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Phospholipid and glycolipid composition of acidocalcisomes of *Trypanosoma cruzi*

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Abstract

Highly purified acidocalcisomes from *Trypanosoma cruzi* epimastigotes were obtained by differential centrifugation and iodixanol gradient ultracentrifugation. Lipid analysis of acidocalcisomes revealed the presence of low amounts of 3 β -hydroxysterols and predominance of phospholipids. Alkylacyl phosphatidylinositol (16:0/18:2), diacyl phosphatidylinositol (18:0/18:2), diacyl phosphatidylcholine (16:0/18:2; 16:1/18:2; 16:2/18:2, 18:1/18:2, and 18:2/18:2), and diacyl phosphatidylethanolamine (16:0/18:2 and 16:1/18:2) were the only phospholipids characterized by electrospray ionization-mass spectrometry (ESI-MS). Incubation of epimastigotes with [³H]-mannose and isolation of acidocalcisomes allowed the detection of a glycoinositolphospholipid (GIPL) in these organelles. The sugar content of the acidocalcisomal GIPL was similar to that of the GIPL present in a microsomal fraction but the amount of galactofuranose and inositol with respect to the other monosaccharides was lower, suggesting a different chemical structure. Taken together, these results indicate that acidocalcisomes of *T. cruzi* have a distinct lipid and carbohydrate composition.

1. Introduction

Acidocalcisomes are acidic calcium-storage organelles found in a diverse range of organisms [1], although they were first defined as such in trypanosomes [2,3].

The chemical composition of acidocalcisomes of *Trypanosoma cruzi* has been analyzed using electron microscopy with elemental analysis, ³¹P NMR, and biochemical analysis. Using X-ray microanalysis, oxygen, magnesium, phosphorus, sodium, potassium, zinc, and calcium were detected in acidocalcisomes of different stages of *T. cruzi* [4,5] while iron was also found in acidocalcisomes of the bloodstream forms [6]. *T. cruzi* acidocalcisomes have high levels of phosphorus in the form of inorganic pyrophosphate (PP_i) and polyphosphate (poly P) [7]. Poly P is a linear chain of a few to many hundreds of phosphate (P_i) residues linked by high-energy phosphoanhydride bonds [8]. *T. cruzi* is especially rich in short chain poly P such as poly P₃, poly P₄, and poly P₅ [9]. ³¹P NMR spectra of purified acidocalcisomes of *T. cruzi* indicated that the poly P has an average chain length of 3.25 phosphates [9]. Based on the total concentration of poly Ps in different stages of *T. cruzi*, the relative volume of the

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acidocalcisomes in these cells (0.86%, 2.3%, and 0.26% of the total cell volume of epimastigotes, amastigotes and trypomastigotes, respectively; ref. [5]), and assuming that these compounds are essentially concentrated in acidocalcisomes, the calculated concentration in the organelles is in the molar range (3-8 M) [1]. This is consistent with the detection of solid-state condensed phosphates by magic-angle spinning NMR techniques and with the very high electron density of acidocalcisomes *in situ* [10]. Other components of these organelles, such as carbohydrates or lipids could be involved in maintaining this physical configuration although this has not been investigated until now. Acidocalcisomes also contain high concentrations of free amino acids. Arginine and lysine, basic amino acids, alone account for almost 90% of the amino acid pool of the acidocalcisomes [11].

The low sulphur content detected by elemental analysis [4] indicates that few proteins are present in acidocalcisomes, and in fact only a few acidocalcisome enzymes have been detected: polyphosphate kinase and a polyphosphatase activities were detected in isolated acidocalcisomes from *T. cruzi* [7]. A small number of proteins have also been identified in the membrane of acidocalcisomes. Several of them have also other cellular localizations such as a vacuolar-type proton pyrophosphatase (V-H⁺-PPase) [12], a vacuolar-type proton ATPase [13], a plasma membrane-type (PMCA)-Ca²⁺-ATPase [14], and an aquaporin [15]. The vacuolar transporter chaperone-1 (VTC-1), however, appears to be present only in these organelles [16].

Two different types of experiments have suggested the presence of carbohydrates in acidocalcisomes and/or acidic compartments of *T. cruzi*. Energy loss spectra measurements gave P:O and N:O ratios compatible with the presence of a sugar rather than of a protein in the matrix of acidocalcisomes [4]. Moreover, reactivity of epimastigotes of *T. cruzi* with an antibody against LPPG (lipopeptidephosphoglycan, the major glycoconjugate of epimastigotes) [17] and with MEST (an antibody against a glycoinositolphosphoceramide present in *Paracoccidioides brasiliensis*; ref. [18], labeled intracellular acidic vesicles in addition to the plasma membrane.

In this work, we have analyzed the phospholipid and glycolipid composition of *T. cruzi* acidocalcisomes. We report that isolated acidocalcisomes of *T. cruzi* possess a distinct chemical composition characterized by the predominance of the phospholipids phosphatidylcholine (GPCho), phosphatidylethanolamine (GPeth), and phosphatidylinositol (GPI_{no}), low amounts of 3 β -hydroxysterols, and the presence of glycoinositolphospholipids (GIPLs).

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), Hanks medium, newborn calf sera, dithiothreitol, and proteinase inhibitors were purchased from Sigma Chemical Co (St. Louis, MO). [³H]-mannose (15 Ci mmol⁻¹), and EN³HANCE[®] were from Perkin-Elmer (Boston, MA). Silicon carbide (400 mesh) was bought from Aldrich (Milwaukee, WI). Iodixanol (40% solution [OptiPrep], Nycomed) was obtained from Life Technologies. All other reagents were analytical grade.

2.2. Isolation of acidocalcisomes

Epimastigotes from the Y strain of *T. cruzi* were grown at 28°C in liver infusion tryptose medium [19] supplemented with 5% newborn calf serum. Epimastigotes (~1-2 × 10¹⁰) were collected by centrifugation, and washed twice in Dulbecco's PBS and once in lysis buffer (125 mM sucrose, 50 mM KCl, 4 mM MgCl₂, 0.5 mM EDTA, 20 mM K-Hepes, 5 mM dithiothreitol, 10 μ M pepstatin, 10 μ M leupeptin, 10 μ M E64 and 10 μ M TLCK, pH 7.2). The cell pellet was

mixed with $1.5 \times$ wet weight silicon carbide, and lysed by grinding in a pestle and mortar for 60 s. The lysate was clarified first by centrifugation at $144 \times g$ for 5 min, then at $325 \times g$ for 10 min. The second pellet was washed under the same conditions, and the supernatant fractions combined and centrifuged for 30 min at $10,500 \times g$. The pellet was resuspended in 3.4 ml lysis buffer and applied to the 34% step of a discontinuous gradient of iodixanol, with 4 ml steps of 20, 24, 28, 34, 37, and 40% iodixanol, diluted in lysis buffer. The gradient was centrifuged at $50,000 \times g$ in a Beckman SW 28 rotor for 60 min. The acidocalcisome fraction pelleted on the bottom of the tube and was resuspended in lysis buffer. Sixteen fractions of 1.5 ml, including the resuspended pellet, were kept at -80°C . The $10,500 \times g$ supernatant was centrifuged again at $105,000 \times g$ for 1 h to obtain the microsomal fraction (scheme 1). The construction of normalized density distribution histograms was carried out as described before [20,21].

2.3. Labeling of epimastigotes

For experiments with radiolabeled compounds, parasites ($\sim 1 \times 10^{10}$) were collected by centrifugation, washed twice in PBS, resuspended in 15 ml of DMEM without glucose, incubated at 28°C for 18 h in the presence of $10 \mu\text{Ci/ml}$ of $D\text{-}[2\text{-}^3\text{H}]\text{-mannose}$, and used to obtain acidocalcisomes as described above. The 16 fractions obtained were extracted with butanol saturated with water ($600 \mu\text{l}$, $3 \times$). The organic phases were washed twice with water, dried in a speed-vac and analyzed by thin layer chromatography on oxalate/EDTA-impregnated Silica-gel plates using chloroform/methanol/acetone/acetic acid/water (90/30/18/27/17, by vol., solvent system A). Radiolabeled lipids were detected by fluorography. The non-radioactive samples were detected by spraying the plates with a solution containing 0.1% orcinol- 5% H_2SO_4 in ethanol and heating at 120°C .

2.4. SDS Electrophoresis and preparation of western blots

Acidocalcisome and microsomal fractions were suspended in lysis buffer and the protein concentration was measured using the BioRad method in the presence of 0.01% sodium dodecylsulfate (SDS). Samples ($2 \mu\text{g}$) were mixed with $10 \mu\text{l}$ of 125 mM Tris-HCl, pH 7, 10% w/v β -mercaptoethanol, 20% w/v glycerol, 4.0% w/v SDS, and 4.0% w/v bromophenol blue as tracking dye, and boiled for 5 min before application to SDS-polyacrylamide gels (10%). Electrophoresed proteins were transferred to nitrocellulose membranes (NitroPure, MSI, Westborough, MA) with a Bio-Rad transblot apparatus. After transfer, the nitrocellulose was blocked with 5% fish gelatin in 0.1% Tween 20-PBS (Tween-PBS) overnight at 4°C . A 1:5,000 dilution of polyclonal rabbit serum against the plasma membrane $\text{H}^+\text{-ATPase}$ (TcHAf) [22] in Tween-PBS containing 2.5% fish gelatin was applied at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with Tween-PBS and incubated with anti-rabbit secondary antibody (1:20,000) conjugated with horseradish peroxidase in Tween-PBS containing 2.5% fish gelatin at room temperature for 60 min. After each detection, blots were incubated for 30 min at 50°C in stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) to remove the antibodies. The membranes were reprobed with a 1:10,000 dilution of polyclonal mouse antibody against TcPPase [23], or a 1:10,000 dilution of polyclonal rabbit antiserum against LPPG [17] in Tween-PBS containing 2.5% fish gelatin, washed, and incubated with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase, respectively, as described above. Immunoblots were visualized on radiographic film using the ECL enhanced chemoluminescence detection kit and according to the instructions of the manufacturer (GE Health Sciences). Similar results were obtained in at least three separate experiments.

2.5. Enzymatic assays and detection of 3β -hydroxysterols

Adenylyl cyclase activity was measured in the total lysate and microsomal and acidocalcisome fractions as described before [24] with some modifications. Briefly, protein ($0.3\text{-}3 \mu\text{g}$, within

the linear range of the reaction) was added to 100 μ l of assay medium (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 0.35 mg ml⁻¹ albumin, 3 mM ATP, 10 mM phosphocreatine, 100 μ g ml⁻¹ U creatine kinase) and incubated for 15 min at 37 °C. Samples were then heated for 3 min at 100 °C, and centrifuged at 14,000 rpm in an Eppendorf centrifuge. The cAMP synthesized was quantified with an enzyme immunoassay cAMP kit as described by its manufacturer (Pharmacia). Hexokinase was measured as described before [12] in a buffer containing 50 mM K-Hepes, pH 7.8, 10 mM MgCl₂, 10 mM glucose, 0.6 mM ATP, 0.6 mM NAD⁺, and 2.5 U ml⁻¹ glucose 6-phosphate dehydrogenase. Absorbance changes were followed at 340 nm. 3 β -hydroxysterols were quantified in lipids extracted from microsomal and acidocalcisome fractions containing similar amounts of proteins. The extracts were dissolved in isopropanol and aliquots were used to determine the sterols with a kit from Boehringer-Mannheim following the manufacturer's instructions. Cholesterol and other 3 β -hydroxysterols are oxidized by cholesterol oxidase [25]. The hydrogen peroxide produced in this reaction oxidizes methanol to formaldehyde in the presence of catalase. Formaldehyde reacts with acetylacetone forming a yellow lutidine-dye in the presence of NH₄⁺-ions.

2.6. Analytical methods

Microsomal and acidocalcisome fractions (20 μ g protein) were extracted with water-saturated butanol (600 μ l, 3 $\times$). The organic phase was washed with water (500 μ l, 2 $\times$), dried in a rotatory evaporator, resuspended in chloroform/methanol/water (10/10/3) and used for thin layer chromatography (TLC). TLC was performed in oxalate/EDTA-impregnated silica-gel 60 plates (Merck) using solvent system A. The plates were prepared by immersion in a solution containing 1.3% potassium oxalate and 2 mM EDTA in methanol/water (2/3, v/v) for 30 min at room temperature. Then plates were allowed to dry overnight and heated at 110°C for 30 min [26]. Radiolabeled lipids were detected by fluorography. The TLC plates were sprayed with EN³HANCE[®] and were exposed to X-Omat A-R5 films (Kodak, Rochester, NY, USA) or blue-sensitive X-ray film (Midwest Scientific, St. Louis, MO) at - 70°C. For the detection of non-radiolabeled lipids, the plate was sprayed with orcinol/H₂SO₄ in ethanol, allowed to dry and heated in a hot plate at 200°C, until the standard phospholipids were well defined. By heating the TLCs GIPLs were detected first as violet spots, but continued heating at 200 °C carbonized all the phospholipids, which were then visualized. Densitometry analysis to compare lipid amounts of microsomal and acidocalcisome samples on the same plate was done with an ISI-1000 digital imaging system (Alpha Inotech Corp.).

To quantify the GIPLs in acidocalcisomes, total lysate and microsomal and acidocalcisome fractions (163 μ g protein) were extracted and analyzed as described above. GIPLs were extracted from the silica plates with chloroform/methanol/water (30/60/20, v/v). The supernatants were dried, hydrolyzed with 6 N HCl at 110 °C for 16 h and analyzed for inositol by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) in a Dionex DX-600 ion chromatograph equipped with a MA-1 column and eluted with 80 mM NaOH. Inositol was quantified by integration of the area under the 10 min peak compared to known amounts of standard inositol. To analyze the neutral sugar content, GIPLs were extracted as described above and hydrolyzed under different acidic conditions: 2 N trifluoroacetic acid for 4 h, 0.02 N trifluoroacetic acid for 2 h, and 4 N HCl for 4 h. After hydrolysis the samples were neutralized and dried with a rotatory evaporator. Neutral sugars were analyzed by HPAEC-PAD in the Dionex DX-600 equipped with a PA-10 column and eluted with 18 mM NaOH. Sugars were identified by the retention time and comparison with known standards. Statistical significance was determined by Student's *t* test.

For the mass spectrometry with electrospray ionization analysis, the microsomal and acidocalcisome fractions in equal amounts of proteins were extracted with chloroform/methanol (1/2, v/v) followed by chloroform and water as described [27]. After centrifugation

at $1,000 \times g$ for 10 min, the upper aqueous phase was removed and the organic phase was washed with water, dried with N_2 , dissolved in chloroform and kept at $-20^\circ C$ under a N_2 atmosphere. The spectra were acquired with a Micromass Q-ToF Ultima instrument. The ESI/MS was done in negative and positive modes and the ESI/MS or ESI/MS/MS spectra corresponded to the signal generated in 1 min. For the generation of spectra in the negative mode, the samples were diluted in chloroform:methanol (1:3, v/v) and were injected with an injection pump at a rate of $1 \mu l/min$ in the ESI source. The spectra in the positive mode were generated in the same way with the addition of 1% acetic acid. The electrospray was done at 4.5 kV. The ESI/MS/MS spectra were obtained by selection of a precursor ion in the first quadrupole (Q1), accelerating the second quadrupole (Q2) and analysis of the resulting ions in the third quadrupole (Q3).

3. Results

3.1. Isolation of microsomal and acidocalcisome fractions

We isolated acidocalcisomes by a modification of an isolation procedure described previously [21]. We have reported before the yield of different markers and the 60-fold purification of the acidocalcisome fraction using this method [21]. The present method used lower concentrations of iodixanol in the gradient steps than those used previously [21]. In addition, the epimastigote sample was added in the middle of the gradient in a 34% iodixanol layer rather than applied to the top of the gradient without added iodixanol. These changes (see scheme 1) resulted in preparations of acidocalcisomes with less glycosomal contamination.

The purity of the acidocalcisome fraction (fraction 16) was evaluated with plasma membrane (adenylyl cyclase; [28]; P-type H^+ -ATPase; [22], and LPPG; [29]), glycosome (hexokinase; [30]), and acidocalcisome (V- H^+ -PPase, [12]) markers. Fig. 1 shows the results of one of these experiments. Microsomal (lanes 1) and acidocalcisome (lanes 2) fractions ($2 \mu g$ protein), were analyzed by SDS-PAGE and western blot (Fig. 1A-C). The same membrane was separately treated with antibodies against P-type H^+ -ATPase (A), V- H^+ -PPase (B), and LPPG (C). Similar results were obtained in at least 3 independent experiments. As expected, fraction 16 was rich in acidocalcisome V- H^+ -PPase (Fig. 1B, lane 2). The antibody against LPPG did not react with this fraction (Fig. 1C, lane 2). Probing with antibodies against the P-type H^+ -ATPase, a marker for the plasma membrane and the prelysosomal compartment of epimastigotes named the reservosome [22], showed a contamination of 8.5-9.9% as estimated by densitometry (Fig. 1A). Reservosomes are rich in sterols and other lipids [31]. To rule out contamination with these organelles we measured 3β -hydroxysterols with the cholesterol oxidase reagent in acidocalcisome and microsomal fractions. Although the activity of this enzyme is lower for ergosterol than for cholesterol [25] it was used for comparative purposes. The amount of total sterols in the acidocalcisomes ($0.9 \mu g$ 3β -hydroxysterol μg^{-1} protein) was 1/3 of that present in the microsomes ($3.3 \mu g$ 3β -hydroxysterol μg^{-1} protein). The adenylyl cyclase activity (in fmol cAMP/ μg protein) in total lysate (99.2 ± 0.4), microsomes (79.6 ± 1.2) and acidocalcisomes (9.3 ± 0.7) suggested a 9.4% contamination. In summary, the acidocalcisome fraction obtained by this method has less than 10% plasma membrane (or reservosome) contamination. Concerning glycosome contamination, we detected only 2% of the hexokinase activity in this fraction (Fig. 1D). We therefore considered that anything with a recovery higher than 10% would indicate that it is located in acidocalcisomes.

3.2. Lipid composition of microsomal and acidocalcisome fractions

The presence of LPPG and a glycoinositolphospholipid (GIPL) in acid vesicles of *T. cruzi* was previously suggested by the use of immunological techniques [17,18]. We therefore investigated the presence of GIPLs and of the inositol phospholipids, their precursors, in the acidocalcisome fraction.

We were interested in the detection of phosphatidylinositol (GPI_{no}) in acidocalcisomes as compared with the microsomal fractions to do MS analysis. Fractions (equal amounts of protein) were extracted with water-saturated butanol as described under Materials and Methods. TLC was used to separate the lipids with solvent A (chloroform/methanol/acetone/acetic acid/water (90/30/18/27/17, by vol.), and orcinol/sulfuric acid (5% in ethanol) reagent was used for detection. Using this method GIPLs were immediately detected as violet spots with low mobility (data not shown). Continued heating of the plates at 200 °C carbonized all the phospholipids, which were then visualized. A high concentration of phosphatidylcholine (GPCho) and GPI_{no} was detected in the acidocalcisome fraction (70% enrichment, by densitometry) (Fig. 2, lane 1). Phosphatidylethanolamine (GPEth) was not detected in appreciable amounts (the standard migrated ahead of GPCho, data not shown).

Both fractions (acidocalcisomes and microsomes) were then analyzed by electrospray ionization and mass spectrometry (ESI/MS) in positive (ESI⁺) and negative (ESI⁻) modes. As expected, the acidocalcisome fraction showed higher concentrations of molecular ions between 700-900 (Figs. 3A and 3B) corresponding to phospholipids. We therefore analyzed them by ESI/MS/MS (Figs. 4 and 5) to confirm the structure of the most abundant phospholipids.

Daughter analysis by MS/MS of the GPEth (M+H)⁺ ion at m/z 716.5 gave the ion at m/z 575 by loss of neutral ethanolamine phosphate (141 D) and at m/z 336 corresponding to 575-C₁₄H₂₉CH=C=O, indicating the presence of palmitic acid (Fig. 4A). In agreement with a GPCho structure, MS/MS of the ions at 758.5 (Fig. 4B) and 782.5 (Fig. 4C) gave the characteristic fragment m/z 184.1 corresponding to positive H₂O₃POCH₂CH₂N⁺(CH₃)₃ [32]. Instead, the (M+Na)⁺ ion at m/z 804.5 lost the choline phosphate as a neutral fragment of mass 184 D and gave an intense [(M+Na)-184]⁺ fragment at m/z 621 (Fig. 4D).

Ions 819.5 and 861.5 (Fig. 3A) were identified by MS/MS as alkylacyl phosphatidylinositol (AAG GPI_{no}, 16:0/18:2) and diacyl phosphatidylinositol (GPI_{no}, 18:0/18:2), respectively. They both showed the characteristic fragment at m/z 241 identified as inositolphosphate -H₂O (Figs. 5B, and 5C). The AAG GPI_{no} showed only one acyl fragment at m/z 279 corresponding to the linoleic acid anion (Fig. 5B), whereas the GPI_{no} (861.5), showed the anions of linoleic and stearic acids at 279 and 283 (Fig 5C).

In addition, chloride adducts of GPCho were detected at 788, 790 and 792 (Fig. 3A). Lipid extracts contain enough chloride for the formation of adducts [32]. The ions at 772 and 756 in the ESI-MS⁻ (Fig. 3A) would be produced from the GPCho ion at 788 by the easy loss of one or two molecules of methane (16 D), respectively. The MS/MS of the three chloride adducts produced as main fragment the linoleic acid anion, thus confirming the GPCho structure. In Fig. 5A the MS/MS of the 790.5 [M+Cl]⁻ ion is shown. The fragment ion at 740 corresponds to the neutral loss of CH₃Cl (50 D). The negative MS/MS spectra of the [M+Cl]⁻ adducts allowed the identification of the fatty acid fragments which were not shown in the positive spectra. The three signals at 788, 790 and 792 observed in the amplified negative ion ESI (Fig. 3A) indicated a microheterogeneity in the C₁₆ fatty acid with respect to unsaturation. The 790 MS/MS spectrum (Fig 5A) showed a peak at m/z 253 corresponding to palmitoleic acid whereas the 788 MS/MS (not shown), with two less mass units, showed the strong peak at 279 of the C_{18:2} anion and a small peak at 251 corresponding to the C_{16:2} fatty acid anion. The MS/MS spectra of the 772 and 756 ions also showed a main fragment of m/z 279 and no fragmentations corresponding to the other common phospholipids (not shown)

In the microsomes, in the negative mode, we observed the following ions: 756.5, 788.5 (GPCho), 819.5 (AAG-GPI_{no}) and 861.5 (GPI_{no}). In the positive mode, the microsomal fraction showed no peaks in the range 700-900 (data not shown). For comparative purposes, we analyzed a more concentrated microsomal extract (300 µg protein). We observed (data not

shown) the following molecular ions: 804.5, 758.5, and 782.5. In the negative mode this larger amount of microsomal fraction gave, in addition, the inositol phosphoceramide ion at 778.5 corresponding to a ceramide with fatty acid (C16:0) and sphingosine (C18:1). We have previously analyzed the inositol phospholipids (IPLs) by ESI/MS in a total extract of epimastigotes at the logarithmic phase of growth, and the IPC with m/z 778 corresponding to N-palmitoylsphingosine was the main IPL, with AAG-GPI_{no} and GPI_{no} also present (unpublished results). Although traces of IPC could be present in acidocalcisomes, the other IPLs are more abundant. In conclusion, the MS pattern for the lipids of the acidocalcisome and microsomal fractions analyzed under the same conditions are quite different and the ion 714.5 (GPEth) (Fig 3B) is unique to the acidocalcisome fraction. Under the conditions used inositolphosphoceramide was not detected in acidocalcisomes.

3.3. Glycolipids in acidocalcisome fractions

In addition to the experiment described in Fig. 2, SDS-PAGE and periodic acid-Schiff staining of acidocalcisome fractions provided preliminary evidence of the presence of a glycoconjugate (data not shown). Epimastigotes were then incubated in the presence of [³H]-mannose and fractionated as described under Materials and Methods. The presence of glycoinositolphospholipids (GIPLs, arrow) in different fractions is shown in Fig. 6. GIPLs are detected in the acidocalcisome fraction (fraction 16). To rule out that the presence of a GIPL in the acidocalcisome fraction was due to glycosome or plasma membrane contamination and taking into account that there is one molecule of inositol per molecule of GIPL, we evaluated the total amount of inositol coming from GIPLs in the different fractions per mg of protein. A similar amount of protein (163 μ g) of the total lysate and microsomal and acidocalcisome fractions were extracted with water-saturated butanol and analyzed by TLC in solvent A. The fraction corresponding to GIPLs were extracted from the silica with chloroform/methanol/water (30:60:20, v/v) and was hydrolyzed with 6 N HCl, at 110 °C for 16 h. The free inositol was analyzed by HPAEC-PAD in the MA1 column with 80 mM NaOH as eluent. A peak at 10.86 min was identified with an authentic sample of inositol. The values obtained are included in (Table 1). The results indicated that the acidocalcisome fraction contained a $28.7 \pm 6.9\%$ of the inositol (GIPLs) of the microsomal fraction. This means that almost a 20% of the GIPLs cannot be justified by contamination by plasma membrane since the plasma membrane marker contamination was less than 10% (see 3.1). The sugars present in the GIPLs were released by acid hydrolysis (2 N trifluoroacetic for total neutral sugars; 4 N HCl for amino sugars and 0.02 N trifluoroacetic acid for galactofuranose) chromatographed on Dionex PA10 and analyzed by HPAEC-PAD. All the galactose was released under the milder acid conditions indicating that it is present as furanose. The results are shown in Table 1. Both fractions contained similar amounts of mannose and glucosamine but approximately 30% less galactofuranose was found in acidocalcisomes. Accordingly the ratio man/galf was higher in acidocalcisomes. In addition, the amount of inositol was lower, 28.7% of the inositol value of the microsomes, suggesting a different structure of the GIPLs. This is in agreement with the lack of reaction of the antibody against LPPG in the acidocalcisome fraction (Fig. 1C).

4. Discussion

Highly purified acidocalcisomes from *T. cruzi* epimastigotes were obtained by differential centrifugation and iodoxanol gradient ultracentrifugation. Lipid analysis of acidocalcisomes revealed the presence of low amounts of 3- β -hydroxysterols and predominance of phospholipids. Glycoinositolphospholipids were detected in these organelles. The isolation of acidocalcisomes by subcellular fractionation and gradient centrifugation was a key step for their definitive characterization. We initially used a Percoll-based method for the separation of acidocalcisomes from various trypanosomatid species [12,33,34], and more recently [21] a iodoxanol(Optiprep)-based method since this compound is soluble and could be removed

completely at the end of the centrifugation run, allowing the acidocalcisome pellet to be resuspended in the buffer of choice. This protocol resulted in a substantial purification of the acidocalcisome compared with the large organelle ($10,000 \times g$) fraction, at least 60-fold as marked by the $V\text{-H}^+\text{-PPase}$ activity [21]. This may be a substantial underestimate, given that an unknown fraction of the $H^+\text{-PPase}$ resides on the cell surface [12], and the Golgi complex [35]. This acidocalcisome fraction was shown to be functionally active, showing H^+ uptake driven by PP_i , Ca^{2+} uptake driven by a vanadate-sensitive $Ca^{2+}\text{-ATPase}$, and a membrane potential generated by the $V\text{-H}^+\text{-PPase}$ activity [21]. The only other organelle that was purified to any extent in these acidocalcisome preparations was the glycosome, evidenced by a 5-fold purification of hexokinase. Lysosomes (marked by α -mannosidase) and mitochondria (marked by alanine and aspartate aminotransferases) were not enriched in this fraction. The acidocalcisome was therefore enriched at least 10-fold more than these other cell compartments by this technique [21]. To decrease glycosome contamination we developed a modification (scheme 1) of that isolation procedure [21]. The results obtained suggest a contamination of the acidocalcisome fraction with plasma membrane of about 10% and with glycosomes of 2%. We therefore considered that a recovery higher than 10% would indicate that the compound is located in acidocalcisomes.

Lipid analysis of acidocalcisomes revealed the presence of low amounts of 3β -hydroxysterols and predominance of phospholipids. The low content of 3β -hydroxysterols found in acidocalcisomes is consistent with their low susceptibility to permeabilization with digitonin, which allowed the measurement of proton and Ca^{2+} uptake by this compartment *in situ* [3]. Alkylacyl phosphatidylinositol (16:0/18:2), diacylphosphatidylinositol (18:0/18:2), diacyl phosphatidylcholine (16:0/18:2; 16:1/18:2; 16:2/18:2; 18:1/18:2 and 18:2/18:2), and diacyl phosphatidylethanolamine (16:0/18:2 and 16:1/18:2) were the main phospholipids identified. Interestingly, we found that linoleic acid occupied position 2 in all the phospholipids analyzed. In this regard, an oleate desaturase of *T. cruzi* has been recently characterized [36]. This converts oleic acid into linoleic acid and is also capable of synthesizing the $C_{16:2}$ fatty acid, now detected in GPCho.

The phospholipid composition of the acidocalcisome fraction differs from that of glycosomes of *T. brucei*, which contain two major phospholipids: phosphatidylcholine and phosphatidylethanolamine in a ratio of approximately 2:1 [37]. These are also the predominant phospholipids of the glycosomes of epimastigotes of *T. cruzi*, which in addition have been shown to possess phosphatidylserine and sphingomyelin, and traces of phosphatidylinositol [38].

Most previous studies have based the identification of phospholipids of *T. cruzi* in their chromatographic migration as compared with known standards and did not report their fatty acid composition [38–41]. In some cases gas liquid chromatography (GLC) was used to characterize the fatty acids in the total lipids and in the phosphatidylinositols [40] or GLC-mass spectrometry to identify the degradation products of inositolphospholipids [39].

Linoleic acid has been identified as a main component of the total lipids of epimastigotes and especially in the phosphatidylinositols [40]. The GPI anchors of trypomastigote mucins contain alkylacyl glycerol with oleic and linoleic acid in position *sn*-2 and it has been proposed that these unsaturated fatty acids are essential for the bioactivity of GPIs [42]. In fact, the GPIs of the non-infective epimastigote mucins contain only saturated fatty acids [42]. Studies with homogenates of epimastigotes [39] and microsomal fractions (this work) showed the presence of linoleic acid in the phosphatidylinositol. This suggests that if these phosphatidylinositols are substrates for the synthesis of GPIs, a remodeling of the fatty acid in a late stage of the biosynthesis pathway would be necessary, as occurs in *T. brucei* [43]. The high proportion of unsaturated fatty acids, the absence of sphingolipids and the low concentration of 3β -

hydroxysterols suggest the absence of lipid rafts in the acidocalcisome fraction. We did not find inositolphosphoceramide, in contrast to its abundant presence in the inositol phospholipids of total homogenates [39]. The predominance of phospholipids in acidocalcisomes is in agreement with recent results reported in the related parasite *Leishmania major* [44,45]. The sphingolipid synthesis pathway is a major route for ethanolamine biosynthesis in these parasites, and promastigotes of *L. major* deficient in either serine palmitoyltransferase or sphingosine 1-phosphate lyase had reduced GPEth and GPCho synthesis and defective acidocalcisomes [44,45]. The presence of GIPLs in the acidocalcisome fractions was determined by TLC analysis of acidocalcisome lipids with chloroform/methanol/acetone/acetic acid/water (90/30/18/27/17) as developing solvent and staining with orcinol/sulfuric acid (5% in ethanol) (Fig. 2), SDS-PAGE and periodic acid-Schiff staining of acidocalcisome fractions (data not shown), and [³H]-mannose incorporation by epimastigotes followed by subcellular fractionation and TLC detection (Fig. 6). These results were confirmed by determination of the inositol released from these glycolipids (Table 1).

A GIPL originally named lipopeptidophosphoglycan (LPPG, [46]) is the major component of the plasma membrane from epimastigote forms. LPPG gives the characteristic reactions of GPI anchors [47] and its complete structure is known [48,49]. Two GIPLs (GIPL A and B) have been isolated from epimastigotes in exponential phase of growth [50]. Both GIPLs differ in the lipid moiety, whereas 1-*O*-hexadecyl-2-*O*-palmitoyl glycerol was identified in GIPL A, ceramides, mainly *N*-palmitoyl and *N*-lignoceryl sphinganine, were found in GIPL B. The ceramides are the same as those found in the LPPG [29]. It is known that antibodies against LPPG recognize the Gal_f residues in these molecules [17]. Although the acidocalcisome GIPLs possess Gal_f they do not react with these antibodies (Fig. 1C). Microheterogeneity was previously reported for the GIPLs of *T. cruzi*. Three species of LPPG have been identified [48] and the main epitope is probably the branching Gal_f structure present in the preponderant component (65%). The GIPLs in acidocalcisomes with a higher ratio Man/Gal_f could lack the branching Gal_f, explaining their lack of reactivity with these antibodies. Accordingly the ratio Gal_f/GlcN was lower than 1.

Taken together, these results indicate that acidocalcisomes of *T. cruzi* have a distinct lipid and carbohydrate composition.

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Fig. 1. Western blot analysis of microsomal and acidocalcisome fractions (A-C) and distribution of hexokinase in iodixanol gradients (D)

Two μg protein from microsomal (lanes 1) and acidocalcisome (lanes 2) fractions were analyzed by SDS-PAGE and western blot with antibodies against P-type H⁺-ATPase (A), vacuolar-type H⁺-PPase (B) and LPPG (C) as described under Materials and Methods. (D), Distribution of hexokinase activity from epimastigotes in iodixanol fractions. Chart shows mean activity $\pm$ SE (as a percentage of the total recovered activity) from 3 independent experiments. Protein distribution in the different fractions is indicated in E. Vacuolar-type H⁺-PPase is concentrated in the acidocalcisome fraction (B), which has low amounts of plasma membrane (P-type H⁺-ATPase (A), and LPPG (C)) and glycosomal markers (hexokinase (D), and a very low amount of protein (E).

Fig. 2. Analysis of phospholipids in the microsomal and acidocalcisome fractions

Microsomal and acidocalcisome fractions (20 µg protein) were extracted with water-saturated butanol as described under Materials and Methods, resuspended in chloroform/methanol/water (10/10/3, v/v) and analyzed by TLC in solvent A. Lane **1**: 2 µg of acidocalcisome protein; lane **2**: 2 µg of the microsome protein. The lipids were detected with orcinol/H₂SO₄ in ethanol and heating at 200 °C on a hot plate until the standards were well shown. GPCho, phosphatidylcholine. Lane **3** is a phosphatidylinositol (GPIIno) standard. There was a 70% enrichment in compounds with the mobilities of GpCho and GpIno in the acidocalcisome fraction with respect to the microsomal fraction as detected by densitometry.

Fig. 3. Negative (A) and positive (B) ion electrospray mass spectrometry of lipid extracts from acidocalcisome fractions

The identities of the major ions are indicated. GPEth, phosphatidylethanolamine, GPCho, phosphatidylcholine, GPI, phosphatidylinositol. The figures in brackets describe the carbon chain length and degree of unsaturation of the acyl, or alkyl chains.

Fig. 4. MS/MS spectra of positive ions of phospholipids in the acidocalcisome fraction

A. Daughter ion spectrum of the $[M+H]^+$ ion of GPEth at 716.5; **B.** Daughter ion spectrum of the $[M+H]^+$ ion of GPCho at 758.5; **C.** Daughter ion spectrum of the $[M+H]^+$ ion of PC at 782.5. **D.** Daughter ion spectrum of the $[M+Na]^+$ ion of GPCho at 804.5. The insets in the figures show the product ion assignments for the phospholipids.

Fig. 5. MS/MS spectra of negative ions of phospholipids in the acidocalcisome fraction
A. Daughter ion spectrum of the $[M+^{35}\text{Cl}]^-$ ion of GPCho at 790.5; **B.** Product ion spectrum of the $[M-H]^-$ ion of AAG-GPIIno at 819.5; **C.** Daughter ion spectrum of the $[M-H]^-$ ion of GPIIno at 861.5. The insets in the figures show the product ion assignments for the phospholipids.

Fig. 6. GPIs distribution in iodixanol gradients

Epimastigotes were pre-labeled with [^3H]-mannose for 18 h in DMEM media without glucose and fractionated as described under Materials and Methods. Same volume (2 ml) of the different fractions of the gradient (**1-16**) was extracted with butanol saturated with water (600 μl , 3 X). The organic phases were washed twice with water, dried in a speed-vac and analyzed by TLC on oxalate/EDTA-impregnated. Silica-gel plates under the solvent system: chloroform/methanol/acetone/acetic acid/water (90/30/18/27/17, by vol.). Radiolabeled lipids were detected by fluorography. **E**, total extract. Only some of the fractions (1-7, 15-16) are numbered for clarity.

Scheme 1.

Table 1

GIPLs were extracted as described under Materials and Methods and hydrolyzed under different acidic conditions: 2 N trifluoroacetic acid for 4 h, 0.02 N trifluoroacetic acid for 2 h, and 4 N HCl for 4 h. After hydrolysis the samples were neutralized and dried with a rotatory evaporator. Sugars were analyzed by HPAEC-PAD in the Dionex DX-600 equipped with a PA-10 column and eluted with 18 mM NaOH. Sugars were identified by the retention time and comparison with known standards. The amount of inositol in the total lysate was 32.2 ± 4.4 pmol/ μ g protein. Results are expressed as means $\pm$ SD (n = 3).

Compound**	Microsomal Fraction (pmol/ μ g protein)	Acidocalcisome Fraction (pmol/ μ g protein)
Mannose (Man)	161.6 ± 4.7	153.6 ± 2.1
Glucosamine (GlcN)	64.4 ± 7.5	56.3 ± 3.2
Galactofuranose (Galf)	53.4 ± 0.7	$38.9 \pm 1.6^*$
Inositol (Ins)	45.3 ± 1.6	$13.5 \pm 5^*$

* Significance as compared with the microsomal fraction ($P < 0.05$).

** The ratios Man/GlcN, Galf/GlcN, and Man/Galf were 2.5, 0.8, and 3.0, respectively (microsomal fraction), and 2.7, 0.6, and 3.9, respectively (acidocalcisome fraction)