Acidocalcisomes are organelles characterized by their acidic nature, high electron density, and a matrix consisting of pyrophosphate (PP$_i$),$^1$ polyphosphate (polyP), calcium, magnesium, and other elements (1, 2). First described in trypanosomatids (3, 4), they are now known to be present in several other microorganisms and are similar to the organelles historically described as "volutin granules" or "polyP bodies" (1, 2).

Several transport systems have been identified in the enclosing membrane of acidocalcisomes; they are a V-H$^+-$ATPase (3–7), a Ca$^{2+}$-ATPase (8, 9), Na$^+$/$\text{H}^+$ and Ca$^{2+}$/H$^+$ exchangers (10, 11), and a proton pyrophosphatase (H$^-$/PPase) (7, 11–14). Not all these pumps and exchangers are necessarily present in all acidocalcisomes. However, the H$^-$/PPase has been found in all cases in the purified organelle (11–13, 15, 16), which makes this pump the marker of choice for acidocalcisomes.

The functions of the acidocalcisome in cell growth and survival are poorly understood. Based on its chemical composition, rich in PP$_i$ and polyP (a linear polymer of hundreds of orthophosphate residues linked by high energy phosphoanhydride bonds), one possible function could be as an energy and phosphate reservoir. PolyP has been shown to have a function in the cellular response to nutrient limitation during the stationary phase of growth and in the chelation of metals (17–19). A critical role for the acidocalcisomal polyP in the adaptation processes of Trypanosoma cruzi to environmental changes has been demonstrated (20). In several protozoan parasites it has been shown that the acidocalcisome is the main calcium storage compartment (1, 2). Because Ca$^{2+}$ signaling is involved in many processes, such as invasion of host cells by different parasites (2, 21), it is possible, although it has not yet been demonstrated, that this compartment could also have a role as a source of releasable Ca$^{2+}$.

Many aspects of acidocalcisomes, such as their transport and enzymatic activities, need further study to understand the physiological importance of these organelles in protozoan parasites. A crucial requirement for these studies is the isolation of the organelles free from interference of other organelles or enzymatic activities. In the present work, we report a modification of the cell fractionation method used for T. cruzi epimastigotes (22) for the purification of acidocalcisomes from Toxoplasma gondii tachyzoites. We also report for the first time the determination of short and long chain polyP in these parasites. Our results indicate that the concentration of polyP changes drastically under alkaline stress or when the cells are incubated with calcium ionophores. Ca$^{2+}$ release from acidocalcisomes is associated with the hydrolysis of polyP.

Toxoplasma gondii tachyzoites were fractionated by modification of an iodixanol density gradient method previously used for acidocalcisome isolation from Trypanosoma cruzi epimastigotes. Fractions were characterized using electron microscopy, x-ray microanalysis, and enzymatic markers, and it was demonstrated that the heaviest (pellet) fraction contains electron-dense vacuoles rich in phosphorus, calcium, and magnesium, as found before for acidocalcisomes. Staining with fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-amino-5-(methylphenoxyl)-ethane-N, N', N''-tetraacetic acid; polyP, polyphosphate; DAPI, 4', 6-diamidino-2-phenylindole; H$^-$/PPase, proton pyrophosphatase; PBS, phosphate-buffered saline.

This paper is available on line at http://www.jbc.org

Received for publication, September 3, 2002, and in revised form, October 11, 2002 Published, JBC Papers in Press, October 11, 2002, DOI 10.1074/jbc.M208990200

Claudia O. Rodrigues‡, Felix A. Ruiz‡, Peter Rohloff, David A. Scott, and Silvia N. J. Moreno§

From the Laboratory of Molecular Parasitology, Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802

This work was supported by National Institutes of Health Grant AI-43614 (to S. N. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ A Burroughs Wellcome Fund New Investigator in Molecular Parasitology. To whom correspondence should be addressed: Laboratory of Molecular Parasitology, Dept. of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Ave., Urbana, IL 61802. Tel.: 217-333-2746; Fax: 217-244-7421; E-mail: s-moreno@uiuc.edu.

1 The abbreviations used are: PP$_i$, pyrophosphate; P$_i$, orthophosphate; BCECF, 2',7'-bis(2-carboxyethyl)-5-((and -6)-carboxyfluorescein; fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)-ethane-N, N', N''-tetraacetic acid; polyP, polyphosphate; DAPI, 4', 6-diamidino-2-phenylindole; H$^-$/PPase, proton pyrophosphatase; PBS, phosphate-buffered saline.

48650
Isolated Acidocalcisomes from Toxoplasma gondii

EXPERIMENTAL PROCEDURES

Culture Methods—Tachyzoites of T. gondii RH strain were cultivated and purified by the method of Moreno and Zhong (5) in bovine thymus cell cultures (ATCC CRU, 1996). Host cells were cultivated in tissue culture flasks using Dulbecco's minimum essential medium supplemented with 10% horse serum. Cells were infected with tachyzoites at a final host-to-parasite ratio of 1:5. Parasites were harvested 2-3 days after infection and purified as described previously (23).

Chemicals and Reagents—Horse serum, DNase, RNase, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (PBS), Hanks' solution, nucodazole, sodium orthovanadate, sodium pyrophosphate, silicone carbide (400 mesh), protease inhibitors mixture, and goat anti-mouse antibodies labeled with horseradish peroxidase were purchased from Sigma. Bafilomycin A1 was from Kamiya Biomedicals, Thousand Oaks, CA. Iodixanol (Optiprep, Nycomed) was from GE Healthcare, Little Chalfont, UK. Coomassie Blue protein assay reagent was obtained from Amersham Biosciences. Coomassie Blue protein assay reagent was obtained from Bio-Rad. Ammoniumthiolenediphosphonate was synthesized by Michael Martin in the laboratory of Dr. Eric Oldfield at the Department of Chemistry, University of Illinois at Urbana-Champaign. [35S]Iodine was from PerkinElmer Life Sciences. The antibodies used for the characterization of subcellular fractions were: anti-SAG1 monoclonal antibody, a gift from Dr. John Boothroyd, Stanford University School of Medicine, OR. The enhanced chemiluminescence detection kit was from Amersham Biosciences.

Isolation of Acidocalcisomes—Tachyzoites (<2 ¥ 10^10 cells) were centrifuged at 500 ¥ g for 10 min, and the cell pellet was washed twice with Dulbecco's PBS and once in lysis buffer (125 mM succrose, 50 mM KCl, 4 mM MgCl2, 0.5 mM EDTA, 20 mM K-Hepes, 5 mM dithiothreitol, protease inhibitors mixture (0.2% w/v), 12 mg/ml DNase, 12.5 mg/ml RNase, and 8 mg/ml nucodazole, pH 7.2). The cell pellet was mixed with 1.8x wet weight silicon carbide and lysed by grinding with a pestle and mortar for not more than 60 s (lysis was checked by microscopy every 15 s). The mixture of silicon carbide and lysed cells was resuspended in ~100 ml of lysis buffer, and the suspension was left for 5 min on ice to allow a part of the silicon carbide to settle. The liquid phase was carefully transferred to a new tube and centrifuged at 36 ¥ g for 5 min. The supernatant was collected and left on ice, whereas the pellet was resuspended in ~50 ml of lysis buffer and centrifuged again under the same conditions. Combined supernatants were centrifuged at 144 ¥ g for 10 min to remove debris and unbroken cells. The supernatant obtained from this last centrifugation was centrifuged at 15,000 ¥ g for 10 min. The pellet was resuspended in 2.7 ml of lysis buffer, homogenized with the aid of a 22-gauge needle about 8–10 times until no clumps were observed, and mixed with 1.3 ml of 60% iodixanol. This mixture (20% iodixanol) was included as the middle layer of a discontinuous gradient, with the other (4/ml) steps containing 10, 15, 25, and 30% iodixanol (diluted in lysis buffer). The gradient was centrifuged at 50,000 ¥ g using a Beckman SW 28 rotor for 36 h. Thirteen fractions were collected corresponding to bands and interfaces (Fig. 1). The acido cereosalisomal fraction (F13) pelleted on the bottom of the tube and was resuspended in lysis buffer. All these procedures were done at 4 °C.

Enzyme Assays—ATPases, pyrophosphatase, and exophosphatase activities were assayed by measuring phosphate release using the EnzCheck phosphate assay kit as described before (11–13) with the microtiter plate modification (11). Acid phosphatase (Iysosome marker, Ref. 24) was assayed in microtiter plates by measuring phosphate release from p-nitrophenylphosphate. Fractions (5 ml) were added to a 50-µl reaction mixture containing 0.1 µM sodium acetate, pH 5.5, and 10 mM p-nitrophenylphosphate and incubated for 30 min at 28 °C. Reactions were stopped by the addition of 150 µM sodium hydroxide (100 µl), and released p-nitrophenol was detected at 405 nm. Protein was determined using the Bio-Rad Coomassie Blue method. All assays were recorded in a PowerWave 340i plate reader (Bio-Tek Instruments).

Cell Volume Determination—The intracellular concentration of polyP was calculated taking into account the cell volume of tachyzoites measured by the [35S]Iodinum exclusion method as previously described by Damper and Patton (36), with some modifications. Cells were washed twice in Hanks' balanced salt solution (HBSS) supplemented with 0.1% (w/v) glucose and 0.05% (w/v) albumin (HBSSA) and then resuspended in HBSSA containing 0.2 mg/ml [35S]Iodinum at final concentrations of 1 ¥ 10^6 and 2 ¥ 10^6 cells/ml. Thirty-µl aliquots were taken from each suspension and transferred to scintillation vials. The suspensions were then centrifuged in Eppendorf tubes at 14,000 ¥ g for 2 min, and three 50-µl aliquots of the supernatant were transferred to scintillation vials. 5 ml of scintillation mixture (0.1% PPO and 0.025% POPOP) was added, and the vials were counted. All cell manipulations were performed on ice and/or with chilled solutions. Cell volume was determined by iodinum exclusion. The difference in radioactivity/ml between the cell suspension and the supernatant was used to calculate the cell volume (differ-
ence divided by radioactivity of supernatant = fraction of volume occupied by cells. Results were expressed as an average of the values obtained in two different suspensions from three independent experiments.

**Cell Treatments**—Spectrofluorometric determinations of tachyzoites loaded with fura 2-AM or BCECF-AM were performed as described previously (5). For the alkaline or ionophore treatments, tachyzoites (2.5 × 10⁶) were washed once with Dulbecco’s PBS and resuspended in 0.55 ml of 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose, 1 mM EGTA, and 50 mM Hepes pH 7.4 (buffer A). At the times indicated, 40 mM NH₄Cl, 1 μM ionomycin, or 1 μM bafilomycin were added. Aliquots of 50 μl were withdrawn at the times indicated and mixed with 500 μl of guanidine isothiocyanate lysis buffer (20) for long chain polyP determination or with 300 μl of ice-cold 0.5 M HClO₄ for short chain polyP determination as described above.

**RESULTS**

**Isolation and Characterization of Acidocalcisomes from T. gondii Tachyzoites**—The method used to isolate acidocalcisomes from various protozoa has been improved in stages (11–13, 22) but has not proved useful for isolation of these organelles from apicomplexan parasites. A new procedure for the isolation of acidocalcisomes from *T. gondii* is depicted in Fig. 1. This method used generally lower concentrations of iodixanol in the gradient steps than those previously used for the purification of acidocalcisomes from *T. cruzi* epimastigotes (22) but with the *T. gondii* sample added in the middle of the gradient in a 20% iodixanol layer rather than applied to the top of the gradient without added iodixanol. This strategy allowed a better separation of the acidocalcisomes from the ghosts present in the intermediate fractions and a better recovery of the pellet fraction at the base of the gradient (fraction 13). When directly applied to Formvar-coated grids and observed by electron microscopy (Fig. 2B), fraction 13 was seen to contain electron-dense structures, as expected for acidocalcisomes (1, 2). The same electron-dense organelles were observed in whole cells when they were applied to EM grids in the same manner (Fig. 2A). X-ray microanalysis of the granules present in fraction 13 (Fig. 3) showed that they have the same chemical composition as acidocalcisomes found in other protozoa (1, 2) and electron-dense vacuoles in whole *T. gondii* (9), indicating that they are acidocalcisomes.

The distribution of different enzymatic activities and organelle markers was compared along with that of the established marker for acidocalcisomes, H⁺-PPase (Figs. 4 and 5). The aminomethylenediphosphonate-sensitive pyrophosphatase activity peaks in three different regions along the gradient (Fig. 4). Observation of these fractions by electron microscopy showed that the only fraction containing the electron-dense vacuoles, as seen in Fig. 2B, was fraction 13. Fractions 1 and 5 contained many cell ghosts produced in the lysis procedure (data not shown).

A V-H⁺-ATPase sensitive to bafilomycin has been described to be present in acidocalcisomes of *T. gondii* (5–6). However, the V-H⁺-ATPase activity seems to have a broader distribution if compared with the H⁺-PPase (Fig. 4). Acid phosphatase activity, a marker for another acidic organelle, the lysosome, did not peak in fraction 13 (Fig. 4).

The acidocalciosomal fraction also lacks markers for other organelles present in *T. gondii*, such as rhoptries (30), micronemes (29), and dense granules (31), as depicted in the Western blots shown in Fig. 5. Likewise, antibodies against the surface antigen of *T. gondii* tachyzoites (SAG1) (32) show that the plasma membrane is distributed mainly in the middle of the gradient (Fig. 5). Antibodies against the H⁺-PPase (Fig. 5) confirm the distribution of this protein, obtained by enzymatic measurements (shown in Fig. 4). The strong reaction of this antibody in fractions 4–12 is because of the presence of plasma membrane fragments (as marked by SAG1) and the high amount of protein in these fractions (Fig 4). The H⁺-PPase has been previously shown to be located on the cell surface of *T. gondii* as well as in acidocalcisomes (7). Importantly though, fraction 13, which contains low amounts of protein, showed a clear reaction with antibodies against the H⁺-PPase and no reaction with antibodies against all other markers, thus indicating that this fraction contained only acidocalcisomes.

**PPi and PolyP Levels in T. gondii Tachyzoites**—*T. gondii* tachyzoites contain high levels of long and short chain polyP, as determined by measuring degradation of polyP with recombinant yeast exopolyphosphatase (rPPX1). Cellular concentrations of short chain polyP (less than 50 phosphate residues) were in the mM range (in terms of Pi residues, 24.0 ± 0.5 mM), whereas values for long chain polyP (700–800 phosphate residues) were in the micromolar range (43.0 ± 0.5 μM), taking into account a calculated cell volume of 16.5 ± 2 μl/10⁶ tachyzoites. Tachyzoites also contained very high levels of PPi (7.95 ± 0.16 mM). Controls of uninfected host cells presented undetectable levels of polyP or PPi (data not shown).

**Localization of PolyP and Polyphosphatase Activity in T. gondii**—The localization of polyP in *T. cruzi* (20) and other unicellular eukaryotes (15, 16) has been investigated using
There was a correlation between Ca\(^{2+}\) and long chain polyP, respectively, in fraction 13, whereas the cause polyP yields were between 30 and 60% for short chain acidocalcisomal fraction of calcisomes of trypanosomatids (20), was also present in the staining of the isolated acidocalcisomes with DAPI, corroborating acidocalcisomal localization of polyPs, acidocalcisomal fraction of these compounds. To further confirm the cation, respectively. These results suggest a preferential acidocalcisomal location of these compounds. To further confirm the acidocalcisomal localization of polyPs, acidocalcisomal fractions were incubated with DAPI, mounted on slides, and examined by epifluorescence microscopy. Fig. 6B shows the strong staining of the isolated acidocalcisomes with DAPI, corroborating the presence of a high content of polyP in these organelles.

An exopolyphosphatase activity, previously described in acidocalcisomes of trypanosomatids (20), was also present in the acidocalcisomal fraction of T. gondii (Fig. 7). This activity was distributed along the gradient with the same pattern as the H\(^{+}\)-PPase (Fig. 4).

Changes in PolyP Levels Induced by Processes That Mobilize Ca\(^{2+}\).—Previous work has demonstrated that alkaline stress results in an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) of T. gondii (5). Therefore, we investigated whether there was a correlation between Ca\(^{2+}\) release from the acidic compartment containing most polyP (acidocalcisome) and polyP hydrolysis in T. gondii. The addition of bafilomycin A\(_{1}\), a specific inhibitor of the vacuolar type H\(^{+}\)-ATPase, or the alkalinizing agent NH\(_{4}\)Cl to tachyzoites resulted in significant decrease in long (Fig. 8A) and short chain (Fig. 8B) polyP. Ionomycin, a Ca\(^{2+}\) ionophore that induces cell acidification and Ca\(^{2+}\) release from intracellular compartments (5) (EGTA was present in the extracellular medium to avoid Ca\(^{2+}\) entry), also induced degradation of long (Fig. 8A) and short chain (Fig. 8B) polyP. However, cytosolic acidification by the addition of propionic acid (5) did not increase [Ca\(^{2+}\)]\(_{i}\). Because acidocalcisomes are the main cellular store of polyP and the total amounts released (~70% of long chain and ~50% of short chain polyP) correspond to the percent polyP present in them (Fig. 7), these organelles are certainly involved in these effects. Taken together, these results suggest that processes that lead to alkalinization of the acidocalcisosomes (NH\(_{4}\)Cl addition or treatment with bafilomycin A\(_{1}\)) and/or result in [Ca\(^{2+}\)]\(_{i}\) increase (5) also result in polyP hydrolysis.

Simultaneous measurements of changes in pH\(_{i}\), [Ca\(^{2+}\)]\(_{i}\), and short and long chain polyP in tachyzoites are shown in Fig. 9. The addition of bafilomycin A\(_{1}\) (in the absence of extracellular Ca\(^{2+}\)) caused a decrease in pH\(_{i}\) and a rise in [Ca\(^{2+}\)]\(_{i}\) (Fig. 9A). The addition of bafilomycin A\(_{1}\) was accompanied by immediate hydrolysis of long and short chain polyP. Subsequent addition of ionomycin resulted in a further acidification and Ca\(^{2+}\) release accompanied by further hydrolysis of long and short chain polyP (Fig. 9, B and C).

**DISCUSSION**

Acidocalcisomes are organelles characterized by their acidic nature and high calcium and phosphorus content (1, 2). They possess several transport systems involved in the maintenance of the acidic environment and the accumulation or release of inorganic ions. Although acidocalcisomes isolated from several organisms have some common characteristics, there are some differences between species and also between different stages within the same species. The isolation of these organelles by subcellular fractionation and gradient centrifugation has been a key step to their final characterization (11–13, 15, 16, 22).

We have modified the previously described gradient (22) for the isolation of acidocalcisomes by expanding the lower concentrations of iodixanol and eliminating the highest concentration.

Changes in PolyP Levels Induced by Processes That Mobilize Ca\(^{2+}\).—Previous work has demonstrated that alkaline stress results in an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) of T. gondii (5). Therefore, we investigated whether there was a correlation between Ca\(^{2+}\) release from the acidic compartment containing most polyP (acidocalcisome) and polyP hydrolysis in T. gondii. The addition of bafilomycin A\(_{1}\), a specific inhibitor of the vacuolar type H\(^{+}\)-ATPase, or the alkalinizing agent NH\(_{4}\)Cl to tachyzoites resulted in significant decrease in long (Fig. 8A) and short chain (Fig. 8B) polyP. Ionomycin, a Ca\(^{2+}\) ionophore that induces cell acidification and Ca\(^{2+}\) release from intracellular compartments (5) (EGTA was present in the extracellular medium to avoid Ca\(^{2+}\) entry), also induced degradation of long (Fig. 8A) and short chain (Fig. 8B) polyP. However, cytosolic acidification by the addition of propionic acid (5) did not increase [Ca\(^{2+}\)]\(_{i}\). Because acidocalcisomes are the main cellular store of polyP and the total amounts released (~70% of long chain and ~50% of short chain polyP) correspond to the percent polyP present in them (Fig. 7), these organelles are certainly involved in these effects. Taken together, these results suggest that processes that lead to alkalinization of the acidocalcisosomes (NH\(_{4}\)Cl addition or treatment with bafilomycin A\(_{1}\)) and/or result in [Ca\(^{2+}\)]\(_{i}\) increase (5) also result in polyP hydrolysis.

Simultaneous measurements of changes in pH\(_{i}\), [Ca\(^{2+}\)]\(_{i}\), and short and long chain polyP in tachyzoites are shown in Fig. 9. The addition of bafilomycin A\(_{1}\) (in the absence of extracellular Ca\(^{2+}\)) caused a decrease in pH\(_{i}\) and a rise in [Ca\(^{2+}\)]\(_{i}\) (Fig. 9A). The addition of bafilomycin A\(_{1}\) was accompanied by immediate hydrolysis of long and short chain polyP. Subsequent addition of ionomycin resulted in a further acidification and Ca\(^{2+}\) release accompanied by further hydrolysis of long and short chain polyP (Fig. 9, B and C).

**DISCUSSION**

Acidocalcisomes are organelles characterized by their acidic nature and high calcium and phosphorus content (1, 2). They possess several transport systems involved in the maintenance of the acidic environment and the accumulation or release of inorganic ions. Although acidocalcisomes isolated from several organisms have some common characteristics, there are some differences between species and also between different stages within the same species. The isolation of these organelles by subcellular fractionation and gradient centrifugation has been a key step to their final characterization (11–13, 15, 16, 22).

We have modified the previously described gradient (22) for the isolation of acidocalcisomes by expanding the lower concentrations of iodixanol and eliminating the highest concentration. These changes allowed the separation of acidocalcisomes from other T. gondii organelles. A similar distribution of the H\(^{+}\)-PPase, with three peaks along the gradient, was found during the isolation of acidocalcisomes from Dicystostelium discoideum (16). In this organism, the activity at the top of the gradient (fraction 1) was associated with markers for the contractile vacuole (16). Similar structures, however, have not been described in apicomplexan parasites. Electron microscopy of fraction 1 showed that it was composed of cell ghosts and other unidentified structures (data not shown). Localization of the H\(^{+}\)-PPase in other compartments has been described in different organisms, in plasma membrane vesicles and the Golgi
apparatus of the trypanosomatid T. cruzi (38), and in plants, the H\textsuperscript{-}/H\textsubscript{11001} -PPase has been shown to be present in the plasma membrane as well as the membrane of the vacuole (the tonoplast) (39).

Other activities known to be associated with acidocalcisomes showed the same distribution pattern as the H\textsuperscript{-}/H\textsubscript{11001} -PPase. Exopolyphosphatase is an enzyme involved in the degradation of polyP and has been shown to be present in different compartments of various cell types (17–19). Part of this enzymatic activity was found in the acidocalcisomal fraction, as occurs in T. cruzi epimastigotes (20). A bafilomycin A\textsubscript{1}-sensitive-V-H\textsuperscript{-}/H\textsubscript{11001} -ATPase activity has been identified in acidocalcisomes and plasma membrane of trypanosomatids and apicomplexan parasites (3–6) as well as in other unicellular eukaryotes (15, 16). Although it has a broader distribution along the gradient, this is similar to that detected for the H\textsuperscript{-}/PPase and exopolyphosphatase activities, showing three peaks of activity. This enzyme could become inactive during acidocalcisome purification, as occurs with the T. cruzi V-H\textsuperscript{-}ATPase (22).

This is the first report of the presence of long chain polyP in an apicomplexan parasite. In addition, our results indicate the presence of high levels of PP\textsubscript{i} and short chain polyP in T. gondii tachyzoites. We also demonstrated that PP\textsubscript{i} and polyP are located preferentially in the acidocalcisomes using two different approaches, by visualization of polyP using DAPI and by the biochemical identification of PP\textsubscript{i} and polyP in isolated acidocalcisomes. A possible function of the polyPs in the acidocalcisome was proposed based on results obtained with T. cruzi (20) in which there is a close association between polyP hydrolysis and intracellular Ca\textsuperscript{2+} increase, suggesting that upon polyP hydrolysis Ca\textsuperscript{2+} bound to polyP is released from acidocalcisomes. The possible roles of polyP and PP\textsubscript{i} in microorganisms have been reviewed (17, 19). Some of their possible functions are to serve as energy stores and/or as chelators of metal ions. PP\textsubscript{i} could be used in place of ATP as an energy donor in several reactions in T. gondii, such as the PP\textsubscript{i}-dependent phosphofructokinase (40) and the acidocalcisomal H\textsuperscript{-}/PPase that can drive proton uptake through cleavage of cytosolic PP\textsubscript{i} (7). Because PP\textsubscript{i} is a charged and polar molecule, the utilization of acidocalcisomal PP\textsubscript{i} for these activities implies the presence of a spe-
A transmembrane transporter that shuttles PPi between intracellular and extracellular compartments has been identified recently in several mammalian tissues (41). A similar channel in the acidocalcisomal membrane would explain PPi release to the cytosol to serve as substrate for the H+-PPase and PPi-dependent phosphofructokinase. A role for polyP as an energy source, however, has been disputed on the basis of its low metabolic turnover as compared with that of ATP (42), and a more important regulatory role has been suggested (43). Long chain polyP, even at relatively low levels, has been shown to be essential for adaptation to various stresses and for survival of bacteria in stationary phase (43). Similar studies have been reported in eukaryotic cells such as yeast (44). We have reported (6) that influx of ammonia into tachyzoites induces a rapid alkalinization of the cells followed by recovery of the cytoplasmic pH. This recovery (6) occurs in parallel with hydrolysis of polyP (Fig. 8, A and B). Because H+ generation from polyP hydrolysis can neutralize up to 2.5 pH units of change in S. cerevisiae (44), a role for polyP hydrolysis in pH recovery from an alkaline load has been suggested (20). 

Ca2+ release from acidocalcisomes by combination of an inhibitor of the V-H+-ATPase (bafilomycin A1) and a calcium ionophore (ionomycin) was associated with short and long chain polyP hydrolysis (Fig. 9). The addition of bafilomycin A1 leads to acidification of the cytosol and alkalinization of the acidocalcisomes by inhibition of the V-H+-ATPase. This would favor Ca2+ release through a Ca2+/H+ exchanger, the presence of which has been demonstrated in acidocalcisomes from other

Fig. 7. Distribution of short chain (SC) and long chain (LC) polyP, PPi, and polyphosphatase activity on iodixanol gradients. Long chain polyP and SC polyP content was compared with the distribution of PPi, and polyphosphatase activity expressed as % of total specific activity. PolyPs and PPi are concentrated in the densest fraction (fraction 13). Protein and density distribution are shown in Fig. 4.

Fig. 8. Effect of different agents on the polyP content of T. gondii tachyzoites. Cells were washed once and resuspended in 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM glucose, 50 mM Tris-HCl, pH 7.4. Then 10 mM sodium propionate (open diamonds), 10 mM NH4Cl (open squares), 1 μM ionomycin (filled diamonds) or 5 μM bafilomycin A1 (filled triangles) was added and long chain (LC, A) or short chain (SC, B) polyP content was determined as indicated under “Experimental Procedures.” Results represent the percent of polyP in treated cells compared with controls (100%). Results are representative of three experiments with data points given as means ± S.E.

Fig. 9. Effect of ionophores on the [Ca2+]i, pHi, and polyP content of T. gondii tachyzoites. Parasites were loaded with fura 2-AM or BCECF-AM and resuspended in 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM glucose, 1 mM EGTA, and 50 mM Tris, pH 7.4. A, at the times indicated 1 μM bafilomycin A1 (Baf) and 1 μM ionomycin (Ion) were added. Intracellular calcium (black line) and pH (gray line) changes were determined as indicated under “Experimental Procedures.” In parallel experiments long (LC, B) and short chain (SC, C) polyP content of the samples was examined as indicated under “Experimental Procedures.” Open squares are controls with no additions. Closed diamonds are after the addition of bafilomycin A1 and ionomycin. Results depicted in panels B and C are from a representative experiment with data points given as means ± S.E.
Isolated Acidocalcisomes from Toxoplasma gondii

microorganisms (10). Alkalization of the acidocalcisomes may result in activation of the polyP-hydrolyzing activities in the organelles, as it has been shown that other acidocalcisomes contain polyphosphatases with an alkaline pH optimum (20). It has been hypothesized that one of the roles of acidocalcisomes is calcium storage for use in intracellular signaling, particularly in invasive parasite stages (1, 2). Enzymes cleaving short and long chain polyPs to P1 in acidocalcisomes may, therefore, indirectly regulate the intracellular Ca2+ concentration.

In conclusion, the isolation of acidocalcisomes provides definitive evidence that they are distinct from other previously recognized organelles present in T. gondii and will allow their further biochemical characterization. Our results indicate that the amount of polyP in acidocalcisomes rapidly decreases under alkaline stress. PolyP hydrolysis is accompanied by an increase in [Ca2+]i of tachyzoites. These effects suggest an important role for acidocalcisomes in the adaptation of T. gondii to environmental changes.

Acknowledgments—We thank David Sibley for antibodies against micronemes and dense granules, John Boothroyd for antibodies against SAG1, Jean Francois Dubremetz for antibodies against rhotries, micronemes and dense granules, John Boothroyd for antibodies against T. gondii and Will allow their further biochemical characterization. Our results indicate that the amount of polyP in acidocalcisomes rapidly decreases under alkaline stress. PolyP hydrolysis is accompanied by an increase in [Ca2+]i of tachyzoites. These effects suggest an important role for acidocalcisomes in the adaptation of T. gondii to environmental changes.

REFERENCES
1. Docampo, R., and Moreno, S. N. J. (1999) Parasitol. Today 15, 443–448
2. Docampo, R., and Moreno, S. N. J. (2001) Mol. Biochem. Parasitol. 133, 151–159
3. Vercesi, A. E., Moreno, S. N. J., and Docampo, R. (1994) Biochim. J. 304, 227–233
4. Docampo, R., Scott, D. A., Vercesi, A. E., and Moreno, S. N. J. (1995) Biochim. J. 310, 1005–1012
5. Moreno, S. N. J., and Zhong, L. (1996) Biochem. J. 313, 655–659
6. Moreno, S. N. J., Zhong, L., Lu, H.-G., de Souza, W., and Benchimol, M. (1997) Biochim. J. 330, 853–860
7. Rodrigues, C. O., Scott, D. A., Bailey, B. N., de Souza, W., Benchimol, M., Moreno, B., Urbina, J. A., Oldfield, E., and Moreno, S. N. J. (2000) Biochim. J. 349, 737–745
8. Lu, H.-G., Zhong, L., de Souza, W., Benchimol, M., Moreno, S. N. J., and Docampo, R. (1998) Mol. Cell. Biol. 18, 2309–2323
9. Luo, S., Viera, M., Graves, J., Zhong, L., and Moreno, S. N. J. (2001) EMBO J. 20, 55–66
10. Vercesi, A. E., and Docampo, R. (1996) Biochim. J. 315, 265–270
11. Rodrigues, C. O., Scott, D. A., and Docampo, R. (1999) Mol. Cell. Biol. 19, 7712–7723
12. Scott, D. A., de Souza, W., Benchimol, M., Zhong, L., Lu, H.-G., Moreno, S. N. J., and Docampo, R. (1999) J. Biol. Chem. 273, 22151–22158
13. Rodrigues, C. O., Scott, D. A., and Docampo, R. (1999) Biochem. J. 340, 759–766
14. Luo, S., Marchesini, N., Moreno, S. N. J., and Docampo R. (1999) FEBS Lett. 460, 217–220
15. Ruiz, F. A., Marchesini, N., Seufferheld, M., Govindjee, and Docampo, R. (2000) J. Biol. Chem. 275, 46196–46203
16. Marchesini, N., Ruiz, F. A., Vieira, M., and Docampo, R. (2002) J. Biol. Chem. 277, 8146–8153
17. Kulaev, I. S., Kulakovskaya, T. C., Andreeva, N. A., and Lichko, L. P. (1999) Prog. Mol. Subcell. Biol. 23, 27–43
18. Kuroda, A., Tanaka, S., Ikeda, T., Kato, J., Takiguchi, N., and Oh Tate, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14264–14269
19. Kornberg, A. (1995) J. Bacteriol. 177, 491–496
20. Ruiz, F. A., Rodrigues, C. O., and Docampo, R. (2001) J. Biol. Chem. 276, 26114–26121
21. Vieira, M., and Moreno, S. N. J. (2000) Mol. Biochem. Parasitol. 106, 157–162
22. Scott, D. A., and Docampo, R. (2000) J. Biol. Chem. 275, 24215–24221
23. Chamberland, S., Kiret, H. A., and Current, W. L. (1991) Antimicrob. Agents Chemother. 35, 903–909
24. Barrett, A. J., Heath, M. F. (1977) in Lysosomes: A Laboratory Handbook (J. T. Dingle, ed.) 2nd Ed., pp. 36–40, Elsevier/North-Holland Biomedical Press, Amsterdam
25. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candi, O. A. (1979) Anal. Biochem. 100, 95–97
26. Clark, J. E., and Wood, H. G. (1987) Anal. Biochem. 161, 290–290
27. Crooke, E., Akira, M., Rao, N. N., and Kernberg, A. (1994) J. Biol. Chem. 269, 6290–6295
28. Wurst, H., Shiha, T., and Kernberg, A. (1995) J. Bacteriol. 177, 898–906
29. Wan, K. L, Carruthers, V. B, Sibley, L. D., and Ajoka, W. J. (1997) Mol. Biochem. Parasitol. 84, 203–214
30. Leriche, M. A., and Dubremetz, J. F. (1991) Mol. Biochem. Parasitol. 45, 249–259
31. Labruyere, E., Lingnau, M., Mercier, C., and Sibley, L. D. (1999) Mol. Biochem. Parasitol. 102, 311–324
32. Bilek, R., and Boothroyd, J. C. (1991) J. Immunol. 147, 3496–3500
33. Serafian, V., Kim, Y., Poole, R. J., and Res, P. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1775–1779
34. Ault-Riche, D., Fraley, C. D., Tzeng, C.-M., and Kornberg, A. (1998) J. Bacteriol. 180, 1841–1847
35. Urbina, J. A., Moreno, B., Vierkotter, S., Oldfield, E., Payares, G., Sanoja, C., Bailey, B. N., Yan, W., Scott, D. A., Moreno, S. N. J., and Docampo, R. (1999) J. Biol. Chem. 274, 33609–33615
36. Damper, D., and Patton, C. L. (1976) J. Protol. 34, 349–356
37. Tijssen, J. P. F., Beekes, H. W., and Van Steveninck, J. (1982) Biochim. Biophys. Acta 721, 384–389
38. Martinez R, Wang, Y., Benaim, G., Benchimol, M., de Souza, W., Scott, D. A., and Docampo, R. (2002) Mol. Biochem. Parasitol. 120, 205–213
39. Maeshima, M. (2000) Biochim. Biophys. Acta 1465, 55–51
40. Peng, Z. Y., and Mansour, T. E. (1992) Mol. Biochem. Parasitol. 54, 223–230
41. Ho, A. M., Johnson, M. D., and Kingsley, D. M. (2000) Science 289, 265–270
42. Chapman, A. G., and Atkinson, D. E. (1977) Adv. Microbiol. Physiol. 15, 253–306
43. Rao, N. N., and Kernberg, A. (1996) J. Bacteriol. 178, 1394–1400
44. Castro, C. D., Meehan, A. J., Koretsky, A. P., and Domach, M. M. (1995) Appl. Environ. Microbiol. 61, 4448–4453