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A proton pumping pyrophosphatase in acidocalcisomes of *Herpetomonas* sp.

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Abstract

Acidocalcisomes are acidic calcium storage organelles found in several microorganisms. They are characterized by their acidic nature, high electron density, high content of polyphosphates and several cations. Electron microscopy contrast tuned images of *Herpetomonas* sp. showed the presence of several electron dense organelles ranging from 100 to 300 nm in size. In addition, X-ray element mapping associated with energy-filtering transmission electron microscopy showed that most of the cations, namely Na, Mg, P, K, Fe and Zn, are located in their matrix. Using acridine orange as an indicator dye, a pyrophosphate-driven H^+ uptake was measured in cells permeabilized by digitonin. This uptake has an optimal pH of 6.5–6.7 and was inhibited by sodium fluoride (NaF) and imidodiphosphate (IDP), two H^+-pyrophosphatase inhibitors. H^+ uptake was not promoted by ATP. Addition of 50 μM Ca^{2+} induced the release of H^+, suggesting the presence of a Ca^{2+}/H^+ countertransport system in the membranes of the acidic compartments. Na^+ was unable to release protons from the organelles. The pyrophosphate-dependent H^+ uptake was dependent of ion K^+ and inhibited by Na^+ *Herpetomonas* sp. immunolabeled with monoclonal antibodies raised against a *Trypanosoma cruzi* V-H^+-pyrophosphatase shows intense fluorescence in cytoplasmatic organelles of size and distribution similar to the electron-dense vacuoles.

Together, these results suggest that the electron dense organelles found in *Herpetomonas* sp. are homologous to the acidocalcisomes described in other trypanosomatids. They possess a vacuolar H^+-pyrophosphatase and a Ca^{2+}/H^+ antiport. However, in contrast to the other trypanosomatids so far studied, we were not able to measure any ATP promoted H^+ transport in the acidocalcisomes of this parasite.

Keywords: Trypanosomatids; H^+-PPase; Herpetomonas; Ca^{2+}; Acidocalcisomes

1. Introduction

The flagellated trypanosomatids of the genus *Phytomonas* and some *Herpetomonas* are parasites of plants. In some cases they parasitize plants without apparent pathogenicity but they can also cause diseases of economic significance in plantations of coconut, oil palm, cassava and coffee [1–4]. These trypanosomatids have also been detected in various edible fruit, such as guavas, pomegranates, peaches and tangerines and in their insect vectors [4]. The parasites live mostly in the xylem and phloem of the infected plants and are transmitted through the bite of phytophagous insects [1–4]. In the biological cycle of these pathogens, several plant-sucking insects act as intermediate hosts and the plant acts as the main host [5]. These parasites have ultrastructural features typical of the family Trypanosomatidae containing kinetoplast,

Abbreviations: EDX, energy dispersive X-ray microanalysis; NEM, *N*-ethylmaleimide; PP_i, pyrophosphate; polyP, polyphosphates; V-H^+-PPase, vacuolar-proton-pyrophosphatase; IDP, imidodiphosphate; PBS, phosphate-buffered saline; AO, acridine orange

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glycosomes, endoplasmic reticulum and one single tubular mitochondrion [4,6,7].

In the last few years a singular intracellular acidic compartment named acidocalcisome was characterized in numerous organisms. These were first described in *Trypanosoma brucei* [8] and *Trypanosoma cruzi* [9], then in *Leishmania amazonensis* [10], *Leishmania donovani* [11], *Plasmodium berghei* [12], *Plasmodium falciparum* [13], *Toxoplasma gondii* [14] and in organisms such as *Chlamydomonas reinhardtii* [15], *Dictyostelium discoideum* [16] and more recently in the bacterium *Agrobacterium tumefaciens* [17]. These acidic organelles are electron dense, posses a surrounding membrane, have variable size, ranging from 200 ± S.E. 90 nm of diameter, and contain very high amounts of Mg, Ca, Na, Zn and short and long chain polyphosphates and low amounts of Cl, K and sulfur [for reviews see 18–21].

Kinetic studies have shown that acidocalcisomes maintain a low internal pH due to the presence of a V-H^+-ATPase [22–24] and a V-H^+-PPase [8,11,13,15,25–27] which pump H^+ into the lumen of the organelle. They also posses a Ca^{2+}–H^+ translocating ATPase, a Ca^{2+}–H^+ exchanger and a Na^+–H^+ exchanger which permit a complex regulation of these ions by the cell It was proposed that acidocalsisomes are involved in mechanisms of Ca^{2+} signaling, osmoregulation, pH homeostasis and energy storage [16,28].

The study of the mechanisms by which a plant parasite (*Herpetomonas* sp.) regulates intracellular H^+ and Ca^{2+} distribution, to maintain cell viability, could pro-

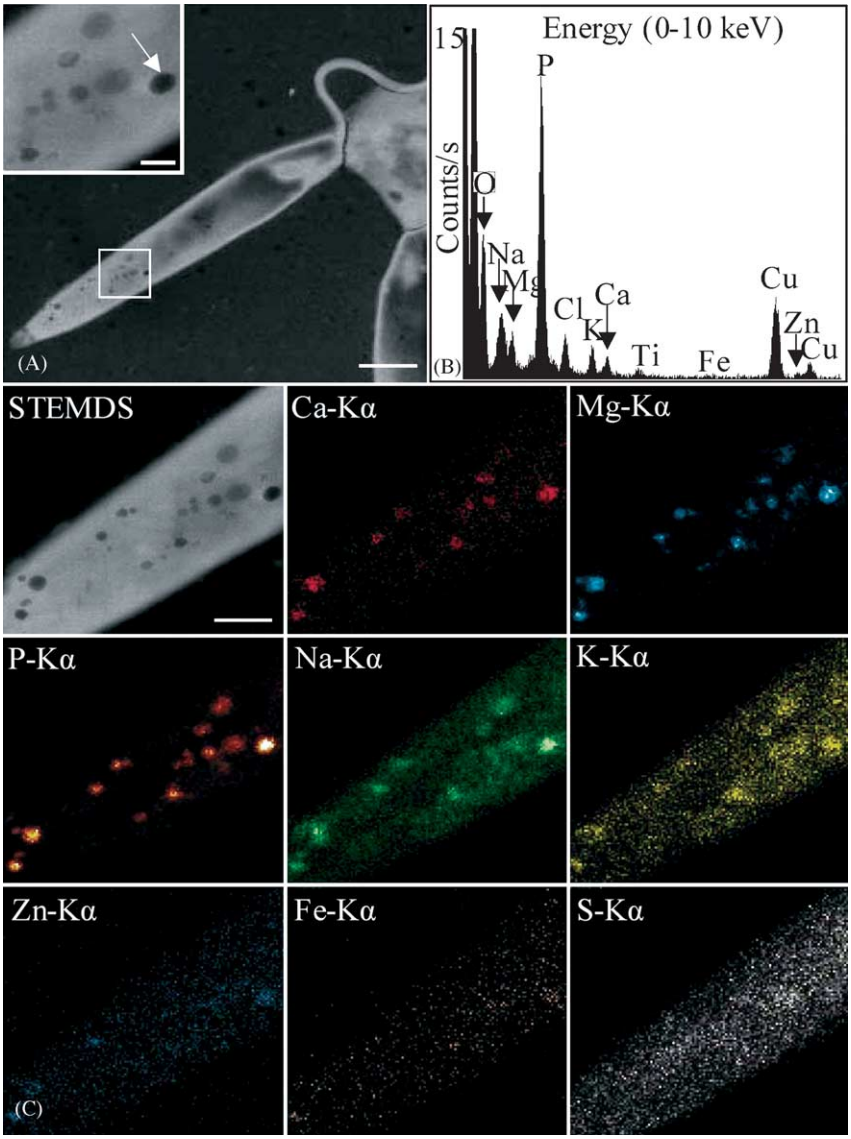

Fig. 1. Presence of acidocalcisomes in promastigotes of *Herpetomonas* sp. (A) Electron spectroscopic imaging of whole unfixed *Herpetomonas* sp. promastigote (Δ*E* between 60 and 80 eV). (B) corresponding X-ray spectrum of the acidocalcisome pointed out in (A). Copper peaks in the spectrum came from 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 (Δ*E* between 60 and 80 eV). Elemental images of the cell displayed in (C) corresponding to: calcium; magnesium; phosphorus; sodium; potassium; zinc; iron; and sulfur. Scale bars: (A) 3.0 μm (inset 400 nm), (C) 800 nm.

vide new insights into the physiology of these parasites. In a previous work, we have identified and characterized two Ca^{2+}-transport systems in *Herpetomonas* sp. permeabilized with digitonin [29]. One, sensitive to antimycin A, is driven by an electrical potential in mitochondria, and a second, vanadate sensitive, is promoted by a SERCA-type (sarcoplasmic–endoplasmic reticulum). Ca^{2+}-ATPase in the endoplasmic reticulum. Since the extramitochondrial Ca^{2+} transport was inhibited by vanadate, but was insensitive to nigericin (H^+/K^+ exchanger), FCCP (H^+ ionophore) or bafilomycin (inhibitor of V-H^+-ATPases), a possible participation of an acidic compartment, such as acidocalcisomes, was not considered in this previous work.

In this report we show that acidocalcisomes are abundant in *Herpetomonas* sp. and that they sustain a H^+ pumping promoted essentially by a V-H^+-PPase.

2. Materials and methods

2.1. Culture method

Herpetomonas sp. promastigotes (CTIOC 13; kindly provided by Dr. Reginaldo Brazil, Instituto Rene Rachour, FIOCRUZ, MG, Brazil) were grown at room temperature (26–28 °C) in a medium containing 20 g/l sucrose, 20 g/l KCl, 3 g/l yeast extract, 3 g/l peptone, 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 70 containing 150 mM NaCl (PBS) and resuspended in the same solution. Protein concentration was determined by the biuret assay [30] in the presence of 02% deoxycolate.

2.2. Electron probe X-ray microanalysis and elemental mapping

Energy dispersive X-ray (EDX) 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 Zeiss/LEO 912 Omega scanning transmission electron microscope. X-rays were collected for 200 s using a Li-drifted Si-detector (front area 30 mm^2) equipped with an ATW atmospheric window. The microscope was operated at 80 kV, in the scanning transmission (STEM) imaging mode, using a tungsten filament, spot size 40 nm and emission current ∼10 mA. Analyses were performed using a Link multichannel energy analyzer and Link ISIS 300 software (Oxford Instruments Wiesbaden, Germany). Electron spectroscopic images were recorded at an energy loss of ∼60 eV with spectrometer slit width of 20 eV.

2.3. Determination of H^+ transport

Variations of H^+ were followed by measuring the changes in the absorbance spectrum of acridine orange [31–33], using a CINTRA 20 spectrophotometer at the wavelength pair 530-493, at room temperature (25 °C). Cells (1 mg/ml) were added to a reaction medium containing 125 mM sucrose, 65 mM KCl, 2 mM $MgCl_2$, 2 mM Pi–Tris, 10 mM Hepes buffer pH 7.2, 1 mg/ml antimycin A, 2 mg/ml oligomycin, 125 mM EGTA, 1 μg/ml acridine orange (AO) and 80 mM digitonina. NH_4Cl (5 mM) was added when indicated. The reaction started with the indicated amount of PP_i. The figures shown are from representative experiments. Each experiment was repeated at least three times with different cell preparations. The assays of H^+ transport inhibition were performed by addition of the inhibitors (NEM, NaF, IDP, vanadate) 2 min before the PP_i.

2.4. Immunofluorescence microscopy

Cells fixed in freshly prepared 4% formaldehyde were allowed to adhere on poly(L)lysine-coated coverslips, permeabilized with 03% Triton X-100 for 3 min, blocked with

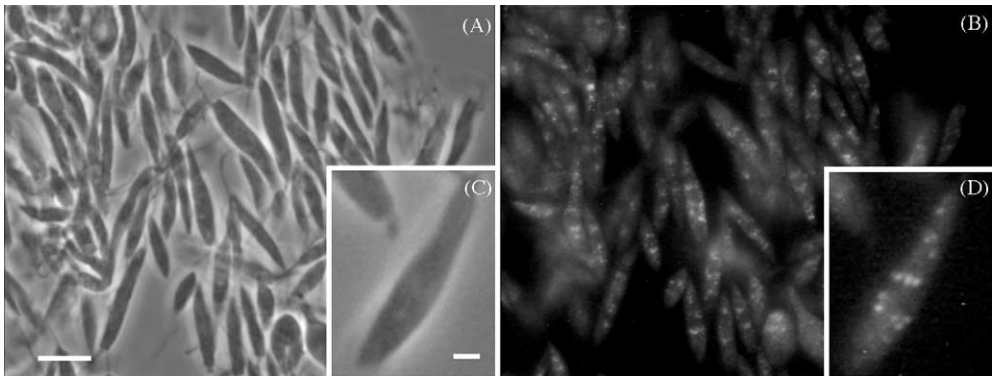

Fig. 2. Indirect inmunofluorescence analysis of V-H^+-PPase in *Herpetomonas* sp. Phase contrast (A and C) and fluorescence (B and D) images of *Herpetomonas* sp. using antibodies raised against a peptide sequence of the *T. cruzi* V-H^+ PPase, as described in Section 2. Images of an isolate cell are shown with a greater amplification in (C) and (D). Panels (B) and (D) show intense labelling of intracellular vesicles. Scale bars: (A) 10 μM, (C) 15 μM.

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 and Alexa fluor-coupled goat anti-mouse IgG secondary antibody (1:300). Images were obtained in a confocal laser-scanning microscope (Zeiss CLSM 310).

2.5. Chemicals

Sodium orthovanadate, antimycin A, oligomycin, carbonylcyanide *p*-trifluoromethoxiphenylhydrazone (FCCP), *N*-ethylmaleimide (NEM), imidodiphosphate (IDP), NaF, ATP, GTP, succinate, arsenazo III, safranine O, digitonin, and calcium ionophore (A23187) were purchased from Sigma

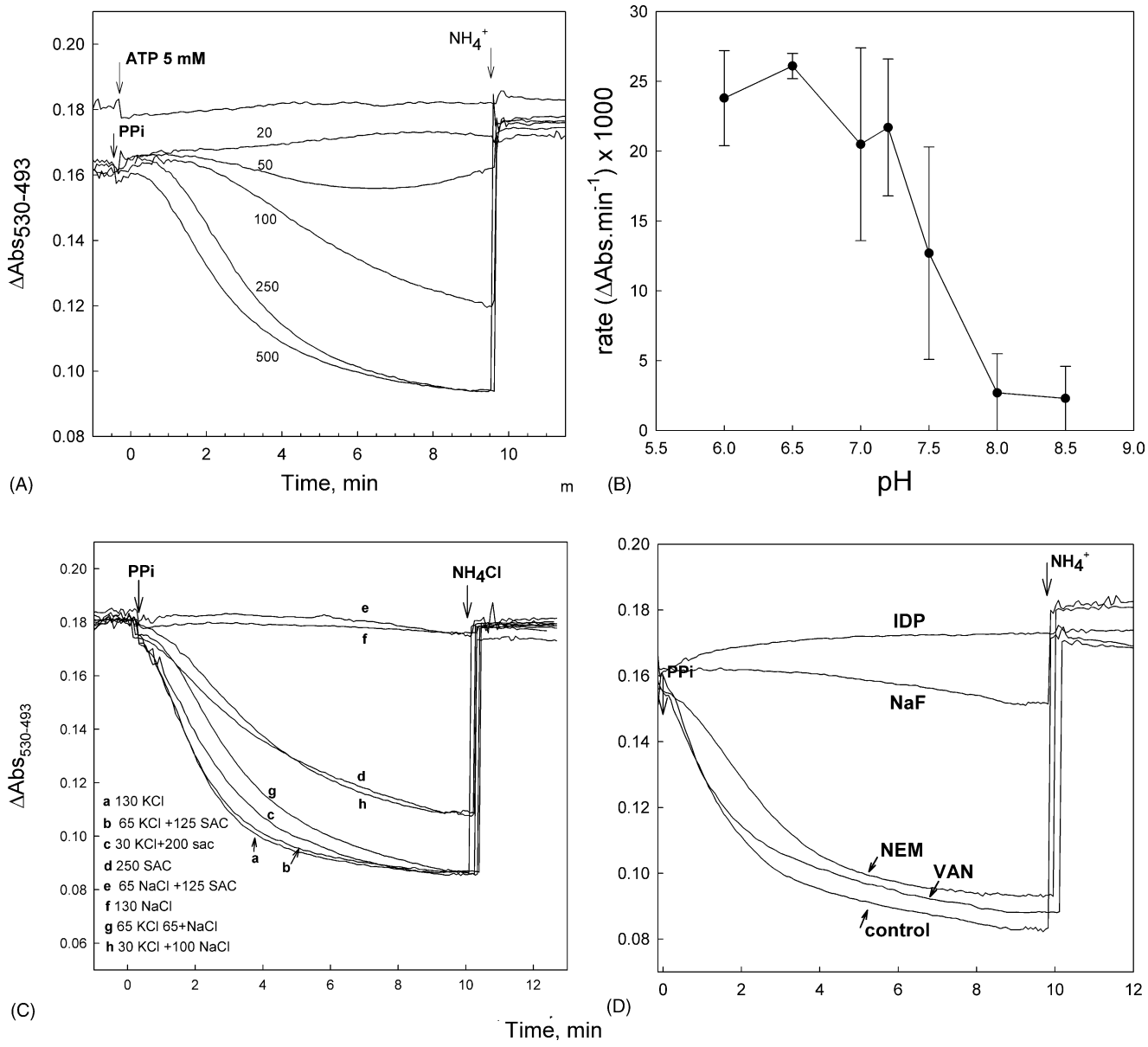

Fig. 3. Proprieties of proton uptake promoted by PP_i in *Herpetomonas* sp. Cells (1 mg/ml) were added to a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES–KOH pH 7.2, 1 μg/ml antimycin A, 2 μg/ml oligomycin, 125 μM EGTA, 1 μg/ml acridine orange and 80 μM digitonin. (A) Dependence on PP_i concentration. The reactions were started with the indicated amount of PP_i (in mM) or 5 mM ATP. (B) pH dependence of the rate of H^+ uptake. Error bars indicate the S.D. of mean values from three separate experiments. (C) Effect of the medium composition. Sucrose, KCl and NaCl were present at the concentrations specified (in mM). (D) Effect of *N*-ethylmaleimide (NEM, 100 μM), sodium orthovanadate (VAN, 1 mM), imidodiphosphate (IDP, 5 mM) and sodium fluoride (NaF, 10 mM). The inhibitors were added 2 min before the PP_i. The H^+ uptake in B, C and D were initiated by addition of 500 μM PP_i. NH_4Cl (5 mM) was added where indicated. All the experiments were repeated at least three times with different cell preparations.

Chemical Co. (St. Louis, MO). All other reagents were analytical grade.

3. Results

3.1. Elemental analysis of Herpetomonas sp.

Acidocalcisomes have been readily analyzed in whole intact cells by transmission electron microscopy [11,31,32]. With whole unfixed *Herpetomonas* sp. this revealed the presence of electron dense organelles of variable size (∼100 nm) (Fig. 1A). Electron probe X-ray microanalysis showed the presence of high amounts of sodium, magnesium, phosphorus, potassium, calcium, and oxygen in the electron dense organelles, indicating that their mass density corresponds to the presence of high amounts of phosphorus-cation groups (Fig. 1B). Minor amounts of zinc and iron were also detected. The distribution of these elements over the cell was assessed by the X-ray elemental mapping of the whole cell (Fig. 1C). Clearly phosphorus, magnesium, calcium, potassium, sodium and zinc were mostly concentrated in the electron-dense organelles. Sodium and potassium were also present in the cytoplasm and in other organelles. Sulfur was uniformly dispersed all over the cell. Iron signal was comparable to background.

3.2. Immunolocalization of the H^+-PPase

The size, distribution and elemental composition of the electron dense organelles was the first indication that they corresponded to acidocalcisomes [18–20]. V-H^+-PPases have been shown to localize in vacuoles of higher plants cells [34,41], and in acidocalcisomes [11,25,31] and plasma membrane of trypanosomatids [23]. We therefore investigated whether it is also present in *Herpetomonas* sp. The localization of the H^+-PPase was tested using monoclonal antibodies raised against a peptide sequence of *T. cruzi* V-PPase which presumably cross-react with the H^+-PPase of diverse trypanosomatids. Results revealed a punctate intracellular staining pattern (Fig. 2) with a size and distribution similar to the electron dense organelles seen in whole cell images (Fig. 1). No fluorescence was observed in control cells incubated in the presence of the secondary antibodies only (data not shown).

3.3. Characterization of a H^+-translocating PPase

Acidocalcisomes are characterized by possessing an active H^+ pumping which is sustained either by the PP_i or ATP hydrolysis. Addition of sodium pyrophosphate to digitonin-permeabilized *Herpetomonas* sp. showed a fast AO uptake from the medium as a result of proton accumulation in an intracellular compartment (Fig. 3A), which could be reverted either by alkalinization with NH_4Cl or by addition of protonophores like FCCP (nor shown). In contrast to what has been previously described for different trypanosomatids, ATP was not capable to promote any H^+ transport (Fig. 3A). The optimal PP_i concentration for the H^+ pumping was 250 μM. Lower concentrations showed a biphasic kinetic with an uptake followed by a slow release.

Considering the rate of decrease of AO absorption immediately after the addition of PP_i, it was found that the optimal pH for the H^+-PPase was around 6.5 (Fig. 3B). Values of pH above 7.2 caused a drastic loss of efficiency. In the absence of monovalent cations (250 mM sucrose to maintain osmolarity) there was a considerable H^+ uptake, which was increased when sucrose was substituted by KCl (Fig. 3C). In contrast, addition of sodium inhibited the H^+ uptake, becoming negligible at 65 mM NaCl. Use of equimolar concentrations (65 mM) of NaCl and KCl resulted in a lower acidification than in the presence of 130 mM KCl or 65 mM KCl/125 mM sucrose. These data suggest that K^+ was an activator of the H^+ pumping while Na^+ was inhibitory.

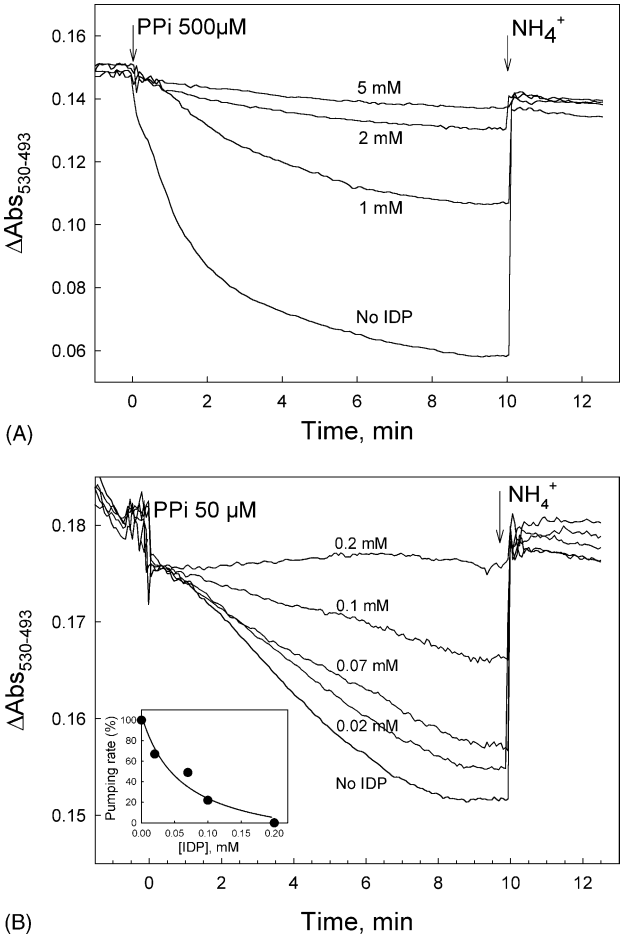

Fig. 4. Inhibition of PP_i-driven proton uptake in *Herpetomonas* sp. by imidodiphosphate (IDP). H^+ uptakes were measured as described in Section 2 with PP_i concentrations 50 μM (A) or 500 μM (B). The IDP concentrations present during the uptake are indicated in the graphics. Inset: residual H^+-transport rates at different IDP concentrations. The values, in percent, were calculated from the slopes of the curves shown in (B).

3.4. Inhibition of the PP_i dependent H^+ uptake

Fig. 3D shows that neither NEM nor vanadate was capable of inhibiting the PP_i dependent H^+ uptake, whereas IDP and NaF were good inhibitors. These two compounds were described as inhibitors of the PPases from plants and other trypanosomatids. IDP is a non-hydrolysable PP_i analogue which inhibits the PPase activity by substrate competition. Fig. 4A shows that it was necessary to apply 5 mM IDP to inhibit H^+ uptake promoted by 500 μM PP_i. However, 0.2 mM was sufficient when 10 times less substrate was used (Fig. 4B). At this low substrate concentration the apparent K_i for the IDP inhibition was about 60 μM (Fig. 4B, inset) which is close to that observed with *Plasmodium chabaudi* [12], *C. reinhardtii* [15], *T. brucei* [26] and *D. discoideum* [16].

3.5. H^+ release from the acidic vacuoles

A H^+/Ca^{2+} exchanger that permit the uptake of Ca^{2+} coupled to the efflux of H^+ was described in acidocalcisome membranes from *T. brucei* and *T. cruzi*. The continuous H^+ pumping by a V-H^+-PPase and/or a V-H^+-ATPase maintains the acidity in the lumen of the organelle and therefore Ca^{2+} can be concentrated.

When *Herpetomonas* sp. cells were incubated with PP_i to allow 10 min of H^+ upload, and then 10 mM NaF was added in order to stop the H^+ pumping, a slow spontaneous release of H^+ was observed (Fig. 5). Addition of 40 mM NaCl did not induce any H^+ efflux suggesting the absence of a H^+/Na^+ exchanger in the acidocalcisomes of *Herpetomonas* sp. or that it may have much lower capacity than in other trypanosomatids [11,27]. In contrast, addition of $CaCl_2$ in excess over EGTA induced rapid loss of H^+ from the organelle, suggesting the presence of a Ca^{2+}/H^+ countertransporting system in *Herpetomonas* sp. acidocalcisomas.

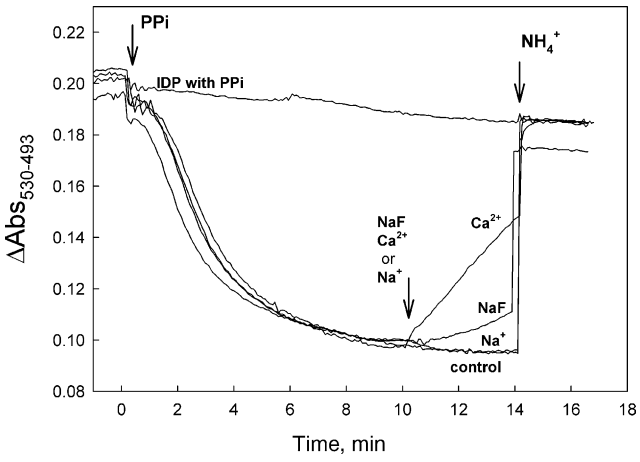

Fig. 5. Effect of NaF, Ca^{2+} and Na^+ on H^+ release. PP_i (500 μM) was added to start the H^+ uptake as described in Section 2. After 10 min, NaF (10 mM), $CaCl_2$ (200 μM), NaCl (40 mM) or NH_4Cl (5 mM) were added. A curve with uptake inhibited from the beginning with 5 mM IDP is also shown.

4. Discussion

Transmission electron microscopy and the distribution of Ca, Mg, P, Na, and Zn in *Herpetomonas* sp. (Fig. 1), determined by electron probe X-ray microanalysis, showed the presence of acidocalcisome-like organelles which have been reported to be rich in these elements [31–38]. This assumption was supported by the finding that the parasites displayed a vacuolar H^+-PPase localized in organelles of same size and distribution (Fig. 2). Moreover, the kinetic properties of the PP_i dependent H^+ pumping were similar to the V-H^+-PPase described for the acidocalcisomes in other parasites and in plant vacuoles [12,25,26,33,39–41]. This activity had a pH optimum of 6.5–7.0 (Fig. 3B), was stimulated by K^+ (Fig. 3C), insensitive to 1 mM vanadate and to 100 μM NEM, whereas it was fully inhibited by NaF (Fig. 3D) and IDP (Fig. 4). It should be remark that the high fluoride concentration required for full inhibition of H^+ uptake is characteristic of V-H^+-PPases [11,12,15,27,33]; soluble PPases have typically higher sensitivities. The lack of response to 100 μM NEM was also found with the H^+-PPase from acidocalcisomes of *P. berghei* [12] and *C. reinhardtii* [15], whereas the enzymes from *T. cruzi* [33,40] and *D. discoideum* [16] are fully inhibited. The cystein C634:AVP1, in the plant vacuolar PPase, was recognized to be the target for the maleimides that irreversible block catalysis [39,41]. While this residue is conserved in most of the V-PPases, mutagenesis of this residue in AVP1 did not interfere with catalysis [42]. Thus, it seems likely that C634:AVP1 may be close to the active site but not directly involved in the catalysis [42].

Recently in *A. thaliana* [43] and *P. falciparum* [44] a second type of V-H^+-PPase named AVP2 and PfVP2, respectively, has been described. However, their activities, like those from bacteria and mitochondria, are insensitive to stimulation by K^+ suggesting that the PP_i dependent H^+ transport observed in *Herpetomonas* may be considered as an AVP1 type.

It was not possible to determine the K_m for PP_i because at low substrate concentrations the H^+ uptake was biphasic (Fig. 3A). This pattern was most likely a consequence of a fast PP_i consumption in the media (probably due to the existence of other PPase activities that rapidly consumes the substrate) combined with an acidocalcisomal leak of H^+, though, like in other organisms, K_m was in the micromolar range.

In contrast to what has been described for acidocalcisomes of other parasites [20,45,46], the acidification of the lumen of the organelle was exerted only by a H^+-PPase. There was no evidence of an ATP-driven H^+ transport (Fig. 3A), suggesting the absence of a H^+-ATPase in the acidocalcisomas. However, it is not ruled out the existence of an ATP-driven H^+ transport in a different organelle, sensitive to digitonin, as was found in *Phytomonas françai* (unpubl. results).

V-PPase may contribute to parasitic cytosolic pHstasis by pumping H^+ into the acidocalcisomes [17,23]. However this organelle accumulates also Ca and other divalent cations such as Zn and Fe which co-precipitates with large amounts of

polyphosphates. For this reason, they have been suggested to participate in several cellular processes such as osmoregulation [37], storage of high-energy polyphosphate for mobilization in case of stress or great energy demands [47,48], in pHstasis [23] and the Ca^{2+} homeostasis [25,49]. We have previously shown that the endoplasmic reticulum of *Herpetomonas* sp. possess a Ca^{2+}-ATPase that supports the Ca^{2+} accumulation into its lumen helping to maintain the low Ca^{2+} levels in the cytosol. In those preparations we were unable to induce IP_3-dependent Ca^{2+} liberation from this pool. We show here that acidocalsisomes possess a Ca^{2+}/H^+ exchanger, which allows the uptake and the release of Ca^{2+} between the cytoplasm and the lumen of the organelle. It is plausible that this mechanism can also be involved in the Ca^{2+} homeostasis in conjunction with the H^+ homeostasis. The study of the mechanisms by which a plant parasite (*Herpetomonas* sp.) regulate intracellular H^+ and Ca^{2+} distribution to maintain cell viability could provide new insights into the physiology of these parasites and, in addition, could be useful for the design of new drugs.

In conclusion the results indicate that *Herpetomonas* sp. possess acidocalcisomes that are able to concentrate phosphorus, magnesium, calcium, potassium, sodium and zinc, however, in contrast with acidocalcisomes of *P. françai* [32], almost no iron was found in their lumen. In addition, they have a NEM-insensitive V-H^+-PPase but, different to other trypanosomatids, they seem to have no H^+-ATPases.

Acknowledgments

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