Functional expression of a vacuolar-type H⁺-ATPase in the plasma membrane and intracellular vacuoles of *Trypanosoma cruzi*

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Acid-loaded *Trypanosoma cruzi* amastigotes and trypomastigotes regained normal cytoplasmic pH (pH_i), as measured in cells loaded with 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), by a process that was sensitive to bafilomycin A₁ at concentrations comparable to those that inhibited vacuolar (V) H⁺-ATPases from different sources. Steady-state pH_i was also decreased by similar concentrations of bafilomycin A₁ in a concentration-dependent manner. The efflux of H⁺ equivalents from amastigotes and trypomastigotes was measured by following changes in the fluorescence of extracellular BCECF. Basal H⁺ extrusion in the presence of glucose was 15.4 ± 2.8 (S.D.) nmol of H⁺/min per 10⁸ amastigotes and 6.37 ± 0.8 nmol of H⁺/min per 10⁸ trypomastigotes. Bafilomycin A₁ treatment significantly decreased the efflux of H⁺ equivalents by amastigotes (8.9 ± 2.2 nmol of H⁺/min per 10⁸ cells), but not by try-

mastigotes (5.1 ± 1.7 nmol of H⁺/min per 10⁸ cells). The localization of the V-H⁺-ATPase of *T. cruzi* was investigated by immunocytochemistry. Confocal and electron microscopy indicated that, in addition to being located in cytoplasmic vacuoles, the V-H⁺-ATPase of different stages of *T. cruzi* is also located in the plasma membrane. However, no labelling was detected in the plasma membrane lining the flagellar pocket of the different developmental stages. Surface localization of the V-H⁺-ATPase was confirmed by experiments involving the biotinylation of cell surface proteins and immunoprecipitation with antibodies against the V-H⁺-ATPase. Taken together, the results are consistent with the presence of a functional V-H⁺-ATPase in the plasma membrane of amastigotes and with an important role for intracellular acidic compartments in the maintenance of pH_i in different stages of *T. cruzi*.

INTRODUCTION

Eukaryotic cells can express H⁺-ATPases in their plasma membranes. These pumps occur in two forms: an electrogenic H⁺-ATPase and an electroneutral K⁺/H⁺-ATPase. The plasma membrane H⁺-ATPase of lower eukaryotes, such as yeast [1] and other fungi [2], is of the P-type; that present in several normal [3–7] and tumoural [8] mammalian cells and in some avian, reptilian, amphibian, fish, mollusc, crustacean, insect and protozoan cells (reviewed in [9]) is of the vacuolar (V-) type. These two types of H⁺-ATPase are involved in the regulation of intracellular pH (pH_i) in many of these cells. In contrast, the H⁺/K⁺-ATPase, which is of the P-type, is present mainly in the gastric mucosa and is involved in acid secretion [10]. The V-H⁺-ATPases are also present in various endocytic and exocytic vesicles, including secretory vesicles, clathrin-coated vesicles, synaptosomes, Golgi, endosomes and lysosomes [11–13]. It is believed that V-H⁺-ATPases have a significant role in the maintenance of the acidic environment found in these vesicles.

Several pathogenic protozoa have developed the ability to live in two distinct hosts, one vertebrate and the other invertebrate. Therefore they require adaptation mechanisms to live in the different environmental conditions found in the two hosts, which include important differences in ionic composition and pH. Among this group of parasites is *Trypanosoma cruzi*, the aetiological agent of Chagas's disease. *T. cruzi* epimastigotes proliferate within the gut of reduviid insects, which has an acidic pH (5.0 to 6.0) [14], and then transform into non-dividing but highly infective trypomastigote forms, which are released into the urine and faeces and inoculated into the vertebrate host. In this host trypomastigotes invade different mammalian cell types occupying

for a few hours an acidic parasitophorous vacuole (pH 4.5–5.5) [15]. They gradually transform into amastigote forms, disrupt the vacuolar membrane and enter into direct contact with the cytoplasm of the host cell, which has a neutral pH. Subsequently the amastigotes transform into trypomastigotes, which are released and reach the bloodstream, which has a pH of approx. 7.4. Therefore during its life cycle *T. cruzi* has to regulate its pH_i in environments with marked pH differences.

In a recent study we investigated the mechanisms involved in pH_i regulation in the epimastigote forms of *T. cruzi* under acid loads, as a function of the ionic composition of the medium and in the presence of different inhibitors [16]. Our results were consistent with an important role for an electrogenic H⁺-ATPase, probably of the P-type, in the regulation of pH_i in these cells [16].

Here we report that different stages of *T. cruzi* possess a V-type H⁺-ATPase that in addition to being located in cytoplasmic vacuoles is located in the plasma membrane. On the basis of the effect of the specific V-H⁺-ATPase inhibitor bafilomycin A₁ we suggest that this enzyme has a role in the regulation of pH_i in *T. cruzi*.

MATERIALS AND METHODS

Culture methods

T. cruzi trypomastigotes and amastigotes (Y strain) were obtained from the culture medium of L₆E₉ myoblasts by a modification of the method of Schmatz and Murray [17], as described previously [18,19]. The final concentration of trypomastigotes and amastigotes was determined with a Neubauer chamber. The contamination of trypomastigotes with amastigotes and inter-

Abbreviations used: BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; pH_e, extracellular pH; pH_i, intracellular pH; V, vacuolar.

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mediate forms or of amastigotes with trypomastigotes or intermediate forms was always less than 5%. *T. cruzi* epimastigotes (Y strain) were grown at 28 °C in a liquid medium consisting of brain-heart infusion (37 g/l), haemin chlorohydrate [20 mg/l dissolved in 50% (v/v) triethanolamine], and 10% (v/v) heat-inactivated newborn calf serum [16]; 5 days after inoculation, cells were collected by centrifugation. The protein concentration was determined by the biuret assay [20] in the presence of 0.2% deoxycholate. L₆E₉ myoblasts were cultured as described previously [21].

Chemicals and solutions

Fetal and newborn calf serum, Dulbecco's PBS, Tween 20, poly-(L-lysine) (70 kDa), aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor and PMSF were purchased from Sigma. Bafilomycin A₁ (purity at least 95% by HPLC) was from Kamiya Biomedicals (Thousand Oaks, CA, U.S.A.). Free 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) or its acetoxymethyl ester (BCECF/AM), and fluorescein-labelled antibodies were from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade. A rabbit antiserum (7058L) reactive to at least six of the eight subunits of the V-H⁺-ATPase from *Dictyostelium discoideum* [22] was a gift from Dr. Kathleen V. Nolte and Dr Theodore Steck (University of Chicago, Chicago, IL, U.S.A.). Gold-labelled goat anti-rabbit antibodies were obtained from Ted Pella (Redding, CA, U.S.A.). Biotin succinimidyl ester, avidin-peroxidase conjugate and the chemiluminescence detection kit were from Amersham Life Sciences (Arlington Heights, IL, U.S.A.).

The standard buffer used contained 135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂ and 10 mM Hepes/Tris, pH 7.4, unless indicated. Buffer A contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose and 50 mM Hepes/Tris, pH 7.4.

pH measurements

pH was measured fluorometrically with BCECF. After the trypomastigotes and amastigotes had been harvested, they were washed twice in buffer A at 4 °C, followed each time by centrifugation at 1000 *g* for 10 min. For pH_i measurements, cells were resuspended to a final density of 10⁹ cells/ml in loading buffer that consisted of buffer A plus 1.5% (w/v) sucrose and 5 μM BCECF/AM. The suspensions were incubated for 30 min at room temperature. Subsequently the cells were washed twice with standard buffer to remove extracellular dye [16]. A 50 μl aliquot of the cell suspension was diluted into 2.5 ml of standard buffer (2 × 10⁷ cells/ml final density) in a cuvette placed in a thermostatically controlled (37 °C) Hitachi F-2000 spectrofluorometer. For extracellular pH (pH_e) measurements a 100 μl aliquot of the cell suspension in standard buffer (unloaded) was centrifuged and the pellet was diluted into a weakly buffered solution containing 0.38 μM BCECF (free acid), 135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose and 0.1 mM Hepes/Tris, pH 7.4. The fluorescence ratio, with wavelengths for excitation set at 505 and 440 nm and for emission at 530 nm were recorded and translated into pH values on the basis of the ratios obtained at standard pH values [16]. Other experimental conditions and calibrations were as described previously [16].

Immunofluorescence microscopy

The parasites (mixed populations of amastigotes and trypomastigotes, or epimastigotes) were fixed for 30 min at room

temperature in a solution containing 4% (v/v) freshly prepared formaldehyde in 0.1 M cacodylate buffer, pH 7.2, washed in Dulbecco's PBS, allowed to adhere for 10 min to coverslips previously coated with 0.1% poly-(L-lysine) and incubated for 5 min in the presence of 0.1% (v/v) Triton X-100. Subsequently they were incubated for 30 min in the presence of 50 mM NH₄Cl to block free aldehyde groups, washed in Dulbecco's PBS, incubated for 3 h in the presence of the rabbit antiserum recognizing the V-H⁺-ATPase (1:50 dilution in Dulbecco's PBS), washed in Dulbecco's PBS/3% (w/v) BSA and incubated in the presence of fluorescein-labelled goat anti-(rabbit IgG) for 60 min (diluted 1:50 or 1:100 in Dulbecco's PBS). The specimens were mounted with *N*-propyl gallate and observed in a Zeiss LSM10 laser-scanning confocal microscope equipped with an argon ion laser for excitation of fluorescein at 488 nm. Optical sections of 0.1 and 0.3 μm were used. Emitted fluorescence was detected by using an FITC filter set, and the corresponding differential-interference contrast images were acquired by using transmitted light. For analysis of live cells, the cells were incubated with 3% (w/v) BSA in Dulbecco's PBS at room temperature for 2 h, incubated with several dilutions of the rabbit antiserum recognizing the V-H⁺-ATPase (1:100, 1:200, 1:400 and 1:800) for 1 h, washed three times with Dulbecco's PBS and incubated at room temperature for 30 min in the presence of FITC-conjugated anti-(rabbit IgG) (1:200), washed three times with Dulbecco's PBS, spread on glass slides and observed with an Olympus BX60 fluorescence microscope.

Electron microscopy immunocytochemistry

The parasites were fixed in a solution containing 4% (w/v) paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, overnight at 4 °C, then infused in 25% (w/v) polyvinylpyrrolidone/2.3 M sucrose for 2 h and plunged into liquid nitrogen [23]. They were then transferred to a cryoultramicrotome (Ultracut Reichert) and cryosections were obtained at temperature range of -80 to -100 °C, then collected on nickel grids coated with Formvar film and carbon. For immunolabelling the cryosections were washed in PBS/3% (w/v) albumin, quenched in 50 mM NH₄Cl for 30 min and subsequently incubated for 3 h in the presence of the rabbit antiserum recognizing the V-H⁺-ATPase (1:50 dilution), washed and incubated in the presence of 10 nm gold-labelled goat anti-(rabbit IgG) (1:50 dilution) for 60 min. In preliminary experiments we used different dilutions of the gold-labelled antibodies, trying to find those associated with intense labelling and low background. These studies showed that labelling density can vary from cell to cell even when the same dilution was used. When the 1:50 dilution was used a more uniform labelling was obtained; this is shown in the micrographs selected. After incubation with gold-labelled antibodies, the specimens were thinly embedded in 3% (w/v) poly(vinyl alcohol)/uranyl acetate (9:1, v/v) [23] and viewed in a Zeiss 902 transmission electron microscope operating at 100 kV.

SDS/PAGE and preparation of Western blots

The electrophoretic system used was essentially that described by Laemmli [24]. *T. cruzi* epimastigotes (2.5 × 10⁸), trypomastigotes (10⁹) or amastigotes (10⁹) were centrifuged at 1000 *g* for 10 min, resuspended in 300 μl of Dulbecco's PBS containing proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF) and frozen at -70 °C. Cells were thawed and homogenized with a Teflon pestle at 4 °C. Aliquots of the *T. cruzi* homogenates were mixed with 40 μl of a

buffer containing 125 mM Tris/HCl, pH 7.0, 10% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol and 4.0% (w/v) SDS, with 4.0% (w/v) Bromophenol Blue as tracking dye, and were boiled for 5 min before application to SDS/10% (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose by the method of Towbin et al. [25], with a Bio-Rad (Richmond, CA, U.S.A.) transblot apparatus. Membranes with transferred proteins were placed overnight in a blocking buffer solution of 5% (w/v) low-fat dried milk in Dulbecco's PBS, pH 7.4. A 1:1000 dilution of rabbit antiserum against *D. discoideum* vacuolar H⁺-ATPase in blocking buffer was applied at room temperature for 60 min. The nitrocellulose was washed four times in Dulbecco's PBS for 15 min each before adding a 1:1500 dilution of goat anti-rabbit IgG in blocking buffer for 30 min. The nitrocellulose was again washed four times (15 min each) with washing buffer. The enhanced chemiluminescence detection kit was used in accordance with the instructions of the manufacturer (Amersham).

Cell surface labelling

Cell surface labelling was performed by a modification of the method of Hart et al. [26]. Epimastigotes were washed three times with 10 mM sodium phosphate (pH 8.6)/150 mM NaCl/0.1 mM MgCl₂/0.1 mM CaCl₂ and then resuspended in 1 ml of the same ice-cold medium (4×10^6 cells/ml). Biotin succinimidyl ester (in dry dimethylformamide) was added to the cells in accordance with the instructions provided by the manufacturer (Amersham) and incubated, with rotation, for 1 h at 4 °C. Cells were washed three times with ice-cold Dulbecco's PBS and lysed in 0.5 ml of ice-cold immunoprecipitation buffer [1% (v/v) Nonidet P40/20 mM Tris/HCl (pH 7.6)/0.1 mM EDTA/1 mM PMSF/2 µg/ml pepstatin/2 µg/ml leupeptin/1 µg/ml soybean trypsin inhibitor]. Cells were kept at 4 °C for 30 min with rotation; the lysate was then centrifuged for 20 min at 10000 g. The supernatant was diluted to 1.5 ml with immunoprecipitation buffer containing 150 mM NaCl, then precipitated with the rabbit antiserum against the V-H⁺-ATPase (1:300 dilution) [22]. After incubation for 16 h at 4 °C, immunocomplexes were bound to Protein A-Sepharose beads by incubating them together for 1 h at 4 °C. The beads were washed five times with immunoprecipitation buffer; bound proteins were eluted in boiling SDS sample buffer. The proteins were separated on an SDS/10% (w/v) polyacrylamide gel and blotted on a nitrocellulose membrane, as above, which was probed with avidin-peroxidase conjugate in accordance with the protocol provided by the manufacturer (Amersham). Proteins were detected by enhanced chemiluminescence (Amersham).

Statistical analysis

Where indicated, the results are expressed as means $\pm$ S.D. for n different experiments. Statistical significance was determined by Student's t test. $P < 0.05$ was taken to be significant.

RESULTS

Bafilomycin A₁ is a specific inhibitor of V-type H⁺-ATPases when used at low concentrations [27]. In a previous report [16] we demonstrated that neither basal pH_i nor recovery from an acid load of epimastigotes was significantly affected by the addition of up to 0.2 µM bafilomycin A₁, although higher concentrations (1–5 µM) caused significant decreases in steady-state pH_i, in the rate of pH_i recovery, and in the final pH_i attained after an acid load. These effects were attributed to the inhibition of the V-H⁺-ATPase of acidic intracellular compartments, or to inhibition of a plasma membrane proton pump with

Figure 1 Effect of bafilomycin A₁ on pH_i recovery of acid-loaded amastigotes (A) and trypomastigotes (B)

Acidification was obtained by preincubation of 5×10^7 cells (12.1 and 13.5 µg of amastigote and trypomastigote protein respectively) with 40 mM NH₄Cl in standard buffer, for 15 min and resuspension in standard buffer containing different concentrations of bafilomycin A₁. The control was without inhibitors present. The curves show the recovery rate from acidification and the final pH_i of the cells after recovery was complete. Other experimental conditions are described in the Materials and methods section. Values are means $\pm$ S.D. for three different experiments.

low sensitivity to this inhibitor [27]. We therefore investigated whether this was also true of the infective stages.

In the nominal absence of bicarbonate and with a pH_e of 7.4 (standard buffer), the mean baseline pH_i of trypomastigotes and amastigotes was 7.32 ± 0.092 ($n = 9$) and 7.35 ± 0.08 ($n = 13$) respectively, in agreement with previous reports [28]. Amastigotes and trypomastigotes were acidified to pH approx. 6.0 with an NH₄⁺ prepulse technique [16]. When resuspended in medium containing bafilomycin A₁, acid-loaded cells showed a dose-dependent inhibition of their recovery rates and in the final pH_i attained (Figures 1A and 1B). Similar concentrations of bafilo-

Figure 2 Effect of bafilomycin A_1 on the steady-state pH_i of amastigotes and trypomastigotes

The cells ($5 \times 10^7/50 \mu\text{l}$ of buffer A) were centrifuged in an Eppendorf centrifuge at 2000 g for 1 min; the pellet was resuspended in 3 ml of standard buffer in the presence of different concentrations of bafilomycin A_1 . The values shown are changes in pH_i (ΔpH_i) after 5 min of incubation in the presence of the indicated concentrations of bafilomycin A_1 indicated in the abscissa. Other experimental conditions are described in the Materials and methods section. Values are means $\pm$ S.D. for three different experiments.

Figure 3 Effect of bafilomycin A_1 on H^+ extrusion by amastigotes (A) and trypomastigotes (T)

The parasites (10^8 cells) were suspended in a weakly buffered solution [135 mM NaCl/5 mM KCl/1 mM $CaCl_2$ /1 mM $MgSO_4$ /10 mM glucose/0.1 mM Hepes/Tris (pH 7.4); final volume 2.5 ml] containing 0.38 μM BCECF (free acid) at 37 $^{\circ}\text{C}$. The fluorescence changes were recorded immediately as described in the Materials and methods section. Bafilomycin A_1 (4 μM ; Baf, solid lines) was added where indicated. Controls are shown with broken lines. Results shown are of a typical experiment. Two other experiments gave comparable results.

mycin A_1 were also able to decrease the steady-state pH_i of these cells suspended in standard buffer (Figure 2).

To investigate whether the intracellular acidification produced by treatment with bafilomycin A_1 (Figure 2) was due to H^+ release from intracellular stores or to inhibition of H^+ extrusion through the plasma membrane, we measured changes in the pH_e

of cells incubated in the presence and the absence of the inhibitor. Figure 3 shows that the rate of extracellular acidification was higher for amastigotes (15.4 ± 2.8 nmol of H^+ /min per 10^8 cells; $n = 6$) than for trypomastigotes (6.4 ± 0.8 nmol of H^+ /min per 10^8 cells; $n = 5$). Addition of 4 μM bafilomycin A_1 significantly diminished the initial rate of H^+ pumping by amastigotes (to 8.9 ± 2.2 nmol of H^+ /min per 10^8 cells; $n = 8$) ($P < 0.0001$), whereas it did not significantly affect the rate of H^+ pumping by trypomastigotes (5.1 ± 1.7 nmol of H^+ /min per 10^8 cells; $n = 6$). These results suggest that inhibition of H^+ extrusion by the $V-H^+$ -ATPase from amastigotes has a role in the intracellular acidification detected upon treatment of the cells with bafilomycin A_1 , whereas H^+ release from intracellular stores is the more important effect of the inhibitor in trypomastigotes.

We then investigated the localization of the $V-H^+$ -ATPase of *T. cruzi* by immunocytochemistry with a rabbit antiserum that recognizes several subunits of *D. discoideum* $V-H^+$ -ATPase and is known to cross-react with $V-H^+$ -ATPases of animal cells [22]. The binding of this antiserum to the various developmental stages of *T. cruzi*, as revealed with fluorescein-labelled secondary antibodies and examined by confocal laser-scanning microscopy, was to the surface and intracellular vacuoles (Figures 4A to 4E). The intensity of labelling varied from cell to cell but surface labelling was in general more intense with amastigotes (Figures 4C, 4D and 4E, small arrows) than with trypomastigotes (Figure 4C, 4D and 4E, large arrows). Careful examination of the cells with an optical section of 0.1 μm showed labelling of some cytoplasmic vacuoles, especially evident in epimastigotes (Figure 4B, arrows). No fluorescence was observed in control cells incubated in the presence of normal serum or only the secondary fluorescein-labelled goat anti-rabbit IgG (results not shown). Living epimastigote cells were also examined. The anti- $(V-H^+$ -ATPase) reactivity was very weak, although it was positive in agreement with the surface location of the enzyme (results not shown).

To analyse in more detail the structures labelled with the antibodies, immunoelectron microscopy was performed on fixed parasites by using anti- $(V-H^+$ -ATPase) serum (Figure 5). Labelling of the cell surface of most of the parasites was observed, this labelling being more intense with amastigotes (Figure 5C). The gold particles were not seen only on the actual cell surface: they were also associated with the inner portion of the plasma membrane. Occasionally a few gold particles were seen associated with the flagellar membrane (Figure 5D). No labelling of the membrane lining the flagellar pocket was observed, although small vesicles located close to it were labelled (Figure 5C, arrowheads). Larger vacuoles, with a mean diameter of 210 ± 60 nm, identified as acidocalcisomes [29,30], were also labelled (Figures 5A and 5B). The labelling was associated with the luminal face of their membrane (Figures 5A and 5B, arrowheads). Usually these vacuoles did not show an inner content and in epimastigotes (results not shown) they were morphologically distinct from the reservosomes, which usually contain intravacuolar inclusions [30–32].

To confirm the surface localization of the $V-H^+$ -ATPase in *T. cruzi*, we labelled epimastigotes with biotin succinimidyl ester, a reagent that couples biotin to lysine residues of exposed proteins. The cells were incubated with the reagent and lysed; the $V-H^+$ -ATPase was immunoprecipitated with the rabbit antiserum. The precipitates were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Biotinylated proteins were detected by peroxidase-conjugated streptavidin and enhanced chemiluminescence (Figure 6). Figures 6(A) to 6(C) show the immunological cross-reactivity of the rabbit antiserum against the $V-H^+$ -ATPase [22] with a protein band present in whole homogenates of *T.*

Figure 4 Indirect immunofluorescence analysis of the presence of V-H⁺-ATPase in different developmental forms of *T. cruzi*

(A–C) Bright-field images (left panels) and fluorescence confocal laser-scanning microscopy images (right panels) of epimastigotes (A, B), and trypomastigotes (large arrows) and amastigotes (small arrows) (C) that were mixed before analysis to facilitate comparisons. (D, E) Fluorescence confocal images of the mixed amastigotes (small arrows) and trypomastigotes (large arrows). The images showed a patch labelling pattern on the surface (arrows in A) and in cytoplasmic vesicles (arrows in B) of epimastigotes. In amastigote and trypomastigote forms, surface labelling was evident, with areas [large arrows in C (right panel), D and E] where labelling of cytoplasmic vesicles was more intense. Optical sections of 0.1 μm (A and B, right panels) or 0.3 μm [C (right panel), D and E] were used. Scale bar, 5 μm .

cruzi amastigotes (Figure 6A), trypomastigotes (Figure 6B) and epimastigotes (Figure 6C) of apparent molecular mass 56 kDa, equivalent to the 53 kDa protein found in *D. discoideum* [22]. No background staining was observed when normal serum was used as a control (Figures 6D to 6F). Figure 6(G) shows one band of a biotinylated protein immunoprecipitated with the same anti-serum. The band had a slightly higher molecular mass (78 kDa instead of 56 kDa). The lack of a perfect correlation between the molecular mass of the protein in Western blots and its molecular mass as revealed with peroxidase-conjugated streptavidin could be attributed to the attachment of biotin molecules to the protein that might have affected its electrophoretic motility. Alternatively, this could be explained by a lower abundance of another subunit in whole homogenates and its enrichment by the biotinylation procedure.

DISCUSSION

Although the presence of a functional V-H⁺-ATPase in the plasma membrane of a number of cells has been postulated, most of the studies have been based on either correlations between increased acid efflux from the cells and hyperpolarization [33,34] or on the effect of vacuolar H⁺-ATPase inhibitors on pH_i and plasma membrane potential [3–7,35–38]. Immunocytochemistry, combining the use of specific antibodies and their visualization both by light microscopy and transmission electron microscopy, has been used for the high-resolution location of V-H⁺-ATPases

in the plasma membrane of renal and toad bladder cells [39], osteoclasts [40], chick embryo chorioallantoic cells [41], rat epididymis cells [42], insect cells [43,44] and fish cells [45]. This technique has also been used to locate V-H⁺-ATPases in macrophage phagosomes [46] and in the contractile vacuoles of *Paramecium multimicronucleatum* [47] and *D. discoideum* [48,49].

Using both confocal laser scanning microscopy and transmission electron microscopy of cryosections probed with previously well-characterized antibodies that recognize different subunits of a V-H⁺-ATPase [22], we showed the presence of this enzyme both in the plasma membrane and in intracellular compartments of all developmental stages of *T. cruzi*. Although a quantitative analysis was not performed, it seems that the density labelling in the plasma membrane was higher in amastigotes (Figure 5C) than in trypomastigotes (Figure 5A) or epimastigotes (results not shown). This correlated with the higher inhibitory effect of bafilomycin A₁ on H⁺ extrusion by amastigotes (Figure 1), although it was not correlated with the presence of similar amounts of protein in immunoblots of the three developmental stages (Figure 6).

Forgacs [50] has shown that the catalytic 'A' subunit of V-H⁺-ATPases has two key thiol residues that are proximal to each other in the tertiary structure; these residues form a disulphide bond under oxidizing conditions, thereby inactivating the enzyme. It has been postulated [9] that the retention of regulatory cysteine residues in the active site during evolution might explain why functional V-H⁺-ATPases are commonly found in the

Figure 5 Immunoelectron microscopy of trypomastigotes (A, B) and amastigotes (C, D) with anti-(V-H⁺-ATPase)

Labelling of the cell surface and cytoplasmic vacuoles (V) is evident. In vacuoles the gold particles are associated mainly with their luminal portion (B). No significant labelling of the membrane lining the flagellar pocket (FP) was observed (C, D). However, small vesicles located close to the flagellar pocket were labelled (arrowheads in C). Abbreviations: F, flagellum; K, kinetoplast; N, nucleus; V, vacuole. Scale bar, 0.2 μ m.

reducing atmosphere of the cytoplasm, where they are expected to be active, rather than in the putative oxidizing atmosphere of many plasma membranes, where they would be inactive. This 'redox modulation mechanism' might obviate the need to invoke two types of enzyme to explain the selective targeting of V-H⁺-ATPases to plasma membranes or endomembranes: a membrane that contains a single form of V-H⁺-ATPase might cycle between the membranes of the cytoplasmic organelles and the cell surface, the enzyme being active only when reducing conditions remove the disulphide bonding restraint [9]. Because amastigotes are free in the reducing atmosphere of the cytoplasm of host cells, our results are in agreement with this hypothesis because they should behave as intracellular 'organelles' and contain a functional plasma membrane-located V-H⁺-ATPase.

The V-H⁺-ATPase is composed of two different structures, the peripheral or catalytic (V₁) and the membrane (V₀) sectors; each is composed of several subunits. Investigation of the structure and function of the pump of clathrin-coated vesicles has revealed that the peripheral sector is composed of polypeptides of 70, 58, 40, 33 and 14 kDa, termed subunits A, B, C, E and F respectively [51]. Recent work has also identified other components of this sector. These include a dimer composed of 57 and 50 kDa polypeptides termed SFD (sub-fifty-eight kDa dimer), subunit D (28 kDa) and two polypeptides, designated G and H, of molecular masses 14 and 15 kDa [51]. In contrast with the subunit structure of the catalytic sector that is generally defined, that of the

membrane sector of V-H⁺-ATPase is largely unknown [52]. One subunit is the proteolipid of 16 kDa responsible for its sensitivity to dicyclohexylcarbodi-imide. The other subunits that were defined as part of the V₀ sector are subunits of 115 and 39 kDa, and a recently identified subunit of 45 kDa [53]. It has been indicated that it is likely that we are still missing subunits of the membrane sector that will be present in all membrane sectors of V-H⁺-ATPases of eukaryotic cells [53]. Using a rabbit antiserum against *D. discoideum* V-H⁺-ATPase [22], we have identified a subunit of 56 kDa in Western blots (Figures 6A to 6C) equivalent to the 53 kDa subunit present in the slime mould. After biotinylation of surface proteins a band of higher molecular mass was detected (Figure 6G), probably due to either the attachment of biotin molecules to the protein detected in whole homogenates with a change in its electrophoretic mobility or the enrichment of another subunit by the biotinylation procedure. In this regard, although some of the subunits of *D. discoideum* V-H⁺-ATPase have been identified [22], the correspondence of these subunits to those present in other V-H⁺-ATPase has not been established. Purification of the V-H⁺-ATPase of *T. cruzi* will help to elucidate its subunit composition and whether the subunit(s) described here correspond to the catalytic or membrane sector of the enzyme.

The V-H⁺-ATPase inhibitor bafilomycin [27] has been used previously to provide evidence for the presence of this enzyme in the plasma membrane of macrophages [4], neutrophils

Figure 6 Specificity of the proton pump antiserum and biotinylation of cell surface proteins

T. cruzi amastigotes, trypomastigotes and epimastigotes were lysed, and the proteins were separated by SDS/PAGE; each lane received 30 μ g of protein. Lanes A (amastigotes), B (trypomastigotes) and C (epimastigotes) show immunoblots probed with a rabbit antiserum against V-H⁺-ATPase. Lanes D–F show the controls probed with normal serum. The proton pump antiserum recognized a *T. cruzi* polypeptide with an apparent molecular mass of 56 kDa. In lane G, epimastigotes were incubated with biotin succinimidyl ester. After lysis of the cells, V-H⁺-ATPases were immunoprecipitated by the rabbit antiserum and the immunoprecipitate was subjected to Western blot analysis. Detection of biotinylation was by streptavidin–peroxidase conjugate and enhanced chemiluminescence. A band of apparent molecular mass 78 kDa was recognized. The migration positions of pre-stained molecular mass standards (in kDa) (Bio-Rad, Hercules, CA, U.S.A.) are shown at the left: myosin (207 kDa), β -galactosidase (137 kDa), BSA (84 kDa), and carbonic anhydrase (44 kDa).

[36,54], several tumour cell lines [8] and the protozoan parasites *Entamoeba histolytica* [38] and *Toxoplasma gondii* [55]. The concentration of the inhibitor used with these intact cells varied between 0.1 μ M [36,54,55] and 1–10 μ M [4,8]. Taking into account the amount of protein used in our experiments, 1 μ M bafilomycin A₁ was equivalent to a concentration of 0.18–0.2 μ mol/mg of cell protein. Because the V-H⁺-ATPase should be a relatively small component of the total cell protein, its sensitivity is within the range determined for the inhibition of the solubilized partly purified V-H⁺-ATPase from *Neurospora crassa* (I_{50} 0.0004 μ mol/mg of protein) [27], and much higher than that of the plasma membrane P-type H⁺-ATPase from *N. crassa* (I_{50} 100 μ mol/mg of protein) or other purified ATPases [27]. In addition, when bafilomycin A₁ was tested in intact rat hepatocytes, cells that do not seem to have a V-H⁺-ATPase in the plasma membrane [56], at concentrations of 1.0–50 μ M, in no case did it have a significant effect on pHi [57]. In contrast, in some studies with intact macrophages [58], a concentration of bafilomycin A₁ as low as 6.25 nM totally inhibited the recovery of cytosolic pH after an acid load. Taking into account these studies [58] and the fact that the V-H⁺-ATPase of *T. cruzi* is also located in intracellular compartments, we cannot rule out the involvement of these compartments in the intracellular acidification that follows the addition of bafilomycin A₁ to different stages of *T. cruzi*. In this regard, the experiments showing the lack of a significant inhibition of H⁺ extrusion from trypomastigotes by bafilomycin A₁ (Figure 3), whereas at similar concentrations (0.12 μ mol/mg of cell protein) it caused significant intracellular acidification (Figures 1B and 2), suggest a role of intracellular acidic compartments in pHi homeostasis. Interestingly, although the role of intracellular compartments in the homeostasis of other ions, such as Ca²⁺, has been well characterized [59], it is generally agreed [60] with few exceptions

[57] that under normal conditions these compartments contribute little to the maintenance of a steady-state pHi although they could have a role under pathological conditions [61,62].

No significant immunolabelling of the plasma membrane surrounding the flagellum was observed (this being especially evident in epimastigote and trypomastigote forms), nor was it in the portion of the plasma membrane lining the flagellar pocket. This is an interesting observation in view of the fact that the flagellar pocket is a region of the parasite where endocytic and exocytic activities have been postulated to take place [63,64]. In the epimastigote and amastigote forms of *T. cruzi*, however, endocytic activity also takes place in a specialized portion of the cell surface located close to the flagellar pocket and known as the cytostome [31,32,63]. The presence of a well-organized layer of microtubules below the plasma membrane enveloping the remainder of the protozoan cell body apparently precludes the process of membrane fission or fusion [63,64]. In other cells the V-type H⁺-ATPase is involved in the acidification of early and late endosomes [12,13]; we therefore expected to find this enzyme in the flagellar pocket. However, the available results suggest that the endocytic process in *T. cruzi*, even that mediated by receptors, does not follow the classical endocytic pathway described for other eukaryotic cells [63,64]. In agreement with these results, by using a cytochemical approach for the detection of Mg²⁺-ATPase, on the basis of the immobilization of the phosphate ions liberated during ATP hydrolysis, this enzyme was found in the plasma membrane but not in the flagellar pocket of *T. cruzi* [65]. Interestingly, small vesicles located close to the flagellar pocket were labelled with antibodies against the V-H⁺-ATPase (Figure 4C, arrows). The reservosomes found in epimastigotes [31,32], which seem to be a late structure of the endocytic pathway that also concentrate lysosomal enzymes synthesized by the protozoan, such as the cysteine proteinase known as cruzipain [66], have a pH of approx. 6.0 [31,32]. However, these organelles were not labelled with antibodies against the V-H⁺-ATPase (results not shown). Taken together, these observations confirm the idea that the plasma membrane of *T. cruzi* presents several well-defined domains and that the flagellar pocket represents a highly specialized portion of the plasma membrane of trypanosomatids [63,64].

We also observed significant labelling of the membrane lining some cytoplasmic vacuoles. The vacuoles did not show an internal organization, thus excluding the possibility that they correspond to the reservosomes found in epimastigote forms [31,32]. Their size and distribution are compatible with the electron-dense vacuoles or acidocalcisomes [28,29], recently described in different stages of *T. cruzi*, that are known to possess a bafilomycin A₁-sensitive H⁺ uptake [28,67].

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