A pyruvate–proton symport and an H⁺-ATPase regulate the intracellular pH of *Trypanosoma brucei* at different stages of its life cycle

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Regulation of intracellular pH (pH_i) and H⁺ efflux were investigated in *Trypanosoma brucei* bloodstream and procyclic trypomastigotes using the fluorescent dyes 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester and free BCECF respectively. pH_i in bloodstream and procyclic trypomastigotes was 7.47 ± 0.06 and 7.53 ± 0.07 respectively. Differences in the mechanisms for the regulation of pH_i were noted between bloodstream and procyclic forms. Procyclic trypomastigotes maintained their pH_i at neutral over a wide range of external pH values from 6 to 8, and in the absence of K⁺ or Na⁺. The H⁺-ATPase inhibitors *N,N'*-dicyclohexylcarbodi-imide (DCCD), diethylstilboestrol and *N*-ethylmaleimide substantially decreased the steady-state pH_i and inhibited its recovery from acidification. The rate of H⁺ efflux in these forms was determined to be 62 ± 6.5 nmol/min per mg of protein, and was substantially decreased by H⁺-ATPase inhibitors. The data support the presence of an H⁺-ATPase as the major regulator of pH_i in procyclic trypomastigotes. In contrast, bloodstream trypomastigotes were unable to maintain a neutral pH under acidic conditions, and their steady-state pH_i and recovery from acidification were unaffected by H⁺-ATPase inhibitors, except for DCCD (100 μM). Their steady-state pH_i was markedly decreased in glucose-free buffer or by ≥ 10 mM pyruvate, whereas procyclic

trypomastigotes were unaffected by similar treatments. The rate of H⁺ efflux in bloodstream trypomastigotes was 534 ± 38 nmol/min per mg of protein, and was decreased in the absence of glucose and by the addition of pyruvate or DCCD. Pyruvate efflux in these forms was calculated to be 499 ± 34 nmol/min per mg of protein, and was significantly inhibited by DCCD, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonic acid and α-cyanohydroxycinnamic acid. The pyruvate analogues β-hydroxy-pyruvate, 3-bromopyruvate, 3-oxoglutarate, oxaloacetate, 3-oxoisovalerate and 3-oxoisohexanoate significantly decreased pH_i, as well as proton and pyruvate efflux, whereas lactate had only a small effect, and no effect was observed with citrate or fumarate. The inhibition by pyruvate analogues of pyruvate efflux, proton efflux and acidification of pH_i supports the hypothesis that pyruvate efflux is accompanied by proton efflux and that this is the major pH_i control mechanism in bloodstream forms. Inhibition by H⁺-ATPase inhibitors of residual H⁺ efflux in the absence of glucose or in the presence of high extracellular pyruvate indicates a minor role for H⁺-ATPase(s) in control of pH_i in bloodstream forms.

Key words: proton efflux, pyruvate efflux, trypanosome.

INTRODUCTION

Trypanosoma brucei, a protozoan parasite transmitted by the tsetse fly in sub-Saharan Africa, is the aetiological agent of African sleeping sickness in humans and nagana in cattle. *T. brucei* 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 procyclic stage replicates within the alimentary tract of the tsetse fly, where the environmental (external) pH (pH_e) is acidic [1], whereas the bloodstream form propagates in the host's blood, where the pH_e is consistently 7.4. Marked differences in metabolism between the two stages have evolved as a result of the very different environments in which they reside. In the host's bloodstream, the concentration of glucose is relatively constant (approx. 5 mM), and energy generation by the bloodstream form occurs exclusively via glycolysis. Glucose enters bloodstream trypomastigotes through a facilitated-diffusion transporter independent of a proton motive force, and glycolysis takes place in a unique organelle, the glycosome [2–7]. The end-product of glycolysis in bloodstream trypomastigotes is pyruvate, which is excreted by a facilitated-diffusion pyruvate

carrier [8–10]. In the procyclic trypomastigote, the major metabolic substrates are amino acids and glucose, which may be taken up via an active transport mechanism driven by the proton motive force. Conventional glycolysis and oxidative phosphorylation occur [11]. Although a pyruvate transporter is present in the procyclic form, pyruvate release is over 100-fold slower than for bloodstream forms due to its further metabolism inside the mitochondrion [12].

The ability of *T. brucei* to regulate its intracellular pH (pH_i) throughout its life cycle is critical to its survival, as most intracellular enzymes function optimally within a narrow pH range. In most higher-eukaryotic cells whose environment is neutral, metabolically generated protons are exchanged for Na⁺ via a Na⁺/H⁺ antiporter [13]. The sodium gradient generated by the Na⁺/K⁺-ATPase drives this exchange. However, at low pH_e, Na⁺/H⁺ exchange is unfavourable and therefore cells that maintain a pH_i more alkaline than their environment frequently utilize ATP-driven proton pumps to regulate their pH_i. Proton pumping is electrogenic and is usually supported by one or more anion- or cation-conductive pathways that serve to partially dissipate the membrane potential, thereby increasing the efficiency of the pump. In addition, the proton motive force

Abbreviations used: BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; α-CHC, α-cyanohydroxycinnamic acid; DCCD, *N,N'*-dicyclohexylcarbodi-imide; DES, diethylstilboestrol; EIPA, 5-(*N*-ethyl)-*N*-isopropylamiloride; H₂DIDS, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonic acid; LDH, lactate dehydrogenase; NEM, *N*-ethylmaleimide; pH_e, external pH; pH_i, intracellular pH.

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generated by these pumps frequently drives nutrient uptake in these cells.

Previous studies in the procyclic form of *T. brucei* using the pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) reported a steady-state pH_i of 7.05 [14] or 7.22 [15], suggesting that this stage maintains a pH_i that is more alkaline than its environment. In addition, Fraser-L'Hostis et al. [15] found that the pH_i of procyclics remained neutral in acidic and alkaline conditions, and was sensitive to the H^+ -ATPase inhibitor *N,N'*-dicyclohexylcarbodi-imide (DCCD), supporting a major role for an H^+ -ATPase in this stage. They also proposed that regulation of pH_i in procyclics may be linked to HCO_3^- and is due in part to an amiloride-sensitive Na^+/H^+ exchanger.

The steady-state pH_i of *T. brucei* bloodstream forms has been investigated by a number of researchers. Using ^{14}C -labelled 5,5-dimethylloxazolidine-2,4-dione or $[^{14}C]$ methylamine, pH_i was calculated to be 7.01 by Thissen and Wang [16], and 7.2 by Nolan and Vooheis [17,18]. Using BCECF, the steady-state pH_i was determined to be 7.08 by Ruben et al. [19], and 7.4 by both Scott et al. [14] and Fraser-L'Hostis et al. [15]. Thus bloodstream forms appear to maintain a pH_i equivalent to or slightly more acidic than the pH of their environment in the bloodstream.

There have been limited studies of the regulation of pH_i in *T. brucei* bloodstream forms. Thissen and Wang [16] noted that the protonophore carbonyl cyanide *m*-chlorophenylhydrazone ('CCCP') had little effect on the pH_i of bloodstream forms at pH 7.4, but a major effect at pH 6.0. This suggests a passive control mechanism at physiological pH_e , but potentially the function of a proton pump in acidic environments, such as the mid-gut of the tsetse fly. They also noted that millimolar concentrations of the ATPase inhibitor *N*-ethylmaleimide (NEM) had no effect on pH_i in bloodstream forms. Ter Kuile et al. [20] also suggested that an energy-dependent pump may be needed for maintenance of pH_i in an acidic environment, but not under physiological conditions in the host. Furthermore, they noted that, at 37 °C, pH_i was not regulated when pH_e was varied, whereas at 22 °C the pH_i remained constant. Fraser-L'Hostis et al. [15] found the pH_i of bloodstream forms to be slightly acidified after treatment with the H^+ -ATPase inhibitors DCCD and NEM, or with the anion blocker 4,4'-di-isothiocyanatodihydrostilbene-2,2'-disulphonic acid (H_2 DIDS) or the Na^+/H^+ antiport inhibitor 5-(*N*-ethyl)-*N*-isopropylamiloride (EIPA), suggesting that three mechanisms, namely an H^+ -ATPase, a HCO_3^- -dependent anionic pathway and a Na^+/H^+ exchanger, may play a role in the regulation of pH_i in these forms. These workers also showed that the addition of pyruvate led to rapid cytosolic acidification, suggesting the possibility that pyruvate is co-transported with protons.

Despite these previous studies, the mechanisms that are important in the regulation of pH_i in *T. brucei* remain unclear. These findings do suggest, however, that there may be significant differences in the regulation of pH_i in procyclic compared with bloodstream forms. If protons accompany pyruvate efflux in bloodstream forms, then the monocarboxylate (pyruvate) carrier may play a substantial role in the regulation of pH_i in this stage. In procyclics, pyruvate production, being at a low level, is unlikely to contribute significantly to pH_i regulation. To investigate further the regulation of pH_i in *T. brucei* bloodstream and procyclic forms and to examine the role of the pyruvate carrier in bloodstream forms, we undertook studies involving pH_i determination and proton and pyruvate efflux measurements as a function of the ionic composition of the medium, under acid loads, and in the presence of different inhibitors. We conclude that an H^+ -ATPase supported by chloride-conductive pathways regulates pH_i in both procyclic and bloodstream forms, and that

a pyruvate-proton symport plays a significant role in the regulation of pH_i in bloodstream trypomastigotes.

MATERIALS AND METHODS

Culture methods

T. brucei bloodstream forms (monomorphic strain 427 from clone MITat 1.4, otherwise known as variant 117) were isolated from infected mice or rats using a DEAE-cellulose column and resuspended in ice-cold phosphate buffer at pH 8.0 [21]. The same strain of bloodstream form was also grown axenically at 37 °C in HMI-9 medium [22] and used in a limited number of experiments to confirm the results obtained with trypomastigotes isolated from rats. *T. brucei* procyclic forms were grown at 28 °C in medium SDM-79 supplemented with 10% (v/v) heat-inactivated fetal calf serum [23]. After 2 or 3 days of inoculation, cells were collected by centrifugation (1500 *g* for 10 min) and washed twice in Dulbecco's PBS, pH 7.5. Procyclic trypomastigotes of strain ILTar 1 were used for all studies reported. Additionally, procyclics transformed from the bloodstream strain described above were used for a limited number of experiments to confirm that differences in pH_i regulation were stage- but not strain-related.

Chemicals and solutions

Dulbecco's modified Eagle's medium, Dulbecco's PBS, fetal and newborn calf sera, DCCD, diethylstilboestrol (DES), NEM, ouabain, lactate dehydrogenase (LDH; type II), NADH, α -cyanohydroxycinnamic acid (α -CHC), pyruvate and pyruvate analogues were purchased from Sigma Chemical Co. Bafilomycin A_1 was from Kamiya Biomedicals (Thousand Oaks, CA, U.S.A.). Omeprazole was a gift from Åstra Hassle AB (Mölnådal, Sweden) through the courtesy of Dr. K. Andersson. Luciferase/luciferin (ATP determination kit), BCECF, BCECF acetoxymethyl ester, H_2 DIDS and EIPA were from Molecular Probes. All other reagents were of analytical grade.

The pH of all inhibitors was adjusted to pH 7.4 before use. The following buffers were adjusted to pH 7.4 unless otherwise indicated: buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose and 50 mM Hepes), loading buffer (135 mM NaCl, 5 mM KCl, 15 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM Hepes/Tris), standard buffer (135 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM Hepes/Tris), choline buffer (140 mM choline chloride, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM Hepes/Tris), sodium-free buffer (135 mM choline chloride, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM Hepes/Tris), potassium-free buffer (135 mM NaCl, 5 mM choline chloride, 5 mM glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM Hepes/Tris), chloride-free buffer (135 mM sodium gluconate, 5 mM potassium gluconate, 5 mM glucose, 1 mM calcium gluconate, 1 mM $MgSO_4$ and 10 mM Hepes/Tris), and NMG buffer (140 mM *N*-methylglucamine, 5 mM glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM Hepes/Tris).

pH_i measurements

pH_i was measured fluorimetrically using BCECF. After harvesting the cells, they were centrifuged as before and resuspended to a final density of 1×10^8 cells/ml in loading buffer containing 0.1 μM BCECF acetoxymethyl ester. The suspensions were incubated for 30 min on ice. Subsequently the cells were washed twice with standard buffer to remove the extracellular dye and

resuspended in buffer A (procyclics) or separation buffer, pH 7.5 [21] (bloodstream forms) to a final density of 1×10^8 cells/ml and kept on ice. For pH_i measurements, a 500 μl (bloodstream forms) or 250 μl (procyclic forms) aliquot of the cell suspension was centrifuged and resuspended into 2.5 ml of the appropriate buffer (final densities 2×10^7 and 1×10^7 cells/ml respectively) in a cuvette placed in a thermostatically controlled Hitachi F-2000 spectrofluorimeter at 30 °C for procyclics or at 37 °C for bloodstream forms. 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 pH values, as described previously [24]. Acid loading was accomplished by 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). Parasites were then centrifuged as before and resuspended in various buffers with or without inhibitors. The recovery rate was calculated from the slope of the trace in the initial 60 s of recovery, and the final pH_i was determined after 10 min. Traces shown are representative of at least three independent experiments conducted on separate cell preparations.

Pyruvate efflux measurements

Pyruvate efflux was measured by following NADH oxidation at 340–430 nm using a dual-wavelength spectrophotometer (Aminco DW2000). A total of 2.5×10^7 (250 μl) *T. brucei* bloodstream forms were preincubated in separation buffer [21] at 37 °C for 5 min before each experiment, centrifuged as before and resuspended in 2.5 ml of assay buffer, which consisted of standard buffer plus 10 mM glucose, 200 μM NADH and 10 units of LDH at 37 °C. Pyruvate efflux was followed for 10 min, and inhibitors were added after 5 min. To rule out inhibition of LDH by the inhibitors, 100 nmol of pyruvate was added to the cuvette at the conclusion of each experiment. The pyruvate analogues 3-bromopyruvate and oxaloacetate were found to behave as substrates for LDH. To measure inhibition of pyruvate efflux by these analogues, parasites were preincubated for 5 min with the analogue, centrifuged and immediately resuspended in assay buffer, and pyruvate efflux was measured during the first 60 s. Calibration was performed using 100 nM increments of pyruvate. Traces shown are representative of at least three independent experiments conducted on separate cell preparations.

Proton efflux measurements

To measure pH_e reflecting proton pumping by parasites, 2.5×10^7 parasites were washed and resuspended in a weakly buffered (0.1 mM Hepes/Tris) standard buffer solution containing 0.38 μM BCECF (free acid) at 37 °C for bloodstream forms and at 30 °C for procyclics. The fluorescence ratio, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm, were recorded and translated into H^+ /min values on the basis of ratios obtained by the addition of known equivalents of HCl [25]. Traces shown are representative of at least three independent experiments conducted on separate cell preparations.

ATP measurements

Portions of 5×10^7 cells in 1 ml of standard buffer were incubated with each inhibitor for 10 min at 37 °C with agitation. The cells were then centrifuged at 8500 *g* for 5 min at 4 °C and the supernatant removed. The pellet was freeze-thawed five times and diluted with 25 mM Hepes, pH 7.8, to a total volume of 475 μl , and then incubated with 5% trichloroacetic acid for

15 min on ice. The suspension was then centrifuged at 8500 *g* for 5 min and the supernatant removed. A 5 μl sample of the supernatant was diluted 1:40 (v/v) in 25 mM Hepes buffer and added to 200 μl of luciferin/luciferase reagent in a cuvette, and the chemiluminescence was recorded in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). Calibration was performed by the incremental addition of ATP to parallel samples.

Data analysis

All results are expressed as means $\pm$ S.D. for *n* independent experiments. Statistical significance was determined by Student's *t* test. Significance was considered at a level of $P \leq 0.05$.

RESULTS

Effects of Na^+ , K^+ and Cl^- on steady-state pH_i and recovery from acidification

The mean baseline pH_i of procyclics and bloodstream trypomastigotes in the nominal absence of bicarbonate and at a pH_e of 7.4 in standard buffer was 7.53 ± 0.06 and 7.47 ± 0.07 respectively (Table 1). Procyclics were able to maintain their pH_i within 0.6 pH units in standard buffer over a range of pH_e values of 6.0–8.0 (Figure 1). Bloodstream forms were unable to maintain a neutral pH_i at non-physiological pH_e , with pH_i ranging from 6.71 at pH_e 6.0 to 7.93 at pH_e 8.0 (Figure 1). These data suggest that procyclics are better than bloodstream forms at regulating their pH_i against a pH gradient, which might be expected given the very different environments in which the two stages reside.

The contributions of the ions Na^+ , K^+ and Cl^- to the maintenance of pH_i in *T. brucei* were determined by measuring steady-state pH_i and recovery of pH_i following acidification in specific ion-free buffers as compared with standard buffer. The absence of Na^+ or K^+ from the buffer had no effect on the pH_i of either procyclic (Figure 2A; Table 1) or bloodstream (Figure 2B; Table 1) trypomastigotes. When Na^+ was replaced by choline (Na^+ -free buffer), a rise of 0.18 pH unit in the final pH_i was noted in bloodstream forms, but this was not found in Na^+ -free NMG buffer (Figure 2B; Table 1). Furthermore, the proton efflux rate of neither stage was decreased in Na^+ -free buffer (results not shown). These results suggest that Na^+ and K^+ are not required for the maintenance of steady-state pH_i in either stage, ruling out a significant role for a Na^+/H^+ antiport or Na^+ -dependent anion exchanger in the regulation of pH_i , contrary to a previous report [15]. EIPA (100 μM), an inhibitor of Na^+/H^+ exchangers, caused

Table 1 Effects of specific ion-free buffers on the steady-state pH_i of *T. brucei*

BCECF-loaded parasites were centrifuged, resuspended in specific ion-free buffers and incubated for 10 min at room temperature prior to each determination. Results shown are means $\pm$ S.D. of a minimum of three experiments. n.d., not determined. Significance of differences compared with standard buffer: * $P \leq 0.05$.

Buffer	Steady-state pH_i	
	Bloodstream forms	Procyclic forms
Standard	7.47 ± 0.07	7.53 ± 0.06
Choline	$7.61 \pm 0.06^*$	7.46 ± 0.08
NMG	7.49 ± 0.09	n.d.
K^+ -free	7.42 ± 0.06	7.49 ± 0.10
Cl^- -free	$7.31 \pm 0.09^*$	$7.28 \pm 0.04^*$

Figure 1 Effect of pH_e on pH_i

BCECF-loaded parasites were incubated for 10 min in standard buffer of varying pH prior to each experiment. Other conditions were as indicated in the Materials and methods section. P, procyclic trypomastigotes (closed symbols); B, bloodstream trypomastigotes (open symbols). Results shown are values obtained in three different experiments for each parasite stage. Significance of differences: $^*P \leq 0.05$.

a slight but significant decrease in the steady-state pH_i of procyclic, but not bloodstream, forms (Table 2). In acidified procyclic trypomastigotes, but not in bloodstream trypomastigotes, EIPA caused a dose-dependent decrease in the final pH_i , but not in the rate of pH_i recovery (results not shown). Although these data apparently support the involvement of a Na^+/H^+ exchanger in the regulation of pH_i in procyclics, the lack of a requirement for Na^+ suggests that EIPA may be having some other effect. We demonstrated recently that EIPA behaves as a protonophore in *T. cruzi* amastigotes and trypomastigotes [26], as has been described in other cells [27], and a similar effect may be occurring here.

The steady-state pH_i in the absence of Cl^- was decreased by 0.25 and 0.18 pH unit in procyclic and bloodstream forms respectively (Table 1). These cells rapidly recovered toward their original pH_i when centrifuged and resuspended in standard

Table 2 Effects of inhibitors on the steady-state pH_i of *T. brucei*

BCECF-loaded parasites were suspended in standard buffer and the pH_i allowed to stabilize. Inhibitors were added 30–60 s after the start of each 10 min determination. Results shown are means $\pm$ S.D. of a minimum of three experiments. Significance of differences compared with respective control: $^*P \leq 0.05$.

Inhibitor	Concentration (μ M)	Steady-state pH_i	
		Bloodstream forms	Procyclic forms
Control	0	7.47 ± 0.07	7.53 ± 0.06
H ₂ DIDS	500	$7.23 \pm 0.11^*$	7.47 ± 0.06
EIPA	100	7.48 ± 0.10	$7.24 \pm 0.13^*$
Bafilomycin	2	7.44 ± 0.05	$7.28 \pm 0.06^*$
DCCD	100	$7.02 \pm 0.07^*$	$6.87 \pm 0.11^*$
DES	100	7.45 ± 0.04	$7.03 \pm 0.15^*$
NEM	1000	7.39 ± 0.07	$7.11 \pm 0.09^*$
Omeprazole	250	7.45 ± 0.06	7.45 ± 0.08
Ouabain	1000	7.50 ± 0.10	7.47 ± 0.06

buffer (results not shown). Acidified procyclic (Figure 2A) and bloodstream (Figure 2B) forms suspended in Cl^- -free buffer recovered their pH_i almost at the same rate as controls, and the final pH_i attained was 0.28 and 0.19 pH unit lower respectively. These data support a role for Cl^- in the maintenance of pH_i in *T. brucei*, where it is likely to function to support electrogenic pumping, as has been observed in other trypanosomatids [24,28–31]. The addition of 25 mM HCO_3^- reduced the acidification that resulted from suspension in Cl^- -free buffer in both stages (results not shown). No significant effect of HCO_3^- was observed in either stage on steady-state pH_i in standard buffer at pH 7.4 (results not shown). However, at pH_e 6, HCO_3^- increased pH_i from 6.71 to 7.07 in bloodstream forms (results not shown). To investigate the possible presence of an Na^+ -dependent or -independent Cl^-/HCO_3^- exchanger, the inhibitor H₂DIDS was used at concentrations up to 0.5 mM. Even though no effect was noted on the pH_i of procyclics, they rapidly became clumped (but remained motile) after a few minutes' exposure to concentrations of H₂DIDS of 250 μ M or more. In bloodstream forms, 0.5 mM H₂DIDS decreased the steady-state pH_i (Table 2)

Figure 2 Effects of specific ion-free buffers on pH_i of acid-loaded parasites

BCECF-loaded parasites were acidified, centrifuged and resuspended in Na^+ -free, K^+ -free, standard, NMG or Cl^- -free buffer. All other conditions were as in the Materials and methods section. Traces shown are from typical experiments. (A) Procyclic trypomastigotes; (B) bloodstream trypomastigotes.

Figure 3 Effects of H^+ -ATPase inhibitors on pH_i of acid-loaded parasites

Bloodstream (b) and procyclic (p) trypomastigotes were acidified, centrifuged and resuspended in standard buffer containing different concentrations of DCCD (A), DES (B), NEM (C) or bafilomycin A₁ (D). Other conditions were as in Figure 2. Results shown are means $\pm$ S.D. for a minimum of three experiments. Significance of differences: * $P \leq 0.05$.

and the final pH_i after recovery from acidification, but did not reduce the rate of recovery following acidification (results not shown). The lack of inhibition by H_2DIDS , in addition to the data demonstrating the maintenance of steady-state pH_i and normal recovery from acidification in the absence of Na^+ or HCO_3^- in procyclic trypomastigotes, suggests that a $\text{Cl}^-/\text{HCO}_3^-$ exchanger is not a significant regulator of pH_i in *T. brucei* procyclic forms.

Effects of H^+ -ATPase inhibitors on steady-state pH_i and recovery from acidification

The effects of various H^+ -ATPase inhibitors on pH_i were determined in parasites at steady state and following acidification. The general H^+ -ATPase inhibitors DCCD (100 μM) and NEM (1 mM) and the plant and yeast H^+ -ATPase inhibitor DES (100 μM), as well as the V-type H^+ -ATPase inhibitor bafilomycin A₁ (2 μM), all markedly acidified the steady-state pH_i of procyclic forms (Table 2). Furthermore, these inhibitors decreased the rate of recovery of pH_i in acidified procyclics in a dose-dependent manner (Figures 3A–3D). In bloodstream forms both the steady-state pH_i (Table 2) and the recovery of pH_i following acidification were significantly decreased by 100 μM DCCD (Figure 3A), but not by up to 100 μM DES (Figure 3B), 1 mM NEM (Figure 3C) or 5 μM bafilomycin (Figure 3D). In addition, there was no effect on the pH_i of either stage by 250 μM omeprazole, an inhibitor of

gastric H^+/K^+ -ATPase, or 1 mM ouabain, an inhibitor of the Na^+/K^+ -ATPase (Table 2). Limited studies on pH_i in axenically grown bloodstream forms and in procyclics transformed from bloodstream forms confirmed these results, supporting the conclusion that the differences observed were stage-related (results not shown). Intracellular ATP levels were determined, to rule out inhibitor effects resulting from decreased ATP levels. ATP levels as a percentage of control in procyclics and bloodstream forms respectively were 96% and 92% for 100 μM NEM, 69% and 61% for 100 μM DCCD, 73% and 84% for 50 μM DES and 85% and 89% for 0.5 mM H_2DIDS . Together these results are consistent with the hypothesis that an H^+ -ATPase is the major regulator of pH_i in *T. brucei* procyclic forms, but not in bloodstream forms.

Effects of inhibitors on proton efflux

Proton efflux was calculated to be 62 ± 6.5 nmol/min per mg of protein in procyclics, and 534 ± 38 nmol/min per mg in bloodstream forms ($n = 3$). In procyclic forms, 25 μM DCCD, 25 μM DES or 50 μM NEM almost completely abolished proton pumping, while 2 μM bafilomycin decreased it by 29% (Figure 4). In bloodstream forms, 100 μM DCCD, 50 μM DES and 1 mM NEM decreased proton efflux by 56%, 17% and 8% respectively (results not shown). These data show that proton efflux in procyclics appears to be entirely the result of H^+ -

Figure 4 Effects of H^+ -ATPase inhibitors on proton efflux of procyclic forms

Procyclic trypomastigotes were centrifuged and resuspended in Hepes/Tris standard buffer containing free BCECF. All other conditions were as indicated in the Materials and methods section. Inhibitors [50 μ M NEM, 25 μ M DES, 25 μ M DCCD or 2 μ M bafilomycin A (Baf)] were added at the times indicated. Traces shown are from typical experiments.

ATPases. In bloodstream forms, where proton efflux was almost 10-fold higher than in procyclics, only a small percentage could be attributed to H^+ -ATPases. This suggests that H^+ -ATPases are present in the plasma membrane of bloodstream forms, but that their overall contribution to proton pumping is minor. The substantial difference in the magnitude of the inhibition by DCCD compared with that by DES or NEM suggests that DCCD may be affecting proton efflux by another mechanism in addition to inhibition of H^+ -ATPases.

Contribution of metabolism to regulation of pH_i

Since there are major differences in metabolism between procyclics and bloodstream forms, we examined whether these

differences contributed to the regulation of pH_i in bloodstream forms. When resuspended in glucose-free standard buffer, bloodstream forms rapidly acidified to pH_i 7.16 ± 0.08 (Figure 5A), whereas the pH_i of procyclics remained similar to that of controls (results not shown). Parasites remained motile during this period of glucose depletion. The subsequent addition of glucose to bloodstream forms resulted in a further drop in pH_i to 7.02 ± 0.05 , followed by recovery to the same pH_i as controls (Figure 5A). Acidified bloodstream forms recovered their pH_i more slowly in glucose-free buffer as compared with controls, and the final pH_i attained was 7.14 ± 0.12 (Figure 5B). The non-metabolizable glucose analogues 6-deoxy-D-glucose and 2-deoxy-D-glucose, when substituted for glucose, had no effect on pH_i , ruling out the presence of a proton-glucose symport as the cause of the initial decrease in pH observed following the addition of glucose (results not shown). Additionally, incubation in a glucose-free buffer significantly reduced proton efflux by 54 % to 246 ± 24 nmol/min per mg of protein in bloodstream forms, but not in procyclics (Figure 6A, trace a; and results not shown). These data suggest that glucose metabolism is linked to the regulation of pH_i and the rate of proton efflux in bloodstream forms.

Role of the pyruvate transporter in regulation of pH_i

The addition of pyruvate to bloodstream forms has been shown previously to result in cytosolic acidification [15]. Pyruvate efflux in bloodstream forms appears to occur by facilitated diffusion through a monocarboxylate (pyruvate) transporter [8–10]. Previously identified monocarboxylate transporters in bacteria, yeast and mammalian cells transport substrates bidirectionally down their concentration gradient in symport with protons, and have an affinity for a limited repertoire of carboxylic acids [32–36]. To investigate the relationship between pyruvate efflux and regulation of pH_i , inhibitors of pyruvate efflux, including pyruvate and various carboxylic acids that compete with pyruvate for the transporter, as well as the mitochondrial pyruvate transport inhibitor α -CHC, were examined for their effects on pH_i and on proton and pyruvate efflux.

The addition of ≤ 10 mM pyruvate to bloodstream forms in the presence of glucose caused rapid acidification, followed by a slow recovery to near-control levels, while a concentration of

Figure 5 Effect of glucose on the pH_i of bloodstream forms

Bloodstream forms were suspended in standard buffer with or without 10 mM glucose (A), or acidified, centrifuged and resuspended in standard buffer with or without glucose and allowed to recover their pH_i (B). Glucose at 10 mM (Glc) was added at the times indicated by the arrows. Traces shown are from typical experiments.

Figure 6 Effects of glucose and pyruvate on proton efflux of bloodstream forms

Bloodstream forms were resuspended in standard buffer with (A, trace b and where indicated in trace a; C and D) or without glucose (A, trace a; B). Other conditions were as in Figure 4. Then 10 mM glucose (Glc), 100 μ M DCCD or 25 mM pyruvate (Pyr) was added at the times indicated. Traces shown are from typical experiments.

Table 3 Effects of various compounds on the steady-state pH_i of *T. brucei*

BCECF-loaded parasites were suspended in standard buffer and the pH_i allowed to stabilize. Inhibitors were added 30–60 s after the start of each 10 min determination. Results shown are means $\pm$ S.D. of a minimum of three experiments. Significance of differences compared with respective control: * $P \leq 0.05$.

Inhibitor	Bloodstream forms		Procyclic forms	
	Concn. (mM)	Steady-state pH_i	Concn. (mM)	Steady-state pH_i
Control	0	7.47 ± 0.07	0	7.53 ± 0.06
Pyruvate	1	7.39 ± 0.08	10	7.50 ± 0.03
	10	$7.34 \pm 0.07^*$	25	$7.38 \pm 0.06^*$
	25	$7.09 \pm 0.06^*$	50	$7.32 \pm 0.06^*$
Oxaloacetate	1	$7.13 \pm 0.13^*$	10	7.39 ± 0.09
	10	$7.06 \pm 0.09^*$	25	$7.42 \pm 0.03^*$
3-Oxoglutarate	1	7.31 ± 0.12	10	7.46 ± 0.05
	10	$7.12 \pm 0.16^*$	25	7.41 ± 0.06
3-Oxoisovalerate	1	$7.14 \pm 0.15^*$	10	7.39 ± 0.09
	10	$7.09 \pm 0.11^*$	25	$7.37 \pm 0.05^*$
β -Hydroxypyruvate	1	$7.12 \pm 0.08^*$	10	$7.25 \pm 0.07^*$
	10	$6.97 \pm 0.09^*$	25	$7.21 \pm 0.06^*$
Lactate	10	7.43 ± 0.05	10	7.42 ± 0.07
	25	$7.22 \pm 0.08^*$	25	$7.35 \pm 0.11^*$
α -CHC	1	$7.23 \pm 0.10^*$	2	7.28 ± 0.16
	2	$7.17 \pm 0.08^*$	4	$7.17 \pm 0.12^*$

Figure 7 Effect of pyruvate on the pH_i of bloodstream forms

Bloodstream forms were suspended in standard buffer (A) or acidified, centrifuged and resuspended in standard buffer and allowed to recover their pH_i (B). Pyruvate was added at the time indicated by the arrows at 10 mM (10) or 25 mM (25) (A), or was present in the resuspension medium at the concentrations indicated (B). Traces shown are from typical experiments.

25 mM or more resulted in rapid acidification to $pH\ 7.09 \pm 0.09$ (Table 3), without recovery (Figure 7A). Pyruvate also inhibited recovery from acidification in a dose-dependent manner in bloodstream forms (Figure 7B). Pyruvate at 25 mM did not induce further acidification in bloodstream forms already acidified by suspension in glucose-free buffer (results not shown). Procyclics were unaffected by < 25 mM pyruvate and mildly acidified by 25–50 mM pyruvate (Table 3). These data strongly suggest that pyruvate in bloodstream forms is transported in symport with protons.

In the absence of glucose, pyruvate had no significant effect on proton efflux by bloodstream forms (Figure 6B, trace b). The addition of 100 μ M DCCD to parasites suspended in glucose-free buffer reduced proton efflux by 73 %, to 136 ± 11 nmol/min per mg of protein (Figure 6B, trace a). In the presence of glucose, the proton efflux rate in bloodstream forms was decreased by 25 mM pyruvate by 37 %, to 314 ± 21 nmol/min per mg of protein (Figure 6C, traces a and b), but was not changed in procyclic forms (results not shown). The addition of both DCCD and pyruvate to bloodstream forms suspended in standard buffer substantially reduced proton efflux by 89 %, to 56 ± 12 nmol/min per mg of protein (Figure 6C, trace a). Furthermore, the addition of 1 mM NEM or 50 μ M DES substantially reduced proton efflux further in the absence of glucose to 219 ± 24 and 198 ± 19 nmol/min per mg of protein respectively, and in the presence of 25 mM pyruvate to 232 ± 14 and 213 ± 30 nmol/min per mg respectively. These data support the existence of two major mechanisms for proton efflux in bloodstream forms.

Pyruvate efflux in bloodstream forms has been shown previously to be inhibited by a number of pyruvate analogues [8,9]. The steady-state pH_i of bloodstream forms was significantly lowered by 10 mM oxaloacetate, 3-oxoglutarate, 3-oxoisovalerate (Table 3), 3-bromopyruvate or β -hydroxypyruvate (results not shown). Lactate at 25 mM slightly acidified bloodstream forms. However, pH_i was not affected by 25 mM citrate or 10 mM fumarate (results not shown). The steady-state pH_i of procyclics was not affected significantly by any of the compounds tested at concentrations of ≤ 10 mM (Table 3). 3-Bromopyruvate at 10 mM significantly decreased the pH_i of bloodstream forms to 6.89 ± 0.12 . Proton pumping in bloodstream forms was also significantly inhibited by 10 mM oxaloacetate, 3-oxoglutarate, 3-

Table 4 Effects of various compounds on proton and pyruvate efflux in bloodstream forms

Bloodstream forms were suspended in 0.1 mM Hepes/Tris standard buffer for proton efflux, or in the assay buffer described in the Materials and methods section for pyruvate efflux. Inhibitors were added 60–120 s after the start of each 10 min determination, and the rate of efflux was calculated before and after addition of inhibitors. For pyruvate efflux determinations using oxaloacetate, parasites were preincubated for 5 min at 37 °C with the inhibitor, centrifuged and resuspended in assay buffer, and the rate was determined for the first 60 s (see the Materials and methods section). Results shown are means $\pm$ S.D. of a minimum of three experiments. n.d., not determined. Significance of differences compared with control: * $P \leq 0.05$.

Inhibitor	Concn. (mM)	Efflux (nmol/min per mg of protein)	
		Protons	Pyruvate
Control	0	534 ± 38	499 ± 34
Pyruvate	10	$352 \pm 24^*$	n.d.
	25	$337 \pm 8^*$	n.d.
Oxaloacetate	10	$325 \pm 22^*$	$269 \pm 28^*$
3-Oxoglutarate	10	$443 \pm 19^*$	$449 \pm 14^*$
3-Oxoisovalerate	10	$417 \pm 33^*$	$429 \pm 29^*$
β -Hydroxypyruvate	10	$192 \pm 31^*$	$75 \pm 41^*$
Lactate	10	$464 \pm 27^*$	484 ± 23
	25	$422 \pm 35^*$	$409 \pm 49^*$
α -CHC	1	470 ± 69	$359 \pm 28^*$
	2	$345 \pm 32^*$	$304 \pm 29^*$

oxoisovalerate or β -hydroxypyruvate, 25 mM lactate (Table 4) and 10 mM 3-oxoisohexanoate (results not shown). Citrate or fumarate at 25 mM had no effect on proton efflux (results not shown), whereas 10 mM 3-bromopyruvate significantly decreased it to 197 ± 54 nmol/min per mg.

The rate of pyruvate efflux was calculated to be 499 ± 34 nmol/min per mg. Pyruvate efflux in bloodstream forms was significantly inhibited by 10 mM oxaloacetate, 3-oxoglutarate, 3-oxoisovalerate or β -hydroxypyruvate, 25 mM lactate (Table 4) and 10 mM 3-oxoisohexanoate (results not shown). 3-Bromopyruvate at 10 mM decreased pyruvate efflux to 45 ± 34 nmol/min per mg, whereas 25 mM citrate or fumarate had no effect (results not shown). A strongly positive correlation

Figure 8 Correlation of pH_i , proton efflux and pyruvate efflux for bloodstream forms

Data and methods are as described in Tables 3 and 4. Agents used were 25 mM lactate (LAC), 10 mM 3-oxoglutarate (GLUT), 10 mM 3-oxoisovalerate (VAL), 2 mM α -CHC (CHC), 10 mM oxaloacetate (ACET) and 10 mM β -hydroxypyruvate (OHPYR).

was calculated between the inhibition of pyruvate efflux and both the decrease in pH_i ($r^2 = 0.71$) and the inhibition of proton efflux ($r^2 = 0.83$) in bloodstream forms (Tables 3 and 4; Figure 8), supporting the contention that pyruvate efflux is accompanied by proton efflux in bloodstream forms.

The mitochondrial pyruvate carrier inhibitor α -CHC has been shown by some investigators [9], but not others [10], to inhibit pyruvate efflux in *T. brucei* bloodstream forms. The addition of 2 mM α -CHC resulted in a decrease in pH_i and prevented full recovery from acidification in bloodstream forms, and a concentration of 4 mM had a similar effect on procyclics (Table 3). α -CHC also decreased proton and pyruvate efflux in bloodstream forms and proton efflux in procyclic forms (Table 4 and results not shown). Because α -CHC appears to affect both procyclics and bloodstream forms, it is unclear if it is affecting the plasma membrane pyruvate carrier or some other metabolic pathway, such as the mitochondrial pyruvate transporter, in procyclics.

We showed that DCCD has a significant effect on pH_i (Table 2) and recovery from acidification (Figure 3A) in bloodstream trypomastigotes, as compared with other H^+ -ATPase inhibitors (Table 2; Figures 3B–3D). We also demonstrated that DCCD affects both the glucose-sensitive (Figure 6C) and the glucose-insensitive (Figure 6B) portion of proton efflux. These data suggested that DCCD may be inhibiting the pyruvate transporter in addition to the H^+ -ATPase. We found that the addition of 100 μ M DCCD significantly reduced pyruvate efflux by 77%, to 144 ± 23 nmol/min per mg of protein (Figure 9A). The addition of pyruvate to the assay following addition of DCCD produced a decrease in absorbance of similar magnitude when compared with controls, demonstrating that LDH in the assay buffer was not being inhibited by DCCD (Figure 9A). We also observed that H_2 DIDS had a significant effect on the pH_i of bloodstream, but not procyclic, forms (Table 2). Stilbene derivatives have been shown to be inhibitory for mammalian monocarboxylate carriers [13], and we suggest that a similar effect may be occurring here. We found that pyruvate efflux was decreased by 34% to 330 ± 39 nmol/min per mg of protein in the presence of 500 μ M H_2 DIDS (results not shown). These data suggest that much of the effect of DCCD and H_2 DIDS on the pH_i of bloodstream forms may be due to direct inhibition of the pyruvate transporter.

Previously it has been argued that, if the downhill transport of pyruvate is coupled with protons, the inward-directed proton

Figure 9 Effects of DCCD and acidic pH on pyruvate efflux of bloodstream forms

(A) Bloodstream forms were suspended in the assay buffer described in the Materials and methods section, then 100 μ M DCCD or 100 nmol of pyruvate (Pyr) was added, as indicated by the arrows. (B) Bloodstream forms were suspended in the assay buffer at pH 7.4 or pH 5.0. Other conditions were as described in the Materials and methods section. Traces shown are from a typical experiment.

motive force would significantly slow down pyruvate efflux, leading to cytosolic acidification [20]. To demonstrate that pyruvate can be transported against a proton gradient, we measured pyruvate efflux at acidic pH. In standard buffer at pH 5.0, pyruvate efflux was only reduced by 21% (to 369 ± 14 nmol/min per mg), suggesting that pyruvate can be transported against a significant proton gradient (Figure 9B).

DISCUSSION

We have demonstrated for the first time that proton efflux in *T. brucei* bloodstream forms is substantial, and sensitive to inhibitors of pyruvate transport (Table 4). Inhibition of pyruvate efflux and proton efflux by pyruvate analogues in bloodstream forms was highly correlated ($r^2 = 0.83$), supporting the co-transport of protons and pyruvate by the plasma membrane pyruvate carrier in this stage (Table 4). Additionally, inhibition of proton efflux by inhibitors of pyruvate transport led to significant cytosolic acidification, supporting a role for the pyruvate carrier in the regulation of pH_i (Table 3). pH_i , proton efflux and pyruvate efflux were substantially decreased by the H^+ -ATPase inhibitor DCCD, but not by NEM or DES, suggesting that the primary effect of DCCD in bloodstream forms is on the pyruvate carrier and not on the H^+ -ATPase (Table 2; Figures 3, 6 and 9). However, when proton efflux was decreased by the omission of glucose (Figure 6B) or the addition of pyruvate (Figure 6C), a significant additional decrease in proton efflux could be observed on addition of DCCD, or of NEM or DES (results not shown). This suggests the presence of a plasma membrane H^+ -ATPase in bloodstream forms, which plays only a minor role in proton efflux and regulation of pH_i when the pyruvate carrier is active. In procyclic forms, we determined that an H^+ -ATPase is the major regulator of pH_i (Table 2; Figure 3) and proton efflux (Figure 4), based on sensitivity to H^+ -ATPase inhibitors.

We found pyruvate efflux to be inhibited by a number of pyruvate analogues, in close agreement with the results of pyruvate uptake experiments by Weimer et al. [8], but less so with the results of pyruvate efflux experiments by Barnard et al. [9]. We noted slight decreases in pH_i , proton efflux and pyruvate efflux in the presence of 25 mM lactate, whereas Weimer et al. [8]

reported no effect on pyruvate uptake. We also noted no effect with 25 mM citrate, in agreement with Weimer et al. [8], whereas Barnard et al. [9] observed a 29% inhibition of efflux with 1.2 mM citrate. We also noted a significant effect of 2 mM α -CHC, in agreement with Barnard et al. [9] but not with Weimer et al. [8]. Many of the pyruvate analogues are extremely acidic and must be neutralized with base before use, and this may have contributed to the observed differences if this procedure was not carried out in other studies. The marked inhibitory effects of β -hydroxypyruvate and 3-bromopyruvate in bloodstream forms suggest that these analogues may be affecting more than just pyruvate transport. Additionally, β -hydroxypyruvate had significant effects on procyclic forms as well. Furthermore, Barnard et al. [9] found that not only did 3-bromopyruvate compete at the carrier with pyruvate, it was actively transported into the parasite, where it significantly and selectively inhibited trypanosomal glyceraldehyde-3-phosphate dehydrogenase.

We found that pyruvate efflux was significantly inhibited by the H^+ -ATPase inhibitor DCCD and by the anion-carrier blocker H_2 DIDS. Neither of these findings are without precedent, as Barnard et al. [9] noted that 100 μ M DCCD or 100 μ M DIDS reduced pyruvate efflux in bloodstream forms by 22% and 14% respectively, although they felt this decrease to be insignificant. Since H_2 DIDS is a known inhibitor of plasma membrane monocarboxylate transporters and DCCD is known to block H^+ transporters, it is reasonable to believe that these inhibitors could be affecting the pyruvate proton symporter [35,37]. Previous studies in bloodstream forms have demonstrated sensitivity to DCCD and H_2 DIDS, which was interpreted to imply the involvement of an H^+ -ATPase and an anion carrier in the regulation of pH_i [15]. However, it appears that at least some of the observed changes may result from direct inhibition of the pyruvate carrier.

Our calculated rate of pyruvate efflux (499 nmol/min per mg of protein) is considerably higher than previously published values for bloodstream forms (214 nmol/min per mg [9]). However, Barnard et al. [9] performed their experiments at room temperature, and we noted a marked increase in pyruvate efflux at 37 °C compared with that at room temperature (results not shown). Additionally, our calculated rate of proton efflux (534 nmol/min per mg) agrees well with the rate of pyruvate efflux, assuming a 1:1 ratio of pyruvate to protons plus additional efflux from an H^+ -ATPase. Furthermore, Seyfang and Duszenko [4] measured glucose uptake in bloodstream forms at 37 °C of 252 nmol/min per mg of protein, which, if converted into two pyruvate molecules per glucose molecule, would approximate very closely to our values.

Since bloodstream forms rely almost exclusively on glycolysis, drugs that inhibit key components of this pathway potentially have potent trypanocidal activity. However, recent modelling studies have suggested that uncompetitive inhibition of pyruvate export by the pyruvate carrier is the only step in the metabolic pathway of glucose in *T. brucei* bloodstream forms that would increase the concentration of a metabolite sufficiently to interfere with the viability of the organism [38]. Additionally, it appears that the pyruvate carrier in bloodstream forms has a limited affinity for lactate, in contrast with many mammalian plasma membrane monocarboxylate carriers, which transport lactate in preference to pyruvate [39]. Furthermore, Barnard et al. [9] have demonstrated that the pyruvate carrier of *T. brucei* can efficiently transport toxic analogues of pyruvate that affect metabolic processes, as exemplified by the effect of 3-bromopyruvate on glyceraldehyde-3-phosphate dehydrogenase. It should be poss-

ible, therefore, to exploit the differences in affinities between the bloodstream-form pyruvate carrier and the mammalian monocarboxylate transporter in the design of drugs that have a greater affinity for the parasite carrier. These drugs could inhibit pyruvate release directly, resulting in the build-up of pyruvate, acidification of the cytosol and inhibition of glycolysis, or they could be toxic pyruvate analogues that preferentially gain access to the parasite via the carrier and react with a critical metabolic enzyme [9,10,38].

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