Vacuolar-type H⁺-ATPase regulates cytoplasmic pH in *Toxoplasma gondii* tachyzoites

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Cytoplasmic pH (pH_i) regulation was studied in *Toxoplasma gondii* tachyzoites by using the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein. Their mean baseline pH_i (7.07 ± 0.06; *n* = 5) was not significantly affected in the absence of extracellular Na⁺, K⁺ or HCO₃⁻ but was significantly decreased in a dose-dependent manner by low concentrations of *N,N'*-dicyclohexylcarbodi-imide (DCCD), *N*-ethylmaleimide (NEM) or bafilomycin A₁. Bafilomycin A₁ also inhibited the recovery of tachyzoite pH_i after an acid load with sodium propionate. Similar concentrations of DCCD, NEM and bafilomycin A₁ produced depolarization of the plasma membrane potential as measured with bis-(1,3-diethylthiobarbituric)trimethineoxonol (bisoxonol), and DCCD prevented the hyperpolarization that

accompanies acid extrusion after the addition of propionate, in agreement with the electrogenic nature of this pump. Confocal laser scanning microscopy indicated that, in addition to being located in cytoplasmic vacuoles, the vacuolar (V)-H⁺-ATPase of *T. gondii* tachyzoites is also located in the plasma membrane. Surface localization of the V-H⁺-ATPase was confirmed by experiments using biotinylation of cell surface proteins and immunoprecipitation with antibodies against V-H⁺-ATPases. Taken together, the results are consistent with the presence of a functional V-H⁺-ATPase in the plasma membrane of these intracellular parasites and with an important role of this enzyme in the regulation of pH_i homeostasis in these cells.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that resides within the host cells in a compartment termed the parasitophorous vacuole [1]. This vacuole is formed by invagination of the host-cell plasma membrane and resists fusion with other cellular compartments including lysosomes and both early and late endosomes [1–3]. During the first hour of infection of macrophage monolayers by live *T. gondii* tachyzoites, the pH of the parasitophorous vacuole has been shown to undergo a slight decrease (pH 6.8) [4]. However, the parasitophorous vacuole is extensively modified by the parasite at later stages of invasion. Recent evidence suggests the presence of a large pore in the parasitophorous membrane that might allow the free exchange of molecules up to 1.2 kDa in size [5]. Consequently the parasitophorous vacuole probably has a neutral pH, as has the cytoplasm of the host cell, at later stages of invasion. Tachyzoites proliferate inside the parasitophorous vacuole until they are released into the extracellular space and into the bloodstream, where the pH is approx. 7.4, to reach and invade other cells. The ability of the parasite to survive in media with different pH values suggests the presence of cytoplasmic pH (pH_i) regulatory mechanisms. In contrast, it has been found that pH stress (pH 6.8–8.2) results in an increase in cyst-like structures and bradyzoite antigen expression by *T. gondii* tachyzoites *in vitro* [6].

There are at least two families of transporters involved in the regulation of pH_i in eukaryotic cells: Na⁺/H⁺ exchangers, which increase pH_i electroneutrally, and Na⁺-dependent or -independent HCO₃⁻ transporters, which alkalinize or acidify the cytosol respectively [7]. In addition, eukaryotic cells can also express H⁺-ATPases in their plasma membranes that could be involved in the regulation of pH_i. These pumps occur in two forms: an electrogenic H⁺-ATPase and an electroneutral H⁺/K⁺-ATPase.

The plasma membrane H⁺-ATPases present in lower eukaryotes, such as yeast and other fungi [8,9] is described as being of the P-type, whereas that present in several normal [10–14] and tumoral [15] mammalian cells is of the vacuolar (V)-type. The H⁺-ATPase in fungal plasma membrane functions physiologically to hydrolyse ATP and to pump H⁺ out of the cell; the resulting electrochemical H⁺ gradient provides energy for an array of secondary transport systems [16]. The H⁺/K⁺-ATPase, which is also of the P-type, is present mainly in the gastric mucosa and is involved in acid secretion [17]. V-type H⁺-ATPases are also present in various endo- and exo-cytic vesicles, including secretory vesicles, clathrin-coated vesicles, synaptosomes, Golgi, endosomes and lysosomes [18–20]. It is believed that the V-type H⁺-ATPases have a significant role in the maintenance of the acidic environment found in these vesicles. Because a V-type H⁺-ATPase is found in endo- and exo-cytic vesicles, it has been suggested [15] that it is likely to be also present in the plasma membrane of most eukaryotic cells, owing to normal membrane recycling mechanisms.

Here we report studies involving pH_i and plasma membrane potential measurements of *T. gondii* tachyzoites as a function of the ionic composition of the medium, under acid loads and in the presence of different inhibitors, as well as cytochemical studies,

Abbreviations used: AM, acetoxymethyl ester; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; bisoxonol, bis-(1,3-diethylthiobarbituric)trimethineoxonol; DCCD, *N,N'*-dicyclohexylcarbodi-imide; ECL, enhanced chemiluminescence; FCCP, carbonyl dicyanide *p*-(trifluoromethoxy)phenylhydrazine; H₂-DIDS, 4,4'-di-isothiocyanatodihydrostilbene-2,2'-disulphonic acid; [K⁺]_o, extracellular K⁺ concentration; [K⁺]_i, intracellular K⁺ concentration; NEM, *N*-ethylmaleimide; NMG, *N*-methylglucamine; pH_o, extracellular pH; pH_i, cytoplasmic pH; P-type, ATPases that split ATP via a covalent phosphorylation reaction intermediate; SAG-1, surface antigen 1; V-type, vacuolar type.

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to identify the pH_i regulatory mechanisms operating in these cells.

MATERIALS AND METHODS

Culture methods

Tachyzoites of *T. gondii* strain RH were cultivated, by the method of Moreno and Zhong [21], in bovine turbinate cells (ATCC CRL 1390). Host cells were cultivated in tissue culture flasks using minimum essential medium supplemented with 10% horse serum. Cells were infected with tachyzoites at a final host-to-parasite ratio of 1:5; parasites were harvested 2–3 days after infection and purified as described previously [22]. The protein concentration was determined by the biuret assay [23] in the presence of 0.2% deoxycholate.

Chemicals

Minimum essential medium, Dulbecco's PBS, Triton X-100, horse serum, *N,N'*-dicyclohexylcarbodi-imide (DCCD), nigericin, *N*-ethylmaleimide (NEM), *N*-methyl-D-glucamine (NMG), gramicidin, Tween-20, poly(L-lysine) (70 kDa), aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor, PMSF, Nonidet P40 and carbonyl dicyanide *p*-(trifluoromethoxy)phenyl-hydrazine (FCCP) were purchased from Sigma. Bafilomycin A₁ and concanamycin A (folimycin) were from Kamiya Biomedicals (Thousand Oaks, CA, U.S.A.). 2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF/AM), 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonic acid (H_2 -DIDS), bis(1,3-diethylthiobarbituric)trimethineoxonol (bisoxonol) and fluorescein-labelled antibodies were from Molecular Probes (Eugene, OR, U.S.A.). A polyclonal antibody recognizing several subunits from both the catalytic and membrane sectors of the V-type H^+ -ATPase from *Dictyostelium discoideum* [24] was a gift from Dr. Kathleen V. Nolta and Dr. Theodore Steck (University of Chicago, Chicago, IL, U.S.A.). All other reagents were analytical grade. A monoclonal antibody against *T. gondii* surface antigen 1 (SAG-1 or p30) was a gift from Dr. John C. Boothroyd (Stanford University School of Medicine, Stanford, CA, U.S.A.). Biotin succinimidyl ester, avidin-peroxidase conjugate and the enhanced chemiluminescence (ECL) detection kit were from Amersham Life Sciences (Arlington Heights, IL, U.S.A.). The resuspension medium (standard buffer) contained 135 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 5 mM glucose and 10 mM Hepes/Tris, pH 7.4; Na^+ -free medium was prepared by iso-osmotic replacement of NaCl by choline chloride; K^+ -free medium was prepared by iso-osmotic replacement of KCl by choline chloride; Cl^- -free medium contained 135 mM sodium gluconate, 5 mM potassium gluconate, 1 mM MgSO_4 , 1 mM calcium gluconate, 5 mM glucose and 10 mM Hepes/Tris, pH 7.4; K^+ medium for pH_i calibrations contained 130 mM KCl, 10 mM NaCl, 1 mM MgSO_4 , 5 mM glucose and 10 mM Hepes/Tris at different pH values.

Spectrofluorometric determinations

After the cells had been harvested, they were washed twice at 500 g for 10 min at room temperature in buffer A [116 mM NaCl/5.4 mM KCl/0.8 mM MgSO_4 /5.5 mM D-glucose/50 mM Hepes (pH 7.4)] [21]. Cells were resuspended to a final density of 10^9 cells/ml in loading buffer, which consisted of buffer A plus 1.5% (w/v) sucrose and 9 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 buffer A to remove extracellular dye. Cells were resuspended to a final

density of 10^9 cells/ml in buffer A and were kept on ice. Parasites were viable for several hours under these conditions. For fluorescence measurements, a 50 μl aliquot of the cell suspension was diluted into 2.5 ml of buffer A (final density 2×10^7 cells/ml) in a cuvette placed in a thermostatically controlled (37 °C) Hitachi F-2000 spectrofluorimeter. For pH_i measurements the fluorescence ratios of BCECF-loaded cells, with excitation at 505 and 440 nm and emission at 530 nm, were recorded and translated into pH values on the basis of the ratios obtained at various pH values [25]. The relationship of BCECF ratio fluorescence as function of pH_i was determined by incubating the cells for 5 min with the H^+/K^+ ionophore nigericin (5.2 μM) in the presence of K^+ -containing solutions of different pH values [25]. Our calibration solution contained 130 mM KCl and was titrated to values of extracellular pH (pH_e) between 6.5 and 7.5. The pH-fluorescence relationship was used as a calibration curve for estimating pH_i in intact tachyzoites. The ionophore nigericin equilibrates K^+ and H^+ across cell membranes. Therefore when extracellular K^+ concentration ($[\text{K}^+]_o$) approximates intracellular K^+ concentration ($[\text{K}^+]_i$) in the presence of nigericin, pH_i approximates pH_e [25]. Traces shown are representative of at least three independent experiments conducted on separate cell preparations. Variations in the values of pH_i between different experiments with different cell preparations were less than 10%. The average data set for each case was therefore superimposable on the traces of the experiments presented and is not shown for clarity.

Acid loading was accomplished by adding 20 mM sodium or potassium propionate to 5×10^7 cells suspended in 2.5 ml of incubation medium [10].

Membrane potential measurements

Membrane potential was measured fluorimetrically with bisoxonol [26–28]. Bisoxonol (0.2 μM) was added to 5×10^7 cells suspended in 2.5 ml of the indicated medium at 37 °C in a Hitachi F-2000 spectrofluorimeter with excitation at 540 nm and emission at 580 nm. The signal was allowed to equilibrate before any subsequent additions. Calibration was done in cells suspended in medium containing 140 mM NMG-Cl, 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 had been achieved, increasing KCl concentrations were added to the medium, and the fluorescent signals were recorded. Under these conditions the transmembrane potential ($\Delta\Psi$) is given by $\Delta\Psi = 59.4 \log [\text{K}^+]_i/[\text{K}^+]_o$, where $[\text{K}^+]_i$ was taken as 120 mM [26]. It was assumed that $[\text{K}^+]_i$ remained constant during brief cell treatments. The relationship between the fluorescence signal and the calculated $\Delta\Psi$ for the different $[\text{K}^+]_o$ values was nearly linear in the range –20 to –80 mV, and although non-linear beyond that range it could still be used for calibration as indicated previously [26,28]. Variations in the values of $\Delta\Psi$ between different experiments with different cell preparations were less than 10%. The average data set for each case was therefore superimposable on the traces of the experiments presented and is not shown for clarity.

Immunofluorescence microscopy

The parasites were fixed for 30 min at room temperature in a solution containing 4% (w/v) freshly prepared formaldehyde in 0.1 M cacodylate buffer, pH 7.2, washed in Dulbecco's PBS, allowed to adhere for 10 min to coverslips previously coated with 0.1% poly(L-lysine) and incubated for 5 min in the presence of 0.1% (v/v) Triton X-100. Subsequently they were incubated for 30 min in the presence of 50 mM NH_4Cl to block free aldehyde

groups, washed in Dulbecco's PBS, incubated for 3 h in the presence of polyclonal antibodies recognizing the V-H⁺-ATPase (1:50 dilution in Dulbecco's PBS), washed in Dulbecco's PBS/3 % (w/v) BSA and incubated in the presence of fluorescein-labelled goat anti-(rabbit IgG) for 60 min (diluted 1:50 or 1:100 in Dulbecco's PBS). Then the specimens were mounted with *n*-propyl gallate and observed in a Zeiss LSM10 confocal laser scanning microscope equipped with an argon-ion laser for excitation of fluorescein at 488 nm. Emitted fluorescence was detected with an FITC filter set; the corresponding differential-interference contrast images were acquired with transmitted light.

SDS/PAGE and preparation of Western blots

The electrophoretic system used was essentially as described by Laemmli [29]. *T. gondii* tachyzoites (10⁹) were centrifuged at 1000 *g* for 10 min, resuspended in 300 μ l of Dulbecco's PBS containing proteinase inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin and 1 mM PMSF) and frozen at -70 °C. Cells were thawed and homogenized with a Teflon pestle at 4 °C, centrifuged at 17900 *g* for 15 min in an Eppendorf centrifuge (5417R) at 4 °C. Aliquots of the *T. gondii* total homogenates or of the 17900 *g* pellets and supernatants were mixed with a buffer containing 125 mM Tris/HCl, pH 7.0, 10 % (w/v) 2-mercaptoethanol, 20 % (v/v) glycerol and 4.0 % (w/v) SDS, with 4.0 % (w/v) Bromophenol Blue as tracking dye, and were boiled for 5 min before application to SDS/10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose by the method of Towbin et al. [30], with a Bio-Rad (Richmond, CA, U.S.A.) transblot apparatus. Membranes with transferred proteins were placed overnight in a blocking buffer solution of 5 % (w/v) non-fat dried milk in Dulbecco's PBS, pH 7.4. A 1:1000 dilution of rabbit anti-(*D. discoideum* V-type H⁺-ATPase) polyclonal antibodies in blocking buffer was applied at room temperature for 60 min. The nitrocellulose was washed four times in Dulbecco's PBS (washing buffer) for 15 min each before adding a 1:2000 dilution of goat anti-(rabbit IgG) in blocking buffer for 30 min. The nitrocellulose was again washed four times with washing buffer for 15 min each. The ECL detection kit was used as described in the instructions of the manufacturer. The membrane was stripped in accordance with the manufacturer's protocol and re-probed with a 1:1000 dilution of a monoclonal antibody against *T. gondii* SAG-1, then the blot was developed with rabbit anti-mouse antibodies (1:2000) with a similar procedure to that described above.

Cell surface labelling

Cell surface labelling was performed by a modification of the method of Hart et al. [31]. Tachyzoites were washed three times with 10 mM sodium phosphate (pH 8.6)/150 mM NaCl/0.1 mM MgCl₂/0.1 mM CaCl₂ and were then resuspended in 1 ml of the same ice-cold medium (4 $\times$ 10⁶ cells/ml). Biotin succinimidyl ester (in dry dimethylformamide) was added to the cells in accordance with the instructions provided by the manufacturer (Amersham) and incubated for 1 h at 4 °C with rotation. Cells were washed three times with ice-cold Dulbecco's PBS and lysed in 0.5 ml of ice-cold immunoprecipitation buffer [1 % (v/v) Nonidet P40/20 mM Tris/HCl (pH 7.6)/0.1 mM EDTA/1 mM PMSF/2 μ g/ml pepstatin/2 μ g/ml leupeptin/1 μ g/ml soybean trypsin inhibitor]. Cells were kept at 4 °C for 30 min with rotation, after which the lysate was centrifuged for 20 min at 10000 *g*. The supernatant was diluted to 1.5 ml with immunoprecipitation buffer containing 150 mM NaCl, then precipitated with polyclonal anti-(V-H⁺-ATPase) antibodies (1:300) [24].

After incubation for 16 h at 4 °C, immunocomplexes were allowed to bind to Protein A-Sepharose beads by incubation for 1 h at 4 °C. The beads were washed five times with immunoprecipitation buffer; bound proteins were eluted in boiling SDS sample buffer. The proteins were separated on a SDS/10 % (w/v) polyacrylamide gel and blotted on a nitrocellulose membrane as above, which was then probed with avidin-peroxidase conjugate in accordance with the protocol provided by the manufacturer (Amersham). Proteins were detected by ECL (Amersham).

Statistical analysis

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

RESULTS

Effect of Na⁺, K⁺ and Cl⁻ on basal pH_i of tachyzoites

In the nominal absence of bicarbonate and with extracellular pH (pH_e) 7.4 (standard buffer), the mean baseline pH_i of *T. gondii* tachyzoites was 7.07 $\pm$ 0.06 (*n* = 5) (Figure 1A) [21]. This steady-

Figure 1 Effects of Na⁺, K⁺, Cl⁻ and H₂-DIDS on the steady-state pH_i of tachyzoites

(A) The cells (5 $\times$ 10⁷ per 50 μ l of buffer A) were centrifuged in an Eppendorf centrifuge at 2000 *g* for 1 min; the pellet was resuspended in 2.5 ml of standard buffer (control), K⁺-free buffer (-K⁺), Na⁺-free buffer (-Na⁺) or Cl⁻-free buffer (-Cl⁻). (B) The cells were resuspended in standard buffer as indicated above and, where indicated by the arrows, 0 (C), 100, 250 or 500 μ M H₂-DIDS was added. Other experimental conditions are described in the Materials and methods section. Traces are representative of three independent experiments conducted on separate cell preparations. Abbreviation: sec, seconds.

Figure 2 Effect of DCCD and NEM on steady-state pH_i of tachyzoites

The cells were treated as described in the legend to Figure 1(B) and, where indicated by the arrows, 0 (control), 10, 25 or 50 μM DCCD (**A**) or 100 or 200 μM NEM (**B**) was added. Traces are representative of three independent experiments conducted on separate cell preparations. Abbreviation: sec, seconds.

state pH_i level, which was usually attained after approx. 2 min, was maintained in either Na^+ -free (7.01 ± 0.06 , $n = 3$) or K^+ -free (7.02 ± 0.06 , $n = 3$) medium (Figure 1). The absence of a requirement for Na^+ rules out the involvement of a Na^+/H^+ exchanger in steady-state pH_i regulation, and the absence of a requirement for K^+ rules out a similar involvement of a K^+/H^+ ATPase. However, when the cells were suspended in Cl^- -free medium the basal pH_i immediately decreased by 0.2 pH units to 6.8 ± 0.04 ($n = 3$) (Figure 1). If these cells were centrifuged and resuspended in the standard buffer they rapidly recovered towards neutrality and attained the pH_i (7.07 ± 0.06) detected in cells suspended originally in that buffer (results not shown). This supports a role for Cl^- anions in supporting basal pH_i levels, as has been suggested for other unicellular eukaryotes [27,28]. Accordingly, the addition of 100–500 μM $\text{H}_2\text{-DIDS}$, an anion-transport blocker, to *T. gondii* tachyzoites suspended in standard buffer caused a dose-dependent cytoplasmic acidification (Figure 1B).

Effect of inhibitors on basal pH_i

In yeast and fungi [8,9,32] as well as in the protozoan parasite *Trypanosoma cruzi* [27], acid extrusion across the plasma mem-

Figure 3 Effect of bafilomycin A_1 on the steady-state pH_i (**A**) and on pH_i recovery of tachyzoites after acidification by sodium propionate (**B**)

(**A**) The cells were treated as described in the legend to Figure 1(B) and, where indicated by the arrow, 0, 0.1, 0.5 or 1.0 μM bafilomycin A_1 (BAF) was added. (**B**) Where indicated by the arrow, 20 mM sodium propionate and 0 (control), 0.1, 0.5 or 1.0 μM bafilomycin A_1 (BAF) were added to cells suspended in standard buffer. Traces are representative of two independent experiments conducted on separate cell preparations. Abbreviation: sec, seconds.

brane is mediated by H^+ -pumping ATPases. We considered that the Na^+ -independent steady-state pH_i observed in *T. gondii* tachyzoites was mediated by ATP-dependent proton extrusion across the plasma membrane. To verify this hypothesis we tested the effects of NEM and DCCD, two general ATPase inhibitors, on their steady-state pH_i . Figure 2 shows that both compounds decreased pH_i in a dose-dependent manner. Because NEM and DCCD can inhibit both P-type and V-type H^+ -ATPases [11], we tested bafilomycin A_1 , which is a more specific inhibitor of V-type H^+ -ATPases when used at low concentrations [33]. Bafilomycin A_1 caused a dose-dependent decrease in the steady-state pH_i of *T. gondii* tachyzoites (Figure 3A), and was effective at concentrations in the range that inhibits V- H^+ -ATPases (approx. 0.1 μM). To investigate further the role of a V- H^+ -ATPase in pH_i regulation, tachyzoites loaded with BCECF were acid-loaded by the addition of propionic acid, which readily enters the cytoplasm but is excluded from acidic compartments [11]. The addition of 20 mM sodium propionate to tachyzoites suspended in standard buffer at pH_e 7.4 resulted in a 0.2 unit decrease in pH_i and a subsequent recovery from this acid load as illustrated in Figure 3(B) (control). When the same experiment was repeated in the presence of different concentrations of bafilomycin A_1 , acid-loaded cells recovered their initial pH_i more slowly than cells suspended in buffer alone. Similar results were obtained with different concentrations (1–10 μM) of concanamycin A (results not shown).

Figure 4 Calibration of $\Delta\Psi$ in tachyzoites

An aliquot of 2×10^7 cells/ml was equilibrated with $0.1 \mu\text{M}$ bisoxonol in the NMG-Cl medium described in the Materials and methods section. The traces show fluorescence intensities (540 excitation and 580 nm emission) after background subtraction after the addition of gramicidin (G, $0.8 \mu\text{M}$) and KCl to give the final indicated concentrations (in mM). The inset depicts the relationship between fluorescence change (ΔFI) in arbitrary units and the calculated $\Delta\Psi$. The dotted line indicates where the initial value of fluorescence (before the addition of gramicidin) intersects with the calculated value of $\Delta\Psi$. Abbreviation: sec, seconds.

Changes in $\Delta\Psi$ associated with changes in pH_i

The $\Delta\Psi$ -sensitive fluorescent probe bisoxonol was used to investigate whether changes in pH_i were accompanied by changes in $\Delta\Psi$, as expected from the operation of an electrogenic H^+ -ATPase as a main mechanism of pH_i regulation. In normotonic conditions at 37°C , and with a pH_e of 7.4, the steady-state $\Delta\Psi$ estimated from the calibration curve (Figure 4) was $-78.5 \pm 5 \text{ mV}$ ($n = 4$), a value compatible with the $\Delta\Psi$ detected in other eukaryotic cells [34]. The contribution of either ionic K^+ or Na^+ conductances to the $\Delta\Psi$ of tachyzoites was investigated. Figure 5(A) shows that the fluorescence intensity of bisoxonol was not affected by the addition of either potassium gluconate or sodium gluconate to the medium. However, major fluorescence changes occurred when the channel-forming agent gramicidin was added. This suggests that neither K^+ nor Na^+ conductances contributed substantially to $\Delta\Psi$, in agreement with the lack of effect of Na^+ or K^+ on steady-state pH_i (Figure 1). In contrast, if the cells were suspended in a Cl^- -free medium (Figure 5B, $-\text{Cl}^-$), they underwent rapid depolarization and reached a $\Delta\Psi$ value lower than that of cells incubated in standard buffer, but similar to that obtained when the cells in standard buffer were treated with the protonophoric agent FCCP (Figure 5B, FCCP). This depolarization in Cl^- -free medium, which would indicate Cl^- release from the cells, is in agreement with the rapid decrease

in pH_i that occurred in the same medium (Figure 1) and suggests a Cl^- requirement for pump-mediated acid removal.

Acid loading of tachyzoites by a propionate pulse at pH_e 7.4 was followed by hyperpolarization from -78.5 ± 5 to $-96 \pm 8 \text{ mV}$ ($n = 3$), as measured with bisoxonol. To test the possibility that the acidification-induced hyperpolarization represented the electrogenic extrusion of H^+ across the plasma membrane [11], the effect of the H^+ -ATPase inhibitor DCCD on the $\Delta\Psi$ response to acid loading was assessed. DCCD ($50 \mu\text{M}$) effectively inhibited the pH_i recovery from acid loading by the propionate pulse method (results not shown) and also completely inhibited the hyperpolarization that followed acid loading in control cells (Figure 6A). These results indicated that the prevention of H^+ extrusion via the H^+ -ATPase eliminated the hyperpolarization that normally followed acid loading. As occurred with the addition of DCCD, the addition of NEM also produced depolarization of the plasma membrane potential (Figure 6A). In agreement with the operation of an electrogenic V- H^+ -ATPase, the addition of bafilomycin A_1 also produced depolarization of the plasma membrane potential after an initial small hyperpolarization. As occurred in the absence of bafilomycin (Figure 5B), the addition of FCCP after bafilomycin also produced depolarization (Figure 6B). Taken together, these results support the concept that the maintenance of pH_i and pH_i recovery after an acid load in tachyzoites are mediated by an electrogenic V- H^+ -ATPase located in the plasma membrane.

Expression of a V-type H^+ -ATPase in the plasma membrane of tachyzoites

We investigated the localization of the V- H^+ -ATPase of *T. gondii* by immunocytochemistry with polyclonal antibodies against different subunits of *D. discoideum* V- H^+ -ATPase that are known to cross-react with V- H^+ -ATPases of animal cells [24]. The binding of these antibodies to the *T. gondii* tachyzoite, as revealed with fluorescein-labelled secondary antibodies, was to the surface and intracellular vacuoles (Figure 7). No fluorescence was observed in control cells incubated only in the presence of the secondary fluorescein-labelled goat anti-(rabbit IgG) (results not shown).

To confirm the surface localization of the V- H^+ -ATPase in *T. gondii* we labelled tachyzoites with biotin succinimidyl ester, a reagent that couples biotin to lysine residues of exposed proteins. The cells were incubated with the reagent and lysed, then the V- H^+ -ATPase was immunoprecipitated with the polyclonal antibodies [24]. The precipitates were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Biotinylated proteins were detected by peroxidase-conjugated streptavidin and ECL (Figure 8E). A protein band of apparent molecular mass 90 kDa was detected (Figure 8E). A similar 90 kDa band has been detected in *D. discoideum* and apparently corresponds to the large transmembrane subunit of V- H^+ -ATPases [24]. Figure 8(A) shows the immunological cross-reactivity of the polyclonal antibodies directed against the V- H^+ -ATPase with several protein bands present in the whole homogenate (lane 1) and particulate fraction (lane 3) of *T. gondii* tachyzoites but absent from the supernatant (lane 2). No background staining was observed when normal serum was used as a control (Figure 8C). Figure 8(B) shows the reaction of the same preparations with polyclonal antibodies directed against the SAG-1 protein of *T. gondii*, which is known to be located in the plasma membrane [35]. A protein band of apparent molecular mass 30 kDa was detected in the whole homogenate (lane 1) and in the particulate fraction (lane 3) but was absent from the supernatant (lane 2). Figure 8(D) shows Coomassie Blue staining of the same preparations shown

Figure 5 Effect of cations and Cl^- on $\Delta\Psi$

(A) Cells ($2 \times 10^7/\text{ml}$) were suspended in NMG-Cl medium, pH 7.4, previously equilibrated with $0.1 \mu\text{M}$ bisoxonol as described in the legend to Figure 4. After signal stabilization had been achieved, increasing concentrations of either potassium gluconate (KG; final concentrations 10, 20, 30, 40 and 50 mM) or sodium gluconate (NaG; final concentrations 10, 20, 30, 40 and 50 mM) were added and $\Delta\Psi$ was measured as described in the legend to Figure 4. Where indicated, $0.8 \mu\text{M}$ gramicidin (GRA) was added. (B) Cells ($2 \times 10^7/\text{ml}$) were resuspended in standard buffer (lower trace) or in Cl^- -free buffer (upper trace). Where indicated, $1 \mu\text{M}$ FCCP was added. Other conditions were as described in the Materials and methods section. Traces are representative of three independent experiments conducted on separate cell preparations. Abbreviation: sec, seconds.

Figure 6 Effect of DCCD, NEM, propionate, bafilomycin A_1 and FCCP on tachyzoite membrane potential

Cells ($2 \times 10^7/\text{ml}$) were resuspended in standard buffer and, where indicated, $50 \mu\text{M}$ DCCD, $200 \mu\text{M}$ NEM or 20 mM sodium propionate (PRO) (A) or $1 \mu\text{M}$ bafilomycin A_1 (BAF) and $1 \mu\text{M}$ FCCP (B) were added; trace C, control without additions. Traces are representative of three independent experiments conducted on separate cell preparations. Abbreviation: sec, seconds.

Figure 7 Indirect immunofluorescence analysis of the presence of V-H⁺-ATPase in tachyzoites

Bright-field (A) and confocal laser scanning fluorescence microscopy images (B) of tachyzoites. Confocal microscopy shows labelling of the cell surface (large arrows) and in cytoplasmic vesicles (small arrows). The optical section was 0.1 μm . Scale bar, 5 μm .

Figure 8 Specificity of the proton pump antibody and biotinylation of cell surface proteins

(A–D) Tachyzoites were lysed and the proteins were separated by SDS/PAGE; each lane received 10 μg of protein. (D) A strip of the gel stained for total protein with Coomassie Brilliant Blue. (A–C) Immunoblots of the proteins transferred to a membrane and probed with antibodies against *D. discoideum* V-H⁺-ATPase (A) or *T. gondii* SAG-1 protein (B), or with normal serum (C). The migration positions of prestained molecular mass standards (Bio-Rad Labs., Hercules, CA) are indicated (in kDa) at the left: myosin (202 kDa), β -galactosidase (137 kDa), BSA (79 kDa), carbonic anhydrase (42 kDa) and soybean trypsin inhibitor (32 kDa). Lane 1, whole homogenate; lane 2, supernatant fraction; lane 3, particulate fraction. The proton pump antibody recognized *T. gondii* polypeptides with apparent molecular masses of 90, 159 and 190 kDa (A), whereas the antibody against *T. gondii* SAG-1 recognized a protein with an apparent molecular mass of 30 kDa. (E) Tachyzoites were incubated with biotin succinimidyl ester. After lysis of the cells, V-H⁺-ATPases were immunoprecipitated by the polyclonal antibody, and the immunoprecipitate was subjected to Western blot analysis. Detection of biotinylation was by streptavidin–peroxidase conjugate and ECL. A band of apparent molecular mass of 90 kDa was detected.

in Figures 8(A) and 8(B). Figure 8(E) shows that the band of biotinylated protein immunoprecipitated with the anti-(V-H⁺-ATPase) antibodies clearly corresponds to one of the bands shown in Figure 8(A). The higher-molecular-mass bands of approx. 159 and 190 kDa shown in Figure 8(A) could be formed by the aggregation of several subunits [36]. Taken together these results confirm the surface localization of the V-H⁺-ATPase.

DISCUSSION

Several lines of evidence support the concept that the maintenance of steady-state pH_i and proton extrusion by acid-loaded *T. gondii* tachyzoites are mainly mediated by a V-type H⁺-ATPase located in the plasma membrane. The most compelling argument is derived from the potent acidifying effect of low concentrations

of DCCD, NEM (Figure 2) and bafilomycin A₁ (Figure 3A) on pH_i and the inhibitory effect of bafilomycin A₁ on the recovery of tachyzoite pH_i after an acid load (Figure 3B). Similar concentrations of DCCD, NEM and bafilomycin A₁ produced depolarization of the plasma membrane potential (Figure 6), and DCCD prevented the hyperpolarization that accompanies acid extrusion after the addition of propionate (Figure 6A), in agreement with the electrogenic nature of this pump. The results also indicate that there are no cation channels present to provide diffusion potential when the pumps fail or are blocked. In addition, using confocal laser scanning microscopy of tachyzoites probed with previously well-characterized antibodies that recognize different subunits of V-H⁺-ATPases [24], we showed the presence of this enzyme in the plasma membrane and in intracellular vacuoles of tachyzoites (Figure 7). The plasma

membrane localization of this enzyme was confirmed by biotin labelling of the plasma membrane (Figure 8).

The lack of effect of the absence of extracellular Na^+ or K^+ on the maintenance of the steady-state pH_i (Figure 1) or in the tachyzoite plasma membrane potential ruled out the possibility that these processes are mediated by a Na^+/H^+ antiporter or a K^+/H^+ -ATPase. As has been shown to occur in *Leishmania major* [28] and *T. cruzi* [27], Cl^- has a role in the maintenance of intracellular pH_i , because in the absence of Cl^- the steady-state pH_i was decreased significantly (Figure 1A) and the anion-transport blocker $\text{H}_2\text{-DIDS}$ produced a dose-dependent intracellular acidification (Figure 1B). As discussed previously [9,28], the activity of the proton pump seems to require electrogenic Cl^- movement for maintaining electroneutrality. Accordingly, in the absence of extracellular Cl^- depolarization of the plasma membrane potential occurred (Figure 5B). This can be explained by Cl^- release from tachyzoites.

The activity of V-H^+ -ATPases has been suggested to be regulated via the oxidation of critical thiol residues [37,38] and by association by adaptins [39]. It has been postulated that when V-H^+ -ATPases are found in the reducing environment of the cytoplasm they are expected to be active, whereas when present in the putative oxidizing environment of many plasma membranes they would be inactive [40]. If this is so, this regulation is likely to occur at the vacuolar/extracellular domains of the enzyme when it is embedded either in the vacuolar membrane or in the plasma membrane respectively, because the catalytic domain of the V-H^+ -ATPase must be at least proximal to the cytoplasm, where the ATP is. Our results are in agreement with this hypothesis. Because *T. gondii* tachyzoites are in contact with the cytoplasm of the cells through the parasitophorous membrane [5] they should behave as 'intracellular organelles' and contain a functional plasma membrane-located V-H^+ -ATPase.

In summary, *T. gondii* tachyzoites maintain their steady-state pH_i by means of a Na^+ -independent process. The pharmacological profile of this mechanism suggests the operation of a H^+ pump. These results are in agreement with the detection of a V-type H^+ -ATPase in the plasma membrane of tachyzoites by immunofluorescence microscopy, immunoblotting and biotin labelling of the plasma membrane. It is conceivable that this pH_i regulatory mechanism is used by tachyzoites to ensure their survival in the slightly acidic environment of the parasitophorous vacuole on invasion [4] or at later stages when the host cells are full of metabolically active acid-producing tachyzoites (i.e. when host cell rupture is imminent). In this regard it has been pointed out that tachyzoites rely heavily on glycolysis for energy production and some 20% of the glucose catabolized by them is converted to lactate under aerobic conditions [41]. The low pH that would prevail in this environment would tend to inhibit a Na^+/H^+ exchanger by competition between Na^+ and H^+ at the external transport site and would decrease the concentration of HCO_3^- necessary for the operation of a $\text{HCO}_3^-/\text{Cl}^-$ exchanger. Under these conditions, proton pumping should be essential for the maintenance of pH_i .

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