Kinetics of pyrophosphate-driven proton uptake by acidocalcisomes of *Leptomonas wallacei*[☆]

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Abstract

In this work, we show the kinetics of pyrophosphate-driven H^+ uptake by acidocalcisomes in digitonin-permeabilized promastigotes of *Leptomonas wallacei*. The vacuolar proton pyrophosphatase activity was optimal in the pH range of 7.5–8.0, was inhibited by imidodiphosphate, and was completely dependent on K^+ and PPi. H^+ was released with the addition of Ca^{2+} , suggesting the presence of a Ca^{2+}/H^+ antiport. In addition, X-ray elemental mapping associated with energy-filtering transmission electron microscopy showed that most of the Ca, Na, Mg, P, K, Fe, and Zn were located in acidocalcisomes. *L. wallacei* immunolabeled with antibodies against *Trypanosoma cruzi* pyrophosphatase show intense fluorescence in cytoplasmatic organelles of size and distribution similar to the acidocalcisomes. Altogether, the results show that *L. wallacei* acidocalcisomes possess a H^+ -pyrophosphatase with characteristics of type I V- H^+ -PPase. However, we did not find any evidence, either for the presence of H^+ -ATPases or for Na^+/H^+ exchangers in these acidocalcisomes.

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The trypanosomatidae family comprises unicellular eukaryotes that are able to infect a broad range of organisms such as mammals, fishes, plants, and insects causing a wide spectrum of diseases of social, economical, and medical importance. *Leptomonas wallacei* is a trypanosomatid parasite that was first isolated by Romeiro et al. [1] from the intestinal tract of the phytophagous hemipteran *Oncopeltus fasciatus* and since then has been used as a model for studies of the interaction between trypanosomatids and the midgut of the in-

sect vector [2,3]. A remarkable characteristic of these parasites is their ability to form cysts. They occur mainly as promastigotes but can differentiate into cystic forms, which suggests that they represent latent forms of the parasite, protecting it from extreme environmental conditions [1], which include variations on the concentration of ionic species, osmotic pressure, and pH, faced by the parasite in the different portions of the insect gut during the course of infection.

Control of intracellular pH in trypanosomatids occurs through the action of proton uptake/release mechanisms located either in the plasma membrane or in intracellular compartments. These include electrogenic proton pumps, proton/ion exchangers, and HCO_3^- and chloride channels [4]. The electrogenic proton pumps comprise the P-type proton ATPase (P-ATPase)

[☆] Abbreviations: PPi, pyrophosphate; polyP, polyphosphates; V- H^+ -PPase, vacuolar-proton-pyrophosphatase; IDP, imidodiphosphate; PBS, phosphate-buffered saline; AO, acridine orange; NH_4Cl , ammonium chloride; NEM, N-ethyl-maleimide; NaF, sodium fluoride.

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[5], the vacuolar proton ATPase (V-ATPase) [6,7], and the vacuolar proton pyrophosphatase (V-PPase) [8–10]. The first one has been shown to localize to the plasma membrane and to intracellular compartments of unknown nature [5,11] and the other two to the plasma membrane and to the acidocalcisomes [6–10].

Acidocalcisomes are calcium-rich acidic organelles first described in *Trypanosoma brucei* [6] and *Trypanosoma cruzi* [12], and then in several other trypanosomatids such as *Leishmania* [10,13,14], *Phytomonas* [11,15], *Herpetomonas* [16], *Crithidia*, *Blastocrithidia*, and *Leptomonas* [17], in apicomplexan parasites such as *Plasmodium* [18,19] and *Toxoplasma* [20–22], in other unicellular organisms such as *Chlamydomonas reinhardtii* [23], *Dictyostelium discoideum* [24], and in the bacteria *Agrobacterium tumefaciens* [25] and *Rhodospirillum rubrum* [26]. Acidification of acidocalcisomes is promoted by a V-type proton ATPase (V-H⁺-ATPase) [27,28] and a V-type pyrophosphatase (V-H⁺-PPase) [8,10]. They also possess a Ca²⁺-H⁺-translocating ATPase [29] for calcium uptake, a Ca²⁺/H⁺ antiporter for calcium release [6], and a Na⁺/H⁺ antiporter for Na⁺ uptake [30]. From the structural point of view, acidocalcisomes are electron dense organelles that contain a large amount of sodium, magnesium, phosphorus, potassium, calcium, iron, and zinc [14,15,31–34]. Phosphorous compounds are present primarily as pyrophosphates and long and short chains of polyphosphates [23] and may participate as a cation-binding component [33,35].

One of the functions attributed to the acidocalcisomes in heteroxenic parasites is a role in the adaptation of the parasites to different environmental challenges such as differences in extracellular pH, ionic composition, and temperature, found in the different hosts in the course of infection. The mechanisms of pH control by acidocalcisomes are particularly important for *L. wallacei* because variations of extracellular pH may lead to differentiation from promastigotes into cystic forms. Therefore, a detailed characterization of the physiologic properties of the mechanisms of proton uptake by acidocalcisomes of *L. wallacei* might be important for the understanding of the mechanisms underlying the environmental adaptation by this parasite.

In a previous work, we reported the structural properties and the elemental composition of acidocalcisomes in *L. wallacei* [17]. In the present work, a pyrophosphate-driven proton uptake in acidocalcisomes of promastigote forms of *L. wallacei* was characterized. The distribution of acidocalcisomes was determined by X-ray elemental mapping associated with energy filtering transmission electron microscopy and the distribution of the V-H⁺-PPase was determined by immunofluorescence microscopy using antibodies raised against a peptide sequence of *T. cruzi* H⁺-PPase. Images showed a labeling pattern in intracellular vacuoles numerically consistent with the dense granules (acidocalcisomes) seen in whole

cells imaged by transmission electron microscopy. Kinetics of H⁺ uptake by the V-H⁺-PPase showed that this activity is dependent on potassium ions, inhibited by IDP, insensitive to sodium and sucrose, and has maximal activity in the pH range 7.5–8.0, suggesting that this enzyme belongs to the type I V-H⁺-PPase. In addition, a Ca²⁺/nH⁺ counter-transporting system that may participate in Ca²⁺ uptake by acidocalcisomes was detected. Neither H⁺-ATPase activity nor a Na⁺/H⁺ exchanger could be detected in intracellular compartments in these cells. Altogether, the data presented here provide evidence that the mechanisms of pH stasis, ion storage, and homeostasis performed by the acidocalcisomes in *L. wallacei* are similar to those so far described for other trypanosomatids which are unable to differentiate into cystic forms.

Materials and methods

Culture method. *Leptomonas wallacei* promastigotes (CT-IOC 194; kindly provided by Dr. Alexandre Romeiro—Instituto de Biofísica Carlos Chagas Filho, UFRJ, RJ—Brazil) were grown at room temperature (25–27 °C) in a medium containing 37 g/L of brain heart infusion (BHI), 1 mg/L folic acid, supplemented with 10 mg/L haemin and 10% (v/v) fetal bovine serum. At 2–3 days after inoculation, cells were harvested by centrifugation, washed twice with 5 mM phosphate buffer, pH 7.0, containing 150 mM KCl (PBS), and resuspended in the same solution.

Electron probe X-ray microanalysis and elemental mapping. Energy dispersive X-ray spectra were recorded from the acidocalcisomes of whole cells dried onto Formvar-coated grids. Control spectra were collected from regions adjacent to the acidocalcisomes and from the Formvar film. Specimens were analyzed in a LEO 912 Omega scanning transmission electron microscope. X-rays were collected for 200 s using a Li-drifted Si-detector (front area 30 mm²) equipped with an ATW atmospheric window. The microscope was operated at 80 kV using a tungsten filament, in the scanning transmission (STEM) imaging mode, spot size was 40 nm and emission current ~10 µA. Analyses were performed using a Link multichannel energy analyzer and Link ISIS 3.00 software (Oxford Instruments, Wiesbaden, Germany).

Determination of H⁺ transport. Variations of H⁺ were followed by measuring the changes in the absorbance spectrum of acridine orange using a CINTRA 20 spectrophotometer at the wavelength pair 493–530, at room temperature (25 °C). Cells (1 mg/ml) were added to a reaction medium containing 125 mM sucrose, 65 mM KCl, 2 mM MgCl₂, 2 mM Pi-Tris, 10 mM Hepes–KOH buffer, pH 7.2, 1, 2 µg/ml oligomycin, 125 µM EGTA, 1 µg/ml acridine orange, and 80 µM digitonin. NH₄Cl (5 mM) was added when indicated. The reaction started with the indicated amount of PPI. The figures shown are representative of at least three experiments.

Immunofluorescence microscopy. Cells fixed in freshly prepared 4% formaldehyde were allowed to adhere to poly(L-lysine)-coated coverslips, permeabilized with 0.3% Triton X-100 for 3 min, and blocked with 50 mM ammonium chloride and 3% bovine serum albumin in PBS. Immunofluorescence was carried out using a 1:100 dilution of monoclonal antibodies raised against a peptide sequence of the *T. cruzi* V-H⁺-PPase [25] and Alexa fluor-coupled goat anti-mouse IgG secondary antibody (1:300). Images were obtained in a confocal laser scanning microscope (Zeiss CLSM 310).

Chemicals. Oligomycin, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), *N*-ethyl-maleimide (NEM), imidodiphosphate (IDP), sodium fluoride (NaF), pyrophosphate (PPI), acridine orange, and digitonin were purchased from Sigma Chemical (St. Louis, MO). All other reagents were of analytical grade.

Results

Electron microscopy characterization of acidocalcisomes

Energy-filtering transmission electron microscopy (contrast tuning) of *L. wallacei* showed many electron

dense vacuoles with an average diameter of 200 nm. As shown before [17], X-ray microanalysis of the dense vacuoles showed a considerable amount of sodium, magnesium, phosphorus, potassium, calcium, iron, zinc, and oxygen (Fig. 1). This elemental composition is consistent with that previously described for the acidocalci-

Fig. 1. Presence of acidocalcisomes in promastigotes of *L. wallacei*. (A) Electron spectroscopic imaging of whole unfixed *L. wallacei* promastigote (ΔE between 60 and 80 eV). (B) Corresponding X-ray spectrum of the acidocalcisome pointed out in (A). Copper peaks in the spectrum came from the support grid and titanium peaks from the specimen holder. Carbon and chlorine signals were similar in the acidocalcisomes and control regions (cytoplasm). (C) Electron spectroscopic image of a portion of a whole promastigote (ΔE between 60 and 80 eV). Elemental images of the cell displayed in (D–K) corresponding to: calcium; magnesium; phosphorus; sodium; potassium; zinc; iron; and sulfur. Scale bars: (A) 3.0 μm (inset 400 nm), (C) 800 nm.

somes of several trypanosomatids [9,10,31,32,36,37]. Elemental mapping showed that, except for sulfur, which was mostly detected in the cytoplasm, most of these elements were restricted to the acidocalcisome matrix (Fig. 1).

Localization of the vacuolar proton pyrophosphatase

Monoclonal antibodies raised against a peptide sequence of *T. cruzi* V-PPase, which has been shown to cross-react with the H^+ -PPase of other microorganisms [16,25], were used to investigate whether this pump was also present in *L. wallacei*. Results revealed labeling on the plasma membrane and an intracellular punctuate staining pattern with number, size, and distribution similar to those of the electron dense organelles seen in whole cell images (Fig. 2).

Pyrophosphate-driven proton uptake in permeabilized cells

Mechanisms of proton uptake by acidocalcisomes in trypanosomatids and apicomplexan parasites have been demonstrated with the use of digitonin permeabilized cells, incubated in the presence of acridine orange [6,20,21]. In experiments of H^+ transport in *L. wallacei*, the addition of pyrophosphate (PPi) to digitonin permeabilized cells incubated in 65 mM KCl plus 125 mM sucrose was followed by a fast decrease in absorbance of acridine orange, indicating proton uptake by intracellular organelles (acidocalcisomes) (Fig. 3). The activity was dependent on pyrophosphate concentration with minimum concentration to detect proton uptake around 20 μ M PPi and maximum activity in the concentration range of 500 μ M PPi. No further activity was detected at higher concentrations. Subsequent addition of nigericin, a K^+/H^+ exchanger, or NH_4Cl induced a fast release of protons from the intracellular vacuoles of the parasite (Fig. 3). Kinetics of pyrophosphate-dependent proton uptake in reaction media adjusted to different pHs showed that the activity was optimal in the pH

Fig. 3. Proton uptake promoted by different concentrations of PPi. The cells (1 mg/ml) were added to a medium containing 130 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, pH 7.2, 2 mM Pi-Tris, 125 μ M EGTA, 1 μ g/ml antimycin A, 2 μ g/ml oligomycin, and 1 μ g/ml acridine orange. Afterwards 80 μ M digitonin was added. Two minutes later, the reaction was started by the addition of the indicated amount of PPi. NH_4Cl (5 mM) was added when indicated.

range 7.5–8.0 (Fig. 4A). No H^+ uptake was detected when, instead of PPi, 2 mM ATP was used as substrate.

Effect of buffer composition on pyrophosphate-dependent proton uptake

The effect of composition of the reaction medium on the activity of the H^+ -PPase was tested by replacing 125 mM sucrose plus 65 mM KCl reaction medium by a medium containing different concentrations of KCl, NaCl, choline-Cl, and sucrose, yet maintaining the osmolarity constant. Results showed maximal activity in a 130 mM KCl reaction medium with decreasing activities in media containing lower concentrations of K^+ (Fig. 4B). Replacement of 130 mM KCl by 130 mM NaCl or choline-Cl resulted in very low H^+ uptake, while 260 mM sucrose was ineffective in activating the V- H^+ -PPase (Fig. 4B).

Fig. 2. Indirect immunofluorescence analysis of V- H^+ -PPase in *L. wallacei*. Phase contrast (A) and fluorescence (B) images of *L. wallacei* using antibodies raised against a peptide sequence of the *T. cruzi* V- H^+ PPase, as described under Materials and methods. Images of an isolate cell are shown with a greater amplification in the insets. (B) Intense labeling of intracellular vesicles. Scale bars: (A) 10 μ M, (A inset) 2.5 μ M.

Fig. 4. Effect of medium composition on PPI-dependent proton uptake. (A) Dependence on pH. Experimental conditions were as described in the legend of Fig. 3 with the same buffer adjusted to different pH values. The values represent the initial rate of proton uptake after the addition of 500 μM PPI. (B) Effect of ionic composition. The cells (1 mg/ml) were added to a medium containing 10 mM Hepes-Tris, pH 7.2, 2 mM Pi-Tris, 125 μM EGTA, 1 $\mu\text{g}/\text{ml}$ antimycin A, 2 $\mu\text{g}/\text{ml}$ oligomycin, 1 $\mu\text{g}/\text{ml}$ acridine orange, and 80 μM digitonin. KCl, NaCl or choline-Cl was included in the media as indicated in the curves. In the curves with 35 and 65 mM KCl, sucrose was added to have a final osmolarity of 260 mOsm. PPI (500 μM) was added when indicated.

Inhibition by IDP and the effect of ionophores

Proton uptake was inhibited by imidodiphosphate (IDP), a non-hydrolysable pyrophosphate analogue, in a dose-dependent manner (Fig. 5A). Full inhibition of the H^+ -PPase activity was observed with 0.5 mM PPI at IDP concentrations in the range of 5–10 mM, 2 mM being enough to substantially inhibit the pump. However, when pyrophosphate was reduced a significant increase in the IDP sensitivity was observed, characterizing a competitive inhibition (Fig. 5B). With 50 μM of PPI, proton release in control cells could be observed within 4 min of reaction, but this did not occur when IDP was added to the reaction medium. This pattern was most likely the consequence of a fast PPI

Fig. 5. Inhibition of proton uptake by imidodiphosphate (IDP). The cells (1 mg/ml) were added to the medium described in Fig. 3. PPI concentration was 50 μM (A) or 500 μM (B). IDP concentrations are indicated in the graphic.

disappearance in the media due to an acidocalcisomal leak of H^+ . Sodium orthovanadate (2 mM), a known P-type ATPase inhibitor, and *N*-ethyl-maleimide (NEM, 100 μM), a thiol reagent, had no significant effect (data not shown). Addition of FCCP, a proton ionophore, completely collapsed the H^+ gradient whereas valinomycin, a K^+ ionophore, slightly stimulated the uptake of acridine orange driven by PPI (Fig. 6), similar to what was described for *T. cruzi* [5], suggesting that the pumping of H^+ is electrogenic.

Presence of cation/ H^+ exchangers in the acidocalcisomes

Cation/proton exchangers have been reported to have a role in the mechanisms of cation uptake and release by the acidocalcisomes. A Na^+/H^+ exchanger that may participate in Na^+ uptake has been described in the acidocalcisomes of *T. brucei* and *Leishmania donovani* [30,38]. Furthermore, a $\text{Ca}^{2+}/\text{H}^+$ counter-transporting ATPase and a $\text{Ca}^{2+}/n\text{H}^+$ exchanger involved in calcium uptake and release by acidocalcisomes have also been described [6,9]. To investigate whether these exchangers were also present in acidocalcisomes of *L. wallacei*,

Fig. 6. Effect of ionophores on proton uptake. The cells (1 mg/ml) were added to the medium described in Fig. 3. No addition (curve a), valinomycin (10 μ M, curve b), FCCP (10 μ M, curve c). PPI (500 μ M) and NH_4Cl (5 mM) were added when indicated.

Fig. 7. Effect of Ca^{2+} and Na^{+} on H^{+} uptake. The cells (1 mg/ml) were added to the medium described in Fig. 3. No addition (a), NaCl (40 mM, curve b) and CaCl_2 (250 μ M, curve c) were added at 15 min. PPI (500 μ M) and NH_4Cl (5 mM) were added when indicated.

250 μ M CaCl_2 (enough to give 125 μ M of free Ca^{2+}) or 40 mM Na^{+} was added to the reaction medium after PPI-driven proton uptake. Results showed that Ca^{2+} could effectively release H^{+} from intracellular organelles, suggesting the presence of a $\text{Ca}^{2+}/n\text{H}^{+}$ counter-transporting system in the acidocalcisomes, whereas Na^{+} was unable to release H^{+} , suggesting that this exchanger is absent in the acidocalcisomes of *L. wallacei* (Fig. 7).

Discussion

In this work, we report the presence of acidocalcisomes in *L. wallacei*, a trypanosomatid parasite that is able to differentiate into cystic forms. Several functions

have been attributed to the acidocalcisomes, such as control of cytosolic pH, ion homeostasis, storage of high energy compounds, osmoregulation, and a role in the biosynthesis of sphingolipids (reviewed in [39]). It has also been postulated that acidocalcisomes have a functional role in the adaptation of heteroxenic parasites such as *Trypanosoma* and *Leishmania* to different environmental conditions encountered in their different hosts during their life cycle, a condition that is mandatory for a successful infection by these parasites [14,39]. Despite being able to infect only a single host, *L. wallacei* also faces different environments during its life cycle. The parasite infects different portions of the insect gut, which have different physicochemical properties such as differences in pH, ionic composition, and osmotic pressure. Variations of such conditions in the extracellular environment are believed to lead *L. wallacei* to differentiate into cystic forms [1]. As acidocalcisomes of *L. wallacei* accumulate several ions, as seen by X-ray elemental mapping, and present a V-H^{+} -PPase activity, it is possible that these organelles might also play a role on the adaptation mechanisms of the parasite to changes in the environmental conditions, functioning in ion homeostasis and pH stasis, being an alternative mechanism of environmental sensing by this parasite.

The V-H^{+} -PPase is an electrogenic proton pump that has so far been described in bacteria [26], microorganisms [8,40], plants (reviewed in [41]), and recently found in insects [42], being absent in mammals. The H^{+} -translocating pyrophosphatase activity characterized here was shown to be located in acidocalcisomes in accordance with previous results found in different trypanosomatids [39]. This activity had optimum pH between 7.5 and 8.0 (Fig. 4A), was stimulated by K^{+} but minimally by Na^{+} or choline $^{+}$ (Fig. 4B), insensitive to NEM and vanadate (data not shown), and fully inhibited by IDP (Figs. 5A and B). The H^{+} pumping activity seems to be electrogenic since increasing the K^{+} permeability with valinomycin, that would collapse any membrane potential generated by the ion pumping, increased the efficiency of the uptake. Two types of V-H^{+} -PPase have been cloned and characterized from *A. thaliana*, AVP1 [43] and AVP2 [44]. AVP1 is stimulated by K^{+} and AVP2 is insensitive to this ion. In *L. donovani*, the V-H^{+} -PPase activity was shown to be dependent on K^{+} [9]. In contrast, the type II H^{+} -PPase insensitive to potassium has been described in *Plasmodium falciparum* [45]. So, the PPI dependent H^{+} transport observed in *L. wallacei* can be considered as an AVP1 type. The release of H^{+} with the addition Ca^{2+} indicates the presence of a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger in acidocalcisomes of *L. wallacei*. However, in contrast to what has been previously observed in *T. brucei* [38] and *L. donovani* [10,30] Na^{+} was incapable of releasing H^{+} , indicating the absence of a $\text{Na}^{+}/\text{H}^{+}$ exchanger in the acidocalcisomes of this parasite.

We were unable to find evidence for other pH regulatory mechanisms in this parasite. It is possible that the H^+ -ATPase complex in this parasite dissociates during permeabilization, losing its peripheral subunits and becoming inactive. However, we do not discard a possible presence of an ATP-driven proton uptake by *L. wallacei* since this has been shown in trypanosomatid parasites analyzed by alternative methods.

Together, these results suggest that the electron dense vacuoles of promastigote *L. wallacei* and the acidocalcisomes described in other trypanosomatids and apicomplexan parasites are representative of the same class of organelles.

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