the base peak at m/z 205 characteristic of 1-monoalkylglycerols [25] and the diagnostic ions at $M-15$, $M-90$ and $M-103$, which are consistent with a $C_{16:0}$ alkyl chain. No other peak appeared by selected ion monitoring at m/z 205.

As only one alkylglycerol was found, the two fatty acids must be part of 1-*O*-hexadecyl-2-*O*-oleoylglycerol and 1-*O*-hexadecyl-2-*O*-linoleoylglycerol respectively.

When the fatty acids from the DAG were analysed by capillary GLC, $C_{18:2}$ and $C_{18:1}$ fatty acids were found in a 1:1 ratio (results not shown). It was not determined whether both fatty acids are present in one molecular species.

Identification of inositol

Inositol was identified in the aqueous phase from the PI-PLC treatments after acid hydrolysis of the inositol phosphate with 4 M HCl. Analysis by GLC (condition a) of the acetylated

sample showed a peak coincident with inositol acetate; no monosaccharides were detected. The inositol is presumably *myo*-inositol, commonly found in GPI anchors.

DISCUSSION

This is the first report of a detailed analysis of the IPL of *T. cruzi*. IPL are the starting materials for the construction of the GPI anchors of proteins and also of the LPPG, a major component of the membrane of epimastigotes. It is interesting that only glycerophospholipids have been identified in the anchor of the *T. brucei* glycoproteins, and in membrane glycoproteins of *T. cruzi*. However, a ceramide phosphoinositol is part of the LPPG, and is linked to the same core oligosaccharide which is characteristic of the known GPIs.

T. cruzi can incorporate [^3H]palmitic acid into cellular IPCs, palmitoyldihydrospingosine being the major species. It was found that 80–85% of the label of the total IPL was incorporated in the IPC. In the non-radioactive sample the unsaturated base sphingosine was found in a higher proportion, which could indicate incorporation of some medium lipids by the parasite. The fact that [^3H]palmitic acid is incorporated into dihydrospingosine and sphingosine (Figure 4) indicates that, at least in part, the long-chain bases are biosynthesized by the parasite. Palmitoyldihydrospingosine has been also found in appreciable amounts in LPPG together with lignoceroyl dihydrospingosine. However the $C_{24:0}$ fatty acid was not detected in the IPC investigated in the present work. It remains to be established at what stage of the biosynthesis of LPPG the $C_{24:0}$ fatty acid is introduced.

It was reported [1] that PI constitutes about 12% of the total phospholipids in *T. cruzi*. The DAG-containing IPL of epimastigote cells have been described as being responsible for an inositol phosphate/DAG signalling pathway [26]. In the present paper we prove that the DAG glycerolipids are the minor components in the pool of IPL with the AAG lipids being more abundant. Dialkylglycerol PI were not found. Dialkyl species have been described in *Leishmania mexicana mexicana* [27], but they were not found in *T. brucei* [28].

We have to consider that phospholipase C also cleaves the lipid from ceramidephosphoinositol, although it is not known if the *T. cruzi* PLC [29] would act on these compounds. In this respect, sphingolipid breakdown products play an important role in cellular regulation [30]. There are few reports on the IPC in protozoa.

Phosphoinositol lipids with ceramide have been identified in *Leishmania* spp. In *Leishmania donovani* [31] the fatty acids were mainly $C_{16:0}$ and $C_{18:0}$, like those in the *T. cruzi* IPC, and the C_{16} and C_{18} sphingosines and dihydrospingosines were the predominant long-chain bases. In *L. m. mexicana* the predominant ceramide had the $C_{16:1}$ base and the minor component the $C_{16:0}$ base. In both the fatty acid was stearic acid [27]. Only the C_{18} long-chain bases were found in *T. cruzi*. GLC–MS of the ceramides released by PI-PLC indicated that the major ceramide (52%) contained the common C_{18} sphingosine and the $C_{16:0}$ fatty acid. This was also found for *L. donovani* [31].

The alkylacyl species, namely 1-*O*-hexadecyl-2-*O*-acylglycerol with $C_{18:2}$ and $C_{18:1}$ as the ester fatty acids are preponderant in the PI molecules. In contrast, in bloodstream and procyclic forms of *T. brucei*, PI is composed mainly of diacylglycerol, although a ceramidephosphoinositol was tentatively identified by its chromatographic behaviour [28].

In agreement, the 1-*O*-hexadecyl-2-*O*-acylglycerol with $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ fatty acids has been identified in the anchor of the glycoprotein IG7 from *T. cruzi* metacyclic forms [16,32]. In the

Figure 8 GLC-MS analysis of the products obtained from the alkylacylglycerophospholipid

(a) Fr 1 was digested with PI-PLC and the AAG and DAG were separated by TLC (solvent D). After saponification, the lipids obtained from the alkylacylglycerol were analysed as trimethylsilyl derivatives using a DB5 capillary column (0.25 mm $\times$ 30 m). (b) Mass spectrum of peak 1 (linoleic acid). (c) Mass spectrum of peak 2 (oleic acid). (d) Mass spectrum of peak 3 (1-*O*-hexadecylglycerol). Abbreviation: %FS, percentage full scale.

Tc85 glycoprotein of the infective trypomastigotes the lyso-alkylglycerol was detected [3], but probably the precursor is the acylated lipid. 1-*O*-Hexadecyl-2-*O*-palmitoylglycerol was characterized in the mucin-like glycoproteins of metacyclic forms [17]

and of epimastigotes (C. Lima and R. M. de Lederkremer, unpublished work) and in a GIPL [10].

Probably a remodelling, as occurs in *T. brucei* [6], takes place for the exchange of the fatty acid in the precursor PI. So far, in

T. cruzi, an IPC has only been found in the LPPG of epimastigote forms [7–9].

In yeast, the lipid moiety of most GPI anchors are ceramides, different from those found in the pool of IPCs [15]. In addition, it was reported that the ceramide is introduced in the GPI by a remodelling step, after addition to the protein [33].

It will be interesting to determine whether, in *T. cruzi*, the PI could be a precursor for the IPC by transfer of the polar head group from PI to ceramide, as reported for *Saccharomyces cerevisiae* [34], or if a remodelling step takes place on the whole GIPL. Interestingly, we have characterized in *T. cruzi* epimastigotes an LPPG-like GIPL with 1-*O*-hexadecylpalmitoyl-glycerol instead of a ceramide [10].

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