Vacuolar proton pyrophosphatase activity and pyrophosphate (PP_i) in *Toxoplasma gondii* as possible chemotherapeutic targets

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The addition of PP_i promoted the acidification of a subcellular compartment in cell homogenates of *Toxoplasma gondii* tachyzoites, implying the presence of a proton-translocating pyrophosphatase. The proton gradient was collapsed by addition of the K^+/H^+ antiporter nigericin, and was also inhibited by addition of the PP_i analogue aminomethylenediphosphonate (AMDP). Both proton transport and PP_i hydrolysis were dependent upon K^+, but Na^+ caused partial inhibition of these activities. PP_i hydrolysis was sensitive in a dose-dependent manner to AMDP, imidodiphosphate, NaF and to the thiol reagent *N*-ethylmaleimide. This activity was unaffected by common inhibitors of phosphohydrolases, except that NaO_3V (sodium orthovanadate) stimulated the activity by 87%. Immunofluorescence microscopy, using antisera raised against conserved peptide sequences of a plant vacuolar pyrophosphatase, suggested that the pyrophosphatase in *T. gondii* tachyzoites was located in the plasma membrane and intracellular vacuoles of the parasite. High-field ^{31}P-NMR spectroscopy showed that $PP_i$was more abundant than ATP in tachyzoites. Bisphosphonates (PP_i analogues), drugs that are used in the treatment of bone diseases, inhibited proton transport and PP_i hydrolysis in tachyzoite homogenates, and also inhibited intracellular proliferation of tachyzoites in tissue culture cells.

Key words: acidocalcisome, apicomplexan, bisphosphonates, ^{31}P NMR.

INTRODUCTION

Toxoplasma gondii is a widespread opportunistic pathogen that causes disease in congenitally infected infants and immunocompromised patients. *T. gondii* belongs to the phylum Apicomplexa, which contains other important parasites of humans and animals, such as *Plasmodium* (the aetiologic agent of malaria), *Eimeria* (the aetiologic agent of coccidiosis in domestic animals), and *Cryptosporidium* (a pathogen that causes diaorrhea). Infections caused by apicomplexan parasites are among the most widespread parasitic diseases in the world and are responsible for heavy socio-economic losses, especially in underdeveloped countries. There is, therefore, considerable interest in developing novel chemotherapeutic approaches, based on unique aspects of parasite structure and metabolism.

In previous work on the calcium and pH homoeostasis of *T. gondii*, the presence of a vacuolar and plasma-membrane-located H^+-ATPase [1], and calcium storage within the cell in an acidic compartment, which was named the acidocalcisome [2], have been demonstrated. Acidic calcium pools have also been found in other apicomplexan [3] and trypanosomatid [4–6] parasites. Acidocalcisomes in trypanosomatids [7–9] have been shown to contain a vacuolar-proton-translocating pyrophosphatase (V-H^+-PPase), an enzyme previously described in detail only in plants [10].

PP_i was for a long time believed to be merely a by-product of biosynthetic reactions (synthesis of nucleic acids, coenzymes, proteins, activation of fatty acids, isoprenoid synthesis), which was subject to immediate hydrolysis by pyrophosphatases. From the early 1960s, however, data have accumulated suggesting an important bioenergetic and regulatory role for this compound [11]. In *T. gondii*, PP_i is used in place of ATP as an energy donor for the pyrophosphate-dependent phosphofructokinase [12,13]. It has also been shown that PP_i analogues are selective inhibitors of this enzyme, and also inhibit intracellular *T. gondii* replication, although no definitive linkage between the two events could be drawn [13].

In the present work, we demonstrate that *T. gondii* tachyzoites possess a V-H^+-PPase with features in common with the trypanosomatid and plant enzymes, and provide evidence that suggests that the location of the enzyme is in the plasma membrane and intracellular vacuoles. Our results also indicate that PP_i is more abundant than ATP in tachyzoites, and that PP_i analogues inhibit the V-H^+-PPase and the intracellular proliferation of these parasites.

MATERIALS AND METHODS

Culture methods

Tachyzoites of *T. gondii* RH strain were cultivated according to Moreno and Zhong [2], in bovine turbinate cells (A.T.C.C.; CRL 1390). Host cells were cultivated in tissue culture flasks using minimal essential medium supplemented with 10% (v/v) horse serum. Cells were infected with tachyzoites to a final ratio of 1:5 (host/parasite); parasites were harvested 2–3 days after infection and purified as described previously [14]. Protein concentrations were measured by using the Bio-Rad Coomassie Blue method.

Abbreviations used: AMDP, aminomethylenediphosphonate; IDP, imidodiphosphate; V-H^+-PPase, vacuolar-proton-translocating pyrophosphatase; KLH, keyhole-limpet haemocyanin; TCA, trichloroacetic acid.

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Chemicals

Dulbecco's PBS, Hanks solution, imidodiphosphate (IDP), nigericin, NaO_3V (sodium orthovanadate), NaF, oligomycin, Na_2MoO_4 (sodium molybdate), sodium pyrophosphate and all protease inhibitors, with the exception of 4-(2-aminoethyl)-benzenesulphonyl fluoride HCl ('AEBSF'), which was from Calbiochem, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bafilomycin A_1 was from Kamiya Biomedicals (Thousand Oaks, CA, U.S.A.). Acridine Orange, 2-amino-6-mercapto-7-methylpurine ribonucleoside, purine-nucleoside phosphorylase (these two components were from the EnzChek phosphate assay kit), and the phosphate standard solution were from Molecular Probes (Eugene, OR, U.S.A.). Aminomethylenediphosphonate (AMDP) [15] 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 [16], were kindly provided by Professor Philip Rea (Plant Science Institute, Biology Department, University of Pennsylvania, PA, U.S.A.). All other reagents were analytical grade.

Preparation of cell homogenates

For proton pumping and PP_i hydrolysis measurements, tachyzoites (approx. 5×10^9 cells for each preparation) were centrifuged and washed twice in buffer A [116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 50 mM Hepes (pH 7.2), 5.5 mM glucose], 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, 10 μM pepstatin A, 10 μM leupeptin, 10 μM E-64, 10 μM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('tosyl-lysylchloromethane'), pH 7.2]. The cell pellet was mixed with approx. twice its wet weight of silicon carbide (Aldrich) and ground using a mortar and pestle until lysis was greater than 90% (generally 30 s). The 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 was resuspended in 1 ml of lysis buffer for use in assays.

For PP_i hydrolysis experiments with various buffers (Table 1), cells (approx. 5×10^9 tachyzoites) were washed in buffer A, and lysed by freeze–thawing five times in a buffer containing 125 mM sucrose, 20 mM Tris/Hepes (pH 7.4), and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin and 1 mM PMSF). The membrane fraction used in the assays was prepared from the homogenate by centrifugation at 2400 ***g*** for 1 min, and the pellet was washed three times in reaction buffer (see below) containing 1 mM dithiothreitol.

Proton pump activity

Pyrophosphate-driven proton transport was assayed by measuring changes in the absorbance of Acridine Orange ($A_{493} - A_{530}$) in an SLM-Aminco DW2000 dual-wavelength spectrophotometer [7]. Cell homogenates were incubated at 30 °C in 2.5 ml of standard buffer [130 mM KCl, 2 mM $MgSO_4$, 10 mM Hepes/KOH, 50 μM EGTA (pH 7.2)] or the buffers described in Table 1. Acridine Orange (3 μM) and various inhibitors where then added 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.

Table 1 Effect of buffer composition on pyrophosphatase activity in *T. gondii* tachyzoites

The rates are given as a percentage of the rate in controls (100%), where 130 mM KCl buffer was used in each experiment. All buffers contained 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 or *N*-methylglucamine chloride buffers respectively. Values are the means ± S.D. of the number of experiments shown in parentheses. Control activities were $(1.6 \pm 0.203) \times 10^3$ $\Delta A_{493-530}$/min for proton transport and 0.012 ± 0.007 μmol of PP_i consumed/min per mg of protein respectively. The PPase activity in *Toxoplasma* is low and there was a large variation in the control activity, measured in 130 mM KCl. Consistent results were obtained by calculating the rate in the various buffers as a percentage of the rate in the 130 mM KCl buffer, in each experiment, and then taking the average.

Experimental conditions	Acidification rate (% of control)	PP_i hydrolysis (% of control)*
130 mM KCl	100 (5)	100 (4)
65 mM KCl/125 mM sucrose	77 ± 4 (5)	71 ± 6 (4)
130 mM NaCl	6 ± 6 (4)	19 ± 14 (4)
65 mM NaCl/125 mM sucrose	35 ± 12 (3)	23 ± 4 (3)
65 mM KCl/65 mM NaCl	42 ± 8 (4)	62 ± 8 (3)
65 mM *N*-methylglucamine chloride/125 mM sucrose	30 ± 10 (5)	8 ± 8 (3)
250 mM sucrose	14 ± 9 (4)	31 ± 8 (4)

* Rates were corrected by subtraction of non-specific activity in the presence of 20 μM AMDP.

Pyrophosphatase assay

Pyrophosphatase activity, in terms of P_i release, was assayed as described previously [7], except that a volume of 0.1 ml in a micro-titre plate and single-wavelength (360 nm) detection (PowerWave 340 microplate scanning spectrophotometer; Biotek Instruments, Winooski, VT, U.S.A.) were used. The reaction mixtures contained 130 mM KCl, 10 mM Hepes/KOH, 2 mM $MgSO_4$, 50 μM EGTA (pH 7.2) (or the buffers described in Table 1), 0.1 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 0.4 unit/ml purine-nucleoside phosphorylase, together with the cell membrane preparation. Activity was calibrated for each buffer with a phosphate standard solution.

SDS/PAGE and preparation of Western blots

T. gondii tachyzoites (1×10^9) were resuspended in 300 μl of 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 of electrophoresis buffer [125 mM Tris/HCl (pH 7), 10% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 4.0% (w/v) SDS, 4.0% (w/v) Bromophenol Blue] and boiled for 5 min before the proteins were separated by SDS/PAGE (10% gel). The proteins were transferred to nitrocellulose [17] using a Bio-Rad Transblot apparatus. The blots were blocked in 5% (w/v) non-fat dried 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 [16] were used. A 1:1000 dilution of antiserum in blocking buffer was applied to the blots at room temperature for 60 min. The nitrocellulose was washed three times for 15 min with 0.1% (v/v) Tween 20 in PBS before the addition of a 1:2000 dilution of goat anti-rabbit IgG in blocking buffer for 30 min. Immunoblots were visualized by autoradiography (Kodak film) using an ECL® detection kit (Amersham).

Immunofluorescence microscopy

Parasites fixed with freshly prepared 4% formaldehyde were adhered to poly-L-lysine-coated coverslips, permeabilized with 0.3% (v/v) Triton X-100 for 3 min, blocked with NH_4Cl and 3% (w/v) BSA in PBS, and prepared for immunofluorescence using a 1:100 dilution of anti-V-H^+-PPase antibody and a FITC-coupled goat anti-rabbit IgG secondary antibody (1:80). Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope and the image analysis system described previously [6,18].

Preparation of perchloric acid extracts, pyrophosphatase treatments and ^{31}P-NMR spectrometry

For NMR spectroscopy, *T. gondii* tachyzoites (2×10^{10}) were washed twice with buffer A and extracted with ice-cold 0.5 M $HClO_4$ (2 ml/g wet weight of cells). After 30 min incubation on ice, the extracts were centrifuged at 3000 ***g*** for 5 min. The supernatants were neutralized by the addition of 0.72 M KOH/0.6 M $KHCO_3$. Precipitated $KClO_4$ was removed by centrifugation at 3000 ***g*** for 5 min, the supernatant was removed and the pH was adjusted to 8.0 with 0.1 M KOH. The samples were divided into two identical portions, 2 mM $MgSO_4$ was added to each, and these were incubated in the absence or presence of yeast inorganic pyrophosphatase (Sigma;10 units/ml, final activity) at 30 °C, pH 7.4. Methylphosphonate was added (0.1 mM final concentration) as an internal NMR shift standard and samples were made 10% (v/v) in ^2H_2O to provide a field-frequency lock. Proton-coupled ^{31}P-NMR spectra were obtained using a 17.625 Tesla Varian INOVA NMR spectrometer, which operates at 303.7663 MHz for ^{31}P (750 MHz for ^{1}H). All NMR experiments were carried out at 25 °C using 14.5 μs 60° pulse widths, 32000 data points and 1280 scans with 1.5 s recycle times. Chemical shifts are reported with respect to external 85% H_3PO_4, using the convention that high-frequency, low-field, paramagnetic or deshielded values are positive (IUPAC convention δ-scale). Both direct referencing versus an external standard of 85% H_3PO_4 (replacement method) as well as use of an internal standard of methyl phosphonate (taken to be 22 p.p.m. downfield from 85% H_3PO_4, at pH 8.0) were used.

Growth inhibition assays

For the invasion and proliferation assays with *T. gondii*, human foreskin fibroblast monolayers were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-calf serum [19], plated in 12-well tissue culture plates and incubated at 37 °C in a 5% CO_2 atmosphere. Cell monolayers were challenged with 1×10^6 tachyzoites per well. After incubation for 1 h at 37 °C in a 5% CO_2 atmosphere, the cultures were washed twice with Hanks solution and the culture medium was replaced in order to remove any remaining extracellular tachyzoites. At this time, 1.0 μCi of [5,6-^{3}H]uracil per well (specific radioactivity 40–50 Ci/mmol; New England Nuclear Research Products) was added, and cultures were incubated for a further 19 h. Incorporation of [^{3}H]uracil into trichloroacetic acid (TCA)-precipitable material was measured at this time. The supernatants were removed from the monolayers by aspiration, the cells were solubilized with 800 μl of 1% (w/v) SDS containing 100 μg of unlabelled uracil/ml, and 2.4 ml of 0.3 M TCA was added. The resulting precipitates were maintained at 4 °C for 15 min and were collected on to glass fibre filters (Whatman GF/C) by using a sampling manifold (Millipore). The filters were washed twice with 0.3 M TCA and once with 95% ethanol, dried, placed in 10 ml of scintillation cocktail (BudgetSolve, Research Products International) and the radioactivity was measured (Packard Tri-Carb 2100 TR). To investigate the effect of antimicrobial agents on the replication of tachyzoites, drugs were added after the *T. gondii* challenge. To investigate the effect on the invasion of human foreskin fibroblasts by tachyzoites, drugs were added only during the challenge period. Subtraction of counts to allow for the labelling of uninfected controls (typically around 300–400 c.p.m. after 19 h incubation) yielded the incorporation that could be ascribed to the intracellular parasites (typically around 25000 c.p.m. after 19 h incubation). Parallel cultures were prepared to determine whether inhibition of growth by antimicrobial agents, as shown by radioactive assay, was similar to that observed microscopically. For the microscopic studies, the cultures were fixed in methanol (1 min) and stained (45 min) with Giemsa stain. *Toxoplasma* present in a minimum of 200 cells in each culture were counted.

RESULTS

PP_i drives proton transport in a subcellular compartment of *T. gondii* tachyzoites

When Acridine Orange was added to cell homogenates of *T. gondii* tachyzoites, some dye accumulated and was retained in the absence of added energy sources (Figure 1). Once a steady state of Acridine Orange accumulation was reached, the addition of 0.1 mM PP_i led to further dye uptake (Figure 1, trace a). This indicated the establishment of a proton gradient (ΔpH) across the membrane of a subcellular compartment, and increasing organelle acidity. The pH gradient collapsed completely after the addition of 1 μM nigericin, and was prevented from occurring by pretreatment with 20 μM AMDP, a PP_i analogue and specific

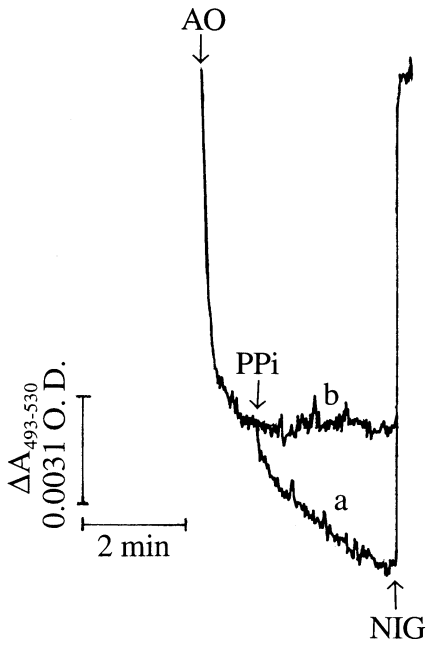

Figure 1 PP_i-driven proton transport in tachyzoite homogenates

Tachyzoite homogenates (21 μg of protein/ml) were added to a buffer containing 130 mM KCl, 2 mM $MgSO_4$, 50 μM EGTA, 10 mM Hepes (pH 7.2) and 3 μM Acridine Orange (AO), in the absence (trace a) or presence (trace b) of 20 μM AMDP; 0.1 mM PP_i and 2 μM nigericin (NIG) were added where indicated (nigericin was added in both experiments but, for clarity, only one line is shown, since the traces were superimposable).

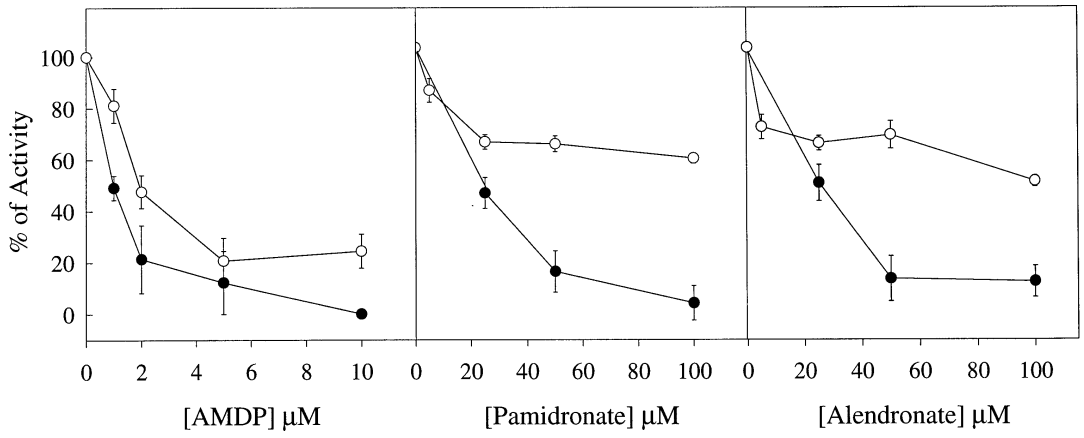

Figure 2 Inhibition of PP_i-dependent proton pumping (●) and PP_i hydrolysis (○) by PP_i analogues (bisphosphonates) in tachyzoite homogenates

Assays were performed in the buffer described in the legend to Figure 1. Aliquots of tachyzoite homogenates, 50 μl (21 μg of protein/ml) for proton transport assays and 10 μl (38 μg of protein/ml) for PP_i hydrolysis assays, were added to the standard reaction mixture in the presence of increasing concentrations of AMDP, pamidronate or alendronate. The percentage activity compared with the control in the absence of inhibitors (100%) is indicated. Control activities were $(1.6 \pm 0.2) \times 10^3$ $\Delta A_{493-530}$/min for proton transport, and $(7.9 \pm 0.5) \times 10^3$ ΔA_{360}/min for PP_i hydrolysis. Error bars indicate means $\pm$ S.E.M. of at least three separate experiments.

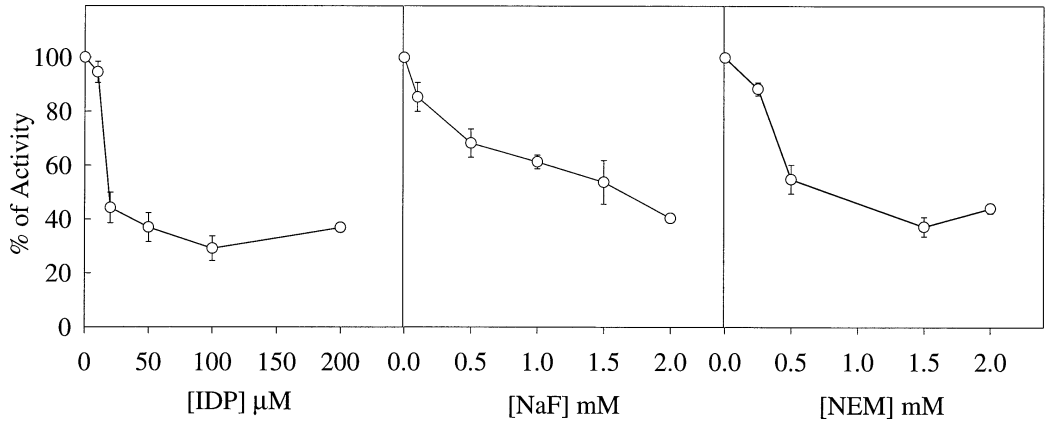

Figure 3 Inhibition of PPi hydrolysis by IDP, NaF and N-ethylmaleimide in tachyzoite homogenates

Assays were performed in the buffer described in the legend to Figure 1. Aliquots of cell homogenates (10 μl, 38 μg of protein/ml) were assayed for PP_i hydrolysis in the standard reaction mixture in the presence of increasing concentrations of IDP, NaF or NEM. The percentage activities are relative to the control in the absence of inhibitor (100%). Control activity was $(7.9 \pm 0.5) \times 10^3$ A_{360}/min for PP_i hydrolysis.

inhibitor of plant vacuolar pyrophosphatases [15] (Figure 1, trace b).

Pyrophosphatase was also assayed in membrane preparations using purine-nucleoside phosphorylase and 2-amino-6-mercapto-7-methylpurine ribonucleoside as co-substrate with phosphate [7]. Control pyrophosphatase activity was 0.012 ± 0.007 μmol of PP_i used/min per mg of protein (means $\pm$ S.D. of results from four separate experiments) and was inhibited by 20 μM AMDP by 53.0 ± 2.1 % (average $\pm$ S.D. of four experiments). The variation in the control pyrophosphatase activity was greater than in the acidification assay (Table 1), because of better specificity in the latter assay. For the pyrophosphatase assay it was necessary to subtract the (variable) fraction of pyrophosphatase activity not inhibited by the specific inhibitor AMDP. Also, in order to assay the pyrophosphatase (phosphate production) activity it was necessary to wash the cell pellets to remove endogenous phosphate, and this introduced another source of error, particularly given the low overall activity. The effects of univalent cations on both acidification rate and AMDP-inhibitable PP_i hydrolysis were generally similar (Table 1). Less activity was detected if KCl in the medium was replaced with NaCl, *N*-methylglucamine chloride, or sucrose. Elevating the concentration of KCl in the buffer to 130 mM increased both activities; whereas increasing the amount of NaCl to 130 mM decreased the acidification rate compared with the rate in the presence of 65 mM NaCl/125 mM sucrose, although the reduction in the rate of PP_i hydrolysis was not significant. Use of a buffer containing equimolar concentrations of NaCl (65 mM) and KCl (65 mM), resulted in less Acridine Orange uptake (and PP_i hydrolysis) than in the presence of 130 mM KCl. These results suggest that K^+ stimulated these activities, especially at low KCl concentrations, whereas Na^+ was inhibitory. These results are in agreement with those obtained with plant [10] and trypanosomatid [7–9] V-H^+-PPases, which are K^+-dependent and are inhibited by Na^+, in contrast with mitochondrial H^+-PPases, which do not require K^+ [20].

Table 2 Effect of ATPase and phosphohydrolase inhibitors on PP_i-hydrolysis

PP_i hydrolysis was assayed as described in the Materials and methods section, using the standard buffer with 130 mM KCl. Control activity was $(7.9 \pm 0.5) \times 10^3$ ΔA_{360}/min. Values are the means $\pm$ S.E.M. of the number of experiments shown in parentheses.

Inhibitor	PP_i hydrolysis (% of control)
None (control)	100 (6)
Na_2MoO_4 (2 mM)	95.7 $\pm$ 4.6 (4)
Bafilomycin A_1 (1 μM)	100.3 $\pm$ 5.6 (4)
Oligomycin (1 μg/ml)	73.7 $\pm$ 7.7 (4)
NaO_3V (500 μM)	186.6 $\pm$ 5.4 (5)

Inhibition of V-H^+-PPase activity in *T. gondii* tachyzoites: effect of bisphosphonate drugs

PP_i hydrolysis and PP_i-induced acidification in the tachyzoite homogenate were inhibited, in a dose-dependent manner, by the PP_i analogue AMDP (Figure 2). PP_i hydrolysis was similarly inhibited by IDP, NaF and the thiol reagent *N*-ethylmaleimide (Figure 3). The effective concentrations of NaF, IDP and AMDP were similar to those that inhibit plant [15,21] and trypanosomatid [7–9] V-H^+-PPase activities. The effect of different H^+-ATPase inhibitors on PP_i hydrolysis by membrane preparations of *T. gondii* tachyzoites was also investigated. Neither bafilomycin A_1 (a specific V-H^+-ATPase inhibitor when used at nanomolar concentrations [22]), oligomycin (a mitochondrial ATPase inhibitor) nor Na_2MoO_4 (a general phosphatase inhibitor) were able to significantly affect this activity (Table 2). NaO_3V (a P-type H^+-ATPase inhibitor [23]), in contrast, stimulated the activity by 87%. The reason for this is not known, but a similar effect has been observed with the H^+-pyrophosphatase activity in purified acidocalcisomes of *T. cruzi* (D. A. Scott and R. Docampo, unpublished work).

Since AMDP is a bisphosphonate, we tested other bisphosphonates used clinically in the treatment of bone resorption disorders [24] for their inhibitory effects on vacuolar acidification and PP_i hydrolysis. Pamidronate (3-amino-1-hydroxypropylene-1,1-bisphosphonate) (Figure 2, middle panel), alendronate (4-amino-1-hydroxybutylidene-bisphosphonate) (Figure 2, right panel), risedronate [1-hydroxy-2-(3-pyridinyl)-ethylidenebisphosphonate] and etidronate (1-hydroxyethylidene-bisphosphonate) (results not shown) were found to inhibit both PPi-dependent vacuolar acidification and, less markedly, PP_i hydrolysis by membrane preparations. The IC_{50} values for PP_i hydrolysis of pamidronate, alendronate and risedronate were 23, 25 and 77 μM respectively. The inhibition of acidification was total with 100 μM pamidronate and alendronate (Figure 2),

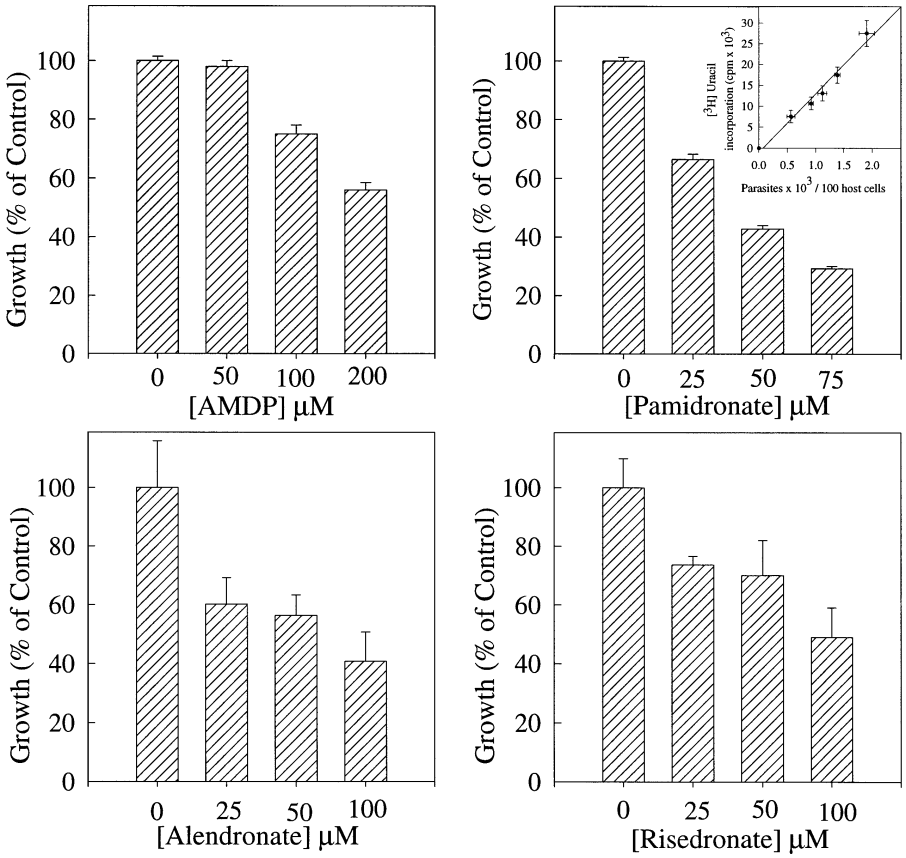

Figure 4 Effects of PP_i analogues (bisphosphonates) on growth of *T. gondii* tachyzoites in human fibroblasts

Tachyzoite growth was quantified by measuring [^{3}H]uracil incorporation after 19 h of infection in the presence of AMDP, pamidronate, alendronate and risedronate at the concentrations shown. The results are expressed as percentage inhibition compared with controls in the absence of drug. Inset: [^{3}H]uracil incorporation, in the presence of various concentrations of pamidronate, correlated closely with the number of parasites per 100 host cells in Giemsa-stained monolayers, under the conditions used. Error bars indicate means $\pm$ S.E.M. of at least three separate experiments.

although residual PP_i hydrolysis activity was detected with similar concentrations of each reagent. This suggests the presence of a bisphosphonate-insensitive pyrophosphatase activity, not involved in proton transport.

PP_i analogues selectively block the intracellular proliferation of *T. gondii*

To test whether bisphosphonate derivatives interfered with parasite development *in vivo*, we monitored the growth of *T. gondii* tachyzoites in human foreskin fibroblasts [19] in the presence of various concentrations of AMDP, pamidronate, alendronate and risedronate. The results are shown in Figure 4. Tachyzoite proliferation was significantly inhibited by pamidronate, as assayed by uracil incorporation and Giemsa staining (inset), with 50 % inhibition at 42 μM. AMDP, however, did not inhibit by as much as 50 %, even at 200 μM. No toxicity to the host cells could be detected at the concentrations of AMDP and pamidronate used, indicating selective activity. Invasion of host cells by the parasite was not affected by these compounds. Alendronate (IC_{50} 37 μM) and risedronate (IC_{50} 95 μM) were also effective in inhibiting intracellular proliferation of *T. gondii*.

Evidence for localization of V-H^+-PPase in the plasma membrane and vacuoles of *T. gondii* tachyzoites

Since the V-H^+-PPase localizes to vacuoles of higher plant cells [10], and to the acidocalcisomes and plasma membranes [7–9] of trypanosomatids, we investigated whether this was also true for *T. gondii*.

The location of the V-H^+-PPase in *T. gondii* was tested using polyclonal antibodies specific for the plant enzyme which cross-react with the V-H^+-PPase of trypanosomatids [7] (Figure 5). Antibody 326 showed cross-reactivity with a strong and wide band of 64 kDa present in *T. gondii* (Figure 5C, lane a). No background staining was observed when normal serum was used as a control (Figure 5C, lane b). Immunofluorescence indicated labelling of vacuoles and of the cell surface (Figure 5A). Intracellular tachyzoites were intensely stained (Figure 5B). No fluorescence was observed in control parasites incubated only in the presence of the secondary fluorescein-labelled goat anti-rabbit IgG, or with pre-immune serum (results not shown), or in the host cells (Figure 5B).

PP_i is abundant in *T. gondii*

Figure 6(A) shows the 303.7663 MHz ^{31}P-NMR spectrum of a perchloric acid extract, at pH 8.0, of tachyzoites of *T. gondii*. The spectrum is dominated by three principal features: a resonance at 3.8 p.p.m., probably associated with P_i, and a principal resonance at -4.2 p.p.m., close to the range of the resonance frequencies of terminal phosphates of nucleotide di- and tri-phosphates and short-chain polyphosphates such as pyro- and tri-polyphosphate, and a resonance at -16.2 p.p.m., which corresponds to the bridging phosphorus of tripolyphosphate. Resonances of much lower intensity were also observed in the region of α-phosphates (-9 to -13 p.p.m.) and β-phosphates (-18 to -20 p.p.m.) of nucleotides. This spectrum differs markedly from those of most other cell and tissue extracts [25,26], which contain prominent resonances due to the α, β and γ-phosphate groups of nucleotides. In the case of *T. gondii* tachyzoites, the integrated intensity of the dominant peak at -4.2 p.p.m. was 12 times higher than the corresponding area in the α-phosphate region (-9.1 p.p.m.) of nucleotides, implying the peak was not of nucleotide origin. The lack of a strong resonance at -22 p.p.m. also rules out the presence of significant amounts of soluble polyphosphates in these extracts [27]. The assignment of the -4.2 p.p.m. resonance to PP_i was supported by 'spiking' the samples with pure sodium pyrophosphate, which resulted in an increase in intensity of this resonance (results not shown), and by treatment with pure yeast inorganic pyrophosphatase. After incubation of a perchloric acid extract of tachyzoites with the enzyme for 60 min, the resonance at -4.2 p.p.m. was decreased, with a concomitant stoichiometric increase in the intensity of the resonance at 3.8 p.p.m., assigned to P_i (Figure 6B). The remaining intensity in the -4.2 p.p.m. region, together with the resonance at -16.2 p.p.m., correspond to the terminal and bridging phosphates of inorganic tripolyphosphate respectively. These data therefore indicate that PP_i is more abundant than ATP in *T. gondii*.

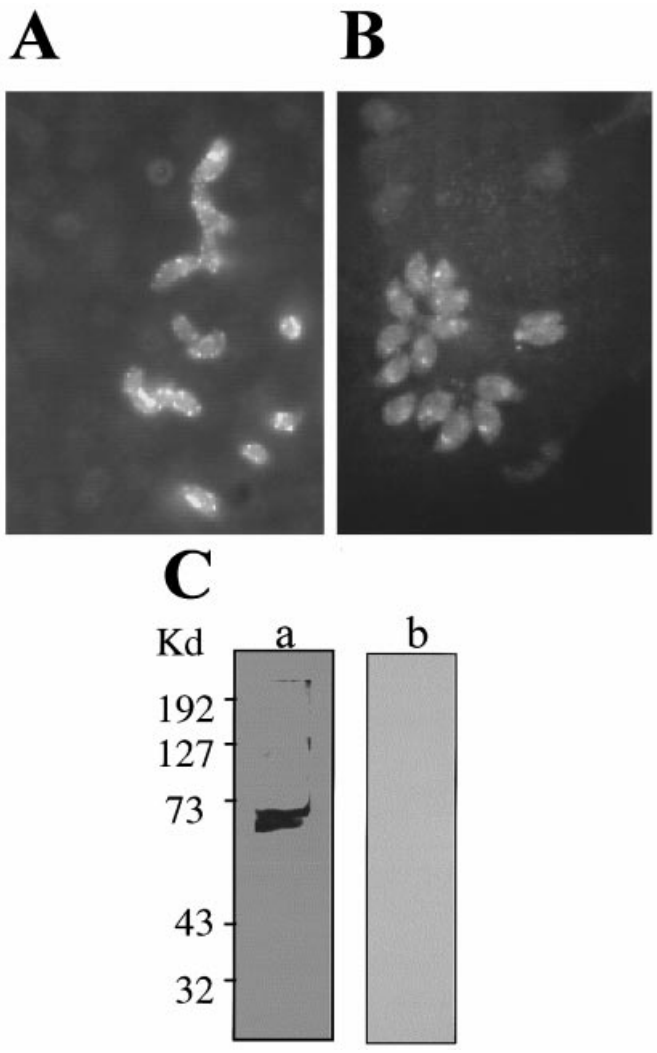

Figure 5 Indirect immunofluorescence analysis of V-H^+-PPase in tachyzoites of *T. gondii*

Fluorescence microscopic images of extracellular (**A**) and intracellular (**B**) tachyzoites. The images show intense labelling of cytoplasmic vesicles, with lighter labelling of the cell surface. Magnification $\times$6200 (applies to **A** and **B**). (**C**) Immunoblot to show V-H^+-PPase, using antisera specific for the plant enzyme. *T. gondii* proteins (10 μg) were separated by SDS/PAGE and transferred to nitrocellulose. Lane a: immunoblot probed with antiserum against V-H^+-PPase (antibody 326). The V-H^+-PPase antibody recognized a polypeptide with an apparent molecular mass of 64 kDa. Lane b: immunoblot probed with normal rabbit serum. Kd, kDa.

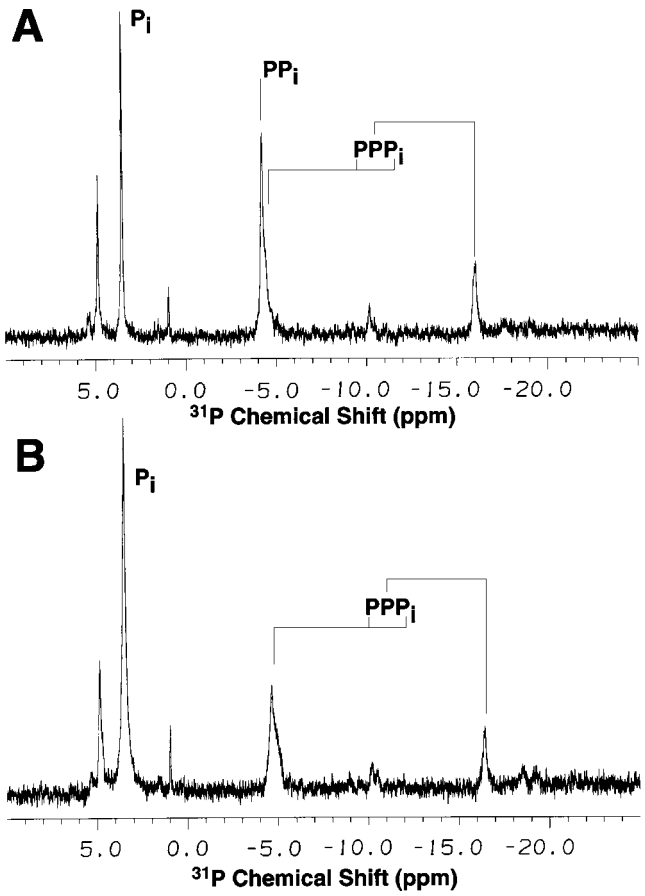

Figure 6 PP_i identified by ^{31}P NMR in *T. gondii* tachyzoites

^1H-coupled 303.7663 MHz ^{31}P-NMR spectra of a perchloric acid extract of tachyzoites incubated in the absence (**A**) or presence (**B**) of inorganic pyrophosphatase (10 units/ml) for 60 min. Note the disappearance of the resonances at −4.2 p.p.m. (PP_i) and the increase in the intensity of the resonance at 3.8 p.p.m. (P_i) upon treatment with inorganic pyrophosphatase. Spectra were recorded at 25 °C using 60 ° (14.5 μs) pulse excitation, a 1.5 s recycle delay and 3Hz line broadening. PPP_i, tripolyphosphate.

DISCUSSION

In the present study, we have identified and characterized an H^+-translocating pyrophosphatase activity in *T. gondii*. Acridine Orange uptake in the presence of PP_i was reversed by the K^+/H^+ exchanger, nigericin, indicating that PP_i induced organelle acidification. PP_i-driven proton transport was blocked by NaF and by the PP_i analogues, AMDP and IDP, and was stimulated by K^+ ions. The H^+-pyrophosphatase was insensitive to bafilomycin A_1, a very specific inhibitor of V-H^+-ATPases [22] and, unlike P-type H^+-ATPases [23], it was stimulated by NaO_3V.

This is the first report demonstrating biochemically the presence of a V-H^+-PPase in *T. gondii*. Until recently, V-H^+-PPases had been found only in plants (including the unicellular alga *Acetabularia* [28], along with a homologous H^+-PP_i synthase in the photosynthetic bacterium *Rhodosporillum* [29]). Now, with the recent discovery of this enzyme in trypanosomatids [7–9] and *T. gondii* (the present work), the range of organisms possessing V-H^+-PPases has been greatly expanded. In addition, a V-H^+-PPase activity has been detected in *Plasmodium falciparum* [30] and *P. berghei* [31]. The apparent lack of a V-H^+-PPase in mammalian cells makes this enzyme a potential target for specific chemotherapy.

In plants, V-H^+-PPases are present in the vacuole membrane (tonoplast) [10] and probably also in some plasma membranes [32,33]. *T. gondii* tachyzoites do not have the large central vacuole of plant cells but they do have several acidic organelles, such as rhoptries [34], lysosome-like vacuoles [35], and acidic calcium-storage organelles called acidocalcisomes [2]. Antibodies to conserved regions of the plant V-H^+-PPase, known to cross-react with the enzyme from *T. cruzi* [7], reacted with a *T. gondii* polypeptide (Figure 5C) of similar molecular mass (64 kDa) to that present in red beet and *T. cruzi* epimastigotes [7]. The results suggested an intracellular and plasma-membrane localization of the V-H^+-PPase (Figures 5A and 5B).

Our results also show that PP_i is more abundant than ATP in *T. gondii* tachyzoites. Inorganic PP_i is the substrate for the PP_i-dependent phosphofructokinase found in several apicomplexan parasites, including *T. gondii* [12,13,36], *Cryptosporidium parvum* [36] and *Eimeria tenella* [36]. This, together with our detection of a proton-translocating pyrophosphatase activity in *T. gondii*, suggests that PP_i could play an important role in the metabolism of these parasites.

Some of the PP_i analogues tested, in the present work, as pyrophosphatase inhibitors were effective in inhibiting *T. gondii* intracellular proliferation. Although there was some correlation between these activities among alkyl N-containing bisphosphonates (pamidronate, risedronate and alendronate), the correlation broke down with AMDP, probably due to the poor penetration of this compound into intact cells. Structure–activity-relationship investigations of bone resorption inhibition, slime mould cell growth and macrophage apoptosis supported the hypothesis that nitrogen-containing bisphosphonates interact with a highly specific target involved with protein prenylation [24]. However, other bisphosphonates, such as clodronate, can be metabolized to a cytotoxic, non-hydrolysable analogue of ATP by mammalian cells [37], and an isolated report indicated that tiludronate inhibits the osteoclast vacuolar H^+-ATPase [38]. In mammalian systems, the nitrogen-containing bisphosphonates used in the present study (pamidronate, alendronate, and risedronate) are not metabolized [37], and act by a different mechanism that leads to osteoclast apoptosis [39]. Recent results by different groups [40,41] have indicated that these bisphosphonates inhibit the prenylation of small GTP-binding proteins that control cytoskeletal reorganization, vesicular fusion and apoptosis, processes involved in osteoclast activation and survival. Nitrogen-containing bisphosphonates have been postulated to act as carbocation transition-state analogues for isoprenoid biosynthesis [42], and have been shown to be active *in vitro* and *in vivo* against *T. cruzi* [43]. Recent studies have indicated that, in addition to being weak inhibitors of the V-H^+-PPase [44,45], they are potent inhibitors of protein prenylation, because they inhibit farnesylpyrophosphate synthase in a very effective manner [45–47]. Since it is already known that protein prenylation is important in the related apicomplexan parasite *P. falciparum* [48], and that the growth of *P. falciparum* in culture is sensitive to protein farnesyltransferase inhibitors [48], it seems most reasonable to suppose that pamidronate, alendronate and risedronate act primarily by inhibiting farnesylpyrophosphate synthase, with less activity against the V-H^+-PPase.

It is interesting to note that macrophages (one of the preferred host cells for *T. gondii*), like osteoclasts, appear to be particularly susceptible to these compounds. The plasma concentration of pamidronate achieved in humans after a single intravenous administration is around 10 μM [49], and the concentration of bisphosphonates is several-fold higher in tissues (including those susceptible to *T. gondii*) than in plasma [50]. These compounds are sequestered in bone over the short term, and are slowly

released from the skeletal deposits, accounting for their prolonged multiple-phase elimination [51]. Alendronate, for example, has estimated half-lives of approximately 300 days in rats and at least 1000 days in dogs [51]. Since a large number of bisphosphonates are already approved for long-term use in treating various bone-resorption disorders, it seems possible that such drugs could be potential chemotherapeutic agents against *T. gondii* infections. The presence of large calcium/PP_i deposits in the acidocalcisomes may facilitate drug uptake, in the same way as bone minerals facilitate bisphosphonate uptake in bone resorption therapy.

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