The role of a H^+-ATPase in the regulation of cytoplasmic pH in *Trypanosoma cruzi* epimastigotes

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Cytoplasmic pH (pH_i) regulation was studied in *Trypanosoma cruzi* epimastigotes using fluorescent probes. Steady-state pH_i was maintained even in the absence of extracellular Na^+ or K^+, but was significantly decreased in the absence of Cl^-. Acid-loaded epimastigotes regained normal pH_i by a process that was ATP-dependent and sensitive to *N*-ethylmaleimide, dicyclohexylcarbodi-imide and diethylstilboestrol, suggesting involvement of a H^+-pumping ATPase. Recovery from an acid load was independent of extracellular Na^+ or K^+ and insensitive to omeprazole, vanadate and low concentrations of bafilomycin A_1. Using the fluorescent probe bisoxonol to measure the membrane potential of intact cells, acid loading of epimastigotes was shown to result in a dicyclohexylcarbodi-imide-sensitive hyperpolarization, which suggests electrogenic pumping of protons across the plasma membrane. Addition of glucose, but not of 6-deoxyglucose, produced a transient cellular acidification of possible metabolic origin, and increased the rate of recovery from an acid load. Taken together, these results are consistent with an important role of a H^+-ATPase in the regulation of pH_i homoeostasis in *T. cruzi*.

INTRODUCTION

Since most intracellular enzymes are extremely sensitive to the concentration of $H^+(OH^-)$, eukaryotic cells need to regulate their cytoplasmic pH (pH_i) within a narrow range [1–6]. In most eukaryotic cells studied to date, pH_i regulation is accomplished by mechanisms involving ion-coupled extrusion of H^+ equivalents [2]. Four main systems that participate in the regulation of pH_i have been identified [1–6]. The Na^+/H^+ antiport promotes the net exchange of external Na^+ for internal H^+ and is driven by the inward Na^+ gradient [3]. Two other pH_i regulatory systems involve translocation of H^+ equivalents across the plasma membrane in the form of the weak base HCO_3^-, in exchange for Cl^- [4,5]. Cl^-/HCO_3^- exchange can be either cation-insensitive or dependent on the presence of Na^+. The latter functions as an acid-extrusion mechanism. In contrast, the Na^+-independent Cl^-/HCO_3^- exchanger is believed to protect the cell against alkalosis [4,5]. In addition to the three systems listed, a fourth pH_i regulatory mechanisms exists in some cells. ATP-dependent proton extrusion is known to occur in some normal and tumour cells [6–10], as well as in lower eukaryotes such as *Saccharomyces cerevisiae* [11] and *Pneumocystis carinii* [12]. In these cases, proton pumping is thought to be mediated by ATPases. These pumps occur in two forms, an electrogenic H^+-ATPase and an electroneutral H^+/K^+-ATPase. The plasma membrane H^+-ATPase of lower eukaryotes such as yeast [11] is of the P-type (sensitive to vanadate), while that present in mammalian cells is of the V- or vacuolar-type (insensitive to vanadate) [6–10]. On the other hand, the H^+/K^+-ATPase, which is of the P-type, is present mainly in the gastric mucosa and is involved in acid secretion [13].

Trypanosoma cruzi has a complex life cycle involving several morphological and functionally different stages that adapt to a variety of conditions imposed by the insect vector and mammalian host environments. The free-living epimastigote propagates naturally in the alimentary tract of reduviid insects, in which the environmental pH is acidic (5.0–6.0) [14]. The trypomastigotes, in contrast, live in the blood (pH 7.4), invade mammalian cells after recruitment of lysosomes [15], whose environment is highly acidic (pH 4.5–5.5), and then escape into the cytosol where the pH is neutral (pH 7.0–7.2) to multiply as amastigotes. At present, nothing is known about the regulation of pH homoeostasis in different stages of *T. cruzi*. Interestingly, it has been recently reported [16] that an extracellular acidic pH stimulates *T. cruzi* trypomastigote differentiation into amastigotes. In *Trypanosoma brucei* bloodstream trypomastigotes it has been suggested that an energy-dependent pump may be needed for maintaining their neutral pH_i (7.0–7.2) in an acidic environment, but not under physiological conditions [17]. On the other hand, the information available on the regulation of pH homoeostasis in the related *Leishmania* spp. is contradictory. While two reports indicate that promastigotes grown in culture maintain a pH_i which is mildly acidic (pH 6.7) [18,19], another report indicates that their pH_i is neutral (pH 7.2) [20]. Some studies suggested that an electrogenic P-type proton pump might provide a mechanism for extrusion of metabolically produced H^+ and an electronmotive force for the acquisition of nutrients [21,22]. In agreement with this hypothesis, isolated parasite plasma membrane had vanadate-sensitive ATPase activity [23–25], and treatment of cells with dicyclohexylcarbodi-imide (DCCD) resulted in mild acidification [19]. However, on the basis of its inhibition by inhibitors of the gastric K^+/H^+-ATPase, it was postulated that the H^+-ATPase previously reported in *L. donovani* [23–25] is in fact a K^+/H^+-ATPase [26].

We report here studies involving pH_i measurements of *T. cruzi* epimastigotes under acid loads, as a function of the ionic

Abbreviations used: pH_i, cytoplasmic pH; pH_e, extracellular pH; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein; DES, diethylstilboestrol; H_2-DIDS, 4,4′-di-isothiocyanatodihydrostilbene-2,2′-disulphonic acid; DCCD, dicyclohexylcarbodi-imide.

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composition of the medium, and in the presence of different inhibitors.

MATERIALS AND METHODS

Culture methods

T. cruzi epimastigotes (Y strain) were grown at 28 °C in a liquid medium consisting of brain/heart infusion (37 g/l), hemin chlorohydrate (20 mg/l, dissolved in 50 % triethanolamine) and 5 % heat-inactivated newborn-calf serum [27]. At 5 days after inoculation, cells were collected by centrifugation. The protein concentration was determined by the biuret assay [28] in the presence of 0.2 % deoxycholate.

Chemicals and solutions

Dulbecco's modified Eagle's medium, fetal and newborn calf sera, DCCD, diethylstilboestrol (DES), *N*-ethylmaleimide, *N*-methyl-D-glucamine and digitonin were purchased from Sigma Chemical Co. Bafilomycin A_1 was from Kamiya Biomedicals, Thousand Oaks, CA, U.S.A.. Omeprazole was a gift from Astra Hässle AB (Mölndal, Sweden) through the courtesy of Dr. K. Andersson. 2′,7′-bis-(2-Carboxyethyl)-5(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM), 4,4′-di-isothiocyanatodihydrostilbene-2,2′-disulphonic acid (H_2-DIDS) and bis-(1,3-diethylthiobarbituric)trimethineoxonol (bisoxonol) were from Molecular Probes. All other reagents were analytical grade.

The resuspension medium (standard buffer) contained 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM Hepes/Tris, pH 7.4, unless indicated. $-Na^+$-medium was prepared by iso-osmotic replacement of NaCl by choline chloride; $-K^+$-medium was prepared by iso-osmotic replacement of KCl by choline chloride; $-Cl^-$-medium contained 135 mM sodium gluconate, 5 mM potassium gluconate, 1 mM $MgSO_4$, 1 mM calcium gluconate and 10 mM Hepes/Tris, pH 7.4.

pH_i measurements

pH_i was measured fluorimetrically using BCECF. After harvesting the cells, they were washed twice at 2000 ***g*** for l0 min at 4 °C in buffer A, which contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 50 mM Hepes, pH 7.4. Cells were resuspended to a final density of $1 \times l0^9$ cells/ml in loading buffer, which consisted of buffer A plus 1.5 % sucrose, and 6 μM BCECF/AM. The suspensions were incubated for 30 min in a 30 °C water bath with mild agitation. Subsequently, the cells were washed twice with the ice-cold resuspension buffer indicated in the Figure legends to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in the same buffer and were kept in ice. For pH_i measurements, a 60 μl aliquot of the cell suspension was diluted into 3.0 ml of the appropriate buffer (final density $5 \times l0^7$ cells/ml) in a cuvette placed in a thermostated (30 °C) Hitachi F-2000 spectrofluorimeter. The fluorescence ratio, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm, were recorded and translated into pH values on the basis of the ratios obtained at various pHs [29]. Other experimental conditions and calibrations were as described before [29]. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program as described before [27].

Acid loading was accomplished by one of two methods: (1) preincubating 1.5×10^7 cells in 60 μl of buffer containing 40 mM NH_4Cl at 30 °C for 15 min (NH_4^+ prepulse technique [1]); or (2) adding 20 mM sodium or potassium propionate to 1.5×10^7 cells suspended in 3 ml of incubation medium [1].

ATP measurements

The cells were washed twice with standard buffer, and then resuspended in the same buffer A (5×10^7 cells in a total volume of 1 ml), and incubated for 5 min at 30 °C in the presence or absence of 10 μM DCCD or 10 μM DES. At the end of the incubation period the cells were centrifuged (2000 ***g***; 5 min), and 1.0 ml of ice-cold 1.0 M $HClO_4$ was added to each cell pellet containing 5×10^7 cells. After 30 min incubation on ice, the extracts were centrifuged (2000 ***g***; 5 min), and the supernatants were neutralized by the addition of 1.0 ml of 0.72 M KOH/0.6 M $KHCO_3$. The precipitated $KClO_4$ was removed by centrifugation (2000 ***g***; 5 min), and the supernatant was kept on ice until use. ATP was measured by the reaction between 3-phosphoglycerate and ATP catalysed by phosphoglycerate kinase, coupled with the oxidation of NADH by glyceraldehyde-3-phosphate dehydrogenase, as described by Adams [30].

Membrane potential ($\Delta\Psi$) measurements

Membrane potential was measured fluorimetrically using bisoxonol. Bisoxonol (0.2 μM) was added to 2×10^8 cells suspended in 3.0 ml of the indicated medium at 30 °C in a Hitachi F-2000 spectrofluorimeter with excitation at 540 nm and emission at 580 nm. The signal was allowed to equilibrate prior to any subsequent additions. Calibration was done with cells suspended in medium containing 140 mM *N*-methylglucamine chloride, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM Hepes/Tris, pH 7.4, and in the presence of the Na^+/K^+ ionophore gramicidin D (0.8 μM). After signal stabilization was achieved, increasing KCl concentrations were added to the medium, the fluorescent signals were recorded, and the membrane potential values were calculated as described before [31,32].

Statistics

All results are expressed as means $\pm$ S.D. for n different experiments. Statistical significance was determined by Student's t test. Significance was considered for $P < 0.05$.

RESULTS AND DISCUSSION

Steady-state pH_i

As has been reported before [27], in the nominal absence of bicarbonate and with an extracellular pH (pH_e) of 7.4 (standard buffer), the mean baseline pH_i of *T. cruzi* epimastigotes was 7.20 ± 0.05 ($n = 4$). This steady-state pH_i level was maintained in either K^+-free (7.19 ± 0.06; $n = 3$) or Na^+-free (7.25 ± 0.06; $n = 3$) medium (Figures 1B and 1C). However, when the cells were suspended in Cl^--free medium the basal pH_i immediately decreased by 0.3 unit to 6.90 ± 0.04 ($n = 3$) (Figure 1D). If these cells were centrifuged and resuspended in the standard buffer, they rapidly recovered towards neutrality and attained the pH_i (7.2) detected in cells suspended originally in that buffer (results not shown). This supports a role for chloride anions in supporting basal pH_i levels, as has been suggested for *Leishmania major* promastigotes [19,32]. The presence of a putative anion conductive pathway is required for dissipating the intracellular proton accumulation and the proton diffusion potential generated by the H^+ pump [32].

Characterization of HCO_3^--independent pH_i recovery

To further investigate the roles of Na^+, K^+ and Cl^- in pH regulation, epimastigotes loaded with BCECF were acidified to pH $\sim$ 6.0 using an NH_4 prepulse [6]. When suspended in K^+-free

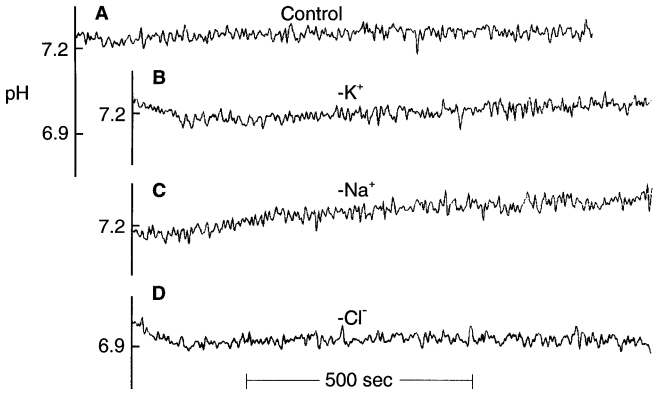

Figure 1 Steady-state pH_i of *T. cruzi* epimastigotes

The cells (1.5×10^7 cells/60 μl of buffer A) were centrifuged in an Eppendorf centrifuge at 2000 ***g*** for 1 min, and the pellet was resuspended in 3 ml of standard buffer (control; **A**), potassium-free buffer ($-K^+$; **B**), sodium-free buffer ($-Na^+$; **C**) or chloride-free buffer ($-Cl^-$; **D**). Other experimental conditions are described in the Materials and methods section. Traces are representative of three independent experiments conducted on separate cell preparations.

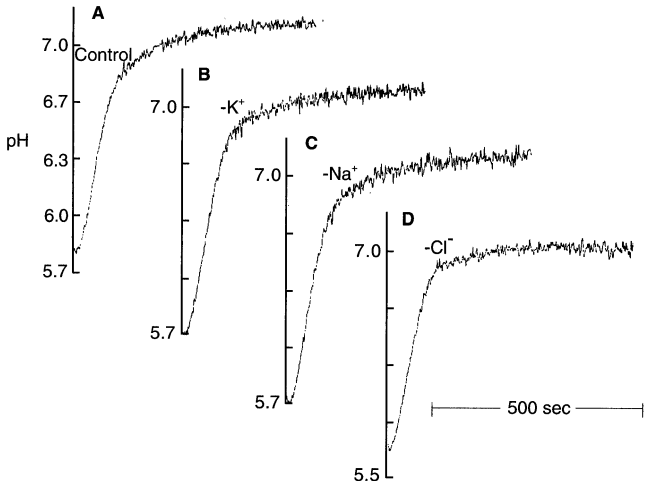

Figure 2 Effect of medium composition on the HCO_3^--independent pH_i recovery of acid-loaded epimastigotes

Acidification was obtained by preincubation of 1.5×10^7 cells with 40 mM NH_4Cl for 15 min and then resuspension in (**A**) standard buffer (control), (**B**) potassium-free buffer ($-K^+$), (**C**) sodium-free buffer ($-Na^+$) or (**D**) chloride-free buffer ($-Cl^-$). Other conditions were as in Figure 1. Traces are representative of three independent experiments conducted with separate cell preparations.

(Figure 2B) or Na^+-free (Figure 2C) medium, acid-loaded cells recovered their initial pH_i at the same rate and up to the same level (Table 1) as cells suspended in standard buffer (Figure 2A). However, cells suspended in Cl^--free medium recovered at the same rate but to a level that was lower than that of controls (Figure 2D and Table 1), confirming the importance of chloride anions in supporting basal pH_i levels in trypanosomatids [19,32]. Figure 3 shows that recovery from an alkaline load produced by addition of 10 mM NH_4Cl was also largely independent of the presence of Na^+ or K^+ in the buffer. Taken together, these results rule out the involvement of a Na^+/H^+ antiporter or a K^+/H^+-ATPase in the maintenance of basal pH_i or in the recovery of the cells from acid or alkaline loads. In agreement with these results, neither basal pH_i nor recovery from an acid or alkaline load was affected by the presence of up to 100 μM omeprazole (an inhibitor of gastric H^+/K^+-ATPase [13]) (results not shown). Although the Na^+/H^+ exchanger inhibitor amiloride [32] (0.5–1.0 mM) apparently decreased the pH_i of epimastigotes, this effect was found to be due to an artifact resulting from interference by amiloride (which because of its positive charge is transported into the cells [33]) with BCECF fluorescence (results not shown). Vanadate, a known inhibitor of P-type ATPases, had no effect on the recovery from an acid load up to a concentration of 0.5–1.0 mM (results not shown). However, vanadate is charged and, as has been postulated in other studies [12,34], is not a potent transport inhibitor in whole cells. The demonstration of intracellular effects of vanadate usually requires membrane permeabilization [35].

Table 1 Effect of different ions on HCO_3^--independent pH_i recovery

Results are means $\pm$ S.D. ($n = 3$) of the initial rate of pH_i recovery from acid loading to pH 6.0. Acid loading was attained by preincubation in 40 mM NH_4Cl for 15 min and resuspension in NH_4^+-free medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Hepes/Tris, pH 7.4). The final pH was measured after 10 min of resuspension in NH_4^+-free medium.

Ion absent	Rate of pH_i recovery (pH unit/min)	Final pH_i
None	0.85 ± 0.05	7.03 ± 0.15
Na^+	0.87 ± 0.03	7.11 ± 0.09
K^+	0.78 ± 0.06	7.11 ± 0.09
Cl^-	0.88 ± 0.08	6.75 ± 0.05

Effect of inhibitors on pH_i recovery from acid loads

Vieira et al. [19] have shown that addition of H_2-DIDS, an inhibitor of Cl^-/HCO_3^- transporters, to *L. major* promastigotes caused a substantial cytoplasmic acidification. However, addition of up to 0.5 mM H_2-DIDS to *T. cruzi* epimastigotes neither caused any cytoplasmic acidification (results not shown) nor affected the recovery of pH_i in acid-loaded cells (Figure 4). Since the recovery of pH_i from an acid load was observed in the nominal absence of HCO_3^-, was not inhibited by H_2-DIDS and was independent of the presence of Na^+ or K^+ in the extracellular medium, these results suggest that the main mechanism for maintenance of pH_i and for recovery from acid loads could involve an ATP-dependent H^+ extrusion mechanism.

Two classes of proton-translocating ATPases that could be involved in pH_i regulation have been described in the plasma membranes of eukaryotic cells: (1) the P-type, such as the H^+/K^+-ATPase present in the luminal membrane of gastric epithelial cells [13] (the presence of which is ruled out by the results shown above) and the H^+-ATPase present in the plasma membranes of fungi [11,12]; and (2) the vacuolar-type H^+-ATPase, present in macrophages [6], osteoclasts [7], hepatocytes [36], epithelial cells [9], secretory cells from the urinary tract [8], intercalating cells of the renal collecting duct [37] and several tumour cells [10]. Bafilomycin A_1 is a very specific inhibitor of vacuolar-type H^+-ATPases when used at low concentrations [38]. Neither basal pH_i nor recovery from an acid load was significantly affected by addition of up to 0.2 μM bafilomycin A_1, although higher concentrations (1–5 μM) caused decreases in the steady-state pH_i (results not shown), in the rate of pH_i recovery (Figure

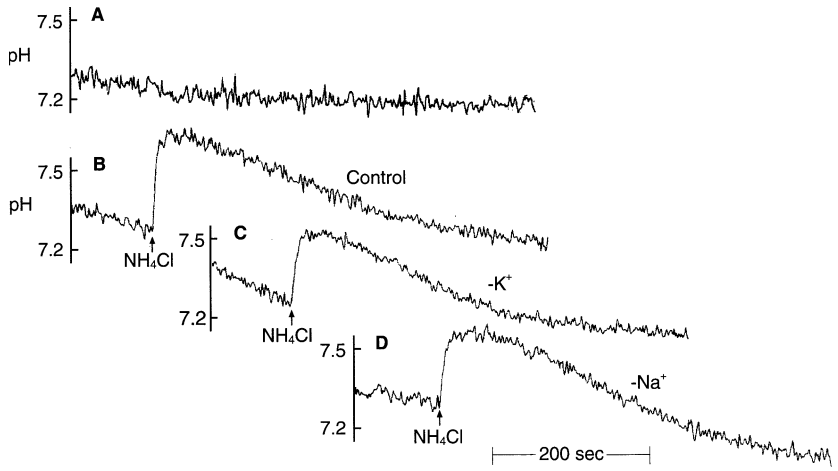

Figure 3 Effect of medium composition on pH_i recovery of epimastigotes following alkalinization by NH_4Cl

Alkalinization was obtained by the addition of 20 mM NH_4Cl where indicated. (**A**) No NH_4Cl added; (**B**) standard buffer (control); (**C**) potassium-free buffer ($-K^+$); (**D**) sodium-free buffer ($-Na^+$). Other conditions were as in Figure 1. Traces are representative of two independent experiments conducted with separate cell preparations.

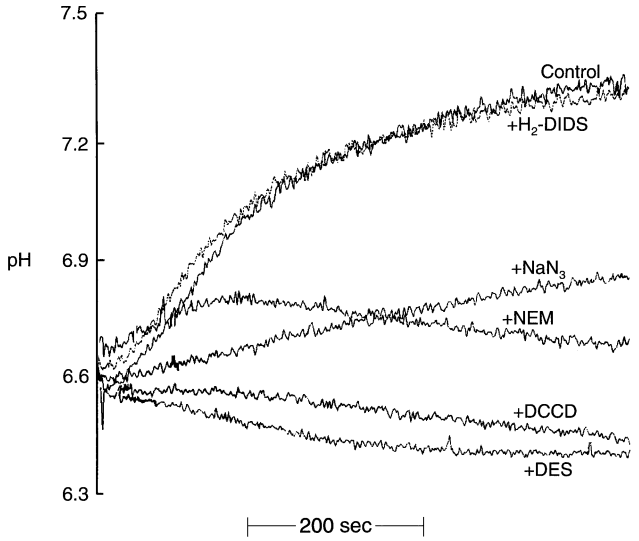

Figure 4 Effect of inhibitors on pH_i recovery in acid-loaded epimastigotes

The cells were treated as described in the legend to Figure 2, and resuspended in standard buffer containing one of the following inhibitors: 100 μM H_2-DIDS, 1 mM NaN_3, 100 μM *N*-ethylmaleimide (NEM), 100 μM DCCD or 25 μM DES. The control lacked inhibitors. Other conditions were as in Figure 2. Traces are representative of four independent experiments conducted with separate cell preparations.

5A) and in the final pH_i attained after an acid load (Table 2). These effects could be due to inhibition of the H^+-ATPase of acidic intracellular compartments [27], or to inhibition of a plasma membrane proton pump with low sensitivity to this inhibitor. In this regard, it has been indicated that bafilomycin A_1, at 1 μM, can decrease the steady-state pH_i of RN1a cells transfected with yeast plasma membrane H^+-ATPase [10].

Recovery was also inhibited in a dose-dependent manner by addition of the general H^+-ATPase inhibitors DCCD (Figure 5B) and *N*-ethylmaleimide (Figure 5D), or by the yeast H^+-ATPase inhibitor [11] DES (Figure 5C). In the presence of these inhibitors the cells could not recover their pH_i after an acid load as well as could the controls, and there was even further acidification in the case of high concentrations of DES (Figure 4), DCCD (Figure 4; Table 2) and NEM (Table 2). At concentrations which significantly decreased both the rate of pH_i recovery and the final pH_i attained after an acid load, DES (10 μM) and DCCD (10 μM) did not significantly decrease cellular ATP levels, thus indicating that their effects were not through ATP depletion but through inhibition of the proton pump. The ATP levels remained essentially constant in standard buffer (1.33 ± 0.20 mM), and fell insignificantly in the presence of either DCCD (1.29 ± 0.10 mM) or DES (1.30 ± 0.30 mM). In contrast, addition of 1 mM NaN_3, an inhibitor of the mitochondrial ATP synthase known to decrease ATP levels in *T. cruzi* when used at that concentration [39], greatly decreased recovery (Figure 4), thus indicating the ATP requirement of this process.

Membrane potential ($\Delta\Psi$) changes associated with pH_i recovery

If an electrogenic H^+-ATPase is responsible for H^+ extrusion in epimastigotes, pH_i recovery is predicted to be accompanied by an alteration in $\Delta\Psi$. To test this hypothesis, the $\Delta\Psi$-sensitive fluorescent probe bisoxonol was used. Since this drug requires an initial period of equilibration, it was not possible to use the NH_4^+ prepulse method of acid loading for these experiments. Instead, epimastigotes were acid-loaded by addition of propionic acid, which readily enters the cytoplasm but is excluded from acidic compartments [6]. Addition of 20 mM sodium propionate to epimastigotes suspended in standard buffer at pH_e 7.4 resulted in a 0.6 unit decrease in pH_i, as illustrated in Figure 6(A). As shown in Figure 7 (upper panel), acid loading by a propionate pulse at pH_e 7.4 was followed by hyperpolarization from -110 ± 10 to -122 ± 16 mV ($n = 3$), as measured with bisoxonol. To test the possibility that the acidification-induced hyperpolarization represented electrogenic extrusion of H^+ across the plasma membrane [6], the effect of the H^+-ATPase inhibitor DCCD on the $\Delta\Psi$ response to acid loading was assessed. DCCD (50 μM) effectively inhibited the pH_i recovery from acid loading by the propionate pulse method (results not shown), and also completely inhibited the hyperpolarization which followed acid loading in control cells (Figure 7, lower panel). These results indicate that prevention

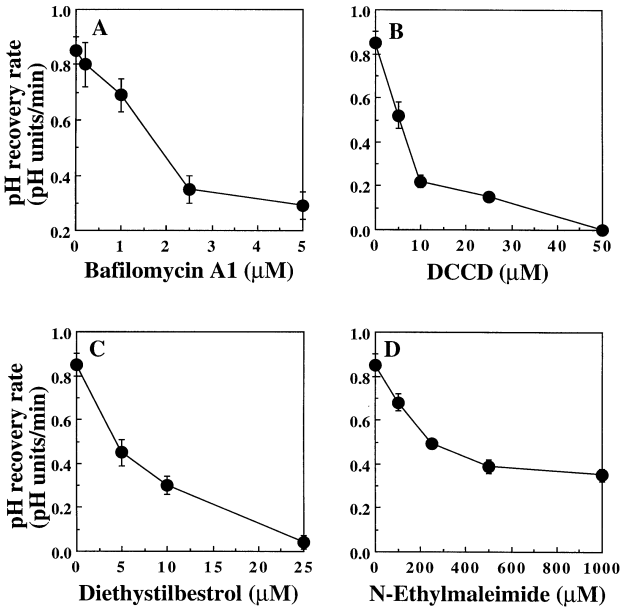

Figure 5 Effects of different concentrations of inhibitors on pH_i recovery in acid-loaded epimastigotes

The cells were treated as described in the legend to Figure 2, and resuspended in standard buffer containing different concentrations of inhibitors: (**A**) bafilomycin A_1, (**B**) DCCD, (**C**) DES, (**D**) *N*-ethylmaleimide.

Table 2 Effects of different inhibitors on the final pH_i attained after recovery from an acid load

Results are means ± S.D. ($n = 3$). The cells were treated as described in the legend to Figure 5, and the final pH_i attained 10 min after the initiation of recovery from an acid load in the absence (none) or presence of different concentrations of inhibitors was measured.

Inhibitor (μM)	Final pH_i
None	7.03 ± 0.15
DES	
5	6.84 ± 0.09
10	6.76 ± 0.09
25	6.63 ± 0.06
DCCD	
5	6.73 ± 0.07
10	6.67 ± 0.05
25	6.59 ± 0.05
50	6.46 ± 0.05
N-Ethylmaleimide	
100	6.82 ± 0.07
250	6.62 ± 0.09
500	6.42 ± 0.08
Bafilomycin A_1	
1	6.95 ± 0.03
2.5	6.77 ± 0.04
5.0	6.64 ± 0.05

of H^+ extrusion via the H^+-ATPase eliminated the hyperpolarization which normally follows acid loading. Taken together, these results support the concept that pH_i recovery in epimastigotes in mediated by an electrogenic H^+-ATPase located in the plasma membrane.

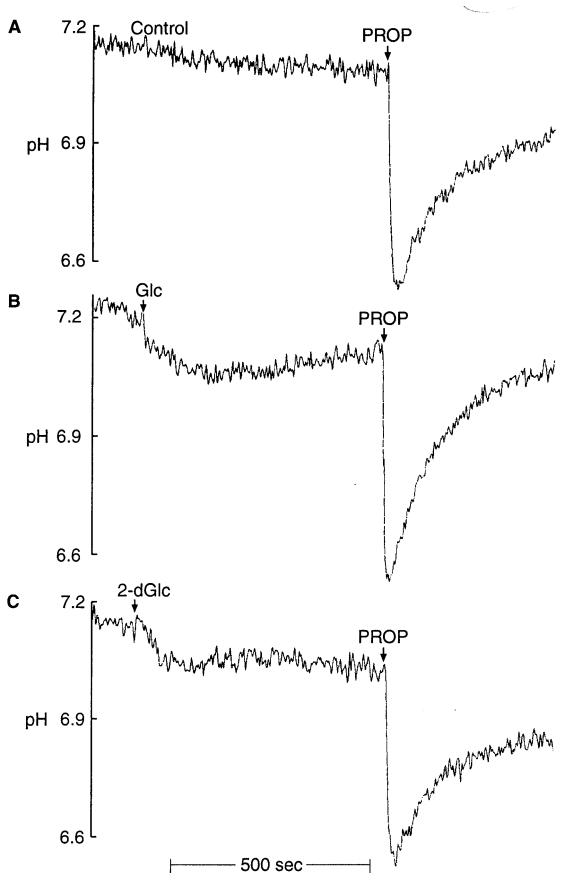

Figure 6 Effect of glucose on the steady-state pH_i of epimastigotes and on their recovery from an acid load

The cells were suspended in standard buffer. Where indicated, 10 mM glucose (Glc), 10 mM 2-deoxyglucose (2-dGlc) or 20 mM sodium propionate (PROP) was added. Other conditions were as in Figure 1. Traces are representative of eight independent experiments conducted with separate cell preparations.

Effect of glucose on steady-state pH_i and recovery from acid loads

Addition of glucose (Figure 6B) or 2-deoxyglucose (Figure 6C) resulted in a significant acidification of the cells. The steady-state pH_i at 250 s after sugar addition was 6.96 ± 0.06 ($n = 24$) in the case of glucose, and 6.98 ± 0.07 ($n = 8$) in the case of 2-deoxyglucose. This acidification could be due to H^+ co-transport, as has been suggested to occur in *L. donovani* promastigotes [9], or to a metabolic effect similar to that found with other cells [40]. The similarity of the initial drop in pH after addition of glucose and 2-deoxyglucose supports H^+–sugar co-transport. However, it is unlikely that any co-transport could explain changes in steady-state pH. Therefore experiments were carried out using 6-deoxyglucose, a glucose derivative that, in contrast to 2-deoxyglucose, cannot be phosphorylated in the 6-position. Addition of 6-deoxyglucose did not affect the steady-state pH_i (results not shown). These results rule out a H^+ co-transport mechanism as the reason for acidification and indicate that formation of glucose 6-phosphate is possibly responsible for this effect, as is the case in yeast [40]. In yeast cells the addition of glucose greatly stimulates proton efflux mediated by the H^+-ATPase [11]. Similar results were observed with *T. cruzi* epimastigotes. After acidi-

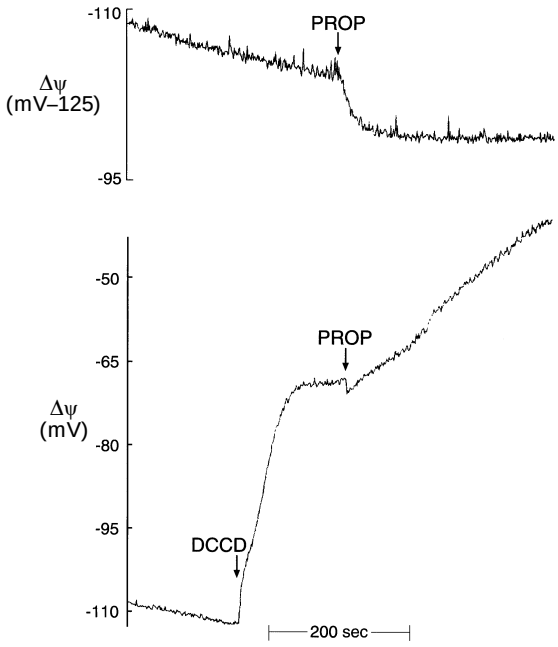

Figure 7 Membrane potential ($\Delta\Psi$) of epimastigotes

The cells were resuspended in standard buffer. Where indicated, 20 mM sodium propionate (PROP) or 100 μM DCCD was added. Other conditions are described in the Materials and methods section. Traces are representative of three independent experiments conducted with separate cell preparations.

fication of the cells with 20 mM propionic acid, the pH_i attained a value of 5.25 ± 0.25 ($n = 12$). The rate of recovery was 0.68 ± 0.18 pH unit/min ($n = 5$; see Figure 6A) in the absence of glucose. This rate was greatly increased by glucose (1.90 ± 0.25 pH units/min; $n = 4$; see Figure 6B) but not by 2-deoxyglucose (0.80 ± 0.22 pH unit/min; $n = 3$; see Figure 6C). Whereas recovery was practically complete at 500 s after addition of propionic acid in the presence of glucose ($pH = 7.1 \pm 0.14$; $n = 4$), it was not attained in the absence of glucose ($pH = 6.5 \pm 0.19$; $n = 5$) or in the presence of 2-deoxyglucose ($pH = 6.2 \pm 0.2$; $n = 3$). This indicates a very specific effect of glucose on proton extrusion, and favours the hypothesis that a H^+-ATPase similar to that in yeast could be responsible for pH_i regulation in trypanosomatids.

In this regard, we have reported previously [41] that labelling of plasma membrane vesicles of *T. cruzi* epimastigotes with [γ-^{32}P]ATP resulted in the labelling of a prominent band of about 100 kDa, which was attributed to a H^+-ATPase activity known to be present in the plasma membrane [42]. When hydroxylamine was added to hydrolyse phosphoanhydrous bonds, the 100 kDa band disappeared from the autoradiograms [41]. These results suggested that the phosphorylation observed in this band corresponded to an acyl phosphate, thus supporting the notion that it corresponded to an H^+-ATPase. These results also indicated that this enzyme belongs to the P-type family of ion pumps [43]. Furthermore, a 105 kDa ATPase which forms a transient phosphorylated intermediate has also been detected in *L. donovani* promastigotes [24,25], and two genes from *L. donovani* that encode a similar P-type ATPase protein of 107 kDa have been cloned and sequenced [44,45].

In summary, *T. cruzi* epimastigotes recover from acid loads by means of a Na^+-independent process. The ATP dependence and pharmacological profile of this mechanism suggest the operation of H^+ pumps. It is conceivable that this pH_i regulatory mechanism was developed by epimastigotes to ensure their survival in the acidic milieu of the host intestine. The low pH_e that prevails in this environment would tend to inhibit Na^+/H^+ exchange by competition between Na^+ and H^+ at the external transport site, and would decrease the concentration of HCO_3^-. Under these conditions, proton pumping might be essential for the maintenance of pH_i. Since an acidic environment is also encountered by the trypomastigotes when they invade the mammalian cells, a similar mechanism should be operative in those forms, and this would constitute an important target for the development of new trypanocidal compounds.

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