Acidocalcisomes and a vacuolar H^+-pyrophosphatase in malaria parasites

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Plasmodium berghei trophozoites were loaded with the fluorescent calcium indicator, fura-2 acetoxymethyl ester, to measure their intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was increased in the presence of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin. Trophozoites also possess a significant amount of Ca^{2+} stored in an acidic compartment. This was indicated by: (1) the increase in $[Ca^{2+}]_i$ induced by bafilomycin A_1, nigericin, monensin, or the weak base, NH_4Cl, in the nominal absence of extracellular Ca^{2+}, and (2) the effect of ionomycin, which cannot take Ca^{2+} out of acidic organelles and was more effective after alkalinization of this compartment by addition of bafilomycin A_1, nigericin, monensin, or NH_4Cl. Inorganic PP_i promoted the acidification of a subcellular compartment in cell homogenates of trophozoites. The proton gradient driven by PP_i collapsed by addition of the K^+/H^+ exchanger, nigericin, and eliminated by the PP_i analogue, aminomethylenediphosphonate (AMDP). Both PP_i hydrolysis and proton transport were dependent upon K^+, and Na^+ caused partial inhibition of these activities. PP_i hydrolysis was sensitive in a dose-dependent manner to AMDP, imidodiphosphate, sodium fluoride, dicyclohexylcarbodi-imide and to the thiol reagent, *N*-ethylmaleimide. Immunofluorescence microscopy using antibodies raised against conserved peptide sequences of a plant vacuolar pyrophosphatase (V-H^+-PPase) suggested that the proton pyrophosphatase is located in intracellular vacuoles and the plasma membrane of trophozoites. AMDP caused an increase in $[Ca^{2+}]_i$ in the nominal absence of extracellular Ca^{2+}. Ionomycin was more effective in releasing Ca^{2+} from this acidic intracellular compartment after treatment of the cells with AMDP. Taken together, these results suggest the presence in malaria parasites of acidocalcisomes with similar characteristics to those described in trypanosomatids and *Toxoplasma gondii*, and the colocalization of the V-H^+-PPase and V-H^+-ATPase in these organelles.

Key words: aminomethylenediphosphonate, calcium, pH, *Plasmodium*.

INTRODUCTION

Plasmodium, the agent responsible for malaria, is an obligate intracellular parasite belonging to the phylum Apicomplexa. Apicomplexan parasites possess several biochemical peculiarities in common with plants, such as the presence of a chloroplast-like organelle, termed the apicoplast [1–3], a functional shikimate pathway [4], and a non-mevalonate pathway of isoprenoid biosynthesis [5]. These recent discoveries have explained the mechanism of action of some antiparasitic compounds [6] and provided new opportunities for the development of novel antimalarial agents [4,6,7].

During its asexual life cycle, *Plasmodium* invades and replicates within erythrocytes. These stages live, proliferate and differentiate inside these cells, where the free Ca^{2+} concentration is normally very low (approx. 0.1 μM) as compared with that in the extracellular medium (approx. 1.0 mM). In addition, erythrocytes have a very low total Ca^{2+} concentration since they are devoid of intracellular Ca^{2+} stores. Greater intracellular Ca^{2+} levels have been postulated to occur in parasitized erythrocytes compared with uninfected cells, and atomic absorption analyses of *P. chabaudi*-infected and non-infected erythrocytes have indicated a general trend of increased Ca^{2+} content in the late stages of development of the malaria parasites [8]. A parasite Ca^{2+} store was postulated to be responsible for this increased Ca^{2+} content of infected erythrocytes [8]. Garcia et al. [9] recently reported an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of fluo-3-loaded *P. chabaudi*, after addition of monensin and 4-chloro-7-nitrobenzofurazan, or of fluo-3-loaded *P. falciparum* after addition of nigericin. Nigericin was also shown to decrease $^{45}Ca^{2+}$ uptake by digitonin-permeabilized *P. chabaudi* [9]. The authors postulated the presence of an acidic Ca^{2+} pool in these parasites [9].

In previous work on the Ca^{2+} and pH homeostasis of trypanosomatids and the apicomplexan parasite *Toxoplasma gondii*, we demonstrated Ca^{2+} storage within the cells, in an acidic compartment that was named the acidocalcisome [10–13]. We also demonstrated that a plant-like vacuolar proton-translocating pyrophosphatase (V-H^+-PPase) was present in acidocalcisomes of *Trypanosoma cruzi* [14], *Leishmania donovani* [15], *Trypanosoma brucei* [16], and *T. gondii* (C. O. Rodrigues, D. A. Scott, B. Bailey, W. De Souza, M. Benchimol, B. Moreno, J. A. Urbina, E. Oldfield and S. N. J. Moreno, unpublished work). Such pyrophosphatases have been previously identified in the energy-coupling membranes of plant vacuoles (tonoplasts) and in chromatophores of prototrophic bacteria [17].

In this report, we demonstrate, by using the fluorescent Ca^{2+} indicator fura-2, that *P. berghei* trophozoites possess a significant amount of Ca^{2+} stored in an acidic compartment similar to the acidocalcisome of *T. gondii* and trypanosomatids, and that these cells also possess a V-H^+-PPase activity with features in common with the trypanosomatid, *Toxoplasma* and plant activities. Our results also suggest that the V-H^+-PPase and the V-H^+-ATPase are colocalized to acidocalcisomes of *P. berghei*.

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; AMDP, aminomethylenediphosphonate; V-H^+-PPase, vacuolar pyrophosphatase; fura-2/AM, fura-2 acetoxymethyl ester; NEM, *N*-ethylmaleimide; DCCD, dicyclohexylcarbodi-imide; IDP, imidodiphosphate.

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MATERIALS AND METHODS

Parasites

P. berghei berghei (strain NK65) was maintained *in vivo* in male Balb/c mice by weekly transfer infection. Blood was collected in PBS (pH 7.4), containing 10 units heparin/ml at approx. 60% parasitaemia. Red blood cells were washed twice in PBS by centrifugation at 4 °C at 1500 ***g*** for 5 min. Washed erythrocytes were diluted 1:2 in PBS, and passed over a powdered cellulose column (Whatman CF11) to remove leucocytes and platelets [18]. Contamination of the resulting preparations with white blood cells was always less than 5%, as determined by using a Neubauer chamber and Giemsa staining. Red blood cells depleted of leucocytes and platelets were washed once, as described above, and resuspended in PBS. Infected erythrocytes were enriched using the Percoll method [19]. Infected erythrocytes within the 70% Percoll interphase were collected. Examination of Giemsa-stained thin blood smears showed a predominance of trophozoites (85–90% of the red blood cells; the rest containing ring stage and schizont forms). To isolate the parasites, infected erythrocytes were lysed with 0.1 mg/ml saponin in PBS at room temperature for 5 min. After centrifugation at 1500 ***g*** for 5 min at 4 °C to remove red blood cell membranes, the parasites were washed five times in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM Hepes, pH 7.2). The parasites were resuspended at 1×10^8 cells/ml in the same buffer. Contamination of the preparation with red blood cells was negligible. Contamination with white blood cells (mostly lymphocytes) was always less than 1%, as determined using a Neubauer Chamber and Giemsa staining [typically we obtained about $(1.2–1.3) \times 10^9$ trophozoites contaminated with $(1.0–1.15) \times 10^7$ white blood cells per mouse]. Control experiments were also done with preparations of white blood cells from control mice in amounts similar to those that could contaminate trophozoite preparations. These numbers of white blood cells were insufficient to detect Ca^{2+} fluxes or any pyrophosphatase activity (results not shown). Protein was measured using the Bio-Rad Coomassie Blue method.

Chemicals

Ionomycin, Triton X-100, imidodiphosphate (IDP), sodium fluoride, monensin, sodium PP_i, protease inhibitors, dicyclohexylcarbodi-imide (DCCD), *N*-ethylmaleimide (NEM), EGTA, and nigericin were purchased from Sigma. Bafilomycin A_1 was from Kamiya Biomedical Co. (Thousand Oaks, CA, U.S.A.). Thapsigargin was from LC Laboratories (Woburn, MA, U.S.A.) Acridine Orange, fura-2/AM (fura-2 acetoxymethyl ester), 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), purine nucleoside phosphorylase, and the standard phosphate solution were from Molecular Probes (Eugene, OR, U.S.A.). Aminomethylenediphosphonate (AMDP) [20] and polyclonal antisera, which had been raised against keyhole-limpet haemocyanin (KLH)-conjugated synthetic peptides corresponding to the hydrophilic loops IV (antibody 324) and XII (antibody 326) of plant V-H^+-PPase [21], were kindly provided by Professor Philip Rea, University of Pennsylvania, PA, U.S.A. All other reagents were analytical grade.

Preparation of cell homogenates

For proton pumping measurements, trophozoites (approx. 5×10^9 cells for each preparation) were centrifuged and washed two times with buffer A, and once with lysis buffer [20 mM Hepes, 50 mM KCl, 125 mM sucrose, 0.5 mM EDTA, 5 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 1 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 10 μM E-64 and 10 μM tosyl-lysylchloromethane, pH 7.2]. The cell pellet was mixed with approx. 2 × wet weight silicon carbide (Aldrich) and ground with a mortar and pestle until lysis was greater than 90% (generally 30 s). This mixture was resuspended in 5 ml of lysis buffer, centrifuged once at 150 ***g*** for 5 min to remove the silicon carbide, and a second time at 580 ***g*** for 10 min to remove unbroken cells. The supernatant obtained was centrifuged at 15000 ***g*** for 10 min and the pellet resuspended in 1 ml of lysis buffer for use in assays.

Spectrofluorometric determinations

After harvesting the cells, they were washed twice at 500 ***g*** for 10 min at room temperature in buffer A. Cells were resuspended to a final density of 1×10^9 cells/ml in buffer A, and 6 μM fura-2/AM. The suspensions were incubated for 30 min in a 30 °C water bath with mild agitation. The cells were then washed twice with buffer A to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in buffer A with or without 1.0 mM $CaCl_2$, and were kept in 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 (2×10^7 cells/ml final density) in a cuvette placed in a thermostat-controlled (37 °C) Hitachi F-2000 spectrofluorometer. For fura-2 measurements, excitation was at 340 and 380 nm and emission was at 510 nm. The fura-2 fluorescence response to $[Ca^{2+}]_i$ was calibrated from the ratio of 340/380 nm fluorescence values, after subtraction of the background fluorescence of the cells at 340 and 380 nm, as described by Grynkiewicz et al. [22]. $[Ca^{2+}]_i$ was calculated by titration with different concentrations of Ca^{2+}-EGTA buffers [10]. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program, as described before [10]. Other experimental conditions and calibrations were as described before [10]. Traces shown are representative of three independent experiments conducted on separate cell preparations. Variations in the values of $[Ca^{2+}]_i$ between different experiments with different cell preparations were less than 10%.

Proton pump activity

PP_i-driven proton transport was assayed by measuring changes in the absorbance of Acridine Orange (A_{493}-A_{530}) in an SLM-Aminco DW 2000 (SLM Instruments, Urbana, IL, U.S.A.) dual-wavelength spectrophotometer [14]. Cell homogenates were incubated at 30 °C in 2.5 ml of standard 130 mM KCl buffer (pH 7.2) or alternate buffers, as described in Table 1, containing in addition, 2 mM $MgSO_4$, 10 mM Hepes, 50 μM EGTA, 3 μM Acridine Orange, and different inhibitors where indicated, for 30 s/1 min prior to the addition of 0.1 mM PP_i (pH 7.2). Each experiment was repeated at least three times with different cell preparations; the Figures and Table show representative experiments.

Pyrophosphatase assay

Pyrophosphatase activity, in terms of phosphate release, was determined according to Scott et al. [14], for assays using different buffers. Reaction mixtures contained 130 mM KCl, 10 mM K-Hepes, 2 mM $MgSO_4$, 50 μM EGTA, pH 7.2 (or other buffers, as described in Table 1), 0.1 mM MESG, 0.4 U/ml purine nucleoside phosphorylase, together with the cell membrane preparation in a total volume of 0.1 ml. Activity was monitored at 30 °C by

the increase in absorbance at 360 nm using a Power Wave 340 plate reader (BioTek Instruments, Winooski, VT, U. S.A), and was calibrated for each buffer with a standard phosphate solution.

SDS electrophoresis and preparation of Western blots

P. berghei trophozoites (1×10^9) were resuspended in 300 μl Dulbecco's PBS (Gibco BRL) containing proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF) and frozen at -70 °C. Thawed cells were homogenized with a Teflon pestle at 4 °C and aliquots (10 μl, approx. 10 μg of protein) were mixed with 10 μl electrophoresis buffer [125 mM Tris/HCl, pH 7, 10 % (w/v) β-mercaptoethanol, 20 % (v/v) glycerol, 4 % (w/v) SDS, 4 % (w/v) Bromophenol Blue], and boiled for 5 min prior to application to 10 % SDS-polyacrylamide gels. Electrophoresed proteins were transferred to nitrocellulose [23] using a Bio-Rad Transblot apparatus. Membranes were blocked in 5 % non-fat dry milk in PBS, and kept overnight at 4 °C. Polyclonal antisera raised against KLH-conjugated synthetic peptides corresponding to the hydrophilic loops IV (antibody 324) and XII (antibody 326) of plant V-H^+-PPase [21] were used. A 1:1000 dilution of antiserum in blocking buffer was applied to blots at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with PBS [containing 0.1 % (v/v) Tween 20], before addition of a 1:10000 dilution of goat anti-rabbit IgG in blocking buffer for 30 min. Immunoblots were visualized on radiographic film (Kodak) using the ECL® chemiluminescence detection kit (Amersham Life Science).

Immunofluorescence microscopy

Parasites released by the saponin treatment were fixed with 4 % formaldehyde (freshly prepared) and adhered to poly-L-lysine-coated coverslips. Following permeabilization with 0.3 % Triton X-100 for 3 min, parasites were blocked with ammonium chloride and 3 % BSA in PBS for 30 min, and prepared for immunofluorescence using a 1:100 dilution of anti-V-H^+-PPase antibody or normal serum in 5 % BSA-PBS, and a FITC-coupled goat anti-rabbit IgG secondary antibody (1:160). Immunofluorescence images were obtained with a Leica TCS SP spectral confocal microscope, as described previously [24,25].

RESULTS

Intracellular Ca^{2+} concentration in trophozoites: effect of thapsigargin

Figure 1(A) shows the excitation spectrum of fura-2 in suspensions of *P. berghei* trophozoites in a reaction medium containing approx. 4.5 μM free Ca^{2+} (trace a). Addition of EGTA resulted in a free Ca^{2+} concentration of < 0.07 nM (essentially Ca^{2+}-free medium), with no significant change in the spectrum observed, thus indicating the absence of extracellular fura-2 (trace b). The excitation spectrum of fura-2, when the cells were lysed with Triton X-100 under these conditions, showed a peak at 380 nm (Figure 1A, trace c). $CaCl_2$ was then added to obtain a free Ca^{2+} concentration higher than 1 μM. The hydrolysis of fura-2/AM was confirmed by the increase in λ_{max} at 340 nm (Figure 1A, trace d). Since the fluorescence intensity at 340 nm was shown to attain its maximum at about 30 min of fura-2 loading with the cells at room temperature, this loading period was used in subsequent experiments.

The concentration of cytosolic calcium in trophozoites was 57.8 ± 3.1 nM ($n = 8$) after 10 min in the absence of extracellular Ca^{2+} (1 mM EGTA added), 116.0 ± 12.1 nM ($n = 5$) after 10 min in the presence of 1 mM extracellular Ca^{2+}, and 77.5 ± 4.5 nM ($n = 8$) after 10 min in the presence of contaminant Ca^{2+} present in the buffer (4.5 μM). These concentrations are in the range

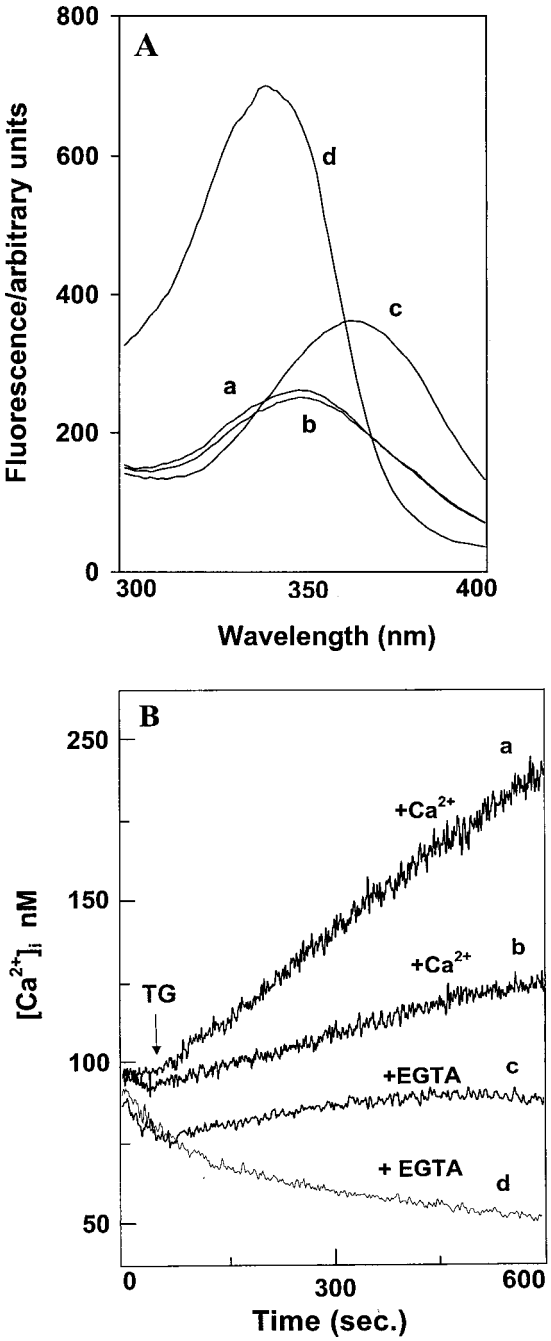

Figure 1 Excitation spectra of fura-2 after loading into *P. berghei* trophozoites (A) and effect of thapsigargin on $[Ca^{2+}]_i$ (B)

(**A**) Cells (2×10^7/ml) were loaded with fura-2/AM (trace a) as described in the Materials and methods section. 1 mM EGTA was then added (trace b), the cells were lysed with 0.02 % Triton X-100 (trace c), and $CaCl_2$ was added to a final Ca^{2+} concentration higher than 1 μM (trace d). (**B**) Cells were incubated in buffer A in the presence of 1 mM EGTA (+EGTA) or 1 mM $CaCl_2$ (+Ca^{2+}), and the $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at 340 nm and 380 nm, as described by Grynkiewicz et al. [22]. The arrow indicates the addition of thapsigargin (TG; 1 μM, only in traces a and c), or the solvent for thapsigargin (traces b and d). The trace in the absence of additions (not shown) was superimposable to that in the presence of the solvent (trace d).

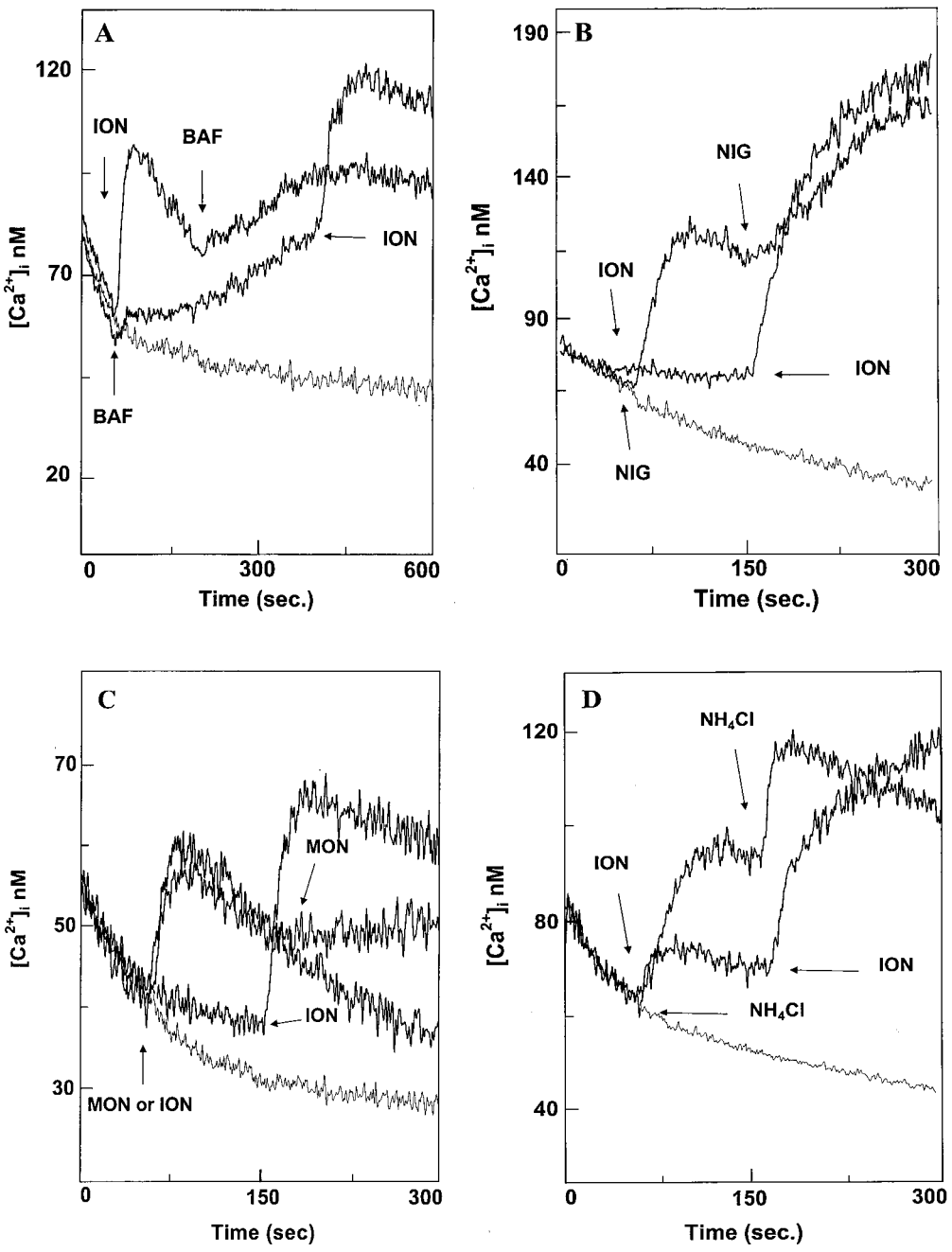

Figure 2 Effect of bafilomycin A_1, nigericin, monensin, NH_4Cl, and ionomycin on trophozoites $[Ca^{2+}]_i$

Cells were loaded with fura-2/AM as described under the Materials and methods section and suspended in buffer A, containing 1 mM EGTA. Bafilomycin A_1 (BAF; 1 μM) in (**A**), nigericin (NIG; 1 μM) in (**B**), monensin (MON; 1 μM) in (**C**), NH_4Cl (10 mM) in (**D**), and ionomycin (ION; 0.25 μM) in (**A–D**), were added where indicated. Pale lines indicate the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added (only one trace is shown in each panel since traces were superimposable).

observed in many studies with eukaryotic cells [22]. We noticed that if the trophozoites were maintained in a buffer without added $CaCl_2$ (only with contaminant Ca^{2+} present) there was a partial depletion of their intracellular Ca^{2+} pools even when they were kept in ice. We then added 1 mM $CaCl_2$ to the suspension buffer and kept them on ice until the experiments were started. All experiments were completed within two hours of the isolation of the parasites from the erythrocytes.

Addition of 1 μM thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase when used at low concentrations [26], increased the $[Ca^{2+}]_i$ (Figure 1B, traces a and c). The effect of this inhibitor was more accentuated in the presence of extracellular Ca^{2+} (Figure 1B, trace a). This could be due to increased Ca^{2+} loss in the presence of EGTA leading to a reduction in the size of the thapsigargin-sensitive Ca^{2+} pool, and resulting in smaller Ca^{2+} rises in the absence of extracellular Ca^{2+} (Figure 1, trace c). In agreement with this hypothesis, the basal Ca^{2+} level decreased with time after suspension of the cells in the buffer containing 1 mM EGTA (Figure 1, trace d) until it reached the steady state indicated above. A Ca^{2+} increase was

also observed over time when the parasites were suspended in medium containing 1 mM $CaCl_2$ (Figure 1, trace b) until it reached the steady state indicated above.

Increase in $[Ca^{2+}]_i$ by bafilomycin A_1, nigericin, monensin and ammonium chloride in trophozoites

Bafilomycin A_1 is an antibiotic which specifically inhibits V-H^+-ATPases [27]. Since acidocalcisomes are characterized by the presence of an ATP-driven H^+ pump to maintain their acidic interior [10–12], we tested the effect of this drug on *P. berghei* $[Ca^{2+}]_i$. Addition of 1 μM bafilomycin A_1 (Figure 2A) increased $[Ca^{2+}]_i$ in trophozoites incubated in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA), indicating the release of Ca^{2+} from intracellular stores. Similar results were obtained when the K^+/H^+ exchanger nigericin (Figure 2B), or the Na^+/H^+ exchanger monensin (Figure 2C) was used instead of bafilomycin A_1. The ability of bafilomycin A_1, monensin, and nigericin to increase $[Ca^{2+}]_i$ in the nominal absence of extracellular Ca^{2+} suggests that a significant amount of Ca^{2+} is stored in an intracellular acidic compartment.

Release of Ca^{2+} by NH_4Cl and the synergistic effect of ionomycin with different alkalinizing agents

Exposure of *P. berghei* trophozoites to 10 mM NH_4Cl resulted in a rapid increase in $[Ca^{2+}]_i$ (Figure 2D). This increase in $[Ca^{2+}]_i$ tended to return to basal levels. Addition of equimolar NaCl, in the absence of an isotonic correction, did not result in increased $[Ca^{2+}]_i$ (results not shown), thus indicating that the effect was not due to changes in osmotic pressure. Ammonium was concluded to be the active ion since $(NH_4)_2SO_4$ produced identical results (results not shown). Taken together, these results indicate that the increase in $[Ca^{2+}]_i$ produced by the different agents tested (bafilomycin A_1, monensin, nigericin, NH_4Cl) was due to release of Ca^{2+} from intracellular stores.

The cellular origin of the mobilized Ca^{2+} was investigated by repeating the above experiments with sequential addition of NH_4Cl, nigericin, monensin, bafilomycin A_1, and ionomycin. Ionomycin binds essentially no Ca^{2+} below pH 7.0, and it cannot carry Ca^{2+} out of acidic compartments because of competition from protons at the inside face of the membrane [28]. Adding ionomycin (0.25 μM) to trophozoites previously exposed to bafilomcyin A_1 (1 μM), nigericin (1 μM), monensin (1 μM), or NH_4Cl (10 mM) caused a second rise in $[Ca^{2+}]_i$ (Figures 2A–2D). If the order of additions were reversed, ionomycin caused an increase in $[Ca^{2+}]_i$ (Figures 2A–2D), and addition of alkalinizing agents caused secondary increases in $[Ca^{2+}]_i$ to levels which were higher than those in the absence of ionomycin. These results suggest that the Ca^{2+} mobilized by ionomycin in the absence of alkalinizing agents comes from different internal pool(s) to that released by alkalinizing compounds with or without ionomycin. In the absence of alkalinizing agents, ionomycin releases a certain amount of Ca^{2+} only from neutral or alkaline compartments, but releases more Ca^{2+} after alkalinizing agents have elevated the pH of acidic compartments. These results are compatible with the pH of a Ca^{2+}-containing intracellular compartment of trophozoites being acidic.

To provide more evidence for the presence of at least two different Ca^{2+} stores, we performed experiments using sequential additions of thapsigargin and nigericin. Adding nigericin (0.5 μM) to trophozoites (Figure 3A) previously exposed to thapsigargin (0.7 μM) caused a second rise in $[Ca^{2+}]_i$. If the order of additions was reversed, nigericin caused an increase in $[Ca^{2+}]_i$; further addition of thapsigargin caused a secondary increase in $[Ca^{2+}]_i$ to a level which was significantly higher to that in the

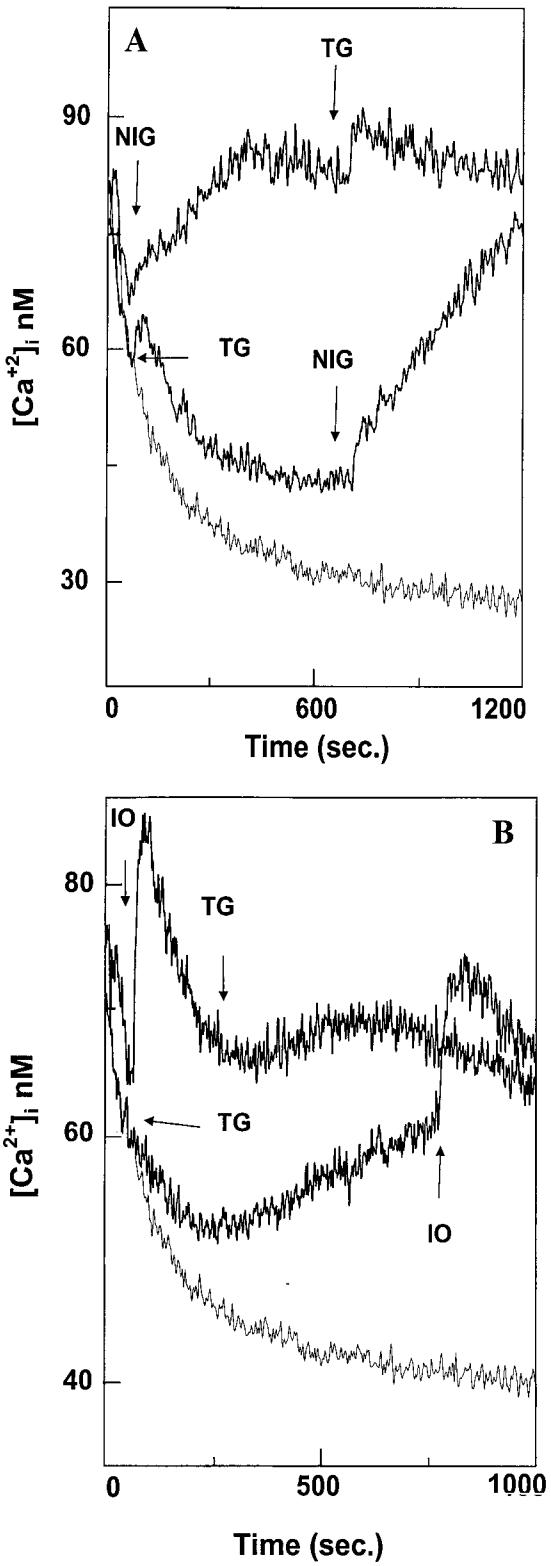

Figure 3 Effects of nigericin, thapsigargin, and ionomycin on Ca^{2+} release from intact trophozoites

Trophozoites were loaded with fura-2/AM and suspended in buffer A, containing 1 mM EGTA. Nigericin (NIG; 1 μM) in (**A**), ionomycin (ION; 0.25 μM) in (**B**), and thapsigargin (TG; 0.7 μM) in (**A**) and (**B**), were added where indicated. Pale lines indicate the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added (only one trace is shown in each panel since traces were superimposable).

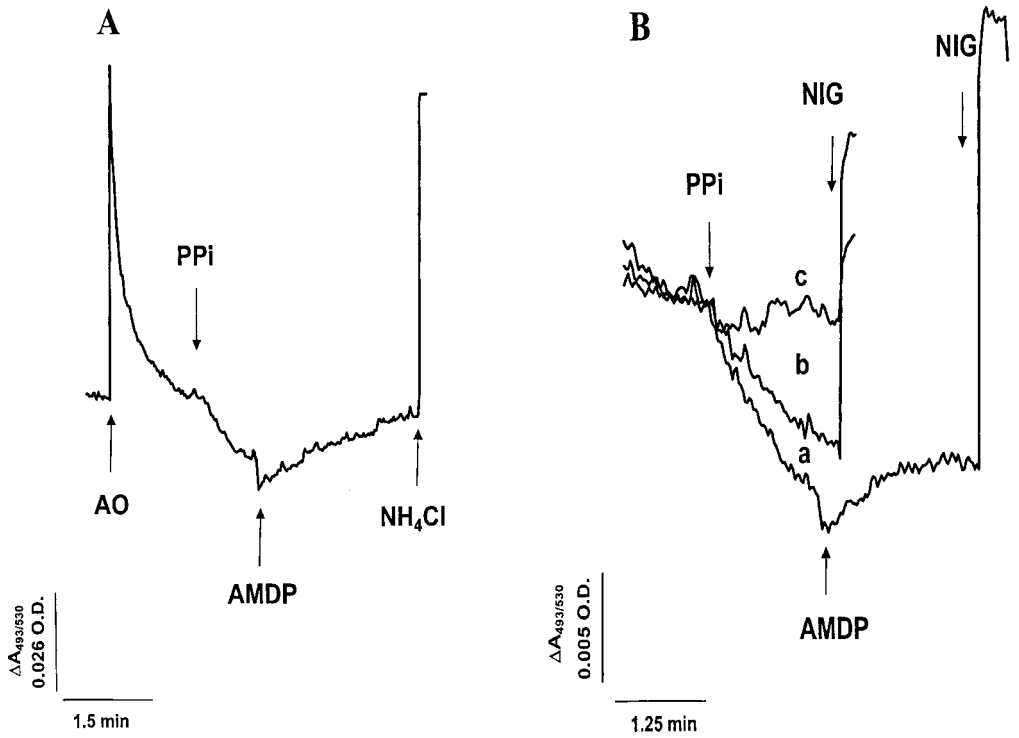

Figure 4 PP_i-driven proton transport in trophozoite homogenates

Homogenates (55 μg protein/ml) were added to a buffer containing 130 mM KCl, 2 mM $MgSO_4$, 50 μM EGTA and 10 mM Hepes (pH 7.2), plus 3 μM Acridine Orange (AO) in the absence (**A**, and trace a in **B**) or in the presence of 2.5 μM (**B**, trace b) or 7.5 μM AMDP (**B**, trace c). Acridine Orange (AO; 3 μM), 0.1 mM PP_i, 10 mM NH_4Cl, 20 μM AMDP (added in **A** and in trace a of **B**), and 1 μM nigericin (NIG), were added where indicated by the arrows.

absence of nigericin (Figure 3A). Sequential additions of ionomycin (0.25 μM) and thapsigargin (0.7 μM), in either order, resulted in independent rises of $[Ca^{2+}]_i$ (Figure 3B), suggesting the presence of a non-acidic, thapsigargin-insensitive Ca^{2+} compartment as has been described recently in *T. gondii* [29]. Taken together, these results indicate the presence of an acidic compartment, sensitive to a H^+/K^+ exchanger (nigericin), a Na^+/H^+ exhanger (monensin) and an inhibitor of the vacuolar H^+-ATPase (bafilomycin A_1), and insensitive to thapsigargin, that contains a significant amount of Ca^{2+} in *P. berghei* trophozoites and which is physiologically similar to that described as the acidocalcisome in trypanosomatids [10,11] and *T. gondii* [12].

PP_i drives proton transport in a subcellular compartment of trophozoites

When Acridine Orange was added to cell homogenates of *P. berghei* trophozoites some dye was accumulated and retained in the absence of added energy sources (Figure 4A). Once a steady state of Acridine Orange accumulation was reached, addition of 0.1 mM PP_i led to further dye uptake, indicating increasing vesicular acidity. Acridine Orange accumulation was prevented in a dose-dependent manner by the PP_i analogue and specific inhibitor of plant vacuolar pyrophosphatases [20], AMDP (Figure 4B, compare trace a with traces b and c). AMDP (20 μM) released Acridine Orange when added after acidification had started (Figures 4A and 4B, trace a). The vesicle pH was neutralized, and Acridine Orange release also occurred after addition of 10 mM NH_4Cl (Figure 4A) or 1 μM nigericin (Figure 4B).

Table 1 Effect of buffer composition on pyrophosphatase activity in *P. berghei* trophozoites

Rates are relative (%) to the 130 mM KCl buffer. All buffers contained, in addition, 2 mM $MgSO_4$, 10 mM Hepes and 50 μM EGTA, and were adjusted to pH 7.2 with KOH, NaOH, or Tris base for KCl, NaCl, and NMG buffers respectively. Values are means $\pm$ S.E.M. from the number of experiments in parentheses. Significance of difference compared with the 130 mM KCl buffer (Student's t test): $^*P < 0.05$. Control activities were 37.5 ± 7.6 $\Delta A_{493\text{-}530}$/min $\times 10^3$/mg protein for proton transport and 0.026 μmol pyrophosphate consumed/min/mg protein for pyrophosphate hydrolysis respectively. NMG, *N*-methylglucamine chloride; ND, not detectable. †Rates were corrected by subtraction of nonspecific activity in the presence of 20 μM AMDP.

Experimental conditions	Pyrophosphate hydrolysis (% of control†)	Acidification rate (% of control)
130 mM KCl	100 (12)	100 (9)
65 mM KCl/125 mM sucrose	117 ± 5.9 (6)	85 ± 18 (3)
130 mM NaCl	35 ± 1.4 (3)*	ND
65 mM NaCl/125 mM sucrose	22 ± 4.8 (3)*	ND
65 mM KCl/65 mM NaCl	58 ± 2.4 (3)*	40 ± 10 (3)*
65 mM NMG/125 mM sucrose	47 ± 4.2 (3)*	ND
250 mM Sucrose	28 ± 3.2 (3)*	ND

Pyrophosphatase was also assayed in membrane preparations by inorganic phosphate detection [14]. Control pyrophosphatase activity was 0.026 ± 0.004 μmol PP_i consumed/min/mg protein (means $\pm$ S.E.M. of results from four separate experiments) and

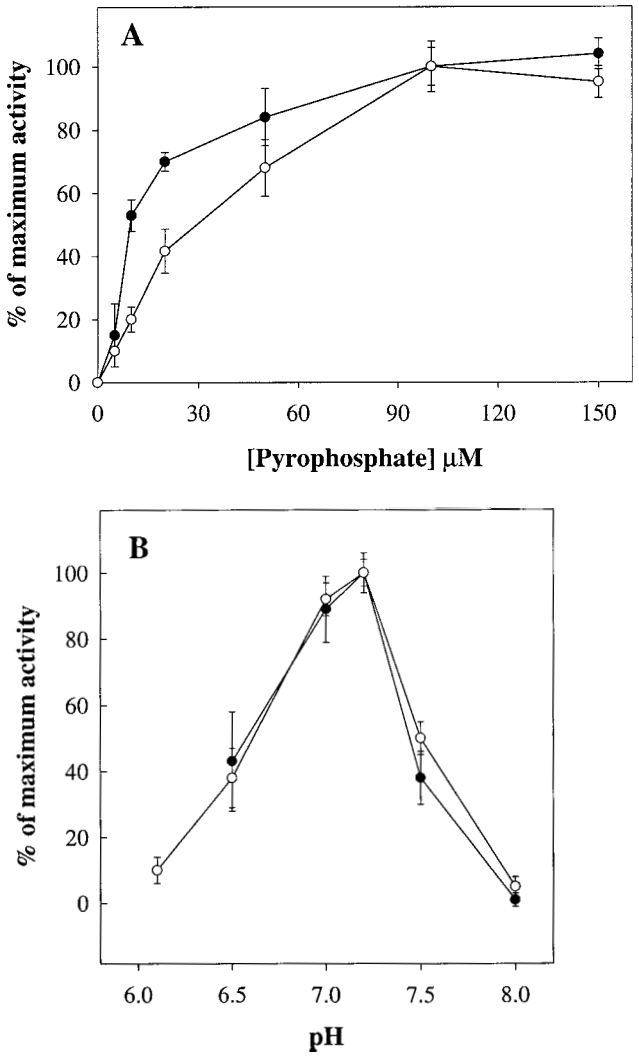

Figure 5 Initial rate of PP_i-dependent proton uptake (○–○) or PP_i hydrolysis (●–●) as a function of PP_i concentration (A) or medium pH (B)

Aliquots of trophozoite homogenates, 55 μg of protein/ml for proton transport assays and 72 μg of protein/ml for PP_i hydrolysis assays, were added to the standard reaction mixture (Figure 4) in the presence of increasing concentrations of PP_i, or incubated in the same buffer adjusted to different pH values. The results are indicated as % of maximum activity. Error bars indicate means ± S.E.M. from at least three separate experiments.

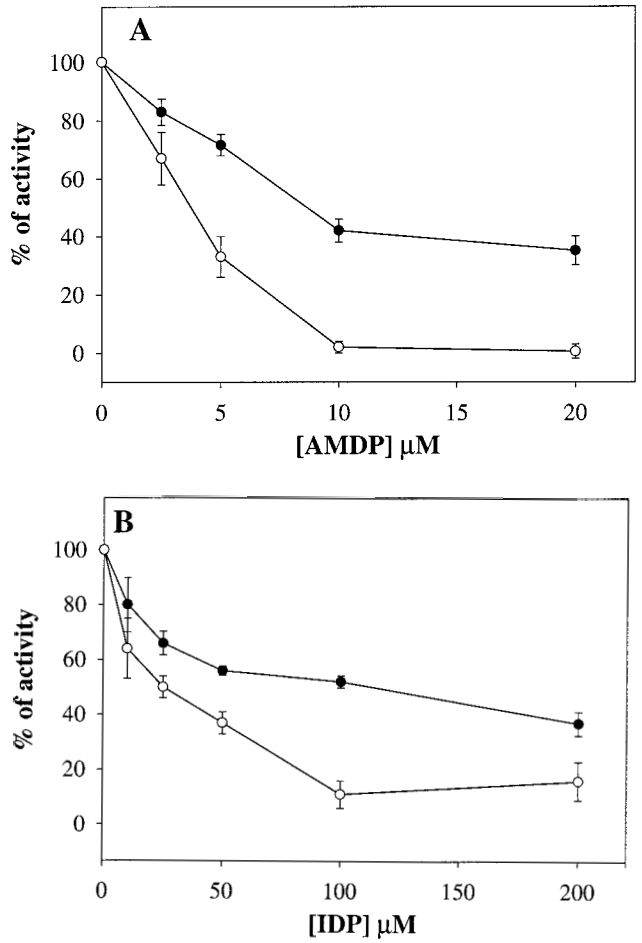

Figure 6 Inhibition of PP_i-dependent proton pumping (○–○) and PP_i hydrolysis (●–●) by PP_i analogues in trophozoite homogenates

Assays were run in the standard buffer described in Figure 4. Aliquots of trophozoite homogenates, 40–80 μg of protein/ml for proton transport assays and 50–90 μg of protein/ml for PP_i hydrolysis assays, were added to the standard reaction mixture in the presence of increasing concentrations of AMDP and IDP. Percent activity compared to the control in the absence of inhibitors (100%) is indicated. Control activities were 37.5 ± 7.6 $\Delta A_{493-530}$/min × 10^3/mg protein for proton transport, and 0.026 μmol PP_i consumed/min/mg protein for PP_i hydrolysis. Error bars indicate means ± S.E.M. from at least 3 separate experiments.

was inhibited by 20 μM AMDP by 68 ± 4% (average ± S.E.M. of three experiments). The effects of monovalent cations on both acidification rate and AMDP-inhibitable PP_i hydrolysis were similar (Table 1). No acidification was detectable if KCl in the medium was replaced with NaCl, *N*-methylglucamine chloride, or sucrose. Use of a buffer containing equimolar concentrations (65 mM) of NaCl and KCl, resulted in lower Acridine Orange uptake and PP_i hydrolysis than in the presence of 130 mM KCl or 65 mM KCl/125 mM sucrose. These results suggest that K^+ was necessary for these activities, whilst Na^+ was inhibitory. Taken together, these results agree with those obtained with plant [17], trypanosomatids [14–16], and *T. gondii* (C. O. Rodrigues, D. A. Scott, B. Bailey, W. De Souza, M. Benchimol, B. Moreno, J. A. Urbina, E. Oldfield and S. N. J. Moreno, unpublished work) V-H^+-PPases, which are largely K^+-dependent. This dependence differentiates V-H^+-PPases from known mitochondrial H^+-PPases, which do not require K^+ [30].

The PP_i concentration dependence of the initial rate of Acridine Orange absorbance decrease and of PP_i hydrolysis in *P. berghei* homogenates is shown in Figure 5(A). Both activities were maximal at about 100 μM PP_i. Figure 5(B) shows the effect of medium pH on the initial rate of PP_i-dependent Acridine Orange absorbance decrease and on PP_i hydrolysis in *P. berghei* homogenates. Both activities were optimal in the range pH 7.0–7.2.

Inhibition of the V-H^+-PPase activity of trophozoites

PP_i hydrolysis and PP_i-induced acidification of the trophozoites subcellular compartment were inhibited, in a dose-dependent manner, by AMDP (Figures 4B and 6A). The inhibition of

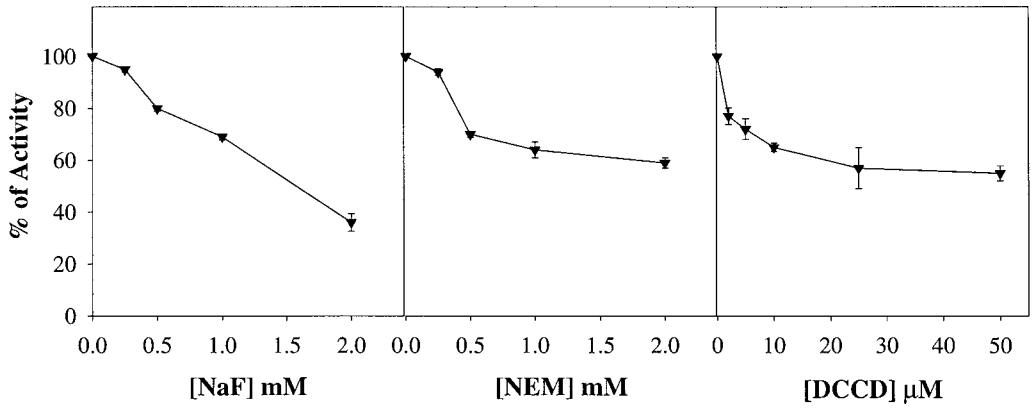

Figure 7 Inhibition of PP_i hydrolysis in trophozoite homogenates by NaF, NEM and DCCD

Assays were run in the standard buffer described in Figure 4. Aliquots of cell homogenates (50–90 μg of protein/ml) were assayed for PP_i hydrolysis in the standard reaction mixture in the presence of increasing concentrations of NaF, NEM, and DCCD. Rates are relative (%) compared to control without inhibitor. Control activity was 0.026 μmol PP_i consumed/min/mg protein for PP_i hydrolysis.

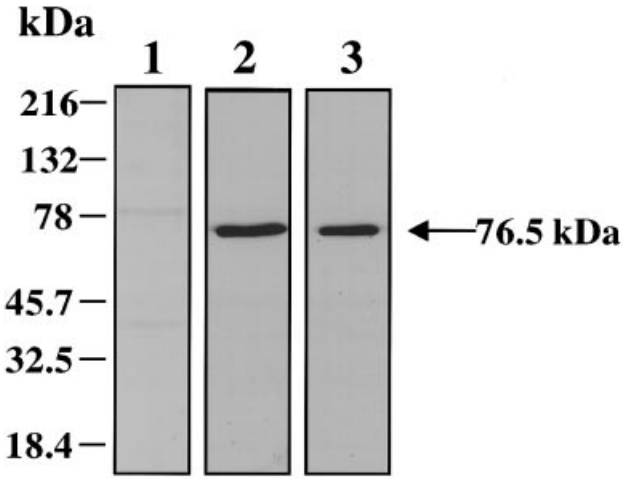

Figure 8 Western blot analysis of the V-H^+-PPase

Panels show the detection of the V-H^+-PPase by immunoblot, using antibodies specific for the plant enzyme. *P. berghei* proteins (10 μg) were separated by SDS/PAGE and transferred to nitrocellulose. Lane 1, immunoblot probed with normal rabbit serum; lanes 2 and 3, immunoblots probed with antibody 326 and 324 respectively. The V-H^+-PPase antibody recognized a polypeptide with an apparent molecular mass of 76.5 kDa.

acidification by AMDP was total at 10 μM, while some residual PP_i hydrolysis activity could be detected even at 20 μM AMDP. This suggests the presence of an AMDP-insensitive pyrophosphatase activity (see also Table 1). Both activities were also inhibited, in a dose-dependent manner, by the PP_i analogue, IDP (Figure 6B), its effect also being more accentuated on the acidification rate than on the PP_i hydrolysis activity.

Figure 7 shows that sodium fluoride, DCCD and the thiol reagent NEM, agents known to inhibit the V-H^+-PPases from plants [17] and trypanosomatids [14–16], were also effective in inhibiting the *P. berghei* pyrophosphatase activity in a dose-dependent manner.

Evidence for localization of V-H^+-PPase in the plasma membrane and vacuoles of trophozoites

Since the vacuolar H^+-translocating pyrophosphatase localizes to vacuoles of higher plant cells [17], and acidocalcisomes [14–16] and plasma membrane [14] of trypanosomatids, we investigated whether this was also the case for *P. berghei*.

The location of the V-H^+-PPase in *P. berghei* was tested using polyclonal antibodies specific for the plant enzyme which cross-react with the H^+-V-ATPase of trypanosomatids [14]. Antibodies 326 and 324 showed cross-reactivity with a band of 76.5 kDa present in *P. berghei* (Figure 8, lanes 2 and 3). No background staining was observed when normal serum was used as a control (Figure 8, lane 1). Immunofluorescence, using the same antibodies (Figure 9A), indicated labelling of intracellular vacuoles (solid arrows) and labelling of the cell surface of some cells (open arrows). No fluorescence was observed in control parasites incubated only with normal serum and secondary fluorescein-labelled goat anti-rabbit IgG (not shown), or in red blood cells present in the preparation (Figure 9A, asterisks).

Release of Ca^{2+} by AMDP and synergistic effect of ionomycin

Exposure of trophozoites to the V-H^+-PPase inhibitor AMDP (40 μM) in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA) resulted in an increase in $[Ca^{2+}]_i$ to a similar level seen in the presence of bafilomycin A_1 (1 μM) (Figure 10A). The effect of AMDP was more rapid than the effect of bafilomycin A_1. Adding ionomycin (0.25 μM) to trophozoites previously exposed to AMDP, caused a second rise in $[Ca^{2+}]_i$ (Figure 10B). If the order of additions were reversed, ionomycin caused an increase in $[Ca^{2+}]_i$, while addition of AMDP caused a secondary increase in $[Ca^{2+}]_i$. These results are similar to those obtained with ionomycin and other alkalinizing agents (Figure 2) and are compatible with the Ca^{2+}-containing, AMDP-sensitive, intracellular Ca^{2+} compartment of trophozoites being the acidocalcisome.

Taken together, these results indicate the presence of an acidic compartment which is sensitive to a H^+/K^+ exchanger (nigericin), a Na^+/H^+ exchanger (monensin), an inhibitor of the vacuolar H^+-ATPase (bafilomycin A_1), and an inhibitor of the vacuolar H^+-PPase (AMDP), and insensitive to thapsigargin. This com-

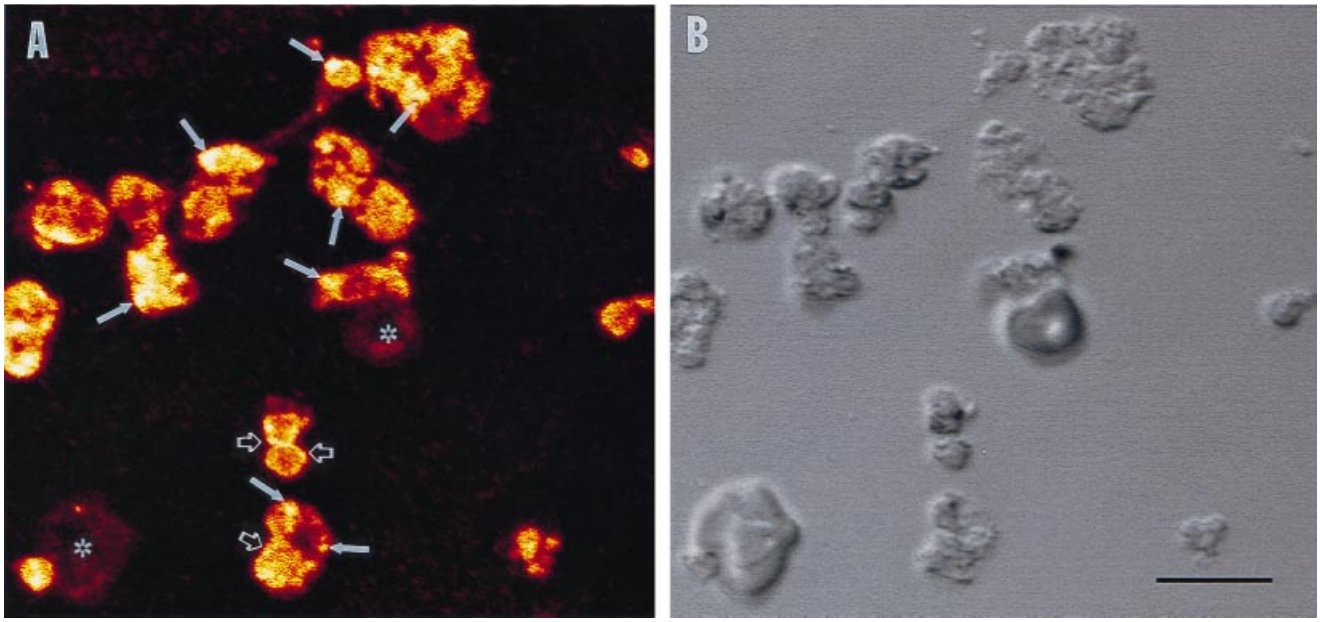

Figure 9 Confocal laser scanning microscopy of V-H$^+$-PPase in trophozoites of *P. berghei*

Fluorescence (**A**) or Nomarsky (**B**) images of a trophozoite preparation before its purification. Trophozoites appear clumped. Panel (**A**) shows intense labelling of cytoplasmic vacuoles (solid arrows) and the plasma membrane of some trophozoites (open arrows) as detected using antibody 324 against the plant V-H$^+$-PPase, as described in the Materials and methods section. No labelling was observed in two red blood cells present in the preparation (asterisks). Bar (for **A** and **B**), 10 μm.

partment contains a significant amount of Ca^{2+} in *P. berghei* trophozoites and is physiologically similar to that described as the acidocalcisome in trypanosomatids [10,11] and *T. gondii* [12].

DISCUSSION

The present study indicates that a considerable amount of Ca^{2+} within *P. berghei* trophozoites is located in an acidic compartment (Figures 2, 3 and 10). Use of the Ca^{2+} ionophore, ionomycin, in combination with monensin, nigericin, NH_4Cl, or specific inhibitors of the V-H$^+$-ATPase (bafilomycin A_1; [27]) or the V-H$^+$-PPase (AMDP; [20]), allowed a large amount of Ca^{2+} to be released (Figures 2 and 10). Ionomycin alone was also effective, which strongly suggests the presence of other, non-acidic Ca^{2+}-storage compartments, some of which are also sensitive to the sarcoplasmic/endoplasmic reticulum-type Ca^{2+}-ATPase inhibitor, thapsigargin (Figure 3). In this regard, genes which encode proteins with homology to sarcoplasmic/endoplasmic reticulum-type Ca^{2+}-ATPases have been cloned from *P. yoelli* [31] and *P. falciparum* [32].

We have also identified and characterized an H$^+$-translocating pyrophosphatase activity in *P. berghei*. Acridine Orange uptake in the presence of PP_i was reversed by the K^+/H^+ exchanger, nigericin, indicating that PP_i induced organelle acidification. This proton-translocating activity was optimal at pH 7.0–7.2 (Figure 5B). The pH optimum of the V-H$^+$-PPase of *L. donovani* acidocalcisomes is between 7.0 and 7.5 [15]. PP_i-driven proton transport was blocked and PP_i hydrolysis was inhibited by the PP_i analogues, AMDP and IDP. PP_i hydrolysis was inhibited by NaF, NEM and DCCD, and both activities were stimulated by potassium ions. All these characteristics are similar to those of plant [16] and trypanosomatid [14–16] V-H$^+$-PPases.

A gene encoding for a protein with homology to inorganic pyrophosphatases has been reported recently in *P. falciparum* [33]. The presence of this enzyme would explain the differences observed in the effect of inhibitors on proton transport and PP_i hydrolysis (Figure 6). V-H$^+$-PPases had been described in detail before only in plants and in phototrophic bacteria [17]. In plants, V-H$^+$-PPases are present in the vacuole membrane (tonoplast) [17] and also in the plasma membrane [34,35]. Use of antibodies to conserved regions of the plant V-H$^+$-PPase known to cross-react with the enzyme from *T. cruzi* [14] suggested a plasma membrane and intracellular localization. The same antibodies used in this study reacted with a *P. berghei* polypeptide (Figure 8) and also suggested an intracellular and plasma membrane localization of the V-H$^+$-PPase (Figure 9). A similar plasma membrane and vacuolar localization of this enzyme has been reported recently in *P. falciparum* [36].

In conclusion, our results indicate that the mechanisms of Ca^{2+} homeostasis and vacuolar acidification in malaria parasites are quite different from those of most mammalian cells [37,38]. In addition to thapsigargin-sensitive and -insensitive neutral Ca^{2+} stores, malaria parasites possess acidocalcisomes carrying vacuolar-type proton pumps. Thapsigargin-insensitive Ca^{2+} stores have been found only in mammalian cells derived from the rat pituitary gland (the adrenal tissue and mast cells), although the role of this previously unrecognized compartment is not known [39]. In mammalian cells Ca^{2+} has also been reported to be present in acidic organelles carrying vacuolar-type proton pumps, such as endosomes, lysosomes and the *trans*-Golgi network, as well as in secretory granules such as chromaffin, pancreatic zymogen, salivary acinar, pituitary, platelet and atrium-specific granules [40]. These organelles contain V-H$^+$-ATPases but do not have V-H$^+$-PPases. A vacuolar-type pyrophosphatase activity was detected in rat liver Golgi fractions [41],

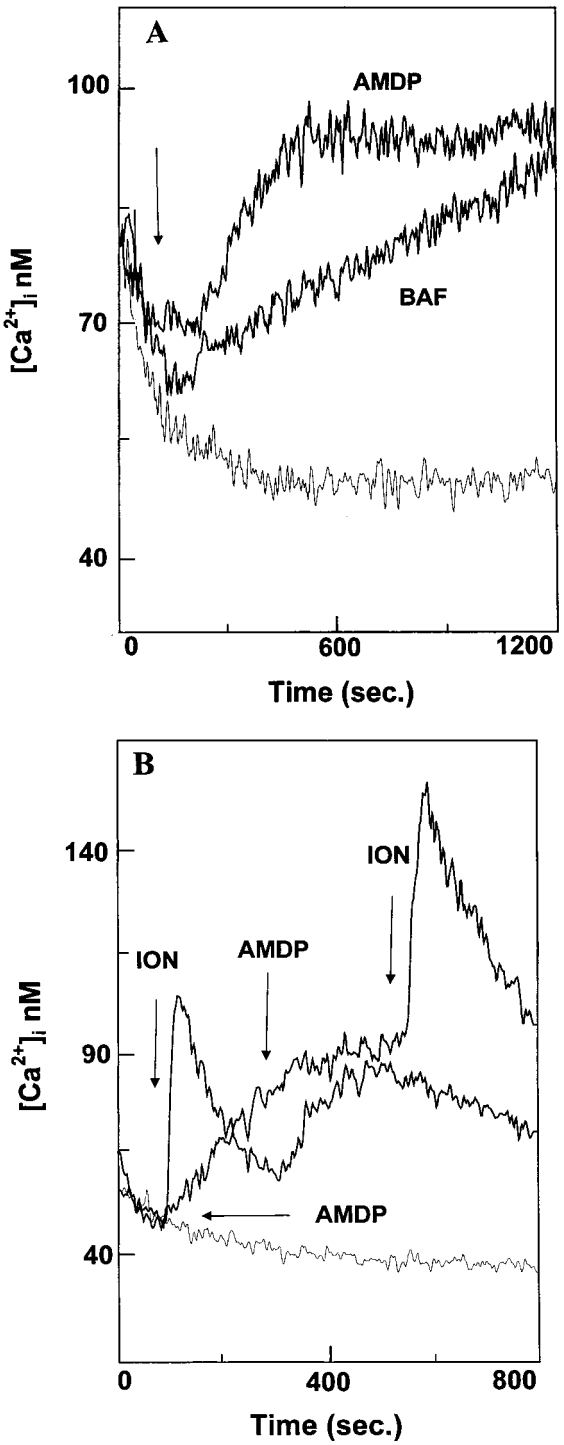

Figure 10 Effect of AMDP, bafilomycin A_1, and ionomycin on Ca^{2+} release from intact trophozoites

Trophozoites were loaded with fura-2/AM and suspended in the same buffer described in Figure 2. Bafilomycin A_1 (BAF; 1 μM), AMDP (40 μM) in (**A**) and (**B**), and ionomycin (ION; 0.25 μM) in (**B**), were added where indicated. Pale lines indicate the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added (only one trace is shown in each panel since traces were superimposable).

but this activity was found using very high concentrations of PP_i (1–3 mM) and appears to be different from the plant or malaria enzyme. The apparent lack of a plant-like V-H^+-PPase in mammalian cells makes this enzyme a potential target for specific chemotherapy. In this regard, we have recently demonstrated that several PP_i analogues currently used for the treatment of bone resorption disorders (bisphosphonates), selectively inhibited the proliferation of different acidocalcisome-containing parasites ([42] and C. O. Rodrigues, D. A. Scott, B. Bailey, W. De Souza, M. Benchimol, B. Moreno, J. A. Urbina, E. Oldfield and S. N. J. Moreno, unpublished work), offering a potential new route to chemotherapy.

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