Presence of a Plant-like Proton-pumping Pyrophosphatase in Acidocalcisomes of Trypanosoma cruzi*

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The vacuum-type proton-translocating pyrophosphatase (V-H\(^+\)-PPase) is an enzyme previously described in detail only in plants. This paper demonstrates its presence in the trypanosomatid Trypanosoma cruzi. Pyrophosphate promoted organellar acidification in permeabilized amastigotes, epimastigotes, and trypomastigotes of T. cruzi. This activity was stimulated by K\(^+\) ions and was inhibited by Na\(^+\) ions and pyrophosphate analogs, as is the plant activity. Separation of epimastigote extracts on Percoll gradients yielded a dense fraction that contained H\(^+\)-PPase activity measured both by proton uptake and phosphate release but lacked markers for mitochondria, lysosomes, glycosomes, cytosol, and plasma membrane. Antiserum raised against specific sequences of the plant V-H\(^+\)-PPase cross-reacted with a T. cruzi protein, which was also detectable in the dense Percoll fraction. The organelles in this fraction appeared by electron microscopy to consist mainly of acidocalcisomes (acidic calcium storage organelles). This identification was confirmed by x-ray microanalysis. Immunofluorescence and immunoelectron microscopy indicated that the V-H\(^+\)-PPase was located in the plasma membrane and acidocalcisomes of the three different forms of the parasite. Pyrophosphate was able to drive calcium uptake in permeabilized T. cruzi. This uptake depended upon proton gradient and was reversed by a specific V-H\(^+\)-PPase inhibitor. Our results imply that the phylogenetic distribution of V-H\(^+\)-PPases is much wider than previously perceived but that the enzyme has a unique subcellular location in trypanosomatids.

T. cruzi is the causative agent of Chagas’ disease, an incurable, chronically debilitating, and deadly disease affecting several million people in Latin America (1). In previous work on the calcium and pH homeostasis of T. cruzi, we have demonstrated the presence of plasma membrane Ca\(^{2+}\)- and H\(^+\)-ATPases (2, 3) and calcium storage within the cell in an acidic compartment that was named the acidocalcisome (4). Acidocalcisomes have also been found in other trypanosomatids (5–9) and in apicomplexan parasites (10). The principal store of calcium in both plants (11) and yeast (12) is the acidic lysosomal-like vacuole. Our recent studies of the T. cruzi acidocalcisome, using immunofluorescence and immunoelectron microscopy (13) and subcellular fractionation and in situ compositional analysis (14) showed that it is similar to the yeast vacuole in containing high levels of phosphate but is dissimilar in being separate from organelles containing lysosomal enzymes.

Several categories of pyrophosphatase (PPase) have been identified. Soluble PPases are present in prokaryote, yeast, and mammalian cells (15). Membrane-bound PPases are found in various organelles. Mitochondria possess a proton-pumping (H\(^-\))PPase that may be dimeric (16) and is oriented to pump protons out into the cytosol (17, 18). A PPase has been purified from chloroplast thylakoids but has not shown to be involved in H\(^-\) pumping (19). Plant vacuoles, including those from chloroplasts and Acetabularia, have a monomeric H\(^+\)-PPase that functions to acidify vacuoles (20, 21) and may be related to the H\(^+\)-PPase present in the chromophores of phototrophic bacteria (21, 22). H\(^+\)-PPases may also be present in the plasma membrane of some plant cells (23, 24). An apparently vacuum-type (V-) H\(^+\)-PPase activity has been detected in rat liver Golgi fractions (25), but the physiological significance of this activity has been questioned on the grounds of low cytosolic pyrophosphate concentrations in this tissue (20).

PPase activity has been little studied in trypanosomatids, although Michels et al. (26) briefly reported the presence of PPase activity at pH 8.4 in the cytosolic fraction of T. brucei and acidic PPase activity associated with lysosomal and flagellar pocket fractions. It was not clear if these PPase activities were distinguished from general phosphatase activity. Here we demonstrate that T. cruzi possesses a V-H\(^+\)-PPase that, like the plant enzyme, is stimulated by K\(^+\) and inhibited by Na\(^+\) and pyrophosphate analogs and that is located in acidocalcisomes and plasma membranes.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Epimastigotes, amastigotes, and trypomastigotes of T. cruzi (Y strain) were cultured as before (4). Cells were washed twice in 0.25 M sucrose before use in experiments.

Assay for Vacuole Acidification—Pyrophosphate-driven vacuole acidification was assayed by measuring changes in the absorbance of acridine orange (A\(_{493} - A_{430}\)) in an SLM-Aminco DW 2000 dual wavelength spectrophotometer (4, 27). Cells (0.1–0.4 mg of protein) were incubated

* The abbreviations used are: PPase, pyrophosphatase; V-H\(^+\)-PPase, vacuolar H\(^+\)-translocating pyrophosphatase; AMDP, aminomethylene diphosphonate.

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at 30 °C in 2.5 ml of 65 mM KCl, 125 mM sucrose, 2 mM MgSO4, 10 mM K-Hepes, 50 μg EGTA, pH 7.2, or alternate buffers as described in the legends to Figs. 1 and 4 plus acridine orange (Molecular Probes, 3 μM) and filipin (Sigma, 3–5 μg/ml) for 5 min before the addition of 0.1 mM sodium pyrophosphate, pH 7.2. The amount of filipin and cells (protein) used for each couplet goat peptide was 0.09 mg total protein/ml, present as described previously (32). Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope and the image analysis system described previously (9, 13).

**Electron Microscopy**—For immunocytochemistry, cells were fixed for 60 min at 4 °C in a solution containing 0.2% glutaraldehyde, 4% freshly prepared formaldehyde, 0.1% picric acid in 0.1 M cacodylate buffer, pH 7.2. After fixation the cells were infused in a mixture of 25% polyvinylpyrrolidone and 2.3 mM sucrose for 2 h before plunging into liquid nitrogen (34). Cryosections were cut at −80 to −100 °C and collected on nickel grids coated with Formvar film and carbon. For immunolabeling, cryosections were washed in phosphate-buffered saline, 3% albumin, quenched in 50 mM NH4Cl for 30 min, incubated for 3 h in the presence of antibodies (1:50 or 1:100 dilution), washed, and incubated with 10-nm gold-labeled goat anti-rabbit IgG (1.50 dilution for 60 min). Sections were thin-embedded in a 9:1 mixture of polyvinyl alcohol and uranyl acetate (34) and observed with a Hitachi 600 transmission electron microscope operating at 100 kV. Controls were carried out using a nonrelated antibody or incubation in the presence of the secondary antibody only. For observation of Percoll fraction 1, a 5-μl sample of the fraction, washed and concentrated as for immunoblots (see above), was placed on a Formvar-coated copper grid, allowed to adsorb for 5–15 min at room temperature, blotted dry, and observed directly by electron microscopy (14). Whole epimastigotes were applied to grids in a similar manner (14). For energy-dispersive x-ray analysis, specimen grids were examined in a Hitachi H-7100F transmission electron microscope at an accelerating voltage of 50 kV. Fine probe sizes were adjusted to cover the organelle in question, and x-rays were collected for 100 s utilizing a thin window (Novar®) detector. Analysis was performed using a Noran Voyager III analyzer with a standardless analysis identification program.

**RESULTS**

V'-PPase activity was detected in filipin-permeabilized epimastigotes of *T. cruzi* using an acridine orange uptake assay in which decrease in absorbance (493–530 nm) indicates increasing vesicular acidity (4, 27) (Fig. 1). The vesicle pH was neutralized, and acridine orange was released by the K' (Na')/H' exchanger nigericin in K' or Na' buffers. Replacement of KCl (Fig. 1, traces d and e) with NaCl (trace c) or choline chloride (trace b) in the assay buffer reduced the acidification rate.

Pyrophosphate-induced acidification was inhibited by potassium fluoride and the pyrophosphate analogs imidodiphosphate and AMPD (Fig. 2a). It was not inhibited by 250 nM concanamycin A (5 nM totally inhibits the *T. cruzi* V'-ATPase) (35) nor 1 mM sodium molybdate, which inhibits *T. cruzi* alkaline phosphatase by the P-type H'-ATPase inhibitor, sodium o-vanadate (500 μM; 25 μM totally inhibits *T. cruzi* activity, Ref. 35) nor by the mitochondrial ATPase inhibitor, oligomycin, at 2.5 μM.

V'-PPase activity was detected by acridine orange assay in amastigotes and trypomastigotes of *T. cruzi*; this activity was inhibited by 20 μM AMPD as indicated by acridine orange release (Fig. 3).

The effects of K' and Na' concentration on H'-PPase activity in epimastigotes were examined in more detail (Fig. 4a). A series of buffers with varied proportions of KCl, NaCl, and sucrose were used, and acidification rates were measured as a proportion of the rate with standard KCl-sucrose buffer (Buffer 9). Replacement of KCl with NaCl (compare Buffers 11, 10, 6, 4, and 2) led to reduced rates when the concentration of NaCl exceeded that of KCl, whereas partial replacement of KCl with sucrose (Buffers 11, 9, 8, and 5) did not reduce the rate, suggesting that NaCl at high concentrations was inhibitory. Total

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2 D. A. Scott, unpublished results.
replacement of KCl by sucrose (Buffer 3) yielded reduced activity. Buffer 12 (with 10 mM extra KCl compared with Buffer 11) was included to show the system was not sensitive to slight differences in tonicity between KCl and sucrose buffers.

PPhase was also assayed in membrane preparations of T. cruzi by inorganic phosphate detection using purine nucleoside phosphorylase and 2-amino-6-mercapto-7-methylpurine ribonucleoside as co-substrate with phosphate for purine nucleoside phosphorylase and 2-amino-6-mercapto-7-methylpurine ribonucleoside as co-substrate with phosphate. The effects of inhibitors on epimastigote activity was not due to the V-type PPase, and therefore, inorganic phosphate detection using purine nucleoside phosphorylase was also assayed in membrane preparations of epimastigotes by imidodiphosphate (IDP), KF, and AMDP. All assays were run in KCl/sucrose buffer (see Fig. 1 legend). Inhibitors were added to assays 4 min before the addition of pyrophosphate. Error bars indicate S.D. of mean values from 3–9 separate experiments. Activity was measured as proton uptake by acridine orange assay using filipin-permeabilized cells (average control activity ± S.D.) = 0.033 ± 0.006 absorbance units/min; average protein = 97 ± 36 μg/ml, n = 11. Note: The amount of cells (protein) used was not linearly related to activity but was constant for each experiment (a) or as phosphate production using washed membrane preparations (average ± S.D. of nine experiments). Activity was measured as proton uptake by acridine orange assay using filipin-permeabilized cells.

Fig. 1. V-H+-PPase activity is present in T. cruzi epimastigotes, measured using acridine orange uptake. Buffers: for traces a, d and e, 65 mM KCl, 125 mM sucrose, 2 mM MgSO₄, 10 mM Hepes, and 50 μM EGTA, adjusted to pH 7.2 with KOH; for trace b, 65 mM choline chloride replaced KCl, pH adjusted with Tris base; for trace c, 65 mM NaCl replaced KCl, pH adjusted with NaOH. Pyrophosphate (0.1 mM) was added at the indicated point (PP) in all traces except trace a, where 3 mM nigericin was added. Nigericin (NIG), also added at other points as indicated, collapsed pH gradients.

T. cruzi. Specific activities in membrane preparations of epimastigotes, amastigotes, and trypomastigotes were, respectively, 0.21 ± 0.11, 0.20 ± 0.04, and 0.09 ± 0.05 μmol of pyrophosphate consumed/min/mg of protein (means ± S.D. of results from 4, 3, and 3 separate experiments, respectively).

To further demonstrate that phosphate release assays and acridine orange assays were detecting the same PPase activity and to study the subcellular location of the enzyme, extracts of epimastigotes were separated on Percoll gradients (Fig. 5). PPhase, as assayed by both methods, was concentrated toward the bottom (dense end) of the gradient (fraction 1), with a smaller peak in the middle of the gradient (fraction 9). Markers for other compartments all peaked further up the gradient in the region of fractions 8–10, except for the cytosolic marker glucose-6-phosphate dehydrogenase, which, as expected, was fairly evenly spread through the gradient. The average amount of each organelle marker found in fraction 1 was under 5% of the total recovered (activity) of that marker, compared with 19 or 22% of pyrophosphatase activity, as measured by H⁺ uptake or phosphate release, respectively.

Examination of the densest fraction (fraction 1) from Percoll gradients by electron microscopy showed round, electron-dense organelles of varying size, up to 200 μm in diameter (Fig. 6c). These were similar to (if somewhat smaller than) the dense organelles found in whole epimastigotes (Fig. 6b and Fig. 2 in Ref. 14), which ranged in size up to 300 μm. These organelles were previously identified as acidocalcisomes (14). To confirm
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Fig. 3. V-H⁺-PPase activity in T. cruzi amastigotes (a) and trypomastigotes (b), assayed by acridine orange uptake. KCl/sucrose buffer was used, as in Fig. 1. In both cases, data were corrected for steadily rising background rates by subtraction of data points for control assays without pyrophosphate addition, using Microsoft Excel. Additions, as indicated: 0.1 mM pyrophosphate (PP), 20 μM AMDP, 3 μM nigericin (NIG). Data are representative of four (amastigote) or five (trypanastigote) experiments.

The location of the H⁻-PPase in T. cruzi was also investigated using polyclonal antibodies specific for the plant enzyme. Antibody 326 showed cross-reactivity with a band of 64 kDa present in T. cruzi (Fig. 7, Panel A, lane a). No background staining was observed when normal serum was used as a control (Fig. 7, Panel A, lane b). A 64-kDa band was also seen in fractions 1–2 from Percoll gradients, corresponding to the major peak of H⁻-PPase activity (Fig. 7, Panel B, cf. Fig. 5). This ran in the same position as the immunoreactive polypeptide from a red beet membrane preparation, which has a Mr of 64.5 kDa (32) (Fig. 7, Panel B, B). Strong reaction to the antibody was also seen in fractions 8–10, although here the bands were of somewhat lower molecular weight. Immunofluorescence indicated labeling of cytoplasmic structures generally found in the posterior and central regions of epimastigotes and cell surface, including the flagellum (Fig. 8, a). Intracellular amastigotes were intensely stained. Careful examination revealed labeling of the amastigote surface and a large number of small cytoplasmic structures mainly located toward the periphery of the cell (Fig. 8, b). No fluorescence was observed in control parasites incubated only in the presence of the secondary fluorescein-labeled goat anti-rabbit IgG or with pre-immune serum (not shown) or in the host cells (Fig. 8, b). Immuno electron microscopy using anti-V-H⁺-PPase antibody (antibody 324; similar results were obtained with antibody 326) confirmed labeling of the cell surface and intracellular vacuoles (Fig. 9). Gold particles (small arrowheads) associated with the membrane lining the cell body, the flagellum (F) and the flagellar pocket (FP) of amastigotes (Fig. 9A), trypomastigotes (Fig. 9B), and epimastigotes (Fig. 9C). The reaction was very strong with the amastigote surface membrane (Fig. 9A). Intracellular vacuoles were also labeled on the luminal face of membranes (Figs. 9; A–E). In some cases, these vacuoles contained electron-dense material occupying part of their interior (Fig. 9; B–D, big arrowheads), typical of acidocalcisomes (13). The acidocalcisome of T. cruzi was identified before biochemically by its insensitivity to the calcium ionophore ionomycin (which is only effective at neutral to alkaline pH; Ref. 36), unless organellar acidity was first neutralized, e.g. by use of nigericin (4). Calcium uptake into the acidocalcisome or into nonacidic compartments in permeobilized trypanosomes may therefore be studied by pretreatment with ionomycin or nigericin, respectively (4, 6). We found that pyrophosphate can drive calcium uptake into the acidocalcisome in digitonin-permeabili-
lized *T. cruzi* epimastigotes (Fig. 10, panel a, trace a), but not into the nonacidic compartment (panel b, trace a). Pyrophosphate-dependent calcium uptake was inhibited by AMDP (panel a, trace b). For comparison, we show ATP-driven Ca\(^{2+}\) uptake assays under the same conditions. H\(^+\)/Ca\(^{2+}\) exchanger assays (panel a, trace c; panel b, trace b) included 20 \(\mu\)M AMDP to block pyrophosphatase activity and 200 \(\mu\)M o-vanadate to inhibit Ca\(^{2+}\)-ATPase activity (4). The vacuolar H\(^+\)-ATPase inhibitor concanamycin A (37) inhibited Ca\(^{2+}\) uptake into the acidic (panel a, trace c) but not into the nonacidic compartment (panel b, trace b). We also ran reactions without preaddition of inhibitors (panel a, traces d and e; panel b, traces c and d). Under these conditions Ca\(^{2+}\)-ATPase activity predominates (4), as shown by the failure of AMDP to block activity, in contrast to total inhibition by 200 \(\mu\)M o-vanadate. We were unable to detect pyrophosphate-driven Ca\(^{2+}\) uptake into Percoll fractions, possibly because free [Ca\(^{2+}\)] was too high. In permeabilized epimastigotes, H\(^+\)-PPase activity was inhibited by the inclusion of 100 \(\mu\)M CaCl\(_2\) in the assay buffer by 25 ± 1% (average ± S.D. of three experiments).

**DISCUSSION**

After our initial discovery of pyrophosphate-induced organelle acidification in *T. cruzi* epimastigotes (Fig. 1), we compared the properties of this apparent V-H\(^+\)-PPase activity with those of the well studied V-H\(^+\)-PPase of plants. Three inhibitors of the plant enzyme were all effective against the trypanosomal activity (Fig. 2). KF inhibited proton uptake with an \(I_{50}\)
of 1–2 mM, and phosphate release with an I_{50} of 2–5 mM. The former value is the same as found for the V-H\textsuperscript{+}-PPase of oat roots (38), whereas mung bean hypocotyl V-H\textsuperscript{+}-PPase has an I_{50} for fluoride of 5 mM (39). The T. cruzi activity, measured by H\textsuperscript{+} uptake, was completely inhibited at this concentration, as is the V-H\textsuperscript{+}-PPase of Ricinus cotyledons (40). The I_{50} values for AMDP and imidodiphosphate for T. cruzi activity were approximately 5 \mu M (2 \mu M by phosphate release assay) and 50 \mu M, respectively (Fig. 2), similar to those found for red beet V-H\textsuperscript{+}-PPase (respectively 3.6 and 24 \mu M; Ref. 41).

In plants, V-H\textsuperscript{+}-PPase activity has been shown to be K\textsuperscript{+}-stimulated; substitution of K\textsuperscript{+} with Na\textsuperscript{+} in assay buffers leads to a 50–100% reduction in activity (30, 38, 40, 42), and this dependence differentiates V-H\textsuperscript{+}-PPase from known mitochondrial H\textsuperscript{+}-PPases that do not require K\textsuperscript{+} (16). Plant V-H\textsuperscript{+}-PPases are also inhibited by other cations such as choline (43) and various buffer cations (44). We found that the T. cruzi activity was weak in Na\textsuperscript{+} buffers compared with K\textsuperscript{+} buffers, and there was no activity in the presence of choline (Figs. 1 and 4). Use of buffers containing various proportions of K\textsuperscript{+}, Na\textsuperscript{+}, and sucrose (Fig. 4) indicated that Na\textsuperscript{+} was inhibitory where its concentration exceeded that of K\textsuperscript{+} and also indicated that proton-pumping (Fig. 4c) and phosphate-release (Fig. 4b) assays were detecting the same V-H\textsuperscript{+}-PPase-type activity. Maximal activity was seen with KCl concentrations as low as 16 mM (Buffer 5 in Fig. 4) but not where KCl was completely removed from the buffer (Buffer 3; this buffer contained only 3 mM K\textsuperscript{+} from its K-Hepes content). This diminishment in activity may have been the result of a lack of either K\textsuperscript{+} or Cl\textsuperscript{−} ions.

Having established the presence of a V-H\textsuperscript{+}-PPase-like activity in T. cruzi (including in the human-infective amastigote and trypomastigote forms, Fig. 3), we then wished to identify its subcellular location. In plants, V-H\textsuperscript{+}-PPases are present in the vacuole membrane (tonoplast) (20) and probably also in some plasma membranes (23, 24). Trypanosomatids do not have a large central vacuole like plant cells, but they do have lysosomes and store calcium in separate acidic organelles called acidocalcisomes (4–9, 13, 14). Subcellular fractionation of lysates of T. cruzi epimastigotes (Fig. 5) showed that most of the PPase activity was present in a dense fraction separate from the location of established organelle markers, including that for the lysosome. A smaller peak of activity was found near the middle of Percoll gradients. It is not possible to assign a location for this latter activity with certainty, because most organelles peaked around this point, but the greater prominence of the peak seen with phosphate release assays compared with H\textsuperscript{+} uptake assays suggests it may be present in plasma membrane fragments that are incompletely sealed and therefore leaky to protons.

Electron microscopy of the densest fraction from the Percoll gradient showed it contained electron-dense organelles similar to acidocalcisomes (Fig. 6, a and b). X-ray microanalysis of these organelles yielded spectra with peaks indicating phosphorus, magnesium, calcium, and zinc (Fig. 6c). Previous x-ray microanalysis of cryosections of epimastigotes showed that only the acidocalcisome has this characteristic elemental composition (14).

Use of antibodies to conserved regions of the plant V-H\textsuperscript{+}-PPase (Figs. 7–9) also indicated a relationship between the plant and trypanosomatid enzymes and allowed further definition of the subcellular location of the protein in T. cruzi. The antibody (326) reacted with a T. cruzi polypeptide of about the same molecular weight as that present in red beet (Fig. 7). The same size of reactive polypeptide was seen in the dense, PPase-containing, fractions of Percoll gradients (Fig. 7, Panel B), but only lower molecular weight bands were seen in the lighter Percoll fractions. These may represent degradation products of the complete polypeptide, which formed despite the presence of a mixture of protease inhibitors in the lysis buffer. Such bands were also often seen in analyses of whole-cell lysates (not shown) and have been observed in preparations from Zea mays (32).

Use of the anti-V-H\textsuperscript{+}-PPase antibodies in immunoelectron microscopy (Fig. 9) indicated both interior membrane and cell surface locations for the V-H\textsuperscript{+}-PPase in T. cruzi. The surface localization was especially prominent in amastigotes (Fig. 9A). In some of the internal organelles labeled, there was a partial content of electron-dense material. This is characteristic of acidocalcisomes, as seen in fixed sections of T. cruzi (13, 14).

Finally, we provide functional evidence for the presence of V-H\textsuperscript{+}-PPase activity in acidocalcisomes (Fig. 10). Pyrophos-
phosphate was capable of driving \( \text{Ca}^{2+} \) uptake in permeabilized epimastigotes in a manner that was dependent upon trans-membrane pH gradients but that did not require \( \text{Ca}^{2+} \)-ATPase activity and, therefore, presumably proceeded via a \( \text{Ca}^{2+}/\text{H}^{+} \) exchanger. We were not successful in detecting pyrophosphate-induced \( \text{Ca}^{2+} \) uptake in subcellular fractions either because of instability of the PPase or the putative \( \text{Ca}^{2+}/\text{H}^{+} \) exchanger or because assay medium-free \( \text{Ca}^{2+} \) was too high. T. cruzi V-H\(^+\)-PPase was found to be inhibited by \( \text{Ca}^{2+} \) at 100 \( \mu \text{M} \); the plant activity is inhibited to a similar extent (43, 45, 46).

We present here the first report of the presence and activity of a V-H\(^+\)-PPase in a lower eukaryote. The location of this enzyme in acidocalcisomes indicates that these organelles are different from any subcellular compartments previously characterized. Various unrelated unicellular eukaryotes have magnesium plus phosphorus plus calcium-containing organelles or inclusion bodies. These have not been studied biochemically except in terms of their elemental analysis (discussed in Ref. 14). Whether any of these organelles possess a membrane V-H\(^+\)-PPase like the T. cruzi acidocalcisome is an interesting topic deserving further investigation. In the case of the cellular slime mold Dictyostelium discoideum, dense granules similar to acidocalcisomes have been analyzed by x-ray microanalysis (47), and membrane-bound pyrophosphatase activity has been described, but this activity was not shown to be associated with proton-pumping and was not stimulated by K\(^+\) (48). Yeast, although it also has similar dense granules (49), definitely lacks a V-H\(^+\)-PPase (43).

Our results provide further evidence that the mechanisms of organellar acidification and calcium uptake in T. cruzi are quite different from those in mammalian cells (50) and therefore represent attractive targets for novel anti-trypanosomatid chemotherapy.

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