Na⁺-dependent pH Regulation by the Amitochondriate Protozoan Parasite * Giardia intestinalis*

Giancarlo A. Biagini‡§§, Leigh A. Knodler‡§, Kevin J. Saliba§, Kiaran Kirk§, and Michael R. Edwards‡

From the ‡School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney 2052, Australia and the §School of Biochemistry and Molecular Biology, Faculty of Science, Australian National University, Canberra 0200, Australia

* Giardia intestinalis is a pathogenic fermentative parasite, which inhabits the gastrointestinal tract of animals and humans. G. intestinalis trophozoites are exposed to acidic fluctuations in vivo and must also cope with acidic metabolic endproducts. In this study, a combination of independent techniques (³¹P NMR spectroscopy, distribution of the weak acid pH marker 5,5-dimethyl-2,4-oxazolidinedione (DMO) and the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF)) were used to show that G. intestinalis trophozoites exposed to an extracellular pH range of 6.9–7.5 maintain their cytosolic pH (pHi) within the range 6.7–7.1. Maintenance of the resting pHi was Na⁺-dependent but unaffected by amiloride (or analogs thereof). Recovery of pHi, from an intracellular acidosis was also Na⁺-dependent, with the rate of recovery varying with the extracellular Na⁺ concentration in a saturable manner ($K_m = 18 \text{ mm}$; $V_{max} = 10 \text{ mU H}^+ \text{ min}^{-1}$). The recovery of pHi from an acid load was inhibited by amiloride but unaffected by a number of its analogs. The postulated involvement of one or more Na⁺/H⁺ exchanger(s) in the regulation of pHi in G. intestinalis is discussed.

† To whom correspondence should be addressed: University of Cambridge, Dept. of Pharmacology, Tennis Crt. Rd., Cambridge CB2 1QJ UK. Tel.: 44 (0) 1223 33402; Fax: 44 (0) 1223 334040; E-mail: Giancarlobiagini@hotmail.com.

‡ Present address: Biotechnology Lab., Rm. 237 Wesbrook Bldg., 6174 University Blvd., University of British Columbia, Vancouver, BC, V6T 1Z3, Canada.

Received for publication, March 27, 2001
Published, JBC Papers in Press, April 10, 2001, DOI 10.1074/jbc.M102728200

Na⁺-dependent pH Regulation by the Amitochondriate Protozoan Parasite * Giardia intestinalis*

* This work was supported by the Australian Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The abbreviations used are: pHi, cytosolic pH; DMO, 5,5-dimethyl-2,4-oxazolidinedione; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; NHE, Na⁺/H⁺ exchanger.

1 The abbreviations used are: pH, cytosolic pH; DMO, 5,5-dimethyl-2,4-oxazolidinedione; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; NHE, Na⁺/H⁺ exchanger.
urements of pH$_i$, allowing the effect of additions of ions and inhibitors to be determined in real time (15).

The aim of the present study was to use a combination of the techniques outlined above to estimate the resting pH of *G. intestinalis* trophozoites (over a range of extracellular pHs), and to investigate the mechanism(s) involved in extruding H$^+$ from the cell, thereby protecting it from intracellular acidosis.

**EXPERIMENTAL PROCEDURES**

**Organism and Culture of *G. intestinalis*—**Portland 1 strain was grown as described previously (16). For each experiment, cells were grown to stationary phase, harvested by centrifugation at 650 × g for 5 min and resuspended in the appropriate buffers. Cells were counted using a hemocytometer.

**Solutions—**Phosphate-buffered saline (PBS, pH 7.2) contained 150 mM NaCl, 5 mM K$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, Na$^+$ - and K$^+$-free medium (NGM-CI solution) contained 140 mM N-methyl-D-glucamine, 0.8 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM HEPES, and 11 mM glucose, and was pH adjusted to 7.4 with HCl. The MES- and HEPES-buffered saline (pH 7.0) used in the $^{31}$P NMR experiments contained 140 mM NaCl, 5 mM MES, 5 mM HEPES, and 1 mM EDTA in 10% (v/v) D$_2$O. The MES- and HEPES-buffered saline, used in the [2$^−$14C] DMO experiments, contained 140 mM NaCl, 20 mM MES, and 20 mM HEPES, with the pH adjusted to range 6.0–7.5. The high K$^+$ medium used in stimulating the BCECF pH$_i$ measurements (see below) contained 120 mM KCl and 20 mM HEPES and was adjusted to pH 6.2, 6.5, 6.8, 7.0, and 7.4 using HCl or NaOH.

**Determination of pH$_i$ by $^{31}$P NMR—**The method adopted for $^{31}$P NMR analysis of *G. intestinalis* trophozoites was based on that described previously (17). Approximately 10$^5$ trophozoites were harvested from 900 ml of culture medium ($60 \times 15$ ml tubes) washed in PBS, and then resuspended in the appropriate MES- and HEPES-buffered saline (1.5 ml). An aliquot of this suspension, containing ~5 × 10$^5$ cells, was transferred to an insert tube (5-mm outer diameter), which was placed inside a 10-mm outer diameter NMR tube for acquisition of spectra. $^{31}$P NMR spectra were obtained at (37 °C) on a Bruker AM-300 spectrometer operating in the Fourier transform mode. Spectra (averaged over 512 scans) were collected using a pulse angle of 60° and a repetition rate of 0.85 s. Chemical shifts are expressed relative to 85% phosphoric acid but were measured against a primary standard of 0.6 M TEP (tris(2-carboxyethyl)phosphine, Ref. 18), contained in a 1-mm internal diameter capillary. A standard titration curve, relating the $^{31}$P NMR chemical shift of P$_i$ to pH was obtained from MES- and HEPES-buffered saline supplemented with either 100 mM NaCl, 20 mM MES, and 20 mM HEPES, and with the pH adjusted to range 6.5–7.5. The high K$^+$ medium used in stimulating the BCECF pH$_i$ measurements (see below) contained 120 mM KCl and 20 mM HEPES and was adjusted to pH 6.2, 6.5, 6.8, 7.0, and 7.4 using HCl or NaOH.

**Determination of pH$_i$, by [2$^−$14C] DMO Distribution—**$G. intestinalis$ trophozoites were harvested by centrifugation and resuspended in MES-containing saline (15) at a density of ~6 × 10$^5$ cells ml$^{-1}$. An aliquot (100 μl) of the equilibrated cell suspension was transferred, in triplicate, to microcentrifuge tubes containing 100 μl of MES- and HEPES-buffered saline at the same pH containing [2$^−$14C] DMO (0.05 μCi) together with unlabeled DMO (to give a final DMO concentration of 1–100 μM) and layered over 100 μl of oil (a mixture of dibutyl phthalate and di-iso-octyl phthalate, 4:1, 1.03 g ml$^{-1}$). The suspension was transferred to a cuvette that was placed in the temperature-controlled chamber of a Perkin Elmer LS-50B luminescence spectrophotometer, maintained at 37 °C. Using a dual excitation microscope apparatus, the fluorescence signal was excited at 465 nm and 440 nm successively, and the fluorescence emission was measured at 535 nm. The ratio of the fluorescence intensity measured using the two excitation wavelengths (495 nm/440 nm) provides a quantitative measure of pH. The luminescence spectrophotometer was linked to a computer, allowing real-time monitoring of pH$_i$. Data was imported into graphics software for analysis. Calibration of the fluorescence signal was performed using nigericin, as described previously for Leishmania major and mammalian tumor cells (19, 20). Linear regression of 3–5 point calibration curves (pH versus the fluorescence intensity ratio) consistently yielded regression coefficients of >0.99. The pH$_i$ traces shown in the results are, in each case, representative of at least three separate experiments.

**Intracellular Buffering Power and H$^+$ Efflux Determination—**The intracellular buffering power of *G. intestinalis* was calculated from the pH$_i$ changes resulting from the addition of a range of concentrations of NH$_4$Cl (16). The buffering power (β, μmol/mmol) is given by β = [NH$_3$]/[ΔpH$_i$, where the intracellular NH$_4^+$ concentration following the addition of NH$_4$Cl, calculated using a pK of 9.21 (in the Henderson-Hasselbach equation) and assuming that [NH$_3$]$_o$ is equal to [NH$_3$]$_i$. ΔpH$_i$ is given for changes in the intracellular pH ranging from 7.0 to 6.2. It was assumed that the buffering capacity of the cell remained approximately constant through the range of 6.2–6.8, as shown for mammalian cells (16).

**Intracellular Buffering Power and H$^+$ Efflux Determination—**The intracellular buffering power of *G. intestinalis* was calculated from the pH$_i$ changes resulting from the addition of a range of concentrations of NH$_4$Cl (16). The buffering power (β, μmol/mmol) is given by β = [NH$_3$]/[ΔpH$_i$, where the intracellular NH$_4^+$ concentration following the addition of NH$_4$Cl, calculated using a pK of 9.21 (in the Henderson-Hasselbach equation) and assuming that [NH$_3$]$_o$ is equal to [NH$_3$]$_i$. ΔpH$_i$ is given for changes in the intracellular pH ranging from 7.0 to 6.2. It was assumed that the buffering capacity of the cell remained approximately constant through the range of 6.2–6.8, as shown for mammalian cells (16).

**Initial rates of pH$_i$ recovery ([ΔpH$_i$/Δt]) for cells suspended in medium of varying Na$^+$ concentration (NGM-CI solution with added NaCl 5–50 mM) and acidified using the NH$_4$Cl prepulse technique were calculated by fitting the initial phase of the pH$_i$ recovery trace to a straight line, using linear regression. The slope of the line, ΔpH$_i$/Δt, was converted to μmol/mmol·min$^{-1}$ using the equation $J_{NH_4} = \beta \times \Delta$H/Dt. The graph of $J_{NH_4}$ versus [Na$^+$] was fitted to the Michaelis-Menten equation using the Enzyme Kinetics 1.11 computer program (Trinity Software).

**Inhibitors—**A number of transport inhibitors were tested for their effect on both the resting pH$_i$ and the recovery of pH$_i$ following an imposed intracellular acid load. These included amiloride (1 mM), hexamethonium (10 mM), 2,4 diaminopyrimidine (2,4 DAP, 50 μM), valinomycin A$_1$ (100 mM), N,N-dicyclohexylcarbodiimide (DCCD, 100 μM), and N-ethylmaleimide (NEM, 200 μM). All experiments, appropriate solvent (ethanol or dimethyl sulfoxide) controls were included (<0.2% v/v).

The valinomycin A$_1$ and valinomycin A$_1$ stocks used in this study were shown to be active in control experiments in which they were tested for their effect on the intracellular pH of the malaria parasite *Plasmodium falciparum* (12) and the yeast *Saccharomyces cerevisiae* (7), respectively (not shown).

**RESULTS**

**31P NMR Studies—**The $^{31}$P NMR spectrum of *G. intestinalis* trophozoites suspended in MES- and HEPES-buffered saline is shown in Fig. 1. At an extracellular pH of 6.5 (the reduction of pH$_i$ from an initial value of 7.0 to 6.5 was because of the generation of acidic metabolic products), there were two P$_i$ resonance peaks, indicating a significant difference between the intracellular and extracellular pH. One attributable to sugar phosphates (2 to 6 ppm) and nucleotides (−2 to 8 ppm) were observed. The largest peak corresponds to the internal standard, TEP. The pH$_i$ estimated from the chemical shift of the intracellular P$_i$ resonance (2.28 ppm) to be 6.7 ± 0.05 (n = 3).

Following the acquisition of the initial spectrum, the suspension was supplemented with either 25 mM arginine, 25 mM...
glucose, or no metabolic substrate, and the extracellular and intracellular pH were monitored. In all cases, pH\textsubscript{i} fell. The total changes in pH\textsubscript{o}, (ΔpH\textsubscript{o}) were 0.52, 0.79, and 0.78 over 60 min in the presence of 25 mM arginine, 25 mM glucose, or no metabolic substrate, respectively (single measurements). As pH\textsubscript{o} fell, pH\textsubscript{i} also decreased, though not to the same extent, reaching a minimum value of 6.4 at an extracellular pH of 5.4 (Fig. 2, open circles).

The generation of acidic metabolic products by the cells confirms their biochemical viability. The ability of the parasites to limit the fall in pH\textsubscript{i} and maintain a substantial transmembrane pH gradient in an acidic medium illustrates their ability to regulate pH\textsubscript{o}.

Determination of pH\textsubscript{o} from [2-14C]DMO Distribution—On addition of [2-14C]DMO to \textit{G. intestinalis} trophozoites suspended in MES- and HEPES-buffered saline, the radiolabel distributed between the intra- and extracellular compartments at a distribution ratio ([DMO]/[DMO]\textsubscript{o}) that was independent of the total DMO concentration over the range 1–100 μM (not shown).

The pH\textsubscript{i} of \textit{G. intestinalis} trophozoites suspended in medium having a pH\textsubscript{o} range of 6.0–7.5 was calculated from the DMO distribution ratio measured at a DMO concentration of 10 μM (Fig. 2, closed squares). The cells maintained a pH\textsubscript{i} between 6.7 and 7.0 over a pH\textsubscript{o} range of 6.0–7.5. The pH\textsubscript{i} estimated using DMO for the pH\textsubscript{o} range 6.0–6.5 was in close agreement with that estimated using 31P NMR (Fig. 2). Decreasing the temperature from 37 to 25 °C had no significant effect on pH\textsubscript{i}, which was maintained at 6.7–6.8 over a pH\textsubscript{o} range of 6.0–7.5 (data not shown).

Determination of pH\textsubscript{o} from BCECF Fluorescence—The pH\textsubscript{i} of \textit{G. intestinalis} trophozoites in PBS (pH\textsubscript{o} = 7.2) estimated using the pH-sensitive fluorescent indicator BCECF was in the range 6.7–7.1 with a mean value of 6.95 ± 0.10 (n = 24). As pH\textsubscript{o} was varied over the range 5.4–7.7, the pH\textsubscript{i} estimated using BCECF varied from 6.3–7.2, correlating well with the estimates made using the 31P NMR and DMO methods (Fig. 2). The fluorescence method proved to be the most straightforward of the three methods tested, allowing on-line monitoring of pH\textsubscript{i} and it was therefore adopted as the method of choice for the further characterization of the pH\textsubscript{i} regulatory mechanisms in these cells.

Na\textsuperscript{+}-dependence of pH\textsubscript{i} Regulation—When \textit{G. intestinalis} trophozoites in PBS (pH\textsubscript{o} = 7.2) were transferred to a Na\textsuperscript{+}- and K\textsuperscript{+}-free medium (NMG-Cl solution) there was a rapid intracellular acidification (Fig. 3A). On addition of NaCl (30 mM) to the medium, the pH\textsubscript{i} recovered.

When cells subjected to an intracellular acid load using the NH\textsubscript{4}Cl prepulse technique were resuspended in NMG-Cl solution there was no recovery of pH\textsubscript{i} (Fig. 3B). Under these conditions, addition of KCl (30 mM) had no effect. By contrast, addition of NaCl (30 mM) resulted in a recovery of pH\textsubscript{i} to its normal resting value.

To quantify the rate of extrusion of H\textsuperscript{+} from \textit{G. intestinalis} trophozoites following the acid-loading procedure it was necessary to measure the intracellular buffering power (β). This was determined to be 32.4 ± 0.8 mM pH\textsuperscript{-1} (n = 3, at pH\textsubscript{o} 6.2–6.3). From this value the rate of pH recovery from an acid load (ΔpH/Δt) could be converted to the rate of extrusion of H\textsuperscript{+} equivalents (see “Experimental Procedures”).

Fig. 4 shows the recovery of pH\textsubscript{i} in \textit{G. intestinalis} trophozoites subjected to an intracellular acidification (via an NH\textsubscript{4}Cl prepulse) then resuspended in medium having a range of Na\textsuperscript{+} concentrations. The rate of pH\textsubscript{i} recovery increased with increasing extracellular Na\textsuperscript{+}. The initial phase of the pH\textsubscript{i} trace shown in Fig. 4A was fitted to a straight line, the slope of which (ΔpH/Δt) was converted to a H\textsuperscript{+} equivalent efflux rate (JH\textsuperscript{+} = β × ΔpH/Δt). JH\textsuperscript{+} was a saturable function of [Na\textsuperscript{+}]\textsubscript{o}, well described by the Michaelis-Menten expression with a K\textsubscript{m} \textsuperscript{+} and V\textsubscript{max} \textsuperscript{+} of 18 ± 7 mM Na\textsuperscript{+} and 10 ± 2 mM H\textsuperscript{+} min\textsuperscript{-1}, respectively (Fig. 4B).

Effect of Inhibitors on pH\textsubscript{i} Regulation—The Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibitor amiloride (up to 2 mM) and its analogues EIPA and HMA (up to 200 μM) had no effect on the resting pH\textsubscript{i} of \textit{G. intestinalis} trophozoites suspended in PBS. As shown in Fig. 5A, the addition of 1 mM amiloride did cause a small, instantaneous decrease in the BCECF fluorescence ratio. However an equivalent decrease was observed when the experiment was repeated for cells killed by heat-fixation (incubation at 60 °C for 15 min) and can therefore be attributed to an optical effect of amiloride rather than an effect on pH\textsubscript{i}. The fluorescence ratio was unaffected by both EIPA and HMA (each at 200 μM).

In contrast to the lack of effect of amiloride on the resting
up to an hour, the pH_{o} decreased significantly and to a greater extent than the pH_{i}. The decrease in pH_{o} was larger for cells to trophozoites, resulted in a slight decrease in the NH_{4} shown by three different techniques to maintain a resting pH in vivo the range 6.0–7.5 (similar to those experienced Leishmania major Ref. 23), without effect on either the resting pH_{i} or the recovery of pH_{i} m.

In suspensions of G. intestinalis the dynamic environment of its host. In the case of G. intestinalis trophozoites, they must withstand exposure to a wide range of pH ranges as found in the gastrointestinal tract. In those parasite; e.g. Trypanosoma brucei promastigotes (pH_{i} 7.0–7.2, Ref. 22), Trypanosoma cruzi epimastigotes (pH_{i} 7.2, Ref. 23), Leishmania major promastigotes (pH_{i} 6.75, Ref. 19), and P. falciparum trophozoites (pH_{i} 7.3, Refs. 12, 24).

In suspensions of G. intestinalis trophozoites incubated for up to an hour, the pH_{i} decreased significantly and to a greater extent than the pH_{i}. The decrease in pH_{i} was larger for cells to which either glucose (ΔpH_{i} = 0.79) or no substrate (ΔpH_{i} = 0.78) were added than for cells to which arginine was added (ΔpH_{i} = 0.52). In the absence of exogenous substrate, it is likely that the trophozoites were reliant on endogenous glucose stores (e.g. glycogen, Ref. 25) and that the metabolism was therefore essentially the same as that occurring in cells supplemented with glucose. Glucose metabolism in G. intestinalis results in the production of the acidic end-product acetic acid (26, 27), whereas arginine catabolism generates ammonia and ornithine, alkaline and neutral products, respectively (6). The pronounced extracellular acidification observed in suspensions in which the cells were utilizing glucose is likely to be due primarily to the production of acetic acid whereas the lesser acidification observed in cells supplemented with arginine probably represents the combined effects of arginine and glucose (glycogen) metabolism.

The buffering capacity of G. intestinalis was estimated to be 32 mM pH^{-1} (at pH 6.2–6.3). This value is in the range reported for other cell types (21) and very close to the value estimated for the protozoan P. falciparum (35 mM pH^{-1}, 24)).

Maintenance of a steady-state pH_{i} in G. intestinalis was shown to be Na^+-dependent with pH_{i} declining sharply in cells suspended in Na^+-free medium (Fig. 3A). The recovery of pH_{i} in cells subjected to an intracellular acid load was also Na^+-dependent (Fig. 3B). Maintenance of the resting pH_{i} and recovery from an acid load were both inhibited by the carboxyl-blocking agent DCCD and unaffected by the two amiloride analogs, EIPA and HMA. However, amiloride itself was found to 1 mM amiloride inhibited the pH_{i} recovery following an imposed intracellular acidification (Fig. 5B). EIPA (50 μM) and HMA (50 μM) were both without effect (not shown).

Addition of the carboxyl-blocking agent DCCD (100 μM), to G. intestinalis trophozoites, resulted in a slight decrease in the resting pH_{i} (of between 0.1–0.2 pH units; Fig. 6A). DCCD also inhibited pH_{i} recovery from imposed intracellular acidification (Fig. 6B). The P-type H^+-ATPase inhibitor vanadate (up to 200 μM), the V-type ATPase inhibitor bafilomycin A_{1} (up to 200 nM), and the general ATPase inhibitor NEM (200 μM) were all without effect on either the resting pH_{i} or the recovery of pH_{i} from an imposed intracellular acidosis (not shown).

**DISCUSSION**

Central to the parasite pathogenicity is its ability to survive the dynamic environment of its host. In the case of G. intestinalis trophozoites, they must withstand exposure to a wide range of pH ranges as found in the gastrointestinal tract. In this study G. intestinalis trophozoites exposed to pH_{i} values in the range 6.0–7.5 (similar to those experienced in vivo) were shown by three different techniques to maintain a resting pH_{i} of 6.7–7.1. Similar values for pH_{i} have been reported for other parasitic protozoa; e.g. Trypanosoma brucei epimastigotes (pH_{i} 7.0–7.2, Ref. 22), Trypanosoma cruzi epimastigotes (pH_{i} 7.2, Ref. 23), Leishmania major promastigotes (pH_{i} 6.75, Ref. 19), and P. falciparum trophozoites (pH_{i} 7.3, Refs. 12, 24).

In suspensions of G. intestinalis trophozoites incubated for up to an hour, the pH_{i} decreased significantly and to a greater extent than the pH_{i}. The decrease in pH_{i} was larger for cells to which either glucose (ΔpH_{i} = 0.79) or no substrate (ΔpH_{i} = 0.78) were added than for cells to which arginine was added (ΔpH_{i} = 0.52). In the absence of exogenous substrate, it is likely that the trophozoites were reliant on endogenous glucose stores (e.g. glycogen, Ref. 25) and that the metabolism was therefore essentially the same as that occurring in cells supplemented with glucose. Glucose metabolism in G. intestinalis results in the production of the acidic end-product acetic acid (26, 27), whereas arginine catabolism generates ammonia and ornithine, alkaline and neutral products, respectively (6). The pronounced extracellular acidification observed in suspensions in which the cells were utilizing glucose is likely to be due primarily to the production of acetic acid whereas the lesser acidification observed in cells supplemented with arginine probably represents the combined effects of arginine and glucose (glycogen) metabolism.

The buffering capacity of G. intestinalis was estimated to be 32 mM pH^{-1} (at pH 6.2–6.3). This value is in the range reported for other cell types (21) and very close to the value estimated for the protozoan P. falciparum (35 mM pH^{-1}, 24)).

Maintenance of a steady-state pH_{i} in G. intestinalis was shown to be Na^+-dependent with pH_{i} declining sharply in cells suspended in Na^+-free medium (Fig. 3A). The recovery of pH_{i} in cells subjected to an intracellular acid load was also Na^+-dependent (Fig. 3B). Maintenance of the resting pH_{i} and recovery from an acid load were both inhibited by the carboxyl-blocking agent DCCD and unaffected by the two amiloride analogs, EIPA and HMA. However, amiloride itself was found...
to inhibit the recovery of pH from an imposed intracellular acidification whereas having no significant effect on the ability of the parasite to maintain its normal resting pH. Amiloride, an inhibitor of Na⁺/H⁺ exchangers in a wide range of cell types is not highly selective and is known to affect a range of biological processes. Nevertheless, the finding that the recovery of pH from an intracellular acidification, observed in the nominal absence of HCO₃⁻ from the medium, is both Na⁺-dependent and amiloride-sensitive is at least consistent with the pathway involved being a type of Na⁺/H⁺ exchanger. The flux of H⁺ via this pathway was a saturable function of the extracellular Na⁺ concentration, with an apparent Kₘ for Na⁺ of 18 mM. This value is comparable with that reported for Na⁺/H⁺ exchanger-mediated H⁺ extrusion from vertebrate cells: e.g. thymic lymphocytes (59 mM, 28), apical membrane cells of amphibian gall bladder (11 mM, Ref. 29) and dog kidney cells (42 mM, Ref. 30).

The finding that the resting, steady-state pH is unaffected by amiloride would suggest that the maintenance of a normal resting pH is achieved via a H⁺-extrusion mechanism that differs from that which plays the major role in the efflux of H⁺ following an intracellular acidification. Although amiloride-insensitive, this pathway is Na⁺-dependent and DCCD-sensitive. The available data are consistent with it being an amiloride-insensitive Na⁺/H⁺ exchanger. Higher eukaryotes are known to have number of Na⁺/H⁺ exchanger isoforms (the NHE family; Ref. 31) and it is not uncommon for vertebrate tissues to express more than one Na⁺/H⁺ isoform, having different Na⁺ affinities, pH dependences and amiloride sensitivities (e.g. NHE1 is known to be sensitive to amiloride whereas NHE3 is resistant to amiloride and amiloride analogues, Ref. 31). Lower eukaryotes, such as S. cerevisiae, also contain more than one putative Na⁺/H⁺ exchanger (e.g. Ref. 32) and although the pharmacology of these different transporters has not been fully elucidated, differences between isoforms might be expected. The possibility of there being more than one Na⁺/H⁺ exchanger serving somewhat different functions in G. intestinalis is therefore not unprecedented.

In some protozoa, the major H⁺ efflux mechanisms are thought to be electrogenic H⁺ pumps (e.g. Refs. 10–12). However, the pronounced Na⁺-dependence of H⁺ extrusion from G. intestinalis trophozoites, together with the lack of significant inhibitory effect of the H⁺-pump inhibitors NEM (a general H⁺ pump inhibitor), baflomycin A₁ (a specific V-type H⁺ pump inhibitor), or vanadate (a specific P-type H⁺ pump inhibitor) on either the resting pH or the extrusion of H⁺ following an intracellular acidification would suggest that such pumps do not play a significant role in pH regulation in G. intestinalis trophozoites.

DCCD is perhaps best characterized as an inhibitor of electrogenic H⁺ pumps and an effect of DCCD on pH is often taken as being indicative of the involvement of this class of proteins in the pH regulation. However, DCCD is a general carboxyl blocking agent, which has been shown to inhibit Na⁺/H⁺ exchangers of higher organisms (e.g. Refs. 33, 34). The effect of DCCD on both the maintenance of the normal resting pH and the recovery from an intracellular acid load is therefore consistent with the postulated involvement of Na⁺/H⁺ exchangers in both processes.

There are, to our knowledge, two previous reports of the presence of a Na⁺/H⁺ exchanger in parasitic protozoa. It has been proposed that in P. falciparum trophozoites a Na⁺/H⁺ exchanger serves as the major pathway for the extrusion of H⁺. The apparent Kₘ for Na⁺ in this system was estimated to be ~6 mM; however, this analysis was complicated by the presence of both a saturable and a linear transport component (24) and, in any case, the role of a Na⁺/H⁺ exchanger in pH regulation in this organism has recently been questioned (12). Leishmania promastigotes have also been proposed to possess an Na⁺/H⁺
exchanger involved in pH control (35); however, this too has been disputed (36).

In summary, the results of the present study demonstrate the presence in *G. intestinalis* trophozoites of active H⁺ extrusion mechanisms that act to maintain the pH₁ within an appropriate range. Available evidence indicates the presence of at least two Na⁺-dependent H⁺ extrusion mechanisms. One is insensitive to amiloride and serves a housekeeping role in maintaining the normal resting pH₁ (in the face of a constant metabolic acid-load). The other is amiloride-sensitive and is activated in response to a cytosolic acidification. The two pathways are postulated to be members of the Na⁺/H⁺ exchanger family.

Na⁺/H⁺ exchangers are ubiquitous from higher eukaryotes to bacteria (31). In higher eukaryotic cells the stoichiometry of the Na⁺/H⁺ exchange is 1:1 (37) whereas in lower eukaryotes and bacteria Na⁺/H⁺ antiporters operate with a Na⁺/H⁺ ratio of 1:2 (38). The biochemical characteristics of *Giardia* are often regarded as being a hybrid of those of eukaryotes and prokaryotes (6), with the antiquity of this organism being the focus of much interest (39). Further characterization of the Na⁺-dependent H⁺ extrusion mechanisms shown in the present study to operate in *Giardia* trophozoites, and the identification of the proteins involved may therefore be of significant interest in terms of the evolution of Na⁺/H⁺ exchangers.

Acknowledgment—We thank George Grossman for his kind assistance with NMR spectroscopy.

REFERENCES

1. Farthing, M. J. G. (1984) in *Giardia: From Molecules to Disease* (Thompson, R. C. A., Reynolds, J. A., and Lymberry, A. J., eds) pp. 15–37, University Press, Cambridge
2. Adam, R. D. (1991) *Microbiol. Rev.* 55, 706–732
3. Warren, K. S. (1989) in *New Strategies in Parasitology* (McAdam, P. W. J., ed) pp. 217–231, Churchill Livington, Edinburgh
4. Fallborg, J., Christensen, L. A., Ingeman-Nielsen, M., Jacobsen, B. A., Abildgaard, K., Rasmussen, H. H., and Rasmussen, S. N. (1990) *Aliment. Pharmacol. Therap.* 4, 247–253
5. Fallborg, J., Christensen, L. A., Ingeman-Nielsen, M., Jacobsen, B. A., Abildgaard, K., Rasmussen, H. H., and Rasmussen, S. N. (1990) *J. Ped. Gastroent. Nut.* 11, 211–214
6. Brown, D. M., Uprcroft, J. A., Edwards, M. R., and Uprcroft, P. (1998) *Int. J. Parasitol.* 28, 149–164
7. Serrano, R. (1988) *Biochim. Biophys. Acta* 947, 1–28
8. DuRose, T. D., Jr., Gitomer, J., and Codina, J. (1999) *Curr. Opin. Nephrol. Hypertens.* 8, 597–602
9. Forgac, M. (1999) *J. Bioenerg. Biomembr.* 31, 57–65
10. Benchimol, M., De Souza, W., Vanderheyden, N., Zhong, L., Lu, H. G., Moreno, S. N., and Docampo, R. (1998) *Biochem. J.* 332, 695–702
11. Moreno, S. N., Zhong, L., Lu, H. G., Souza, W. D., and Benchimol, M. (1998) *Biochem. J.* 330, 853–860
12. Saliba, K. J., and Kirk, K. (1999) *J. Biol. Chem.* 274, 33213–33219
13. Padan, E., Zilberstein, D., and Schuldiner, S. (1981) *Biochim. Biophys. Acta* 650, 151–166
14. Glaser, T. A., Baataz, J. E., Kreisheimer, G. P., and Mukkada, A. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 7602–7606
15. Thomas, J. A., Kolbech, P. C., and Langworthy, T. A. (1982) in *Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Fractions* (Nuccitelli, R., and Deamer, D. W., eds) A. R. Liss Inc., New York
16. Knodler, L. A., Edwards, M. R., and Schofield, P. J. (1994) *Exp. Parasitol.* 78, 117–125
17. Grote, R., Edwards, M. R., Norton, R. S., and O'Sullivan, W. J. (1999) *Mol. Biochem. Parasitol.* 42, 109–118
18. Kirk, K., Raffos, J. E., and Kuchel, P. W. (1986) *J. Magn. Reson.* 70, 484–487
19. Vieira, L. L., Lavan, A., Daggers, F., and Cabantchik, Z. I. (1994) *J. Biol. Chem.* 269, 16254–16259
20. Thomas, J. A., Buchsbaum, R. N., Zinnia, A., and Racker, E. (1979) *Biochemistry* 18, 2210–2218
21. Roos, A., and Boron, W. F. (1981) *Physiol. Rev.* 61, 296–434
22. Ter Kuile, B. H., Wiemer, E. A. C., Michel, P. A. M., and Opperdoes, F. R. (1992) *Mol. Biochem. Parasitol.* 55, 21–28
23. Vanderheyden, N., Benaim, G., and Docampo, R. (1996) *Biochem. J.* 318, 103–109
24. Bosiso, A., Ghigo, D., Turrini, F., Nissani, E., Pescarmona, G. P., and Ginsburg, H. (1993) *J. Cell. Physiol.* 154, 527–534
25. Lanfredi-Rangel, A., Diniz, A. J., and de Souza, W. (1999) *Parasitol. Res.* 85, 951–955
26. Edwards, M. R., Gilroy, F. V., Jimenez, B. M., and O'Sullivan, W. J. (1989) *Mol. Biochem. Parasitol.* 45, 39–48
27. Schofield, P. J., Edwards, M. R., and Kranz, P. (1991) *Mol. Biochem. Parasitol.* 45, 39–48
28. Grinstein, S., Cohen, S., and Rothstein, A. (1984) *J. Gen. Physiol.* 83, 341–369
29. Weinman, S. A., and Reuss, L. (1982) *J. Gen. Physiol.* 80, 299–321
30. Kral, M. J., and Saier, M. H. (1981) *J. Biol. Chem.* 256, 10820–10824
31. Vazquez, L. M., and de Souza, W. (1999) *Parasitol. Res.* 88, 1053–1058
32. Nass, R., Cunningham, K. W., and Rao, R. (1997) *J. Biol. Chem.* 272, 26145–26152
33. Kinless, J. L., Wehrle, J., Wilkins, N., and Sacktor, B. (1987) *J. Biol. Chem.* 262, 7092–7097
34. Igarashi, P., and Aronson, P. S. (1987) *J. Biol. Chem.* 262, 860–868
35. Jiang, S., Andersen, S., Winget, D., and Mukkada, A. (1994) *J. Cell. Physiol.* 159, 60–66
36. Zilberstein, D and Shapira, M. (1994) *Annu. Rev. Microbiol.* 48, 449–470
37. Orlowski, J., and Shull, G. (1996) in *The Na⁺/H⁺ Exchanger* (Fliegel, L., ed) pp. 1–20, Chapman and Hall, New York
38. Taglicht, D., Padan, E., and Shuldiner, S. (1993) *J. Biol. Chem.* 268, 5382–5387
39. Embley, T. M., and Hirt, R. P. (1998) *Curr. Opin. Genet. Dev.* 8, 624–629
Na\textsuperscript{+}-dependent pH Regulation by the A mitochondriate Protozoan Parasite \textit{Giardia intestinalis}

Giancarlo A. Biagini, Leigh A. Knodler, Kevin J. Saliba, Kieran Kirk and Michael R. Edwards

\textit{J. Biol. Chem.} 2001, 276:29157-29162.  
doi: 10.1074/jbc.M102728200 originally published online April 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102728200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 13 of which can be accessed free at http://www.jbc.org/content/276/31/29157.full.html#ref-list-1