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pH Regulation in the Intracellular Malaria Parasite, *Plasmodium falciparum*

H^+ EXTRUSION VIA A V-TYPE H^+-ATPase*

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The mechanism by which the intra-erythrocytic form of the human malaria parasite, *Plasmodium falciparum*, extrudes H^+ ions and thereby regulates its cytosolic pH (pH_i), was investigated using saponin-permeabilized parasitized erythrocytes. The parasite was able both to maintain its resting pH_i and to recover from an imposed intracellular acidification in the absence of extracellular Na^+, thus ruling out the involvement of a Na^+/H^+ exchanger in both processes. Both phenomena were ATP-dependent. Amiloride and the related compound ethylisopropylamiloride caused a substantial reduction in the resting pH_i of the parasite, whereas EMD 96785, a potent and allegedly selective inhibitor of Na^+/H^+ exchange, had relatively little effect. The resting pH_i of the parasite was also reduced by the sulfhydryl reagent *N*-ethylmaleimide, by the carboxyl group blocker *N*,*N*′-dicyclohexylcarbodiimide, and by bafilomycin A_1, a potent inhibitor of V-type H^+-ATPases. Bafilomycin A_1 blocked pH_i recovery in parasites subjected to an intracellular acidification and reduced the rate of acidification of a weakly buffered solution by parasites under resting conditions. The data are consistent with the hypothesis that the malaria parasite, like other parasitic protozoa, has in its plasma membrane a V-type H^+-ATPase, which serves as the major route for the efflux of H^+ ions.

Malaria, one of the most important infectious diseases in the world today, is caused by parasitic protozoa of the genus *Plasmodium*. These are unicellular, eukaryotic organisms, which, during the course of their complex lifecycle, invade the red blood cells of their vertebrate host. Having entered a red cell, the invading parasite lies dormant for some hours (the ring stage), after which it begins a period of rapid growth (the trophozoite stage) followed by division (schizogony), resulting in the generation of 20–30 new parasites.

The metabolic and biosynthetic activity of the malaria trophozoite is intense. The parasite is wholly reliant on glycolysis as its energy source, and it consumes glucose and produces lactic acid at a rate some 100 times higher than does a normal, uninfected erythrocyte (1, 2). The high metabolic activity of the parasite generates a substantial intracellular acid load. In addition, in the *in vivo* situation, malaria infection commonly gives rise to a pronounced extracellular acidosis (3). For the parasite to remain viable, it must therefore have an effective means of protecting its intracellular pH (pH_i)[1] from both intra- and extracellular acid loads.

Eukaryotic cells extrude H^+ via a variety of different mechanisms. Plant cells, yeast, various protozoa, and a number of invertebrate and vertebrate cell types have in their plasma membrane H^+-ATPases that utilize energy derived from the hydrolysis of ATP to pump H^+ ions from the cell cytosol (4). These are either P-type ATPases (so-called because they form an acyl-phosphate intermediate during their reaction cycle) or V-type ATPases (so-called because they were first described on the membranes of intracellular vacuoles) (4). In cells of higher eukaryotes, it is more common for the main H^+ extrusion mechanisms to be "secondary active transporters" that utilize the (inward) transmembrane Na^+ gradient to energize the efflux of H^+. The most prominent and best understood of these are the Na^+/H^+ exchangers (NHEs; Refs. 5 and 6).

In early studies of the pH_i (and transmembrane potential) of the rodent malaria parasite, *Plasmodium chabaudi*, obtained from malaria-infected rats, it was reported that the pH_i of the parasite was largely unaffected by variations in the extracellular pH (in the range 6.5–7.2) but was decreased by the H^+ pump inhibitors *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and vanadate (7). These findings led to the proposal that the malaria parasite extrudes H^+, and thereby regulates its cytosolic pH, via a P-type H^+-ATPase, of the sort that operates in the plasma membrane of yeast and plant cells (7, 8).

More recently, however, Bosia *et al.* (9) reported that, in *Plasmodium falciparum*, the most virulent of the four plasmodial strains that are infectious to humans, the maintenance of the parasite's cytosolic pH and the ability of the parasite to recover from an intracellular acidification are both dependent on the presence of Na^+ in the extracellular medium. Furthermore, both processes were shown to be inhibited by amiloride and ethylisopropylamiloride (EIPA), inhibitors of the NHEs of higher eukaryotes. On the basis of these data, it was proposed that the parasite extrudes H^+ by means of an NHE, linked to the operation of a Na^+ pump, and it was argued that in *P. falciparum* it is unlikely that a H^+ pump makes a significant contribution to the net efflux of H^+ (9).

The sensitivity of the intracellular pH of *P. falciparum* to the NHE inhibitor EIPA has been confirmed in single-cell studies with parasites within intact erythrocytes (10), and Bray *et al.* (11) have also reported that in isolated *P. falciparum* parasites

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[1] The abbreviations used are: pH_i intracellular pH; AM, acetoxymethyl ester; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EIPA, ethylisopropylamiloride; DMO, 5,5-dimethyloxazolidine-2,4-dione; NEM, *N*-ethylmaleimide; NHE, Na^+/H^+ exchanger; NMDG, *N*-methyl-D-glucamine; pH_o extracellular pH.

the recovery of the cytosolic pH from an imposed acidification is Na^+-dependent.

Following on from the original proposal of an NHE at the parasite surface it has, within the last 2 years, been proposed that: (i) the parasite NHE mediates the uptake of the antimalarial agent chloroquine across the parasite plasma membrane (12); (ii) alterations in the NHE play a central role in the phenomenon of chloroquine resistance (10); and (iii) the parasite NHE is encoded by *cg2* (13), a gene identified earlier as being involved in chloroquine resistance (14). All three proposals have been disputed (11, 15, 16). However, the basic premise that the parasite has an NHE in its plasma membrane has not been challenged.

It is unclear whether the reported differences between the rodent parasite, *P. chabaudi* (postulated to extrude H^+ via a H^+ pump; Ref. 7), and the human parasite, *P. falciparum* (postulated to extrude H^+ via an NHE acting in concert with a Na^+ pump; Ref. 9), reflect genuine differences between the two parasite species or whether they reflect the somewhat different methodologies used in the different studies. The aim of the present work was to investigate in detail the H^+ extrusion mechanism(s) of *P. falciparum*. The data are inconsistent with a role for an NHE in mediating the efflux of H^+ from the intracellular parasite but instead provide compelling evidence for the involvement of a V-type H^+-ATPase in both the maintenance of resting pH_i and the recovery from an intracellular acidification.

EXPERIMENTAL PROCEDURES

Materials—The free-acid and acetoxymethyl ester (AM) forms of the fluorescent pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) were obtained from Sigma and Molecular Probes, respectively. [^{14}C]5,5-Dimethyloxazolidine-2,4-dione ([^{14}C]DMO), used for the estimation of pH_i, was obtained from Amersham Pharmacia Biotech. All inhibitors were purchased from Sigma with the exception of EMD 96785 (17, 18), which was a generous gift from Dr. Norbert Beier, Merck KGaA. Stock solutions of amiloride, EIPA, EMD 96785, and bafilomycin A_1 were prepared in dimethyl sulfoxide (Me_2SO); DCCD was dissolved in ethanol; *N*-ethylmaleimide (NEM) and vanadate were dissolved in water. The vanadate solution was boiled until colorless before use to ensure that the vanadate was present in monomeric form (19). All experiments in which inhibitors were used included appropriate solvent controls. In all cases the final concentration of Me_2SO or ethanol was ≤0.13% v/v.

In the course of this study, cells were suspended in a variety of different solutions, the compositions of which are specified in Table I.

Parasite Culture—The chloroquine-resistant (IC_{50} = 83 ± 17 nM) *P. falciparum* strain FAF-6 (derived from the ITG2 strain; Ref. 20) was cultured under 1% O_2, 3% CO_2, 96% N_2 in RPMI 1640 culture medium, supplemented with D-glucose (20 mM), hypoxanthine (200 μM), HEPES (25 mM), gentamicin sulfate (25 mg/liter), and the serum substitute Albumax II (0.5% w/v, Life Technologies, Inc.; Ref. 21). The cultures were synchronized by hemolysis of mature, trophozoite-stage parasitized erythrocytes by suspension in a sorbitol solution (5% w/v; Ref. 22) and confirmed as being free of mycoplasma contamination using a polymerase chain reaction method (23, 24).

Cell counts were made using an improved Neubauer counting chamber.

Permeabilization of Parasitized Erythrocytes Using Saponin—All the experiments in this study were carried out using trophozoite-stage parasites (36–40 h after invasion) "isolated" from their host erythrocytes by treatment of parasitized cell suspensions with saponin, a plant-derived detergent that renders cholesterol-containing membranes freely permeable to macromolecules. Treatment of parasitized erythrocytes with saponin permeabilizes both the plasma membrane of the host erythrocyte and the parasitophorous vacuole membrane, in which the intracellular parasite is enclosed (25, 26). The cells were incubated in the presence of saponin (0.05% w/v) for ≤30 s at room temperature, then washed by centrifugation, and resuspended in culture medium without Albumax II (adjusted to pH 7.1 to match the estimated pH of the cytosol of the trophozoite-infected human erythrocyte; Ref. 10). The isolated parasites were stored in this media for up to 3 h before experimentation.

In a study such as this, there are obvious concerns about the viability (and, in particular, the membrane integrity) of the saponin-freed parasites, and the system has therefore been characterized in some detail. More than 95% of the parasites isolated in this way retained the ability to exclude trypan blue (27). The isolated parasites maintained an intracellular ATP concentration of approximately 2.5 mM (see Fig. 2). The rate of incorporation of [^{14}C]isoleucine into protein[2] and the rate of phosphorylation of the essential vitamin pantothenic acid (27) were the same in the isolated parasites as in intact parasitized erythrocytes. The isolated parasites accumulated the K^+ congener $^{86}Rb^+$ to concentrations ~20-fold higher than the extracellular medium, as well as accumulating the lipophilic membrane potential probe [^{3}H]tetraphenylphosphonium to concentrations ~100-fold higher than those in the extracellular solution.[3] These data are consistent with the parasite plasma membrane remaining intact and able to generate and maintain both transmembrane ion gradients and a substantial, inward negative, membrane potential. The results obtained in the present study provide further evidence for the membrane integrity of the isolated parasites.

pH Measurements Using BCECF—The pH_i of the isolated parasites was measured using the pH-sensitive fluorescent indicator BCECF. The indicator was loaded into saponin permeabilized parasitized erythrocytes as the acetoxymethyl ester (BCECF-AM). The neutral ester readily enters the parasite cytosol, where the ester groups are removed by esterases, rendering the molecule charged and impermeant (28). Loading was achieved by incubating isolated parasites suspended at a cell density of 0.9–2.1 × 10^8 cells/ml in culture medium without Albumax II, pH adjusted to 7.1, and containing 1 μM BCECF-AM, for 10 min at 37 °C. The cells were then washed (five times) by centrifugation (20 s at 14,000 × *g*) and resuspension in the culture medium (without Albumax II), then maintained in this medium, at a cell density of 3.7–7.1 × 10^6 cells/ml and at 37 °C, for no more than 3 h before use.

Immediately before beginning pH_i recording, an aliquot of cells was centrifuged and resuspended in 1.5 ml of the appropriate solution (see Table I) at a final cell density of 5.6–10.7 × 10^6 cells/ml. The suspension was transferred to a cuvette, which was placed in the temperature-controlled chamber of a Perkin-Elmer LS-50B spectrofluorometer, maintained at 37 °C. Using a dual excitation "Fast Filter" accessory, the sample was excited at 440 nm and 495 nm successively and the fluorescence measured at 520 nm. The ratio of the fluorescence intensity measured using the two excitation wavelengths (495 nm/440 nm) provides a quantitative measure of pH_i. The spectrofluorometer was linked to a computer, allowing the real time monitoring of pH_i. Data were imported into graphics software for analysis.

Inspection of the BCECF-loaded parasites by fluorescence microscopy revealed that the indicator was confined to, and uniformly distributed in, the parasite cytosol, while remaining excluded from the intracellular food vacuole.

Calibration of pH_i was achieved using nigericin, as has been described previously for malaria parasites (11, 28). At the conclusion of each experiment, cells were suspended in a high-K^+ saline (solution G; Table I) at a pH of 6.8, 7.1, or 7.8, to which was added nigericin (30 μM), an ionophore that catalyzes the exchange of K^+ for H^+. The addition of nigericin to cells suspended in a medium containing K^+ at a similar concentration to that in the cell cytosol causes pH_i to equilibrate rapidly with the pH of the suspending medium. Linear regression of the 3-point calibration curve (pH *versus* the fluorescence intensity ratio) consistently yielded regression coefficients >0.99.

In one series of experiments, BCECF was used to monitor the extracellular pH (pH_o) in a weakly buffered suspension of isolated parasites, using a method similar to that described by Benchimol *et al.* (29). Isolated parasites were suspended in solution F to which was added the membrane-impermeant, free-acid form of the indicator (0.38 μM). The final cell density was 1.1–1.8 × 10^7/ml. Changes in the fluorescence intensity ratio (495 nm/440 nm) provided an estimate of pH_o.

Acidification of the Parasite Cytosol—In experiments designed to investigate the ability of the parasite to respond to an intracellular acidification, pH_i was reduced using the NH_4^+ prepulse technique (10, 11, 30). Cells suspended in solution A were exposed briefly (<2 min) to 40 mM NH_4Cl, after which they were centrifuged (90 s, 14,000 × *g*), resuspended in the appropriate NH_4^+-free solution, and then returned to the spectrofluorometer cuvette. This procedure consistently resulted in a decrease in pH_i of 0.4–0.5 pH units from the resting pH_i.

pH Measurements Using [^{14}C]DMO—In order to exclude the possibility of artifacts associated with the estimation of pH_i using the fluorescent indicator BCECF, estimates of the resting pH_i of isolated par-

[2] R. E. Martin and K. Kirk, unpublished data.

[3] R. J. W. Allen, K. J. Saliba, and K. Kirk, manuscript in preparation.

TABLE I
Composition of solutions used in this study

Solution	Description	NaCl	KCl	$MgCl_2$	Glucose	HEPES	NMDG Cl	Choline Cl	pH
					mM				
A	Saline	125	5	1	20	25			7.1
B	Na^+-free (choline)		5	1	20	25		125	7.1
C	Na^+-free (NMDG)		5	1	20	25	125		7.1
D	Glucose-free	135	5	1		25			7.1
E	Glucose-free/Na^+-free (choline)		5	1		25		135	7.1
F	Weakly buffered saline	125	5	1	20	0.1			7.2
G	High K^+		130	1	20	25			6.8/7.1/7.8

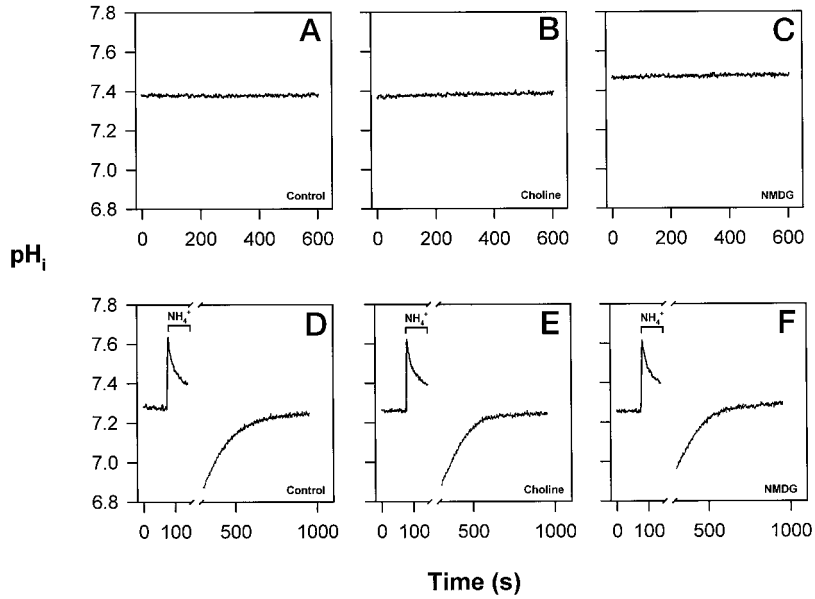

FIG. 1. **Na^+ independence of the maintenance of resting pH_i (A–C), and the recovery from an imposed intracellular acidification (D–F), by isolated *P. falciparum* parasites.** *Panels A*, *B*, and *C* show pH_i traces for parasites suspended at $t = 0$ in solutions A (containing Na^+ as the major cation), B (containing choline as the major cation), and C (containing NMDG as the major cation), respectively (see Table I). In *panels D*, *E*, and *F*, the cells were subjected to an acid load using the NH_4^+ prepulse technique (see "Experimental Procedures"). In each case the cells were suspended in solution A until the time of removal of the NH_4^+, at which point they were washed and resuspended in solution A (*panel D*), B (*panel E*), or C (*panel F*). The *breaks* in the *traces* correspond to the period during which the cells were washed by centrifugation to remove the NH_4^+. pH_i was monitored using BCECF (see "Experimental Procedures"). The *traces* are representative of those obtained from at least three separate cell preparations.

asites were also made from the measured distribution of the weak acid [^{14}C]DMO (7). Saponin-permeabilized parasitized erythrocytes were incubated with 0.25 μCi/ml [^{14}C]DMO (specific activity 54 mCi/mmol) for 15 min at 37 °C at a cell density of 0.6–2.2 $\times$ 10^8/ml in the appropriate solution. Three 200-μl aliquots of the suspension were then transferred to microcentrifuge tubes containing 250 μl of oil (a 5:4 mixture of dibutyl phthalate:dioctyl phthalate). The tubes were centrifuged, thereby sedimenting the cells below the oil, and the cell pellets were processed for scintillation counting as described previously (27).

The intracellular water volume was estimated using [^3H_2O] (27), thereby allowing the calculation of the intracellular concentration of [^{14}C]DMO ($[DMO]_i$) and hence the transmembrane [^{14}C]DMO distribution ratio, $[DMO]_i/[DMO]_o$, where $[DMO]_o$ is the extracellular DMO concentration. The pH_i was calculated using the formula: $pH_i = \log_{10}((([DMO]_i/[DMO]_o) \times (10^{pK_a} + 10^{pH_o}) - 10^{pK_a})$ where pH_o is the extracellular pH, and pK_a (for DMO) is 6.3.

Measurement of Intracellular ATP—The concentration of ATP within the parasite was measured using firefly luciferase (31). Aliquots (200 μl) of suspensions of isolated parasites were transferred to microcentrifuge tubes containing 50 μl of perchloric acid (30% w/v) layered beneath 250 μl of the oil mixture described above. The tubes were centrifuged (2 min, 14,000 $\times$ *g*), to sediment the cells through the oil into the acid, thereby terminating ATP synthesis/utilization. The samples were then placed on ice until further processing. The aqueous supernatant solution was removed by aspiration and any solution remaining on the sides of the tubes removed by rinsing the tube four times with water. The oil was aspirated and the perchloric acid neutralized with 0.5 ml of 0.5 M NaOH, followed by a 10-min centrifugation at 14,000 $\times$ *g*. A 15-μl aliquot of the supernatant solution was then transferred to a scintillation vial containing 3.6 ml of buffered solution (20 mM HEPES, 25 mM $MgCl_2$, and 5 mM Na_2HPO_4, pH 7.4). A 20-μl aliquot of firefly luciferase (diluted in water) was placed in the cap of the vial. The tube was sealed with the cap and the reaction started by inverting the tube 10 s before placing the vial in the scintillation counter and measuring the photon emission.

A calibration curve was obtained using ATP concentrations in the range 1.3–21 nM in the scintillation vial.

RESULTS

Effect of Extracellular Na^+ Replacement on pH_i—As illustrated in Fig. 1*A*, isolated parasites suspended in normal physiological saline (solution A; pH 7.1) maintained a steady resting pH_i. The pH_i estimated using BCECF under these conditions ranged between 7.22 and 7.40, with a mean value of 7.29 $\pm$ 0.01 ($n = 15$; $\pm$ S.E.; Table II). This value is very similar to that of 7.33 $\pm$ 0.06 ($n = 10$; $\pm$ S.E.) calculated from the measured distribution of the weak acid [^{14}C]DMO (Table II).

As illustrated in Fig. 1 (*A–C*), suspension of the parasites in a solution in which Na^+ was replaced with choline (solution B) had no significant effect on pH_i (estimated using BCECF), whereas replacement of Na^+ with NMDG in the extracellular solution (solution C) actually caused a slight increase in the resting pH_i. Very similar results were obtained using the [^{14}C]DMO distribution method for the estimation of pH_i (Table II).

Parasites subjected to an intracellular acidification (achieved via the NH_4^+ prepulse technique; see "Experimental Procedures") recovered their resting pH_i within 5–10 min (Fig. 1, *D–F*). Their ability to do so was unaffected by the replacement of Na^+ with either choline or NMDG in the extracellular medium. This situation contrasted with that in rat hepatoma (HTC) cells, which are known to have a functional NHE (32) and which were shown to recover from an NH_4^+-induced acid load when bathed in solution A but not in solution B or C (data not shown).

In isolated parasites, both the maintenance of the resting pH_i and the extrusion of H^+ from the cytosol following an intracellular acid load therefore occur via mechanisms that are independent of the presence of Na^+ in the extracellular

TABLE II

pH_i of isolated parasites suspended in media of different compositions

pH_i was estimated using the fluorescent pH indicator BCECF, or from the equilibrium distribution of [^{14}C]DMO in cells suspended in the different media for ≥15 min. The composition of the different suspending solutions is given in Table I. The pH_i values are those averaged from the number of experiments shown in parentheses, with each experiment carried out on a different day. The errors are S.E., and the *p* values are those derived from paired *t* tests comparing pH_i estimates for cells in solutions B–D with those for cells in solution A.

Suspending solution	Description	pH_i			
		BCECF	*p*	[^{14}C]DMO	*p*
A	Saline (control)	7.29 ± 0.01 (15)		7.33 ± 0.06 (10)	
B	Na^+-free (choline)	7.31 ± 0.04 (5)	0.26	7.31 ± 0.08 (6)	0.43
C	Na^+-free (NMDG)	7.36 ± 0.03 (7)	0.07	7.41 ± 0.09 (6)	0.05
D	Glucose-free	7.06 ± 0.01 (6)	0.0001	6.94 ± 0.05 (4)	0.013

medium.

Effect of ATP Depletion on pH_i—The malaria parasite is wholly reliant on glycolysis for the generation of ATP (33). As shown in Fig. 2*A*, on suspension of isolated parasites in glucose-free medium (solution D), there was a rapid decline of the intracellular ATP concentration and a progressive decrease in pH_i. On restoration of the glucose to the medium, the intracellular ATP concentration and pH_i both recovered back to their original starting values. The dependence of the maintenance of the resting pH_i on an adequate supply of glucose, and hence ATP, was confirmed using the [^{14}C]DMO distribution method (Table II).

Very similar behavior was seen for cells suspended in glucose-free medium, containing choline in place of Na^+ (solution E), with the cells undergoing an initial acidification followed by a full recovery of pH_i upon the addition of glucose (data not shown). This rules out the involvement of the transmembrane Na^+ gradient in the extrusion of H^+ from the parasite cytosol.

Fig. 2*B* shows the effect of glucose-deprivation on the ability of the parasites to recover from an imposed intracellular acidification. The ability of glucose-deprived parasites to recover their pH_i following an acid load was significantly impaired, consistent with H^+ extrusion under these conditions being via an ATP-dependent mechanism.

Effect of Inhibitors on pH_i—Fig. 3 shows the effects of a range of transport inhibitors on the resting pH_i of isolated parasites, monitored using BCECF. As has been shown previously (9), the NHE inhibitors amiloride (500 μM) and EIPA (200 μM) both caused a marked, progressive acidification of the parasite cytosol (Fig. 3, *B* and *C*, respectively). However, EMD 96785, a more potent and more selective inhibitor of at least some of the mammalian NHE isoforms (17, 18), caused only a slight (although significant) decrease in pH_i when added at a concentration (500 μM) more than 3 orders of magnitude higher than its IC_{50} values for the inhibition of mammalian NHE1 and NHE2 (Refs. 17 and 18; Fig. 3*D*). At the same concentration, EMD 96785 caused a complete inhibition of the recovery of the pH_i of mammalian HTC cells following an acid load (data not shown).

DCCD (50 μM) and NEM (1 mM), both broad specificity protein-reactive agents that have been shown previously to inhibit ATP-dependent H^+ pumps in a variety of systems, caused a progressive decrease in pH_i (Fig. 3, *E* and *F*, respectively). Vanadate (500 μM), a compound that inhibits P-type ATPases (including P-type H^+ pumps), exerted a somewhat complex effect, causing an initial rise in pH_i, followed by a decrease in pH_i to a value below the initial resting value (Fig. 3*G*). Bafilomycin A_1 (100 nM), a potent inhibitor of V-type H^+-ATPases (34), caused a pronounced decrease in the resting pH_i (Fig. 3*H*).

The effect of amiloride, DCCD, and bafilomycin A_1 on the resting pH_i were tested using the [^{14}C]DMO distribution method, and the results, together with those obtained with BCECF, are given in Table III. Amiloride (500 μM), DCCD (50 μM), and bafilomycin A_1 (100 nM) all caused a significant decrease in pH_i as estimated from the distribution of [^{14}C]DMO. The effects of amiloride and EIPA on the resting pH_i were also observed in cells suspended in Na^+-free media (solutions B and C; Table III), consistent with their effect on pH_i being independent of any effect on an NHE.

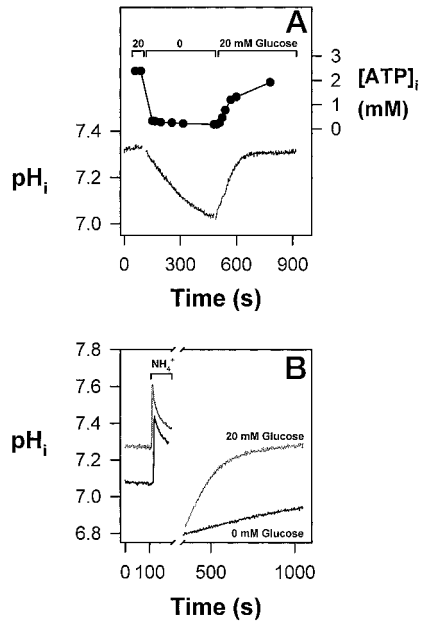

FIG. 2. **ATP dependence of the maintenance of the resting pH_i (*A*) and the recovery of pH_i from an imposed intracellular acidification (*B*).** *A*, cells were suspended in solutions containing glucose at the concentrations indicated (solutions A and D). The *closed circles* show the ATP concentrations within the parasite, and the *lower trace* shows the pH_i, monitored using BCECF. *B*, cells in normal saline (solution A; *upper/gray trace*) or glucose-free saline (solution D; *lower/black trace*) were subjected to an acid load using the NH_4^+ prepulse technique, and the recovery of pH_i from the acid load was monitored using BCECF.

In experiments in which amiloride and bafilomycin A_1 were added to cells, both separately and together, the inhibitory effects were found not to be additive. In cells treated with bafilomycin A_1 (100 nM), there was no further decrease in pH_i following the addition of amiloride (500 μM). In cells treated first with amiloride, the subsequent addition of bafilomycin A_1 caused pH_i to decrease further, to the level seen with bafilomycin A_1 alone. These data (not shown) are consistent with bafilomycin A_1 and amiloride affecting the same H^+ extrusion mechanism, with 500 μM amiloride exerting a lesser inhibitory effect than 100 nM bafilomycin A_1.

Bafilomycin A_1 (100 nM) was tested for its effect on the extrusion of H^+ from parasites following an intracellular acidification. As shown in Fig. 4, the V-type H^+-ATPase inhibitor completely inhibited the recovery of pH_i following an acid load.

Acidification of the Extracellular Medium—The finding that bafilomycin A_1 caused a decrease in the resting pH_i as well as

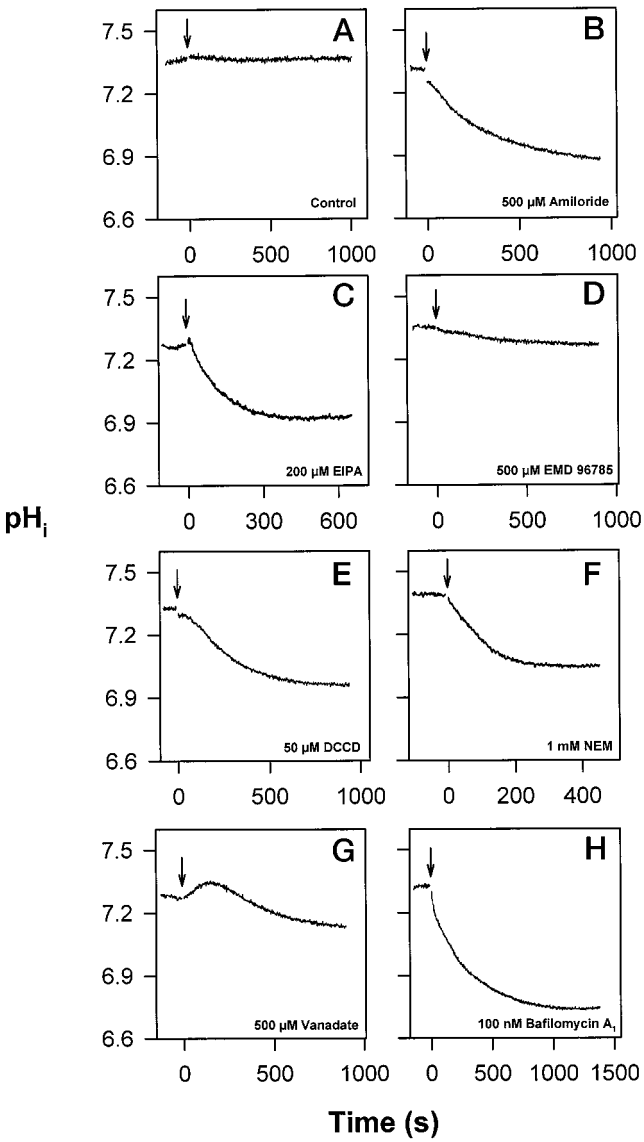

FIG. 3. **pH_i of *P. falciparum* parasites following the addition (at the point indicated by the *arrow*, $t = 0$) of: *A*, a combination of the different solvents used for the inhibitors (Me_2SO, ethanol and water), each at the maximum concentration used; *B*, amiloride (500 μM); *C*, EIPA (200 μM); *D*, EMD 96785 (500 μM); *E*, DCCD (50 μM); *F*, NEM (1 mM); *G*, vanadate (500 μM); *H*, bafilomycin A_1 (100 nM).** pH_i was monitored using BCECF. The *traces* shown are representative of those obtained from at least three separate cell preparations.

blocking the recovery of pH_i from an imposed intracellular acidification are consistent with the involvement of a V-type H^+ pump in the extrusion of H^+ from the parasite cytosol.

There is now substantial evidence for the presence of V-type H^+ pumps in the plasma membranes of a range of different cell types (4), including a number of parasitic protozoa (29, 35, 36). Nevertheless, it remains a theoretical possibility that the effect of bafilomycin A_1 on pH_i is due to the inhibition of the V-type H^+-ATPase on the membrane of intracellular organelles (such as the parasite's digestive food vacuole), rather than due to inhibition of the movement of H^+ ions across the parasite plasma membrane.

To test the hypothesis that a bafilomycin A_1-sensitive H^+-ATPase mediates the extrusion of H^+ from the parasite cytosol across the plasma membrane, into the extracellular medium, isolated parasites were suspended in a weakly buffered solution (solution F) and the pH_o monitored using BCECF (added to the suspension as the membrane-impermeant free acid). As shown in Fig. 5, the parasites induced a progressive acidification of the external medium, consistent with their extruding H^+. Bafilomycin A_1 reduced the rate of acidification of the extracellular solution, consistent with it inhibiting the H^+ extrusion mechanism in the parasite plasma membrane.

DISCUSSION

In this study it was shown that trophozoite-stage malaria parasites within saponin-permeabilized human erythrocytes extrude H^+ ions via a Na^+-independent mechanism. Their ability to maintain their resting pH_i and to recover from an imposed intracellular acidification was not inhibited by the replacement of Na^+ with either choline or NMDG (Fig. 1). These data are inconsistent with the involvement of an NHE in the extrusion of H^+ from the parasite.

The ATP dependence of H^+ extrusion (Fig. 2) and the finding that this effect is independent of the maintenance of a transmembrane Na^+ gradient are consistent with H^+ efflux being via a H^+-ATPase. The experiments with a range of inhibitors (Fig. 3) lend further support to this view. DCCD and NEM, both effective (albeit nonspecific) inhibitors of both P- and V-type H^+-ATPases, inhibited the ability of the parasite to maintain its resting pH_i (Fig. 3, *E* and *F*). The complex effects of the P-type H^+-ATPase inhibitor vanadate on pH_i (an initial increase in pH_i, followed by a decrease; Fig. 3*G*) might argue against the direct involvement of a P-type H^+-ATPase in H^+ extrusion. By contrast the very striking effects of the potent (and perhaps specific) V-type H^+-ATPase inhibitor bafilomycin A_1 (34) on the extrusion of H^+ from the cell cytosol (Fig. 3*H*) and into the external medium (Fig. 5) under resting conditions, as well as the recovery of pH_i following an imposed intracellular acidification (Fig. 4), are consistent with a protein of this type being present on the parasite plasma membrane and playing the major role in the extrusion of H^+ from the parasite cytosol.

Although V-type H^+-ATPases were first described on the membranes of intracellular vacuoles (and named accordingly), there is now abundant evidence that they are present on the plasma membranes of a wide variety of cell types (4). These include the parasitic protozoa *Entamoeba histolytica* (35), *Trypanasoma cruzi* (29), and *Toxoplasma gondii* (36). *T. gondii* is, like the malaria parasite, an apicomplexan, and the two organisms might therefore be expected to show some similarities in their physiology.

The malaria parasite is known to have a bafilomycin A_1-sensitive V-type H^+-ATPase on the membrane of the intracellular food vacuole in which host cell hemoglobin, ingested by the parasite via an endocytotic feeding process, undergoes digestion (37). V-type ATPases are multi-subunit complexes and *P. falciparum* homologues of two of the subunits (A and B) have been cloned (VAP-A (38) and VAP-B (39), respectively). In immunofluorescence experiments with antibodies raised against VAP-B, both trophozoite- and schizont-stage parasites showed a general fluorescence over the whole cell, with labeling not confined to the membrane of the food vacuole (39). As pointed out (39), these data are consistent with the V-type H^+-ATPase playing roles additional to the acidification of the parasite's food vacuole.

Mikkelsen and colleagues have previously investigated the membrane potential and pH_i of rodent malaria parasites (*P. chabaudi*). Using both intact, parasitized erythrocytes (8), and parasites freed from their host cell membrane by N_2 cavitation (7), it was shown that DCCD (10 μM) caused a marked depolarization of the parasite plasma membrane. Vanadate (50 μM) was shown to have a similar effect in isolated parasites (7). It was also found that in isolated parasites (suspended in medium

TABLE III

Effect of inhibitors on pH_i of isolated parasites

pH_i was estimated using the fluorescent pH indicator BCECF (at the point at which pH_i stabilized following the addition of inhibitor), or from the equilibrium distribution of [^{14}C]DMO in cells exposed to the different inhibitors for 15 min. The pH_i values are those averaged from the number of experiments shown in parentheses, with each experiment carried out on a different day. The errors are S.E., and the *p* values are those derived from paired *t* tests comparing pH_i estimates for inhibitor-treated cells in solutions A–C with those for inhibitor-free cells in the same solutions (see Table II). ND, not determined.

Suspending solution	Inhibitor/description	pH_i			
		BCECF	*p*	[^{14}C]DMO	*p*
A	Saline (control)	7.29 ± 0.01 (15)		7.33 ± 0.06 (10)	
A	Amiloride (500 μM)	6.98 ± 0.03 (4)	0.011	7.04 ± 0.15 (8)	0.013
C	Amiloride (500 μM)/Na^+-free (NMDG)	7.15 ± 0.04 (4)	0.004	7.11 ± 0.05 (4)	0.024
A	EIPA (200 μM)	6.95 ± 0.02 (3)	0.012	ND	
B	EIPA (200 μM)/Na^+-free (choline)	6.95 ± 0.04 (3)	0.015	ND	
A	EMD 96785 (500 μM)	7.21 ± 0.03 (4)	0.019	ND	
A	NEM (1 mM)	7.03 ± 0.02 (3)	0.018	ND	
A	DCCD (50 μM)	7.01 ± 0.02 (4)	0.006	6.58 ± 0.14 (10)	0.001
A	Vanadate (500 μM)	7.15 ± 0.06 (4)	0.09	ND	
A	Bafilomycin A_1 (100 nM)	6.70 ± 0.03 (3)	0.003	6.88 ± 0.10 (4)	0.016

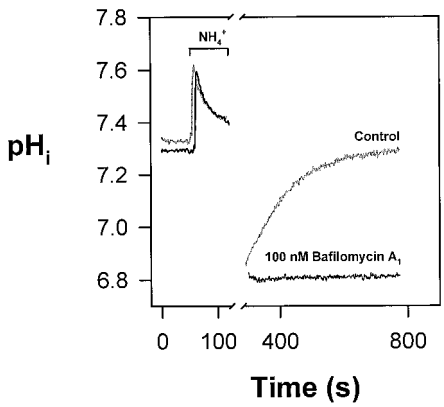

FIG. 4. **Effect of bafilomycin A_1 (100 nM) on the recovery of pH_i from an intracellular acidification, imposed using the NH_4^+ prepulse technique.** The bafilomycin A_1 was added to the cells at the point of resuspension in NH_4^+-free solution A (*i.e.* at the point corresponding to the *break* in the lines). The *traces* shown are representative of those obtained from three separate cell preparations (*lower/black trace*, bafilomycin A_1; *upper/gray trace*, control).

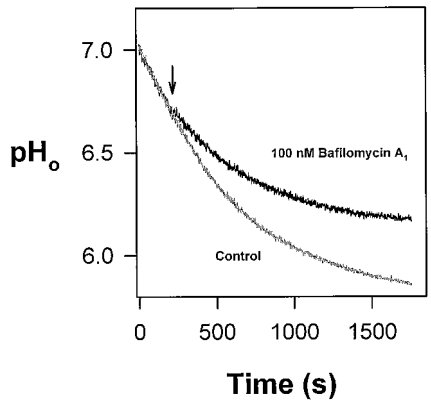

FIG. 5. **Effect of bafilomycin A_1 (100 nM) on the acidification of a weakly buffered extracellular solution (solution F; Table I) by isolated *P. falciparum* parasites.** The *arrow* indicates the point of addition of either bafilomycin A_1 in Me_2SO (*upper/black trace*) or an equivalent volume of Me_2SO (*lower/gray trace*). The *traces* shown are representative of those obtained from four separate cell preparations.

of pH 6.7) DCCD (5 μM) and vanadate (50 μM) caused pH_i to decrease by 0.30 and 0.23 pH units, respectively, following an incubation of unspecified length (7). On the basis of these data, it was postulated that *P. chabaudi* has in its plasma membrane an electrogenic H^+-ATPase similar to that found in yeast (now known to be a P-type ATPase; Ref. 40). The results obtained previously using DCCD with *P. chabaudi* freed from their host cells by N_2 cavitation are very similar to those obtained here with *P. falciparum* isolated using saponin. There is, however, some discrepancy between the results obtained with vanadate in the two systems. The finding by Mikkelsen *et al.* that vanadate caused a decrease in pH_i (7) contrasts with the finding in the present study that vanadate caused an initial *increase* in pH_i (in the 5 min following its addition) followed by a subsequent decline (Fig. 3*G*). The initial rise in pH_i observed to follow the addition of vanadate is consistent with it entering the cell and increasing the pH_i, either via a "weak-base" effect or via an effect on one or more intracellular processes. Whether the apparent discrepancy between the two studies might be due to methodological differences or due to fundamental differences between *P. falciparum* and *P. chabaudi* is unclear.

Although consistent with recent findings with other parasitic protozoa (29, 35, 36), and with at least some of the early data on *P. chabaudi* (7, 8), the results of this study are at odds with some, although not all, of the recent data relating to the mechanism of H^+ extrusion from *P. falciparum*. In experiments with *P. falciparum*-infected human erythrocytes permeabilized with Sendai virus and then stuck onto poly-L-lysine-coated coverslips, Bosia *et al.* (9) found that the ability of the parasite to maintain a resting pH_i of around 7.3, and its ability to recover from an imposed intracellular acidification, were inhibited by exposure of the cells to Na^+-free medium. There has, to our knowledge, been no other demonstration of the maintenance of resting pH_i of *P. falciparum* being Na^+-dependent, although Bray *et al.* (11) reported that in single-cell microfluorescence experiments on parasites freed from their host cell using a peptide-induced hemolysis method and stuck onto poly-L-lysine-coated coverslips, the ability of the parasite's pH_i to recover from an imposed cytosolic acidification was impaired in the absence of extracellular Na^+.

The reason for the discrepancy between the results of the present study (showing H^+ extrusion from the parasite to be via a Na^+ independent mechanism) and those obtained previously with *P. falciparum* parasites (indicating Na^+ dependence of H^+ extrusion) are unclear. There are some differences in the methodologies used in the different studies. The saponin permeabilization treatment used in the present work has been shown previously to permeabilize both the host erythrocyte membrane and the parasitophorous vacuole membrane in which the intracellular parasite is enclosed (25, 26); it therefore gives solutes in the suspending medium free access to the parasite plasma membrane. It is unclear whether the same is true of the Sendai virus- or peptide-induced hemolysis methods used previously. This raises the possibility that in the earlier studies the parasites remained within an intact parasitopho-

rous vacuole, and that the Na^+ dependence observed relates to the movement of H^+ ions across this membrane. However, this is at odds with the prevailing view that the parasitophorous vacuole membrane is freely permeable to low molecular weight solutes (41, 42).

Another methodological difference lies in the fact that, in our study (like that of Mikkelsen *et al.* (Ref. 7)), the cells were in suspension, whereas in previous studies of *P. falciparum* the parasites were stuck to poly-L-lysine-coated coverslips (9, 11). In the course of this work, we repeated a number of the key experiments using cells on poly-L-lysine-coated coverslips. In our hands, the data obtained using this method were far less reproducible than those obtained using cells in suspension, although in the majority of experiments the parasites on coverslips were found to maintain their resting pH_i and recover from an imposed intracellular acidification in the absence of extracellular Na^+.

Bosia *et al.* (9) also reported that amiloride and EIPA caused an acidification of parasites under resting conditions, as well as inhibiting recovery from an acid load. Similarly, Wunsch *et al.* (10), using microfluorescence measurements of the pH_i of parasites within intact erythrocytes, have reported that EIPA prevents the recovery of pH_i from an imposed acidification. These findings are entirely consistent with the observations in the present study that amiloride and EIPA interfered with H^+ extrusion from the parasite under resting conditions (Fig. 3, *B* and *C*; Table III). However, whereas Bosia *et al.* and Wunsch *et al.* interpreted the effects of these compounds on pH_i as being due to inhibition of an NHE at the parasite surface, the data obtained here are consistent with both compounds influencing pH_i via mechanisms unrelated to any effect on an NHE.

The finding that the effects of amiloride and bafilomycin A_1 on pH_i were not additive is consistent with amiloride acting as an inhibitor of the V-type H^+-ATPase. Both amiloride and EIPA are notoriously nonspecific and have already been shown to exert effects on *P. falciparum* via mechanisms independent of any effect on an NHE (11). Amiloride has been shown previously to inhibit the generation of a H^+ gradient by a V-type H^+-ATPase in the plasma membrane of insect cells, with an IC_{50} of approximately 0.7 mM (43). The finding in the present study that the decrease in pH_i caused by the addition of 500 μM amiloride was approximately half that caused by the addition of 100 nM bafilomycin A_1 (Fig. 3; Table III) are consistent with the same being true in *P. falciparum*. The effects of amiloride and EIPA contrast with those of the more potent and selective NHE inhibitor, EMD 96785, which had little effect on the resting pH_i (Fig. 3*D*).

In conclusion, the data presented here are inconsistent with a significant role for an NHE in the efflux of H^+ across the plasma membrane of the human malaria parasite *P. falciparum*. Instead, they are consistent with the hypothesis that the major mechanism for the efflux of H^+ from the parasite is a V-type H^+-ATPase. This hypothesis is consistent with the reported subcellular distribution of the V-type H^+-ATPase B-subunit within the parasite (39), and with the recent reports of the presence of V-type H^+-ATPases in the plasma membranes of other protozoa, including the apicomplexan *T. gondii* (36). The contribution of the V-type H^+-ATPase to the parasite membrane potential and the physiological role(s) of the transmembrane H^+ electrochemical gradient are presently under investigation.

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