Two types of H^+-ATPase are involved in the acidification of internal compartments in *Trypanosoma cruzi*

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ATP-driven acidification of internal compartments of *Trypanosoma cruzi* epimastigotes was assayed spectrophotometrically with Acridine Orange and cells permeabilized with filipin. H^+-ATPase activity was not inhibited fully by either 500 nM concanamycin A or 500 μM orthovanadate, but a combination of 5 nM concanamycin A and 25 μM vanadate completely inhibited activity, suggesting the operation of separate V-type (concanamycin-sensitive) and P-type (vanadate-sensitive) H^+-ATPase activities in the permeabilized cells. This was supported by different kinetics of Acridine Orange uptake seen in the presence of the different inhibitors, and by different optimal protein (cell) concentrations for the two apparent activities. The use of different buffers further distinguished the ATPases. The V-H^+-ATPase activity was stimulated by K^+ and inhibited by a lack of anions or the replacement of Cl^- with gluconate. The P-type H^+-ATPase activity was not affected by a lack of Cl^- or K^+ but was substantially inhibited in a largely anion-free buffer. This inhibition could be annulled by the addition of the K^+ ionophore valinomycin, which probably acted via the establishment of a countercurrent efflux of K^+ from the compartment containing the P-type H^+-ATPase and the relief of the potential difference generated by the electrogenic proton pump. Valinomycin showed some stimulation of P-type activity in all buffers tested, but its effects on V-H^+-ATPase activity were at best transient except in a K^+-free buffer, which suggested that the V-H^+-ATPase was located in an organelle with relatively low [K^+] that was different from that which accommodated the P-type activity. On the basis of acidity and K^+ content, these organelles might correspond, in part at least, to the acidocalcisomes (V-H^+-ATPase activity) and the reservosomes (P-type activity) previously identified in these cells. Both activities could also be found in the human-infective forms of the parasite, amastigotes and trypomastigotes, but the P-type activity was relatively weak in these cells types, which is correlated with a lack of reservosomes in these forms.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas' disease, encounters a wide range of pH values in its life cycle. The gut of the reduviid insect vector could, by analogy with the Lepidoptera [1], be extremely alkaline, whereas, in the mammalian host, trypomastigotes of *T. cruzi* invade host cells via lysosomes [2]. Mechanisms to cope with this varied environmental pH and maintain cytosolic pH homoeostasis might involve the use of proton pumps (H^+-ATPases) on both the plasma membrane and internal membranes.

Four classes of H^+-ATPase transfer protons across cell membranes. The H^+/K^+-ATPase secretes protons across gastric mucosa in exchange for K^+ ions [3]. It is a single-polypeptide enzyme, of the E_1E_2 or P-type (as it has a phosphorylated intermediate) class of ATPase. Other H^+-ATPases are unidirectional, electrogenic H^+ pumps [4,5]. Two of these are multicomponent enzymes: the mitochondrial F_1F_0-ATPase, and the vacuolar (V)-ATPase. V-H^+-ATPases are ubiquitous in eukaryotes [6,7]. They function in the acidification of lysosomes and other acidic components of endocytic and secretory systems [4,5]. They are also present in the plasma membrane of some insect cells [1] and acid-secreting cells in vertebrates, such as macrophages [8], osteoclasts [9] and certain epithelial cells [1,10], and in the contractile vacuole membranes of amoebae of the slime mould *Dictyostelium* [11] and the ciliate *Paramecium* [12]. The final class of H^+-ATPase is also a P-type enzyme, present in the plasma membrane of fungi and plant cells [13], where it functions in the maintenance of cytosolic pH and the uptake of nutrients via proton symports [13,14]. Other cellular locations have not been reported for non-mutated cells, with the possible exception of vacuoles in lemons, where a vanadate-sensitive H^+-ATPase might work with a V-H^+-ATPase to establish the very low vacuolar pH [15]. P- and V-type H^+-ATPases can be distinguished by the use of inhibitors. Orthovanadate is a specific inhibitor of P-type H^+-ATPases, at least at concentrations less than 100 μM [4,5,7]. Bafilomycin A_1 and concanamycin A are both specific V-H^+-ATPase inhibitors at nanomolar concentrations [16].

The importance of (P-type) plasma membrane H^+-ATPase activity in the maintenance of intracellular pH in *T. cruzi* has been demonstrated [17]. Intracellular V-H^+-ATPase activity has been inferred in *T. cruzi* [18] and *T. brucei* [19] from the sensitivity of organelle acidification to the specific V-H^+-ATPase inhibitor bafilomycin A_1. In a related trypanosomatid, *Leishmania donovani*, plasma membrane H^+-ATPase activity has been found [20], and genes probably encoding P-type H^+-ATPases have been identified in this and other trypanosomatids [21].

A matter related to pH control in *T. cruzi* is the control of intracellular Ca^{2+} concentration. Ca^{2+} concentrations vary 20000-fold between mammalian cell cytoplasm and the extracellular space [22], and *T. cruzi* lives in both environments (as amastigotes and trypomastigotes respectively). It stores Ca^{2+} in an acidic compartment that we have named the acidocalcisome [18], which is at least partly analogous to the yeast vacuole. In recent work to identify the acidocalcisome morphologically, we used X-ray microanalysis to analyse unstained cryosections of *T.*

Abbreviations used: P-type, vanadate-sensitive; V-type, concanamycin-sensitive.

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cruzi epimastigotes. This study demonstrated the presence of two compartments that showed an increase in potassium content after treatment with the H^+/K^+ ionophore nigericin; they were therefore probably acidic. One class of acidic compartments (the acidocalcisome) had high levels of calcium, phosphorus, magnesium and zinc, whereas the other type of organelle was rich in iron. Both compartment types were, in untreated cells, deficient in potassium compared with the cytosol. This was especially true of the calcium-containing vacuoles [23].

In the present study we examined the control of pH in compartments of permeabilized *T. cruzi*, excluding the mitochondrion (the F_1F_0 H^+-ATPase was inhibited by oligomycin). We further characterized the V-H^+-ATPase activity and discovered a P-type H^+-ATPase functioning to acidify organelle(s) that are likely to be different from those containing the V-H^+-ATPase.

MATERIALS AND METHODS

Materials

Bafilomycin A_1 and concanamycin A (folimycin) were bought from Kamiya Biomedical (Thousand Oaks, CA, U.S.A.). Acridine Orange was from Molecular Probes, (Eugene, OR, U.S.A.). Other specialist chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Culture methods

T. cruzi, Y strain, epimastigotes, amastigotes and trypomastigotes were cultured as described before [18]. Cells were washed twice and resuspended to three times the wet weight in 0.25 M sucrose, then stored on ice before use in experiments.

H^+-ATPase assay

The ATP-driven acidification of internal compartments of *T. cruzi* was followed by monitoring the uptake of Acridine Orange at 493–530 nm in an SLM-Aminco DW2000 dual-wavelength spectrophotometer. A decrease in absorbance indicated increasing vesicular acidity [24]. The standard assay buffer contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes, 2 mM $MgSO_4$, 50 μM EGTA, 3 μM Acridine Orange, 2.5 μM oligomycin, and filipin and cells (protein) in amounts as noted in figure and table legends; the pH was adjusted with KOH. Filipin was added to stirred assay mixtures in the spectrophotometer 4 min before the start of experiments (5 min before the addition of 1 mM ATP). Assays were run at 30 °C for epimastigotes and 37 °C for amastigotes and trypomastigotes. Alternative assay buffers contained, in place of the 125 mM sucrose and 65 mM KCl in the standard buffer, either 250 mM sucrose ('low salt' buffer; this buffer contained approx. 3 mM K^+ from K-Hepes), 125 mM sucrose and 65 mM potassium gluconate ('no Cl^-' buffer), 125 mM sucrose and 65 mM NaCl ('no K^+' buffer; the pH in this case was adjusted with NaOH), or 130 mM KCl ('high K^+' buffer). Results shown in figures are representative of data from three or four experiments done with separate cell preparations, except where otherwise noted.

RESULTS

Presence of both V- and P-type H^+-ATPase activity in permeabilized *T. cruzi* epimastigotes

Previously, in permeabilized *T. cruzi*, we identified, by using Acridine Orange uptake assays, an H^+-ATPase that was inhibited by bafilomycin A_1, indicating that it was a V-type enzyme [18]. However, dependent on the amount of cells used in the assay (see below), bafilomycin did not inhibit all H^+-ATPase activity within these cells. It was found that neither the P-type inhibitor orthovanadate (Figure 1A, traces b and c) nor the V-type inhibitor concanamycin A (Figure 1B, traces b, c and d) alone substantially inhibited H^+-ATPase activity. Indeed, high concentrations of vanadate caused an initial stimulation of proton uptake (Figure 1A, trace c), possibly via the inhibition of other P-type cation ATPases pumping protons out of, rather than into, organelles, as we noted previously for Ca^{2+}-ATPase activity [18]. The apparent stimulation by 500 nM concanamycin A in Figure 1(B), trace d, was not reproducible. However, a combination of the inhibitors (Figure 1C, trace a) abolished activity, with minimal concentrations for total inhibition being approx. 5 nM concanamycin A and 25 μM vanadate (Figure 1C). In most experimental runs, the inhibitor (vanadate or concanamycin), which had not been added before the start of the run, was added at the point indicated in the figure and led to the release of Acridine Orange from vacuoles, indicating deacidification of the vesicles. The H^+/K^+ ionophore nigericin was added at the end of experiments to neutralize acidic compartments completely and release Acridine Orange. Bafilomycin A_1 (40 nM) could substitute for 5 nM concanamycin A in completely inhibiting H^+-ATPase activity in combination with 25 μM vanadate (results not shown). The likelihood that there are two separate H^+-ATPases in these cells was supported by different patterns of uptake: in the presence of vanadate (V-H^+-ATPase operating), Acridine Orange uptake reached a maximum (minimum absorbance) in 5–7 min (Figure 1A); in the presence of concanamycin A (P-type-H^+-ATPase operating), uptake proceeded for approx. 15 min before tailing off (Figure 1D). In subsequent experiments, 'V-type activity' refers to H^+-ATPase activity in the presence of 100 μM orthovanadate, and 'P-type activity' to H^+-ATPase activity in the presence of 5 nM concanamycin A.

The dependence of activity on the quantity of cells or protein added to the reaction cuvette differed for the two types of H^+-ATPase. P-type activity increased, up to a limit, with increasing addition of cells or protein (Figure 2A). In contrast, V-type H^+-ATPase activity was generally optimal at lower cell or protein concentrations (Figure 2B). The quantity of cells and concentration of filipin that gave the best activity were tested in each experiment (see figure and table legends; there was generally an approximate correlation between optimal cell and filipin concentrations, as shown in Figure 2).

The effects of three divalent cation salts on the H^+-ATPase activities were tested (Table 1). Ca^{2+}, Zn^{2+} and Fe^{2+} salts were tested at 100 μM, representing an excess over the 50 μM EGTA present in the assay buffer. These particular cations were chosen as they are stored in large amounts in vacuoles in *T. cruzi* epimastigotes [23,25]. All inhibited the acidification assay under both P-type and V-type conditions (the V-type activity being generally slightly less inhibited), which might be due to direct effects on the ATPases, or due to indirect effects, e.g. via the stimulation of proton release from organelles by H^+/M^{2+} exchangers.

Effect of buffer composition on ATP-mediated acidification rates

To examine further the differences between the P-type and V-type H^+-ATPases, and to ascertain whether they are in separate compartments, the effects of changes in the composition of the assay buffer were tested, along with the effects of the K^+ ionophore valinomycin (Figures 3 and 4 for V-type and P-type

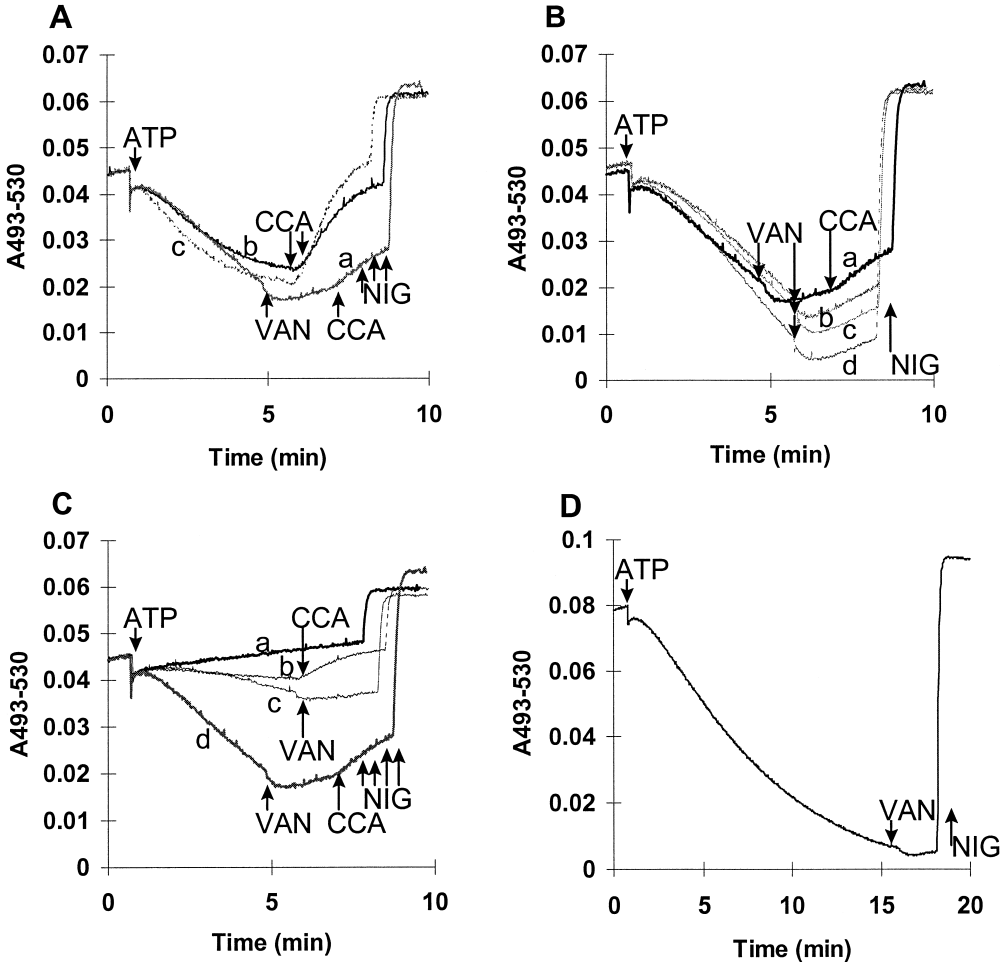

Figure 1 Both a V-H+-ATPase and P-type-H+-ATPase are operative in *T. cruzi* epimastigotes

Acridine Orange uptake assay conditions ($A_{493} - A_{530}$) were as described in the Materials and methods section, with the addition, 3 min before the start of each experimental run, of vanadate and/or concanamycin A. (**A**) Trace a, no additions; trace b, 25 μM vanadate; trace c, 500 μM vanadate. (**B**) Trace a, no additions [as in (**A**)]; trace b, 5 nM concanamycin A; trace c, 50 nM concanamycin A; trace d, 500 nM concanamycin A. (**C**) Trace a, 25 μM vanadate and 5 nM concanamycin A; trace b, 25 μM vanadate and 1 nM concanamycin A; trace c, 10 μM vanadate and 5 nM concanamycin A; trace d, no additions [as in (**A**)]. (**D**) Concanamycin A (5 nM) (note different time scale here). ATP (1 mM K-ATP) was added at the point shown. Further additions during the course of the experiment were made as indicated: CCA, 5 nM concanamycin A; VAN, 50 μM vanadate [15 μM in (**C**), trace c]; NIG, 4 μg/ml nigericin. Filipin at 10 μg/ml was used in this experiment. Protein (250 μg/ml) was used in each assay. Results are representative of four independent experiments. Mean $\pm$ S.D. acidification rates for these experiments were: with 25 μM vanadate (V-type activity), 0.0115 $\pm$ 0.0043 absorbance units/min; with 5 nM concanamycin A (P-type activity), 0.00576 $\pm$ 0.00145 absorbance units/min.

respectively; results are summarized in Table 2). Alteration of the anions or cations used in different buffers might have direct or indirect effects on organelle acidification. The ions might directly inhibit or stimulate the H+-ATPase, or they might have indirect effects because H+-ATPases are electrogenic [4]. That is, H+-ATPases, by the unidirectional pumping of protons, will establish a potential difference across the membrane of the compartment in which they are sited (which will inhibit further H^+ uptake) unless there is a balancing uptake of anions or expulsion of cations. Buffer ions might be involved in these balancing reactions directly or they might stimulate or inhibit ion pumps or channels involved in these reactions.

Acidification of organelles mediated by the two H+-ATPases was affected in different ways by different buffers. The V-H+-ATPase (Figure 3) was apparently stimulated by an increasing concentration of K^+; contrast traces b ('no K^+' buffer), d (standard buffer, 70 mM K^+) and e ('high K^+' buffer, 135 mM K^+). This activity was inhibited by the replacement of the Cl^- with gluconate ('no Cl^-' buffer) (Figure 3, trace a) (see also Table 2), which might have been due to either (1) the compartment in which the V-H+-ATPase was sited requiring the uptake of Cl^- ions to balance the uptake of protons, or (2) the V-H+-ATPase being directly stimulated by Cl^-, or inhibited by gluconate present in 'no Cl^-' buffer. Inhibition by gluconate is suggested by contrasting the effects of 'low salt' buffer, which not only lacked Cl^- (and gluconate) but also contained only 3 mM K^+ (compared with 70 mM K^+ in the 'no Cl^-' buffer) and yet yielded the same activity as the 'no Cl^-' buffer (Figure 3, traces c and a, and Table 2). Nevertheless there does seem to be some requirement for an anion in the buffer (either as a permeable counterion or to stimulate the V-H+-ATPase) for optimal proton pumping, as activity in 'low salt' buffer was less than in 'no K^+' buffer (Table 2; the only anion in 'low salt' buffer was the Hepes anion at approx. 3 mM).

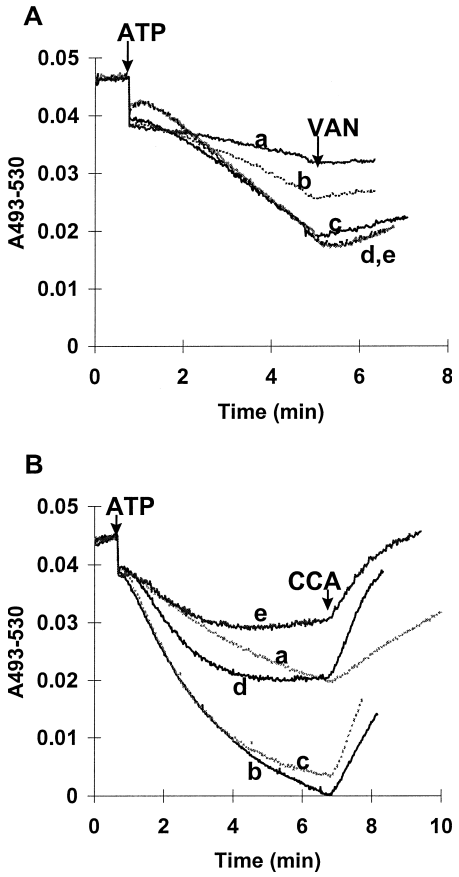

Figure 2 Effects of adding different quantities of *T. cruzi* epimastigotes (measured as protein) on apparent H^+-ATPase activity

Acridine Orange assays included 5 nM concanamycin A for P-type activity (**A**) or 100 μM vanadate for V-type activity (**B**). Protein and filipin concentrations (respectively in both panels): traces a, 30 and 1.5 μg/ml; traces b, 60 and 3 μg/ml; traces c, 110 and 6 μg/ml; traces d, 220 and 12 μg/ml; traces e, 340 and 18 μg/ml. Traces d and e are virtually superimposed in (**A**). ATP (1 mM), concanamycin A (CCA, 5 nM) and vanadate (VAN, 100 μM) were added at the indicated points. Results are representative of three independent experiments. Mean $\pm$ S.D. acidification rates for these experiments (with optimal protein concentration) were P-type activity, 0.00576 ± 0.00065 absorbance units/min; V-type activity, 0.0142 ± 0.0042 absorbance units/min.

Table 1 Effects of divalent cation salts on ATP-mediated uptake of Acridine Orange

Salts (100 μM) were added to the standard reaction mixture 3 min before the addition of 1 mM ATP. Maximum rates of Acridine Orange uptake (change in $A_{493} - A_{530}$ per min) subsequent to ATP addition were measured, and the percentage inhibition was calculated by comparison with control runs without salt addition. Values shown are means $\pm$ S.D. for three experiments. Average reaction rates (means $\pm$ S.D.) of controls were: V-H^+-ATPase, 0.0151 ± 0.0062 absorbance units/min; P-type-H^+-ATPase, 0.00790 ± 0.00410 absorbance units/min. The filipin concentration used was 5 μg/ml in all cases. Protein concentrations used were 70–80 μg/ml for V-type and 120–140 μg/ml for P-type assays.

	Inhibition of ATP-driven Acridine Orange uptake (%)	
Salt	V-H^+-ATPase	P-type-H^+-ATPase
$CaCl_2$	57 ± 18	79 ± 7
$ZnCl_2$	94 ± 9	100
$FeSO_4$	62 ± 7	87 ± 1

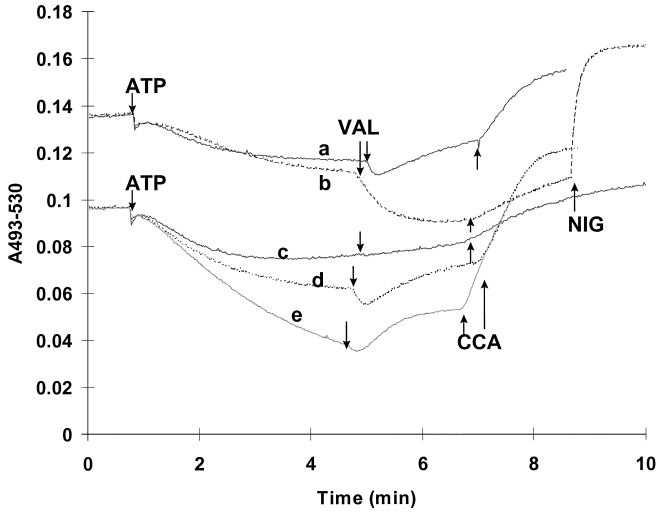

Figure 3 Effect of varied buffer composition and valinomycin addition on V-H^+-ATPase activity, as measured by uptake of Acridine Orange

Vanadate (100 μM) was added 3 min before the start of each assay. Buffers were as described in the Materials and methods section. Trace a 'no Cl^-' buffer; trace b, 'no K^+' buffer; trace c, 'low salt' buffer; trace d, 'standard' (KCl/sucrose) buffer; trace e, 'high K^+' buffer. ATP (1 mM K-ATP, except in 'no K^+' buffer, where Na-ATP was used) was added where indicated. Further additions during the course of runs were: VAL, 1 μM valinomycin; CCA, 5 nM concanamycin A; NIG, 4 μg/ml nigericin. Filipin at 10 μg/ml was used in this experiment. The cellular protein concentration in assays was 240 μg/ml. Data are offset for clarity.

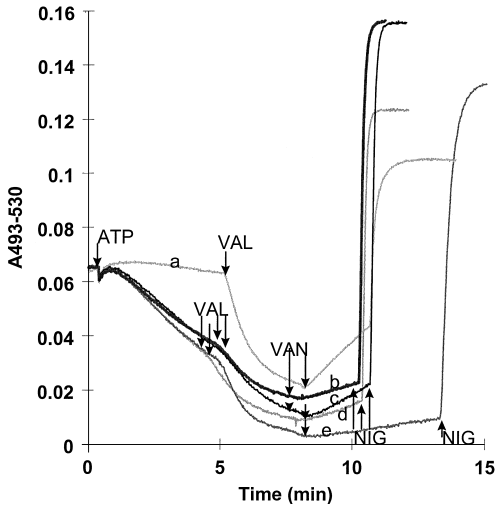

Figure 4 Effect of varied buffer composition and valinomycin addition on P-type-H^+-ATPase activity

Trace a, 'low salt' buffer; trace b, 'standard' (KCl/sucrose) buffer; trace c, 'high K^+' buffer; trace d, 'no Cl^-' buffer; trace e, 'no K^+' buffer. Conditions were as described in the legend to Figure 3, except that preparations were preincubated with 5 nM concanamycin A in place of vanadate, and 370 μg/ml protein was used. VAN indicates the addition of 100 μM vanadate during experimental runs.

The K^+ ionophore valinomycin would be expected to relieve any potential difference built up across the membrane of an H^+-ATPase-containing vesicle caused by the lack of counterions by

Table 2 Quantification of the effects of different buffer conditions on the rate of Acridine Orange uptake by H$^+$-ATPases

See the Materials and methods section for the composition of buffers. Rates of absorbance decrease ($A_{493} - A_{530}$) were taken at 2 min after addition of 1 mM ATP (K-ATP in all cases except 'no K$^+$', where Na-ATP was used) and the percentage inhibition or stimulation of activity compared with control rates was calculated (results are means ± S.D. for the number of experiments in parentheses). Average control reaction rates (means ± S.D.) were: V-H$^+$-ATPase ($n = 3$), 0.0105 ± 0.0027 absorbance units/min; P-type-H$^+$-ATPase ($n = 4$), 0.00820 ± 0.00122 absorbance units/min. The filipin concentration used was 10 μg/ml. Protein concentrations in assays were 240–420 μg/ml (V-type) and 290–410 μg/ml (P-type). Differences between effects of buffers on V-type and P-type activities were significant at $^*P < 0.05$, $^{**}P = 0.01$, $^{***}P < 0.01$ by t test.

Buffer	Inhibition (−) or stimulation (+) of rate (% of standard buffer)	
	V-H$^+$-ATPase	P-type-H$^+$-ATPase
'No Cl$^-$'	−66 ± 13 (3)	+4 ± 18 (3)***
'Low salt'	−59 ± 7 (3)	−83 ± 7 (4)**
'No K$^+$'	−24 ± 18 (3)	+13 ± 18 (4)*
'High K$^+$'	+72 ± 43 (3)	−1 ± 14 (3)

allowing a countercurrent efflux of K$^+$ ions from the vesicle. However, valinomycin addition here (Figure 3) showed only transient stimulation of proton uptake, except in the 'no K$^+$' buffer (trace b), which suggested that the acidifying compartment contained relatively little K$^+$ compared with the external buffer. Valinomycin was without effect in the 'low salt' buffer (Figure 3, trace c), which provides further evidence that there might be a direct requirement for anions for V-H$^+$-ATPase activity.

In contrast, with the P-type H$^+$-ATPase activity (Figure 4), Acridine Orange uptake was similar in all buffers except 'low salt' (trace a), suggesting that there was no direct or indirect requirement for Cl$^-$ but that an anion was required to balance proton uptake. This was supported by the results with valinomycin: addition of this greatly stimulated acidification in 'low salt' buffer, as, presumably, the membrane potential built up across the membrane of the acidifying compartment in the absence of anions was relieved by K$^+$ efflux. There was also some stimulation of acidification by valinomycin in the other buffers used, even 'high K$^+$' (Figure 4, trace c), suggesting that the compartment in which the P-type H$^+$-ATPase is located is relatively rich in K$^+$ (and is a different compartment from that possessing the V-H$^+$-ATPase).

Both H$^+$-ATPases are also present in trypomastigotes and amastigotes of *T. cruzi*

Both of the H$^+$-ATPases found in epimastigotes could also be detected in the human-infective stages of *T. cruzi*, trypomastigotes (Figure 5A) and, in some experiments, amastigotes (Figure 5B). In both cases, and especially in amastigotes, the P-type activity (concanamycin A-insensitive) seemed to be relatively weak, but it still required the addition of a combination of concanamycin A and vanadate to abolish total H$^+$-ATPase activity. The data for amastigotes (Figure 5B) represent the best results from seven experiments in which H$^+$-ATPase activity was detectable. It was not detectable in a further four experiments. This does not necessarily correspond to a lack of H$^+$-ATPase activity, as amastigotes are difficult to permeabilize successfully with either filipin or digitonin, which we used previously [18]. All amastigote results had to be corrected for a steadily rising baseline by running parallel experiments without the addition of ATP.

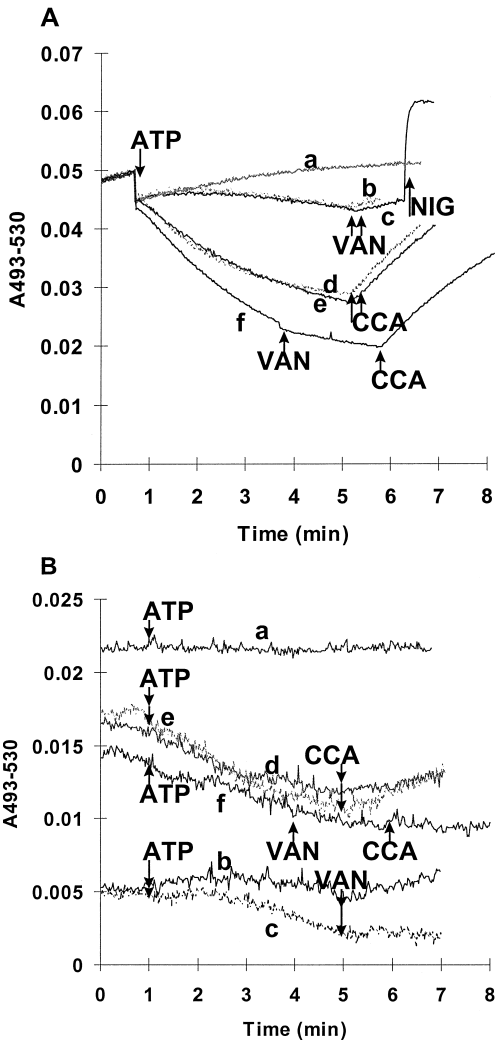

Figure 5 H$^+$-ATPase activity in trypomastigotes (A) and amastigotes (B) of *T. cruzi*

Assays were as in Figure 1. The filipin concentration was 5 μg/ml; the protein concentration was 130 μg/ml (**A**) or 150 μg/ml (**B**). Prior addition of inhibitors: trace a, 25 μM vanadate and 500 nM [400 nM in (**B**)] concanamycin A; trace b, 500 nM [400 nM in (**B**)] concanamycin A; trace c, 5 nM concanamycin A; trace d, 500 μM vanadate; trace e, 25 μM vanadate; trace f, none. Additions during assays were as in Figure 1. Data in (**B**) are offset for clarity and have been corrected for a steadily rising background absorbance by the subtraction of data from parallel experimental runs without the addition of ATP. Mean ± S.D. acidification rates for trypomastigotes were: with 25 μM vanadate (V-type activity), 0.00747 ± 0.00296 absorbance units/min; with 5 nM concanamycin A (P-type activity), 0.00175 ± 0.00090 absorbance units/min. See the text for comments on amastigote activity.

DISCUSSION

In the present paper we have shown that both P-type and V-type H$^+$-ATPases are detectable in permeabilized *T. cruzi*. The primary evidence is that neither concanamycin A nor vanadate is sufficient alone to inhibit ATP-driven H$^+$ transport into vacuoles of permeabilized cells (as measured by Acridine Orange spectrophotometry), but that a combination of these compounds prevents this activity completely (Figure 1). Concanamycin A, as far as it has been tested, is a universal inhibitor of V-type H$^+$-

ATPases at low nanomolar concentrations [16]. Here, the use of concanamycin A at 500 nM did not inhibit to a greater extent than at 5 nM (Figure 1B). Vanadate has been found to inhibit certain V-type isoforms, notably the osteoclast V-H^+-ATPase, but only at concentrations over 100 μM [7,9,26]. We found that 500 μM vanadate was no more inhibitory than 25 μM (Figure 1A), but that the latter concentration in combination with 5 nM concanamycin A was sufficient to inhibit the H^+-ATPase activity totally (Figure 1C). Both types of H^+-ATPase were also detected in the human-infective forms of *T. cruzi*, amastigotes and trypomastigotes (Figure 5), although for both forms the P-type activity was apparently poor, and amastigotes were difficult to permeabilize; baselines were unstable, requiring the subtraction of background rates without the addition of ATP (Figure 5B).

Additional evidence for the presence of separate P-type and V-type H^+-ATPases in permeabilized epimastigotes was provided by the different kinetics of Acridine Orange accumulation in the two systems. The V-type activity (in the presence of vanadate) tended to tail off within 5 min (Figure 1A) but the P-type activity showed more extended linearity (Figures 1B and 1D). Furthermore the two activities showed differing dependence on the number of epimastigotes (amount of protein) added to the reaction cuvette (Figure 2). The P-type activity (Figure 2A), as might be expected, increased with increasing protein up to a certain limit. In contrast, the V-type activity was inhibited by the addition of too much protein (Figure 2B). The reasons for this effect were not explored greatly, but it is not likely to be due to the presence of divalent cations, even though these did inhibit the H^+-ATPase (Table 1), as the assay buffer contained 50 μM EGTA. Various endogenous control mechanisms exist for V-H^+-ATPases, including dissociation of the soluble ATPase portion of the complex from the membrane-integral proton pumping section [27], inactivation by oxidation (cross-linking) of cysteine residues [27,28], stimulation by cAMP and protein kinase A [29] and stimulation via a protein kinase C pathway [30]. It is therefore quite conceivable that there are intracellular inhibitors of V-H^+-ATPase activity in *T. cruzi* epimastigotes (possibly released from internal compartments by the action of filipin) that are diluted out as fewer cells are used per assay. The use of filipin, and the similarly sterol-binding reagent digitonin, are the only methods we have found successful for the permeabilization of epimastigotes. (We have also tried α-toxin, streptolysin O and electroporation.)

The use of buffers of varied composition provides evidence for the location of the two types of H^+-ATPase in separate compartments (Figures 3 and 4, and Table 2). As noted in the Results section, interpretation of these results is complicated by possible direct or indirect effects of the buffer components on the H^+-ATPases. The apparent V-H^+-ATPase activity is stimulated by K^+ ions. This is either a direct effect on the ATPase or a stimulation of an accessory ion channel (e.g. an anion channel). Direct K^+ stimulation on V-H^+-ATPase activity has been described for the enzyme from tobacco hornworm midgut [31]. The effects of anion variation on the V-H^+-ATPase is somewhat ambiguous, but the poor activity in the 'low salt' buffer and the failure of valinomycin to stimulate activity in this buffer (Figure 3, trace c) implies a direct requirement for an anion for V-H^+-ATPase activity (because valinomycin, by allowing K^+ efflux from vacuoles, should by-pass any indirect anion requirement). In other organisms, Cl^- often serves as a counterion to balance V-H^+-ATPase activity (e.g. in rat liver multivesicular bodies [32], yeast vacuoles [33] or *Dictyostelium* acidosomes [34]) but has been found in oat vacuoles to stimulate the V-H^+-ATPase directly [35,36]. Weak valinomycin effects on the V-H^+-ATPase activity in all buffers except that lacking K^+ imply that the compartment possessing the V-H^+-ATPase does not contain much K^+, as valinomycin will cause K^+ to move only down a concentration gradient [37].

The results with alternative buffers with the P-type H^+-ATPase (Figure 4) are different from those obtained with V-type activity. They seem quite straightforward: replacement of K^+ with Na^+, or Cl^- with gluconate, has no effect on activity, but use of the 'no salt' buffer substantially inhibited activity. This inhibition was largely reversed by the addition of valinomycin, implying a requirement for the uptake of some sort of anion to balance H^+ influx. Valinomycin also stimulated the rate of proton pumping (Acridine Orange uptake) in the other buffers used, suggesting that the P-type H^+-ATPase is located in a compartment relatively rich in K^+.

In summary, the V-H^+-ATPase is in a compartment with unknown anion requirements for balancing H^+ uptake (confused by probable direct effects of anions on the V-H^+-ATPase) and containing relatively little K^+, whereas the P-type activity is in a compartment where an anion channel (or channels) of broad specificity balance(s) H^+ uptake, and one that contains more K^+.

The identity of these two compartments is an interesting question. In morphological work with X-ray microanalysis in combination with ionophore treatment, we have identified two apparently acidic compartments, one (the acidocalcisome) with large amounts of phosphorus, calcium, magnesium and zinc and a low potassium content, the other with more potassium (and a high iron content [23]). On the basis of potassium content, it is tempting to speculate that acidocalcisome corresponds to the V-H^+-ATPase-containing compartment identified here, whereas the iron-rich vacuole is the P-type H^+-ATPase-containing compartment. An acidic compartment that has been identified in epimastigotes of *T. cruzi* by electron microscopy of stained specimens is the 'reservosome' [38,39], which accumulates endocytosed proteins and LDL [38]. Interestingly, it has been found by immunocytochemistry not to contain a V-H^+-ATPase (M. Benchimol, W. de Souza, N. Vanderheyden, L. Zhong, H.-G. Lu, S. N. J. Moreno and R. Docampo, unpublished work) and is relatively scarce in trypomastigote forms of *T. cruzi* [40], which corresponds to the relatively poor P-type H^+-ATPase activity in the latter forms seen here (Figure 5A). Therefore the P-type H^+-ATPase/iron-rich/reservosome compartment might be the same organelle.

The finding here of an internal P-type H^+-ATPase, as well as the previous detection of such activity on the plasma membrane [17] of *T. cruzi* epimastigotes, suggests that there might be differentially targeted isoforms of the enzyme. Isoforms of P-type H^+-ATPases are found in plants [41,42], where they are expressed in a cell-specific or tissue-specific manner; in yeast, where expression of the two isoforms is controlled by external pH [43]; and, most pertinently, a tandem pair of probable H^+-ATPase genes has been found in *L. donovani*, where, at least at the mRNA level, they are differentially expressed between the amastigote and promastigote stages of the parasite [21].

Our discovery of the internal P-type activity is a novel finding that requires more investigation. P-type H^+-ATPases have the theoretical capacity to create larger potential differences and/or larger pH gradients across membranes than V-type pumps [44], and the two kinds of pump might work in tandem to produce a pH of 2 in the fruits of lemon [15,45], although in this case it was suggested that the vanadate-sensitive/non-bafilomycin A_1-sensitive activity might be composed of partial V-H^+-ATPase complexes [15]. Otherwise, intracellular P-type H^+-ATPases have been found before only in secretion-deficient yeast [46] or the even more unnatural situation of yeast expressing plant H^+-ATPase genes [47]. It remains to be clarified whether in *T. cruzi*

the internal P-type enzyme is active *in vivo* or is in a storage vesicle destined for the plasma membrane.

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