Characterization of a Vacular Pyrophosphatase in *Trypanosoma brucei* and Its Localization to Acidocalcisomes

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Inorganic pyrophosphatase promoted the acidification of an intracellular compartment in permeabilized procyclic trypanomastigotes of *Trypanosoma brucei*, as measured by acridine orange uptake. The proton gradient generated by pyrophosphatase was collapsed by addition of nigericin or NH$_4$Cl. Pyrophosphate-driven proton translocation was stimulated by potassium ions and inhibited by KF, by the pyrophosphate analogs imidodiphosphate and aminomethylendiphosphonate (AMDP), and by the thiol reagent p-hydroxymercurobenzoate at concentrations similar to those that inhibit the plant vacuolar H$^+$-pyrophosphatase (PPase). The proton translocation activity had a pH optimum around 7.5 and was partially inhibited by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (10 M) and unacted by bafilomycin A$_1$ (40 nM), concanamycin A (5 M), sodium o-vanadate (500 M), oligomycin (1 M), N-ethylmaleimide (100 M), and KNO$_3$. AMPD-sensitive pyrophosphate hydrolysis was detected in both procyclic and bloodstream trypomastigotes. Measurements of acridine orange uptake in permeabilized procyclic trypanomastigotes in the presence of different substrates and inhibitors suggested the presence of H$^+$-ATPase, H$^+$-PPase, and (ADP-dependent) H$^+$/Na$^+$ antiport activity in the same compartment. Separation of bloodstream and procyclic trypanomastigote extracts on Percoll gradients yielded fractions that contained H$^+$-PPase (both stages) and H$^+$/Na$^+$ exchanger (procyclics) activities but lacked markers for mitochondria, glycosomes, and lysosomes. The organelles in these fractions were identified by electron microscopy and X-ray microanalysis as acidocalcisomes (electron-dense vacuoles). These results provide further evidence for the unique nature of acidocalcisomes in comparison with other, previously described, organelles.

African trypanosomiasis (sleeping sickness), caused by the *Trypanosoma brucei* group of parasitic protozoa, occurs in 36 countries in sub-Saharan Africa, where it is a public health problem with a major impact on social and economic development. It has been estimated that about 300,000 new cases occur annually (41). In addition, *T. brucei* causes a similar disease in livestock, thereby making a large part of the African continent unsuitable for agricultural development. There is therefore considerable interest in developing novel chemotherapy, based on unique aspects of the structure and metabolism of these early-branching eukaryotes.

Organelle acidification is an essential function in eukaryotes. Acidification is required for lysosomal enzyme activity, for early-branching eukaryotes.

The known range of organisms possessing V-H$^+$-PPases was recently greatly expanded by our discovery of this activity in *Trypanosoma cruzi* (38). One of the key questions we addressed in that work was the location of the V-H$^+$-PPase, which had to be different from that in plants, as trypanosomatids lack a plant-like central vacuole. Our results showed that much of the activity was associated with a vesicle rich in calcium, phosphorus, and magnesium, which we had previously identified as the acidocalcisome (37). This organelle was first described as the inclusion vacuole in *Trypanosoma cyclops* (45). We initially defined the acidocalcisome in intact or permeabilized *T. brucei* (35, 42) functionally as an organelle that was acidic and that imported Ca$^{2+}$ by the action of a vanadate-sensitive Ca$^{2+}$-ATPase. Acidity appeared to be generated and sustained by a bafilomycin A$_1$-sensitive V-H$^+$-ATPase and was important for Ca$^{2+}$ retention, since alkalization induced by nigericin, NH$_4$Cl, or bafilomycin A$_1$ treatment was followed by Ca$^{2+}$ release (35, 42–44). Na$^+$ was shown to collapse ATP-induced proton gradients and to induce release of Ca$^{2+}$ (43, 44). The latter effect was not additive with the Ca$^{2+}$-releasing effects of nigericin, implying that an Na$^+/H^+$ antiport activity is also associated with acidocalcisomes (43, 44). This activity was inhibited by the antioxidant 3,5-dibutyl-4-hydroxytoluene but unacted by amiloride analogs (43, 44).

Subsequently, acidocalcisomes were detected in other trypanosomatids, i.e., *T. cruzi* (12, 37) and *Leishmania amazonensis* (23), and in the apicomplexan parasite *Toxoplasma gondii* (30). As X-ray microanalysis of unfixed cryosections of *T. cruzi* epimastigotes indicated the presence of calcium within the inclusion vacuoles, we inferred that these were the acidocalcisomes (37). An acidic interior for these organelles was suggested by an increase in their potassium content after treatment with the K$^+$/H$^+$ ionophore nigericin (37). This is sup-
ported by results from T. brucei, where use of weak bases, which can cause swelling of acidic vesicles, had this effect on inclusion vacuoles (7). Subsequently, as noted above, we found that the H\(^+\)-PPase is associated with the calcium- and phosphorus-containing organelles. These demonstrated a unique density, being much more dense even than the most dense organelle previously found in trypanosomatids, the glycosome (38).

In this work, we found that both bloodstream and procyclic trypomastigotes of T. brucei possess a V\(^+\)-PPase with features in common with the T. cruzi and plant activities and used this activity as a marker for the purification of acidicalcosomes. The purified organelles were shown to possess Na\(^+\)/H\(^+\) exchange activity and to generate a PP\(_i\)-dependent membrane potential. In permeabilized cells, it was confirmed that Na\(^+\) could diminish proton gradients established via H\(^+\)-ATPase activity. Na\(^+\) had the same effect on PP\(_i\)-generated proton gradients if ADP was present. Together, these data suggest the colocalization of H\(^+\)-ATPase and H\(^+\)-PPase activities and provide evidence that the isolated acidicalcosome is the same organelle as that identified initially on a functional basis. (This work was presented in partial fulfillment of the requirements for the Ph.D. thesis of C.O.R.)

MATERIALS AND METHODS

Culture methods. T. brucei procyclic forms (ILTar 1 or MITat 1.4 procyclics) were grown at 28°C in medium SDM-79 (5) supplemented with 10% heat-inactivated fetal calf serum. At 2 to 3 days after inoculation, the cells were collected by centrifugation, washed twice in 0.25 M sucrose, and resuspended in the same solution before use in experiments. T. brucei bloodstream forms (monomorphic strain 427 from clone MITat 1.4, otherwise known as variant 117) were isolated from infected mice or rats as described previously (10). The final concentration of cells was determined by using a Neubauer chamber. Protein (except for Percoll fractions [see below]) was measured by using the Bio-Rad Coomassie blue method.

Chemicals. Aprotinin, ADP, ATP, digitonin, dithiothreitol, Dulbecco’s phosphate-buffered saline, imidodiphosphate (IDP), leupeptin, N\(_\text{N}\)\,-dicyclohexyl-carbodiimide, nigericin, valinomycin, ADP, ATP, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, sodium orthovanadate, pepstatin, phenylmethylsulfonyl fluoride, carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone, carbonyl cyanide m-chlorophenylhydrazone, nigericin, and the standard phosphate solution were from Sigma Chemical Co., St. Louis, Mo. Baillonomycin A and concanamycin A were from Kamiya Biomedicals, Thousand Oaks, Calif. Acridine orange, Oxonol VI, 2-amino-6-mercaptop-7-methylbenzimidazole ribonucleotide (MESG), purine nucleoside phosphorylase (PNP), and the standard phosphate solution were from Molecular Probes, Eugene, Oreg. Aminomethylendiphenylphosphonate (AMDP) (50) was kindly provided by Philip Rea, University of Pennsylvania. All other reagents were analytical grade.

Proton pump activity. PP\(_i\)-driven proton transport was assayed by measuring changes in the absorbance of acridine orange (\(\Delta A_{480}\) – \(\Delta A_{530}\)) in an SLM-Aminco DW 2000 dual-wavelength spectrophotometer (38). Cells were incubated at 30°C in 2.5 ml of standard 65 mM KCl–125 mM sucrose buffer (pH 7.2) or alternate buffers (see Table 3), containing, in addition, 2 mM MgSO\(_4\), 10 mM HEPES, 50 \(\mu\)M EGTA, 30 mM MgSO\(_4\), 50 \(\mu\)M EGTA, 100 \(\mu\)M HEPES, 10 mM MgCl\(_2\), 50 \(\mu\)M EGTA, 1 mM phosphoenolpyruvate, 5 mM ATP, pyruvate kinase at 14 U/ml, lactate dehydrogenase at 20 U/ml, and 0.3 mM NADH, pH 7.6. Other enzymes were assayed as described previously (31, 38, 40) in 100-ml reaction mixtures. All assays, except for proton transport, were performed by using a PowerWave 340 spectronic reader (Bio-Tek Instruments) at 30°C. PP\(_i\) was assayed (by determining phosphate release) at the single wavelength of 360 nm. Protein was determined fluorometrically as described by Bohler et al. (2).

Electron microscopy. For observation of Percoll fraction 1, the fraction was washed in 0.25 M sucrose and a 5-\(\mu\)l sample was placed on a Formvar-coated copper or nickel grid, allowed to adsorb for 5 to 15 min at room temperature, blotted dry, and observed directly by electron microscopy (38). Whole trypomastigotes were applied to grids in a similar manner (37, 38). Energy-dispersive X-ray analysis was done at the Electron Microscopy Center, Southern Illinois University. Specimen grids were examined in a Hitachi H-7100FA transmission electron microscope at an accelerating voltage of 50 kV. Fine probe sizes were adjusted to cover the electron-dense granules (or a similar area of the background), and X-rays were collected for 100 s by utilizing a thin-window (Norvar) detector. Analysis was performed by using a Noran Voyager III analyzer with a standardless analysis identification program. Conventional electron microscopy (see Fig. 7) was done as previously described (37).

RESULTS

PP\(_i\) drives proton transport in permeabilized procyclic trypomastigotes. As reported previously (35, 42), when procyclic trypomastigotes were permeabilized with digitonin, some acridine orange was accumulated and retained in the absence of added energy sources (Fig. 1A). Once a steady state of acridine orange accumulation was reached, addition of 0.1 mM PP\(_i\), led
during permeabilization, prior to the addition of 0.1 mM PPi. The results are expressed as ΔΔ493-530/min × 10^3. Error bars indicate SEs of mean values of at least three separate experiments.

to further dye uptake (trace a). This indicated the establishment of a proton gradient (ΔpH) across the membrane of an intracellular compartment and increasing organelle acidity. This gradient collapsed completely after the addition of 1 μM nigericin or 20 mM NH₄Cl (data not shown). Replacement of the KC1 in the buffer with NaCl (trace c) or N-methylglucamine chloride (trace e) reduced the acidification rate. More KC1 in the buffer slightly increased the amount of acridine orange accumulated (trace b), while more NaCl further decreased the acidification rate (trace d compared with trace c). Use of a buffer containing equimolar (65 mM) concentrations of NaCl and KC1 resulted in slightly more acridine orange uptake (trace f) than in the presence of 130 mM KC1 (trace b). These results indicate that the PPase was stimulated by K⁺, similar to plant (33) and T. cruzi (38) V-H⁺-PPases. This dependence differentiates V-H⁺-PPases from known mitochondrial H⁺-PPases, which do not require K⁺ (25).

As occurs with plant vacuoles (27), the protonophore FCCP (Fig. 1B) or carbonyl cyanide m-chlorophenylhydrazone (data not shown) only partially dissipated PPase-induced proton gradients. This suggested that there was a positive (inside) membrane potential of sufficient magnitude to drive partial proton efflux in the presence of the ionophores but that further proton release was inhibited by the relative impermeability of the vacuole membrane(s) to countercurrent cation, or concurrent anion, flow (required to maintain charge neutrality). A PPase-dependent membrane potential was demonstrated in isolated acidocalcisomes (see below). Addition of valinomycin after FCCP completed the collapse of the proton gradient. Membranes become permeable to K⁺ in the presence of valinomycin (a K⁺ ionophore), and the action of the combination FCCP plus valinomycin resembled that of nigericin (a K⁺/H⁺ exchanger) (Fig. 1A). In agreement with this explanation, when the order of additions was reversed, valinomycin slightly in-

TABLE 1. Effects of ATPase inhibitors on PPase-driven proton transport in permeabilized procyclic trypanosomatid protists of T. cruzi

<table>
<thead>
<tr>
<th>Inhibitor (concn [μM])</th>
<th>PPase-dependent H⁺ transport (ΔΔ493-530/min × 10^3)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>35±2</td>
<td>100</td>
</tr>
<tr>
<td>Concancamycin A (0.005)</td>
<td>34±2</td>
<td>87</td>
</tr>
<tr>
<td>Baflomycin A (0.040)</td>
<td>38±3</td>
<td>97</td>
</tr>
<tr>
<td>Sodium o-vanadate (500)</td>
<td>45±0.5</td>
<td>114</td>
</tr>
<tr>
<td>DCCD (50)</td>
<td>35±0.7</td>
<td>90</td>
</tr>
<tr>
<td>NBD-Cl (10)</td>
<td>25±2.1</td>
<td>65</td>
</tr>
</tbody>
</table>

*Proton transport was assayed by using acridine orange, as described in Materials and Methods, in the standard buffer described in the legend to Fig. 1B. Inhibitors were added after 3 min of permeabilization, prior to the addition of 0.1 mM PP. The values are means ± SEs of three experiments.
of higher plant cells (33), we investigated whether they also colocalize in *T. brucei*. We approached this first by measuring acridine orange uptake in permeabilized cells in the presence of different substrates.

Addition of ATP after a steady state of acridine orange accumulation was reached led to further uptake of acridine orange (Fig. 4A, trace a). PPi addition further stimulated acridine orange uptake, at a rate faster than that obtained with ATP but to an extent lower than that generated by PPi alone (Fig. 4A, trace b). When the order of additions was reversed, PPi caused fast accumulation of acridine orange but addition of ATP did not lead to further accumulation of the dye (Fig. 4A, trace b). This was not due to a deficiency of acridine orange in the medium, as addition of more of the dye did not have any effect (data not shown). In both cases, acridine orange was immediately released by addition of NH4Cl. These results suggest that the ATP-driven and PPi-driven proton pumps are located in the same compartment.

Na+ ions added to digitonin-permeabilized *T. brucei* procyclic trypomastigotes were previously shown to reduce ATP-generated proton gradients, suggesting the operation of an Na+/H+ exchanger in these cells (43). Colocalization of the V-H+ -ATPase and the V-H+ -PPase in *T. brucei* procyclic trypomastigotes might therefore be demonstrated by comparing Na+-dependent proton efflux from (nonmitochondrial) compartments previously acidified with either ATP or PPi. *T. brucei* procyclics were permeabilized first in Na+-free medium containing mitochondrial inhibitors, and either ATP or PPi was added. In agreement with previous results (43, 44), partial release of acridine orange, accumulated after ATP addition, could be induced by the addition of 40 mM NaCl (Fig. 5A, trace a). However, 40 mM KCl was ineffective (data not shown), indicating that the effect was not due to changes in osmotic pressure and that Na+ was the active cation.

Initial experiments, in which acridine orange accumulation was driven by PPi instead of ATP, indicated that addition of either 40 mM NaCl (Fig. 5A, trace b), 40 mM KCl, or 40 mM choline chloride (data not shown) resulted not in acridine orange efflux but in slight acidification. However, we reasoned that ADP produced by hydrolysis of ATP could have stimulated the Na+/H+ exchanger. In agreement with this, addition of ADP after NaCl stimulated acridine orange release from the compartment previously acidified by either PPi (trace b) or ATP (trace a). ADP addition alone produced a slow acridine

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**TABLE 2. Effects of different potassium salts on PPi-dependent proton transport in permeabilized procyclic trypomastigotes of *T. brucei***

<table>
<thead>
<tr>
<th>Potassium salt</th>
<th>PPi-dependent H+ transport (ΔA493-530/min × 103)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (control)</td>
<td>39.2 ± 2.2</td>
<td>100</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>38.8 ± 2.7</td>
<td>99</td>
</tr>
<tr>
<td>K2SO4</td>
<td>48.7 ± 4.0</td>
<td>124</td>
</tr>
<tr>
<td>KNO3</td>
<td>35.4 ± 3.9</td>
<td>90</td>
</tr>
<tr>
<td>KHCO3</td>
<td>32.5 ± 3.9</td>
<td>90</td>
</tr>
</tbody>
</table>

*Proton transport was assayed by using acridine orange, as described in Materials and Methods, in the standard buffer described in the legend to Fig. 1B except that KCl was replaced with the other salts. The values are means ± SEs of three experiments.
FIG. 5. Effects of NaCl and ADP on ATP- and PP\textsubscript{i}-dependent proton uptake by permeabilized procyclic trypanosomes. Pro cyclic trypanosomes (0.1 mg of protein/ml) were incubated in a reaction mixture containing 65 mM KCl, 125 mM sucrose, 2 mM MgCl\textsubscript{2}, 250 \mu M EGTA, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM HEPES (pH 7.2), 16 \mu M digitonin, antimycin A at 1 \mu g/ml, and oligomycin at 2 \mu g/ml. In all of the panels, trace a represents proton uptake by 1 mM ATP and trace b represents proton uptake by 0.1 mM PP\textsubscript{i}. (A) NaCl (40 mM) was added before addition of 1 mM ADP. (B) ADP (1 mM) was added prior to the addition of 40 mM NaCl. (C) BHT (20 \mu M) was added prior to the addition of 1 mM ADP and 40 mM NaCl. Acridine orange (AO; 3 \mu M), ATP (1 mM), PP\textsubscript{i} (0.1 mM), ADP (1 mM), NaCl (40 mM), BHT (20 \mu M), and NH\textsubscript{4}Cl (20 mM) were added where indicated by the arrows. O.D., optical density.

orange release which was greatly stimulated by addition of NaCl (Fig. 5B). This proton release by ADP appeared to be due to a direct effect of the nucleotide on the Na\textsuperscript{+/H\textsuperscript{+}} exchanger and not to an inhibitory effect on the V-H\textsuperscript{+}-PPase, since the rapid release of protons was detected only when both ADP and Na\textsuperscript{+} were present. Addition of ADP before PP\textsubscript{i} did not cause any inhibition of proton transport (Fig. 4B, trace b), whereas it did inhibit ATP-dependent H\textsuperscript{+} uptake (Fig. 4C, trace b).

To confirm the mode of action of ADP, we tested its effect in the presence of 3,5-dibutyl-4-hydroxytoluene (BHT), an antioxidant that has been shown to inhibit the Na\textsuperscript{+/H\textsuperscript{+}} exchanger in T. brucei (44). Addition of BHT before ADP did interfere with the slow acridine orange release promoted by ADP (Fig. 5C). However, the fast release of protons caused by NaCl was suppressed, suggesting that ADP directly affected the Na\textsuperscript{+/H\textsuperscript{+}} exchanger.

Detection of PP\textsubscript{i} activity in both procyclic and bloodstream trypanomastigotes and effects of buffer composition. PP\textsubscript{i} was also assayed in membrane preparations of T. brucei by inorganic-phosphate detection by using PNP and MESG as cosubstrates with phosphate (48). In contrast to the results obtained from H\textsuperscript{+}-pumping assays of permeabilized preparations, this activity was detected in both procyclic and bloodstream trypanomastigotes (Table 3). Total PP\textsubscript{i} activity in membrane preparations of procyclic and bloodstream trypanomastigotes was inhibited by 20 \mu M AMDP by 75\% \pm 4\% and 58\% \pm 4\% (average \pm standard error [SE] of five and eight experiments, respectively). The lower inhibition by AMDP of PP\textsubscript{i} hydrolysis compared to PP\textsubscript{i}-dependent H\textsuperscript{+} transport (Fig. 2) could indicate the presence of other non-H\textsuperscript{+}-translocating or AMDP-sensitive PPases or nonspecific phosphatase activities in these preparations. The effects of monovalent cations on AMDP-inhibitable activity in procyclic trypanomastigotes were, in general, similar to those found in the acridine orange assay, and no activity was detectable if KCl in the medium was replaced with N-methylglucamide chloride. When membranes from bloodstream trypanomastigotes were used, the activity decreased as the KCl concentration increased (Table 3). Specific activities in membrane preparations of procyclic and bloodstream trypanomastigotes were, in general, similar to those found in the acridine orange assay, and no activity was detectable if KCl in the medium was replaced with NaCl (21). The V-H\textsuperscript{+}-PPase could be detected by proton uptake in fractions from both bloodstream and procyclic forms, as well as by phosphate release, and as assayed by both methods, was concentrated toward the bottom (dense end) of the gradient (fraction 1), with another peak in the middle of the gradient (fraction 9), as found with T. cruzi (38). Electron microscopic examination of fractions 8 to 11 showed that they contained mitochondria, membranes, and ghosts, some of which had trapped organelles, including electron-dense vacuoles (data not shown). Digitonin treatment reduced H\textsuperscript{+}-PPase activity in fractions from bloodstream forms by 75 to 50\% (in all portions of the gradient where activity was detectable) but had no consistent effect on H\textsuperscript{+}-PPase activity in fractions from procyclies, thus explaining why this proton-pumping activity could not be detected in digitonin-permeabilized bloodstream forms. Markers for other compartments and general ATPase activity (ATP hydrolysis) all peaked further up the gradient, in the region of fractions 6 to 11.

![Table 3. Effect of buffer composition on PPase activity in T. brucei procyclic and bloodstream trypanomastigotes](http://mcb.asm.org/)

<table>
<thead>
<tr>
<th>Buffer concn(s)</th>
<th>Pro cyclic trypanomastigotes</th>
<th>Bloodstream trypanomastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 mM KCl, 125 mM sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>130 mM KCl</td>
<td>114.7 \pm 16.3</td>
<td>69.3 \pm 12.6</td>
</tr>
<tr>
<td>65 mM NaCl, 125 mM sucrose</td>
<td>11.6 \pm 2.6</td>
<td>11.4 \pm 1.7</td>
</tr>
<tr>
<td>130 mM NaCl</td>
<td>28.3 \pm 2.1</td>
<td>37.4 \pm 6.9</td>
</tr>
<tr>
<td>65 mM NMG, 125 mM sucrose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>65 mM KCl, 65 mM NaCl</td>
<td>69.6 \pm 10.3</td>
<td>64.6 \pm 8.9</td>
</tr>
</tbody>
</table>

*PPase activity was assayed by the PNP-MESG method as described in Materials and Methods. Rates are relative percentages compared to that obtained with the 65 mM KCl–125 mM sucrose buffer. In all cases, AMDP (20 \mu M)-sensitive rates are indicated. All buffers contained, in addition, 2 mM MgSO\textsubscript{4}, 10 mM HEPES, and 50 mM EGTA and were adjusted to pH 7.2 with KOH, NaOH, or Tris base for the KCl, NaCl, and N-methylglucamide chloride (NMG) buffers, respectively. The values are means \pm SEs of three experiments. Control activities for procyclic and bloodstream trypanomastigotes were 0.116 \pm 0.048 and 0.038 \pm 0.006 \mu mol/min mg of protein, respectively.
pumping ATPase activity could be detected in any of the fractions (data not shown). The average amount of each organelle marker found in fraction 1 was under 5% of the total recovered activity of that marker, compared with 22 to 30% (H\textsuperscript{+} transport) or 16 to 18% (P\textsuperscript{i} release) of H\textsuperscript{+}-PPase activity, and since most of the recovered protein was in fractions 8 to 11 (Fig. 6), this method allowed significant purification of the H\textsuperscript{+}-PPase activity.

Examination of fraction 1 by transmission electron microscopy with conventional fixation, dehydration, and staining procedures showed round organelles of various sizes, up to 200 μm in diameter, containing an electron-dense region (Fig. 7B). These were similar to organelles found in sections of procyclic (Fig. 7A) or bloodstream trypomastigotes (not shown) and described previously as inclusion vacuoles (45) or polyphosphate bodies (7).

Transmission electron microscopy without fixation and staining of fraction 1 from procyclic (Fig. 8A) or bloodstream (Fig. 8D) trypomastigotes showed homogeneously electron-dense organelles (of various sizes, up to 200 μm in diameter, containing an electron-dense region (Fig. 7B)). These were similar to organelles found in sections of procyclic (Fig. 7A) or bloodstream trypomastigotes (not shown) and described previously as inclusion vacuoles (45) or polyphosphate bodies (7).

When they were submitted to the electron beam, the organelles showed changes in their internal structure leading to the appearance of a sponge-like structure (Fig. 8D, inset) as occurs with T. cruzi acidocalcisomes (24). To confirm this identification, X-ray microanalysis was performed on the fraction 1 preparations (Fig. 8B and E). The spectra shown were the ones that yielded the most counts in 100 s (out of 10 spectra obtained for each stage), but all other spectra taken from dense organelles were qualitatively similar: phosphorus counts were about twofold greater than calcium counts, which were about twofold greater than magnesium counts. Zinc was present in some spectra (not shown). We also performed X-ray microanalysis of acidocalcisomes in whole parasites as described in Materials and Methods. Because of the small size of these parasites, the electron-dense acidocalcisomes could be easily identified. Acidocalcisomes were submitted to X-rays after adjustment of the fine probe size to cover their area. Spectra obtained from acidocalcisomes in whole trypomastigotes (Fig. 9C) were similar to those taken from the isolated organelles (Fig. 8B and E), except that more sodium was detected in whole procyclic trypomastigotes and more magnesium was detected in whole bloodstream trypomastigotes. Peaks for calcium, phosphorus, magnesium, and zinc were not present in spectra taken from the fraction 1 sample back-
ground (Fig. 8C and F) or in the background from the whole-cell preparations (data not shown). Peaks for copper arise from the grid, and peaks for silicon arise from traces of Percoll in the specimens.

**Functional aspects of isolated acidocalcisomes—membrane potential and presence of Na\(^+\)/H\(^+\) antiport.** The generation of a PPI\(^-\)-dependent membrane potential (ΔΨ) was demonstrated in acidocalcisomes isolated from procycles by the shift in Oxonol VI absorbance (Fig. 10A, trace a). This membrane potential was dissipated by FCCP (dashed line), and its generation was prevented by AMDP (trace b).

Na\(^+\)/H\(^+\) antiport activity was detectable in isolated procyclic
FIG. 8. Electron microscopy and X-ray microanalysis of the dense fraction containing PPase activity. Panels A to C, procyclic trypomastigotes; D to F, bloodstream trypomastigotes. (A and D) Direct observation of Percoll fraction 1. Scale bar (for A and D), 2 μm. The inset in panel D shows a fourfold-higher magnification of the sponge-like structure of the acidocalcisomes after submission to the electron beam. (B and E) X-ray microanalysis spectra of dense organelles in fraction 1. (C and F) X-ray microanalysis spectra of the background to fraction 1 preparations.
acidocalcisomes, as shown in Fig. 10B. Following organelle acidification by PP\textsubscript{i}, addition of either NaCl or KCl at 40 mM produced equivalent further increases in acidification. This may be attributable to the addition of more chloride, which could act as a counterion to H\textsuperscript{+}, reduce the membrane potential, and allow the generation of a greater proton gradient. This is in agreement with the experiment whose results are shown in Fig. 1, traces a, b, and f, in which the presence of more chloride increased the extent of acidification after PP\textsubscript{i} treatment of permeabilized procyclics, and with similar results obtained with permeabilized procyclics (Fig. 5A, trace b). ADP addition after KCl treatment had no effect (Fig. 10B, dashed line). However, ADP addition after NaCl treatment caused a transient release of protons, indicating that the ADP-stimulated Na\textsuperscript{+}/H\textsuperscript{+} antiport was present in this fraction.

**DISCUSSION**

In this study, we have identified and characterized an H\textsuperscript{+}-translocating PPass in permeabilized procyclic trypomastigotes of *T. brucei*. Like ATP-dependent proton transport (35, 42–44), acridine orange uptake in the presence of PP\textsubscript{i} was reversed by the K\textsuperscript{+}/H\textsuperscript{+} exchanger, nigericin, or by alkalinization of the acidic compartment with NH\textsubscript{4}Cl.

Several observations suggest that the H\textsuperscript{+}-PPase activity is distinct from that of known H\textsuperscript{+}-ATPases. First, as occurs with plant vacuolar PPases (33), PP\textsubscript{i}-driven proton transport was blocked by KF and by the PP\textsubscript{i} analogs IDP and AMDP but was stimulated by potassium ions and was only partially reversed by protonophores. Second, the H\textsuperscript{+}-PPase was insensitive to bafilomycin A\textsubscript{1} and concanamycin A, two specific inhibitors of V-H\textsuperscript{+}-ATPases (13). Third, unlike P-type H\textsuperscript{+}-ATPases (15)
the H\(^{+}\)-PPase was insensitive to sodium o-vanadate. Fourth, like the plant vacuolar PPase, the proton-translocating activity required a permeant anion and was not inhibited by NO\(_3\)\(^{-}\), as are many V-H\(^{+}\)-ATPases (15).

The presence of a PPase activity in the cytolytic, lysosomal, and flagellar-pocket fractions of bloodstream trypomastigotes of \textit{T. brucei} was reported recently (29). However, these assays were performed with 0.1 M Tris/HCl buffer, pH 8.4, supplemented with 5 mM magnesium acetate (cytolytic fraction) or with 0.1 M sodium acetate, pH 3.9, without magnesium (lysosomal and flagellar-pocket fractions) and with 2 mM tetradsodium PP, as the substrate. The lack of potassium ions and the suboptimal pH conditions (cf. Fig. 1A and 3B and Table 3) meant that the H\(^{+}\)-PPase was probably not detected in these experiments, and the possibility that general phosphatases could account for the activities was not ruled out.

We investigated the relationship of V-H\(^{+}\)-PPase to V-H\(^{+}\)-ATPase activity in \textit{T. brucei}. In plants, these two activities are located in the same membrane—the vacuolar tonoplast (33). We made sequential additions of ATP and PP, to permeabilized procyclic trypomastigotes (Fig. 4) and measured the effects of ADP and NaCl on acridine orange release from acidic compartments (Fig. 5). The lack of additivity of acidification induced by ATP or PP (Fig. 4A) and the essentially identical effects of NaCl on acridine orange release following the addition of either potassium pump substrate (allowing for ADP inhibition of the H\(^{+}\)-ATPase; Fig. 4C and 5) suggest that both H\(^{+}\) pumps are present in the same vacuole. Although no proton-pumping ATPase activity could be detected in isolated acidocalcisomes, this could be attributed to the loss of essential subunits of the vacuolar H\(^{+}\)-ATPase during the fractionation procedure as occurs during the purification of Dictyostelium discoideum endosomes (32). Unfortunately, we could not confirm the localization of the V-H\(^{+}\)-ATPase by using antibodies because no specific antibodies against integral membrane subunits of the \textit{T. brucei} V-H\(^{+}\)-ATPase are available and none of the subunits have been purified. Heterologous antibodies used previously with \textit{T. cruzi} (24) were not useful against the \textit{T. brucei} enzyme (data not shown).

Proton-translocating PPase activity was undetectable in bloodstream trypomastigotes in permeabilization experiments. This was probably due to low activity compared to procytols plus digitonin rendering internal membranes of bloodstream forms leaky to protons. Previously, we were able to detect Ca\(^{2+}\) uptake driven by ATP in acidocalcisomes of digitonin-treated bloodstream forms, but only intermittently (35). Other data indicate that internal membranes, as well as plasma membranes, of trypanosomes can be permeabilized by digitonin, depending upon the concentration used and the incubation time (6). Digitonin permeabilizes membranes by a specific interaction with \(3\beta\)-hydroxysterols (11). Although procytols can synthesize sterols (8), bloodstream forms are dependent upon uptake of host lipoprotein, which has to be broken down by proteases in endosomes or lysosomes to release cholesterol (9). This likely results in a different distribution of sterols in cellular membranes in these forms. The greater susceptibility of bloodstream forms to digitonin was confirmed by treatment of membrane fractions isolated on Percoll gradients. Digitonin had no consistent effect on proton uptake by procytolic fractions but reduced the rate of proton uptake by fractions from bloodstream forms by an average of 40\%. Here, the finding of H\(^{+}\)-ATPase (but not H\(^{+}\)-PPase) activity in permeabilized bloodstream forms suggests that this pump is also located in vacuoles, which are more resistant to digitonin than are acidocalcisomes. Our results obtained with \textit{T. cruzi} (37, 38) indicate that acidocalcisomes are distinct from lysosomes, which presumably possess V-H\(^{+}\)-ATPase activity.

V-H\(^{+}\)-PPase activity was used as a marker for the purification and characterization, by X-ray microanalysis, of acidocalcisomes from both procyclic and bloodstream stages of \textit{T. brucei} (Fig. 6 to 8). These results, together with those of our previous work with other trypanosomatids (34, 37, 38), demonstrate that the acidocalcisomes correspond to the inclusion vacuoles described by Vickerman and Tetley (45) in \textit{T. cyclops} and to electron-dense vacuoles of other trypanosomatids (14, 20, 49). Such vacuoles were shown to contain large amounts of phosphorus, calcium, magnesium, sodium, and in some cases (14, 20, 45), zinc, as examined by X-ray microanalysis of intact (14, 20, 49) or cryosectioned (20) cells. These inclusion vacuoles, also termed polyphosphate bodies, are not accessible to horseradish peroxidase labeling via endocytosis, indicating that they do not belong to the endocytic apparatus (7). Similar results were obtained by using gold-transferrin to label the endosomes of \textit{T. cruzi} (37). In this study, we also measured ATPase activity in fractions of the Percoll gradients used to purify the acidocalcisomes and found that the acidocalcisome-containing fraction had negligible ATPase activity. However, this does not rule out the presence of the V-H\(^{+}\)-ATPase in this fraction, as no proton-pumping ATPase activity could be detected in any \textit{T. brucei} fraction, possibly due to the loss of some essential subunits during the fractionation procedure (as occurs during the purification of endosomes from \textit{D. discoideum} [32]), or else because of enzyme oxidation (17).

Examination by electron microscopy of fractions 8 to 11 showed that they contained other organelles and ghosts, some of which had trapped acidocalcisomes. This explains, at least in part, the detection of V-H\(^{+}\)-PPase activity in these fractions. The material loaded onto the Percoll gradient had been clarified following lysis by centrifugation at only 500 \(\times\) g for 10 min (38). This ensured the maximum yield of acidocalcisomes in the bottom fraction but also failed to remove all of the cell ghosts from the lysate. In \textit{T. cruzi}, the V-H\(^{+}\)-PPase has also been localized to the plasma membrane, as examined by immunofluorescence and immunoelectron microscopy using antibodies against peptide sequences present in the plant enzyme (38). However, these antibodies do not cross-react specifically with the \textit{T. brucei} enzyme (data not shown) and we were unable to confirm a plasma membrane localization.

We were able to link the acidocalcisome, as detected in permeabilized cells, with the purified organelle by assaying for ADP-stimulated Na\(^{+}\)/H\(^{+}\) exchange activity in the latter (Fig. 10B). ADP caused proton release in the presence of Na\(^{+}\), but not when only K\(^{+}\) was present, similar to the results obtained with permeabilized cells (Fig. 5). Figure 5A, trace b, shows that addition of NaCl slightly increased acidification in permeabilized cells, and only after addition of ADP was acridine orange released. Similar results were obtained with isolated acidocalcisomes (Fig. 10B). In the absence of ADP, KCl had the same effect as NaCl, which indicates that the increased acidification observed was due to the Cl\(^{-}\) added and not to the cation added. These results imply that the acidocalcisome detected under both circumstances is the same organelle. In addition, the isolated organelles possess the two characteristics that we used to define the acidocalcisomes (42): they are acidic, as demonstrated by the PP\(^{-}\)-dependent proton transport (Fig. 10), and they contain large amounts of calcium, as detected by X-ray microanalysis (Fig. 8).

For the first time, we have shown that purified acidocalcisomes are able to generate a PP\(^{-}\)-dependent membrane potential (Fig. 10A). In plants, V-H\(^{+}\)-PPases catalyze inward electrogenic H\(^{+}\) translocation from the cytosol to the vacuole.
lumen to establish an inside-acid pH difference (ΔpH) and an inside-positive electrical potential difference (ΔΨH+) which is employed to energize a wide range of secondary, ΔΨ- and/or ΔpH-coupled, transport processes (33).

In conclusion, our results further indicate that acidocalcisomes are novel organelles with no counterpart in mammalian cells. Analysis of their role in parasite survival and pathogenesis is likely to lead to the discovery of novel targets for anti-trypansomomatid chemotherapy.

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REFERENCES


