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A proton pumping pyrophosphatase in the Golgi apparatus and plasma membrane vesicles of *Trypanosoma cruzi*

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Abstract

The proton pumping pyrophosphatase (H^+-PPase) is an enzyme that has been identified in membranes of plant vacuoles, in the Golgi complex of plants and *Chlamydomonas reinhardtii*, and more recently in acidocalcisomes of different trypanosomatids and apicomplexan parasites. Immunofluorescence and immunoelectron microscopy studies using antibodies against the plant enzyme also suggested a plasma membrane localization in different stages of *Trypanosoma cruzi*. In this report we provide immunogold electron microscopy evidence of the presence of the H^+-PPase in the Golgi complex and plasma membrane of epimastigotes of *T. cruzi*. Pyrophosphate promoted acidification of plasma membrane vesicles as determined using acridine orange. This activity was stimulated by K^+ ions, inhibited by the pyrophosphate analogs imidodiphosphate (IDP) and aminomethylenediphosphonate (AMDP) by KF, NaF and DCCD, and it had different responses to ions and inhibitors as compared with the activity present in acidocalcisomes. Surface localization of the H^+-PPase was confirmed by experiments using biotinylation of cell surface proteins and immunoprecipitation with antibodies against H^+-PPase. Taken together, these results are consistent with the presence of a functional H^+-PPase in the plasma membrane of these parasites.

Keywords: Acidocalcisomes; Aminomethylenediphosphonate; *Trypanosoma cruzi*; Pyrophosphatase; Plasma membrane

1. Introduction

The H^+-translocating pyrophosphatase (H^+-PPase) is an electrogenic proton pump that acidifies intracellular compartments in plant cells and unicellular eukaryotes. In plant cells the enzyme localizes mainly to the vacuole membrane (tonoplast) [1], while in several trypanosomatids and apicomplexan protozoa, it localizes to the acidocalcisomes, which are acidic vacuoles rich in calcium similar to the polyphosphate bodies present in a wide variety of microorganisms [2].

A novel type of H^+-PPase (AVP2, or type II H^+-PPase) has been identified in *Arabidopsis thaliana* [3]. This protein has only 35% amino acid sequence identity to the vacuolar H^+-PPase encoded by AVP1, or type I H^+-PPase. The use of green fluorescent protein(GFP)-tagging has suggested the presence of type II H^+-PPase in the Golgi apparatus of *A. thaliana* [4]. Using immunoelectron microscopy, other authors had previously reported the presence of H^+-PPase polypeptides in the Golgi apparatus of maize roots [5], cauliflower inflorescence cells [6], pea cotyledons [7] and *Chlamydomonas reinhardtii* [8].

Abbreviations: AMDP, aminomethylenediphosphonate; DCCD, *N*,*N*′-dicyclohexylcarbodi-imide; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; GFP, green fluorescent protein; H^+-PPase, proton translocating pyrophosphatase; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride.

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The H^+-PPase has also been reported to be associated with the plasma membrane in plants and unicellular eukaryotes [9]. H^+-PPase activity was initially measured in plasma membrane-enriched fractions obtained by sucrose density gradient centrifugation of Jerusalem artichoke tubers [10] and radish seedlings [11], as well as by phase-partitioning of germinating cotyledons of castor beans [12] or maize roots [5]. These observations were initially dismissed as reflecting tonoplast contamination, detectable in these preparations [13]. More recently, immunogold electron microscopy has provided evidence for the presence of H^+-PPase polypeptides in the plasma membrane of several plants such as phloem sieve elements in cotyledons and roots of castor bean seedlings [14], developing pea cotyledons [7], and suspension-cultured tobacco BY-2 cells [15]. H^+-PPase polypeptides have also been detected by immunological techniques in the plasma membrane of unicellular eukaryotes, such as *C. reinhardtii* [8], *Trypanosoma cruzi* [16], *Toxoplasma gondii* [17,18], *Plasmodium berghei* [19] and *P. falciparum* [20,21].

While the localization of the H^+-PPase in the Golgi apparatus is possibly a consequence of its traffic to vacuoles [4], or acidocalcisomes [16], the physiological significance of its presence in plasma membranes is unclear at present [9]. Some authors have presented calculations which place doubt upon the possibility that a plasma membrane-located H^+-PPase could be engaged in pumping protons [22]. It has also been suggested that in *Chlamydomonas* the H^+-PPase is localized in the plasma membrane as a result of the fusion of the contractile vacuole with the plasma membrane [8].

In this report we provide immunoelectron microscopy evidence of the presence of *T. cruzi* H^+-PPase in the Golgi apparatus and in the plasma membrane. In addition, we present surface biotinylation evidence and biochemical experiments of the proton pumping activity of the H^+-PPase present in plasma membrane vesicles of *T. cruzi*. The activity of this enzyme has different characteristics from those of the enzyme located in acidocalcisomes.

2. Materials and methods

2.1. Culture methods

T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose (LIT) medium [23] supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5% heat-inactivated newborn bovine serum. Five days after inoculation (stationary phase of growth), epimastigotes were collected by centrifugation, and washed twice in a medium containing 11 mM KCl, 140 mM NaCl and 75 mM Tris–HCl, pH 7.6. The final concentration of cells was determined using a Neubauer chamber. Protein concentration was determined by the BioRad Coomassie blue assay.

2.2. Isolation of plasma membrane vesicles and acidocalcisomes

Plasma membrane vesicles were prepared as reported before [24]. Briefly, after a final wash in a medium containing 400 mM mannitol, 10 mM KCl, 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), soybean trypsin inhibitor (0.15 mg ml^{-1}), leupeptin (10 μg ml^{-1}) and 20 mM Hepes/KOH (pH 7.6), the cell pellet was mixed with acid-washed glass beads (75–120 μm in diameter) at a ratio of 1:4 (wet wt. per wt. of beads). The cells were disrupted by abrasion in a chilled mortar until 90% disruption was achieved, as determined under an optical microscope. This generally took about 5–7 min. The resulting mixture was resuspended in 10–20 ml of the same buffer. The glass beads, unbroken cells and large debris were removed by centrifugation at $1000 \times g$ for 15 min at 4 °C. The supernatant was subjected to differential centrifugation, first at $16\,000 \times g$ for 30 min at 4 °C and then at $105\,000 \times g$ for 1 h at the same temperature. The resulting pellet was resuspended in about 3 ml of a medium containing 150 mM KCl, 2 mM β-mercaptoethanol and 75 mM Hepes (pH 6.8). The suspension was then gently passed three times through a Dounce homogenizer (AA; Thomas Scientific, Swedesboro, NJ, USA) immersed in an ice-cooled water bath. As reported previously, this preparation was highly enriched in plasma membrane vesicles, as inferred by its specific binding of ^{125}I-labelled concanavalin, which was 12–14-fold enriched with respect to the entire homogenate and its enrichment in 5′-nucleotidase, a plasma membrane glycosylphosphoinositol (GPI)-anchored protein (see Section 3). This fraction was also devoid of succinate:cytochrome *c* reductase activity, thus indicating the absence of mitochondrial contamination. 5′-nucleotidase [25] and other subcellular markers [16,26] were assayed as described in the references. Isolation of acidocalcisomes was done exactly as described before using iodixanol gradient centrifugation [26].

2.3. Chemicals

ATP, 5′-AMP, imidodiphosphate (IDP), sodium fluoride, potassium fluoride, sodium pyrophosphate, protease inhibitors, *N,N′*-dicyclohexylcarbodiimide (DCCD), *N*-ethylmaleimide (NEM), ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), sodium orthovanadate, protease inhibitor cocktail (P 8340), and nigericin were purchased from Sigma Chemical Co (Saint Louis, MO, USA). Bafilomycin A_1 was from

Kamiya Biomedicals (Thousand Oaks, CA, USA). Acridine Orange, 2-amino-6-mercapto-7-methylpurine ribonucleoside, purine nucleoside phosphorylase, and the standard phosphate solution were from Molecular Probes, Inc (Eugene, OR, USA). The ECL protein biotinylation module, the Hybond ECL nitrocellulose membranes, and the ECL chemiluminescence kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Aminomethylenediphosphonate (AMDP) [27] and polyclonal antisera, which had been raised against KLH-conjugated synthetic peptides corresponding to the hydrophilic loops IV (antibody 324) and XII (antibody 326) of plant H^+-PPase [28], that are conserved in the *T. cruzi* enzyme [29], were kindly provided by Professor Philip Rea, University of Pennsylvania. These antibodies have been shown to react specifically with *T. cruzi* H^+-PPase as suggested by the identification of only one band of the expected size in Western blots [16,26], the correlation between antibody reactivity and enzymatic activity in epimastigote fractions in Percoll gradients [16], and the detection of the same protein band in yeast, which are devoid of H^+-PPase, when they were transformed to express the recombinant *T. cruzi* enzyme [29]. All other reagents were analytical grade.

2.4. 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 DW 2000 dual wavelength spectrophotometer [16]. Plasma membrane vesicles were incubated at 30 °C in 2.5 ml standard 130 mM KCl buffer (pH 7.2) or alternate buffers, as described in Table 1 (containing in addition 2 mM $MgSO_4$, 10 mM Hepes, 50 μM EGTA, 3 μM acridine orange, and different inhibitors where indicated) for 30 s 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. Fig. 2 shows representative experiments.

Table 1
5′-Nucleotidase activity in subcellular fractions of *T. cruzi* epimastigotes

Subcellular fraction	Specific activity (μmol min^{-1} mg $protein^{-1}$)
Homogenate	0.85 ± 0.01
1000 × *g* pellet	0.94 ± 0.02
16 000 × *g* pellet	1.05 ± 0.01
105 000 × *g* pellet	5.40 ± 0.01

5′-nucleotidase activity was measured as described in Section 2. No activity was detected in the 105 000 × *g* supernatant. Values are mean ± S.E.M. of four different experiments.

2.5. Pyrophosphatase activity

Pyrophosphatase was assayed by measuring released phosphate using the EnzChek phosphate assay as described before [16,26]. Reaction mixtures contained: 130 mM KCl, 10 mM Hepes, pH 7.2, 2 mM $MgSO_4$, 50 μM EGTA, 0.1 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 0.4 U ml^{-1} purine nucleoside phosphorylase, 0.2–0.4 mg of vacuole fraction and PP_i as indicated in a total volume of 0.1 ml. Activity was recorded at 360 nm and 30 °C in a PowerWave 340i plate reader (Bio-tek Instruments, Winooski, VT).

2.6. Immunoelectron microscopy

Cells were fixed for 60 min at 4 °C in a solution containing 0.2% glutaraldehyde, 4% freshly prepared formaldehyde, 0.8% picric acid in 0.1 M cacodylate buffer, pH 7.2. After fixation the cells were washed in PBS-1% albumin and incubated for 60 min in the presence of 50 mM NH_4Cl, dehydrated at −20 °C in an ethanol series, and infiltrated at the same temperature in Unicryl [30]. Polymerization was carried out for 72 h at −20 °C by ultraviolet irradiation. Thin sections were collected on 300 mesh nickel grids, incubated in the presence of antibodies recognizing H^+-PPase (1:50 or 1:100 dilution), washed in PBS-albumin, and incubated with 10 nm gold-labeled goat anti-rabbit IgG (1:50 dilution for 60 min), washed in distilled water, stained with uranyl acetate and lead citrate, and observed with a Hitachi 600 transmission electron microscope operating at 100 kV. Controls were carried out using a non-related antibody or incubation in the presence of the secondary antibody only.

2.7. Cell surface labeling

Cell surface labeling was performed using the Amersham protein biotinylation module following the manufacturer specifications. Briefly, *T. cruzi* epimastigotes (3×10^8 cells) were washed three times with ice-cold Dulbecco's PBS at 4 °C and were then resuspended in 1 ml of ice-cold bicarbonate buffer (40 mM $NaHCO_3$, pH 8.6). Biotinylation reagent (40 μl) was then added and the cells were incubated at 4 °C for 30 min. Cells were then washed twice with ice-cold Dulbecco's PBS and lysed in 1 ml of ice-cold lysis buffer (1% Nonidet P-40, 25 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, and 1:100 protease inhibitor cocktail). Cells were kept at 4 °C for 1 h with rotation, and the lysate was centrifuged for 10 min at 3000 × *g*. The supernatant was diluted to 1.0 ml, and incubated with 50 μl of protein A-conjugated agarose beads with rotation for 1 h at 4 °C for preclearance. The beads were removed by centrifugation at 6000 × *g* for 1 min. Aliquots (200 μl) of the supernatant were mixed with 10 μl of new protein

A-conjugated agarose beads and incubated at 4 °C for 16 h with normal serum or polyclonal antibody against the H^+-PPase antibody (1:300). The beads were washed three times with ice-cold lysis buffer and bound proteins were eluted in boiling SDS sample buffer. The proteins were separated on a 10% SDS-polyacrylamide gel and blotted onto a Hybond ECL nitrocellulose membrane. The membrane was probed with streptavidin-peroxidase conjugate (1:1500) for 30 min at room temperature. Proteins were visualized by ECL.

3. Results

3.1. Immunogold localization of H^+-PPase polypeptides in plasma membrane and Golgi apparatus of T. cruzi

The location of the H^+-PPase was investigated using polyclonal antibodies known to cross react with the *T. cruzi* enzyme [16,26]. Immunoelectron microscopy using anti- H^+-PPase antibody confirmed labeling of the cell surface, including the flagellar membrane (arrows), and intracellular vacuoles identifiable as acidocalcisomes (Fig. 1) [16,26]. In addition, the Golgi apparatus revealed labeling with the antibodies. Although some gold particles were seen at the membrane of the adjacent endoplasmic reticulum, gold was absent from *cis*- and median cisternae (the lower part of the Golgi body in Fig. 1). By contrast, PPase immunogold staining was very dense over the *trans* cisternae and the vesicular elements lying at the *trans* pole of the Golgi apparatus (Fig. 1, arrows).

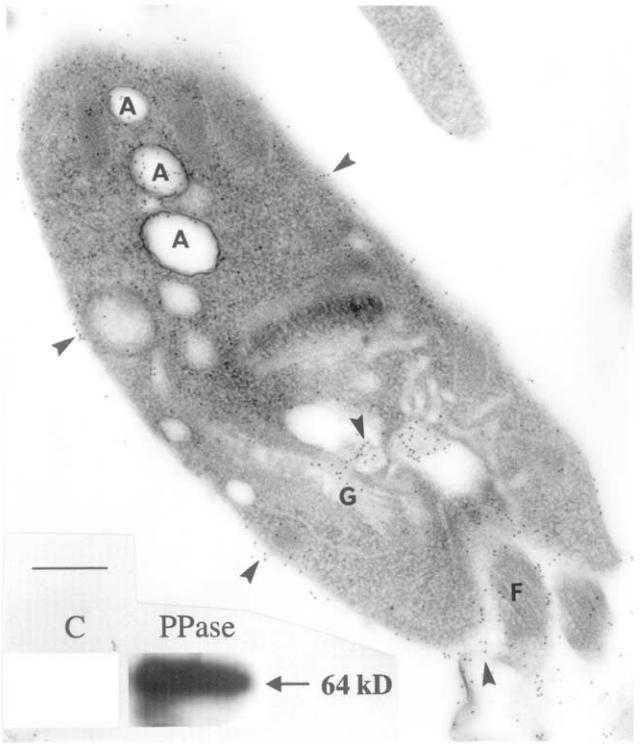

Fig. 1. Immunoelectron microscopy of epimastigotes of *T. cruzi* with anti-H^+-PPase. Labeling of the plasma membrane, including the flagellar membrane (arrows), and acidocalcisomes (A) is evident. Labeling of the Golgi apparatus is also shown (G). Bar: 0.25 μm. Inset. Epimastigotes were incubated with biotin succinimidyl ester. After lysis of the cells, H^+-PPase was immunoprecipitated by anti-H^+-PPase antibody, and the immunoprecipitate was subjected to Western blot analysis. Detection of biotinylation was by streptavidin-peroxidase conjugate and ECL. A band of apparent molecular mass of 64 kDa was detected (PPase) that was absent when immunoprecipitation was performed with normal serum (C, control). Other experimental details in Section 2.

3.2. Further evidence for localization of H^+-PPase in the plasma membrane of epimastigotes.

To confirm the surface localization of the H^+-PPase in epimastigotes, we labeled these cells with biotin succinimidyl ester, a reagent that couples biotin to lysine residues of exposed proteins. The cells were incubated with the reagent, lysed, and the H^+-PPase was immunoprecipitated with a polyclonal antibody or with normal serum. The precipitated proteins were electrophoresed and blotted, and biotinylated proteins were visualized by peroxidase-conjugated streptavidin and ECL. Only one protein band of 64 kDa was detected (Fig. 1, inset), which corresponds to the *T. cruzi* H^+-PPase described before [16,26]. Fig. 1(inset) also shows the negative control immunoprecipitated with normal serum.

3.3. Isolation of plasma membrane vesicles

The development of a method based on cell disruption by abrasion in a chilled mortar followed by resuspension in an hypertonic buffer and differential centrifugation has proven to be ideal for obtaining a plasma membrane fraction from *T. cruzi* [24,31] and other trypanosomatids [32–34] composed of tightly closed vesicles suitable for determination of ion transport. This membrane fraction consists of empty closed vesicles with a diameter between 0.2 and 0.9 μm with no microtubules attached to the membranes [31]. The utility of the method was assessed by assaying marker enzymes as indicated under Section 2. The activity of 5′-nucleotidase, a plasma membrane marker of *T. cruzi* [25] was enriched in this, the 105 000 × *g* pellet, fraction (Table 1). The lower enrichment in 5′-nucleotidase (5-fold) as compared with specific binding of ^{125}I-labeled concanavalin (12–14-fold) [24] could be due the presence of an active enzyme during its transport through the secretory pathway until it reaches the plasma membrane. The plasma membrane fraction was devoid of acidocalcisomes, as examined by direct transmission electron microscopy of air-dried fractions [16,26] (data not shown). Under these conditions acidocalcisomes

would be seen as very electron-dense spheres of a mean diameter of around 200 nm. This is in agreement with the high density of acidocalcisomes, which remain in the $16\,000 \times g$ pellet [16,26]. The $105\,000 \times g$ pellet also showed vanadate-sensitive ATP-dependent acridine orange accumulation (Fig. 2A) which is compatible with the presence of a vanadate-sensitive H^+-ATPase, a marker of *T. cruzi* epimastigote plasma membrane [31], which is absent from acidocalcisomes [26].

3.4. H^+-PPase activity in plasma membrane vesicles

Addition of 0.1 mM PP_i to plasma membrane vesicles ($105\,000 \times g$ pellet) of *T. cruzi* epimastigotes resulted in acridine orange accumulation until a steady state was reached (Fig. 2B). This proton gradient was collapsed by the addition of 6 μM aminomethylenediphosphonate (AMDP) (Fig. 2B), a specific inhibitor of plant vacuolar pyrophosphatases [27], or by 10 mM of the weak base NH_4Cl (Fig. 2C). The vesicle pH was neutralized and acridine orange was also released after addition of 1 μM of the K^+/H^+ ionophore nigericin (data not shown). KCl greatly stimulated this proton pumping activity in a concentration-dependent manner (Table 2, Fig. 3A). Replacing 130 mM KCl with 130 mM NaCl, 250 mM sucrose, or 65 mM choline chloride/125 mM sucrose in the buffer resulted in loss of most, or all, of the proton pumping activity (Table 2). These results suggest that K^+ was necessary for this activity. Fig. 3B shows the effect of medium pH on the initial rate of pyrophosphate-dependent acridine orange absorbance decrease in epimastigote plasma membrane vesicles. Activity was optimal at pH 7.2. Pyrophosphatase was also assayed in plasma membrane vesicles by inorganic phosphate detection [16,26]. Pyrophosphatase activity was 0.39 ± 0.04 (mean ± S.D. of six separate experiments) and 11.3 ± 2.3 (mean ± S.D. of six separate experiments) μmol PP_i consumed min^{-1} mg $protein^{-1}$ in plasma membrane vesicles and acidocalcisomes, respectively.

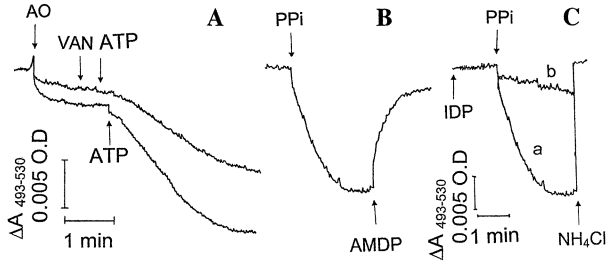

Fig. 2. ATP (A) and PP_i (B, C) driven proton transport in plasma membrane vesicles of epimastigotes. Plasma membrane vesicles (0.4 mg protein ml^{-1}) were added to standard reaction medium containing 130 mM KCl, 125 mM sucrose, 2 mM $MgSO_4$, 10 mM Hepes, and 50 μM EGTA, adjusted to pH 7.2 with KOH. **A.** Addition of 1 mM ATP resulted in a decrease of the absorbance of acridine orange (AO, 3 μM), indicating acidification. This effect was inhibited by sodium orthovanadate (VAN, 20 μM). **B**. Acridine orange (3 μM) taken up after addition of 0.1 mM PP_i was released by 6 μM AMDP. **C**. Plasma membrane vesicles were incubated in the absence (line *a*) or presence (line *b*) of 0.3 mM imidodiphosphate (IDP). The pH gradient was collapsed after addition of 10 mM NH_4Cl.

Table 2
Effect of buffer composition on H^+-PPase activity of epimastigotes of *T. cruzi*

Experimental conditions	Plasma membrane vesicles (% of control)	Acidocalcisomes (% of control)*
130 mM KCl	100 ± 8.9 (9)	100 ± 2.9 (3)
16 mM KCl/228 mM sucrose	17.0 ± 4.3 (7)*	71.0 ± 16.4 (4)*
33 mM KCl/194 mM sucrose	30.5 ± 3.0 (7)*	86.9 ± 4.0 (4)
65 mM KCl/125mM sucrose	42.4 ± 9.6 (7)*	80.0 ± 4.0 (3)
33 mM KCl/65 mM NaCl/64 mM sucrose	57.8 ± 15.1 (6)*	38.4 ± 9.7 (4)*
16 mM KCl/114 mM NaCl	45.7 ± 4.9 (3)*	33.4 ± 9.8 (3)*
130 mM NaCl	13.2 ± 2.1 (7)*	19.2 ± 2.3 (3)*
65 mM Choline chloride/125 mM sucrose	ND (3)	18.9 ± 2.0 (3)*
250 mM sucrose	7.2 ± 0.9 (3)*	37.1 ± 4.7 (3)*

Rates are relative (%) to the 130 mM KCl buffer. All buffers contained, in addition, 2 mM $MgS0_4$, 10 mM Hepes and 50 μM EGTA, and were adjusted to pH 7.2 with NaOH, KOH or Tris base for NaCl, K^+ salts and sucrose buffers, respectively. Values are mean ± S.E. of the number of experiments indicated in parentheses. *$P < 0.05$ (Student's *t*-test). Control activities were 0.39 ± 0.003 and 5.27 ± 0.15 $\Delta A_{493-530}$ min^{-1} mg of $protein^{-1}$ for the plasma membrane vesicles and the acidocalcisomes, respectively. ND, not detectable.

3.5. Inhibition of the H^+-PPase activity of plasma membrane vesicles

Pyrophosphate-induced acidification of the epimastigote plasma membrane vesicles was inhibited, in a dose-dependent manner, by AMDP (Fig. 4B). The inhibition of acidification by AMDP was maximal at 10 μM, while some residual proton pumping activity could still be detected even at 30 μM AMDP. This suggests the presence of an AMDP-insensitive pyrophosphate-dependent acidification. The activity was also inhibited, in a dose-dependent manner, by the pyrophosphate analog imidodiphosphate (IDP) (Fig. 2C Fig. 4C). Fig. 4 also shows that sodium and potassium fluoride (Fig. 4A), the thiol reagents p-hydroxymercuribenzoate (Fig. 4D), and *N*-ethylmaleimide (Fig. 4E), and DCCD (Fig. 4F), all agents known to inhibit the H^+-PPases from plants [1,9], trypanosomatids, and apicomplexan parasites [2], were also effective in inhibiting the plasma membrane pyrophosphate-dependent proton pumping activity in a dose-dependent manner.

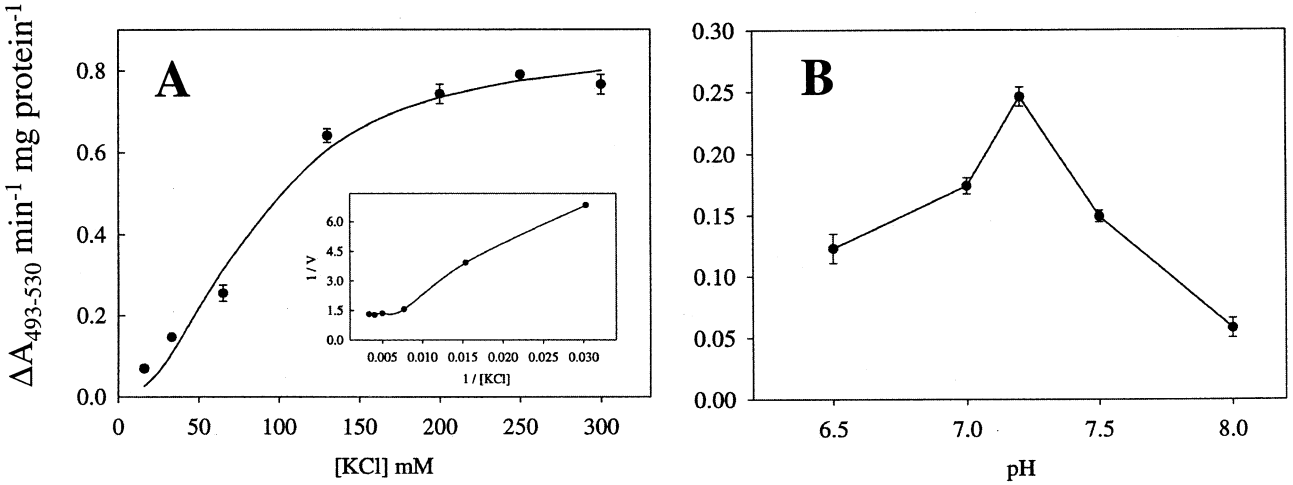

Fig. 3. Initial rate of PP_i-dependent proton uptake as a function of KCl concentration (A) or medium pH (B). Aliquots of plasma membrane vesicles (0.4 mg protein ml^{-1}) were added to the standard reaction mixture (Fig. 2) in the presence of increasing concentrations of KCl (and decreasing concentrations of sucrose to maintain the same osmolarity of the solutions) (A), or incubated in the same buffer adjusted to different pHs (B). Error bars indicate mean ± S.E.M. from at least three separate experiments. The inset in A represents the linear transformation, by double reciprocal plot, of the curve.

3.6. Differences between plasma membrane vesicles and acidocalcisomal pyrophosphate-dependent H^+-transport

As mentioned above, in contrast to acidocalcisomal H^+-PPase activity [16], pyrophosphate-dependent proton pumping was not completely abolished in plasma membrane vesicles by 30 μM AMDP. In addition, H^+-PPase activity in the latter fraction was more dependent upon K^+ than the activity in acidocalcisomes (Table 2). Furthermore, Na^+ clearly inhibited the proton pumping activity of acidocalcisomes since replacement of K^+ by increasing concentrations of Na^+ resulted in lower activity, while this effect was not evident with plasma membrane vesicles (Table 2).

Table 2 shows that activity in plasma membrane vesicles was more dependent upon the presence of KCl than the activity in acidocalcisomes. Assays where KCl was wholly replaced by NaCl or choline chloride indicate that this is not solely a chloride effect, but that the activity is stimulated by K^+. Addition of NaCl to the assay buffer while the KCl concentration was kept constant (16 and 33 mM KCl buffers) resulted in a reduction of the activity for acidocalcisomes and an increase in the activity for plasma membrane vesicles. This implies that Na^+ was inhibitory for the acidocalcisome activity, while it had little effect on the plasma membrane activity, or else any inhibition by the Na^+ ion in this case was balanced by stimulation of the activity by the extra Cl^- added. DCCD inhibition of the H^+-PPase activity of plasma membrane vesicles (IC_{50} = 82 μM) and acidocalcisomes (IC_{50} = 200 μM) [32] was within the same order of magnitude although the plasma membrane activity was slightly more sensitive.

4. Discussion

In this study, we have identified an H^+-translocating PPase activity in plasma membrane vesicles of *T. cruzi*. Acridine orange uptake in the presence of pyrophosphate was reversed by the weak base NH_4Cl or the K^+/H^+ exchanger nigericin. As occurs with the acidocalcisomal H^+-PPase [16,29], pyrophosphate-driven proton transport was inhibited by the pyrophosphate analogs AMDP and IDP and by the PPase inhibitors KF, NaF, DCCD, NEM, and p-HMCB (Figs. 2 and 4), and was stimulated by potassium (Table 2 and Fig. 3A). In contrast to acidocalcisomal PP_i-dependent proton pumping [16], the activity in plasma membrane vesicles was not completely abolished by AMDP and it was inhibited more by the complete absence of K^+. Na^+ inhibited the acidocalcisomal activity but apparently did not affect the plasma membrane vesicle activity (Table 2). DCCD was a more effective inhibitor of H^+-PPase activity of plasma membrane vesicles than of acidocalcisomes. Only one gene encoding an H^+-PPase has been described in *T. cruzi* [29] and no examples of type II H^+-PPase genes have so far been described in trypanosomatids. It is not known whether these differences could be attributed to the presence of other isoforms of the enzyme or differences in the membrane composition of the different compartments where the enzyme is located.

The results obtained using plasma membrane vesicles are in agreement with the detection of H^+-PPase polypeptides in the plasma membrane of epimastigotes by immunoelectron microscopy (Fig. 1) and biotin labeling of the plasma membrane (Fig. 1, inset). In

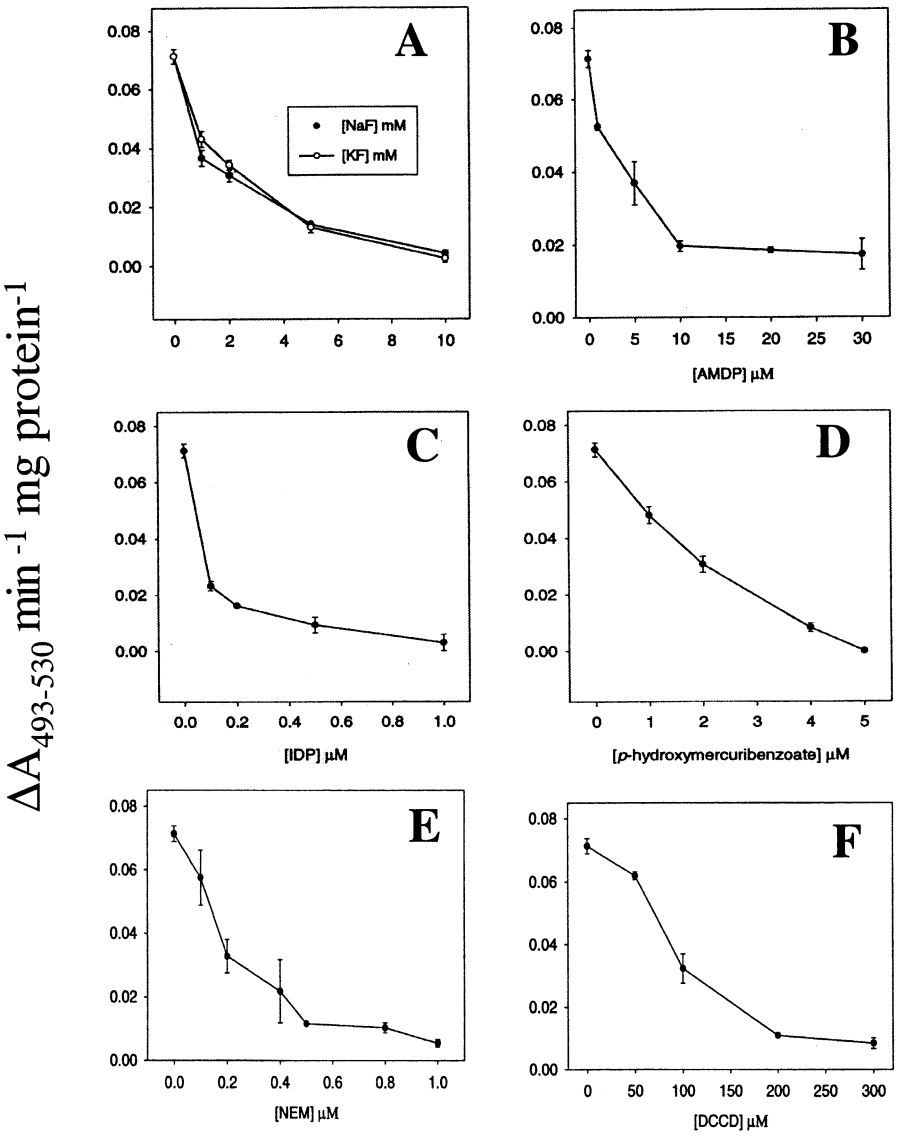

Fig. 4. Inhibition of PP_i-dependent proton pumping by PP_i analogues and other inhibitors in plasma membrane vesicles. Assays were run in the standard buffer described in Fig. 2. Aliquots of plasma membrane vesicles (0.4 mg ml^{-1}) were added to the standard reaction mixture in the presence of increasing concentrations of NaF and KF (A), AMDP (B), IDP (C), *p*-hydroxymercuribenzoate (D), *N*-ethylmaleimide (E), and DCCD (F). Error bars indicate mean ± S.E.M. from at least three separate experiments.

addition, the H^+-PPase was detected in *trans* cisternae elements of the Golgi apparatus (Fig. 1). It can be expected that the PPase, being an integral membrane protein, is initially synthesized at the endoplasmic reticulum before being transported via vesicles to other endomembranes such as the Golgi apparatus. The lumen of the endoplasmic reticulum is not acidic, but there is considerable evidence for the acidification of the Golgi apparatus, in particular the *trans* cisternae [8]. The labeling of the *trans* Golgi cisternae with H^+-PPase antibodies suggests that, as has been proposed in the case of *C. reinhardtii* [8], it may function to acidify this organelle.

The significance of the presence of a functional H^+-PPase in the plasma membrane of *T. cruzi* is unknown. A proton pump has been postulated to be involved in the regulation of intracellular pH homeostasis and in the generation of a membrane potential in *T. cruzi* epimastigotes [35]. Although this pump was postulated to be a P-type ATPase, some of the compounds used to inhibit its activity, such as *N*-etylmaleimide and DCCD [35], are also able to inhibit the plasma membrane H^+-PPase activity (Fig. 4) and we cannot rule out that some of their effects could have been due to inhibition of proton pumping by the H^+-PPase. PP_i is a byproduct of several biosynthetic reactions and another possible function of the plasma membrane H^+-PPase could be to serve to degrade cytosolic PP_i if soluble pyrophosphatases are not present in *T. cruzi* as occurs in some photosynthetic plant tissues [1].

In conclusion, this is the first report of a functional H^+-PPase in plasma membrane vesicles of an organism different from plants. Since H^+-PPases are apparently absent from vertebrates, analysis of the role of the H^+-PPase in the plasma membrane and Golgi apparatus of *T. cruzi* could provide useful information to facilitate the development of new antiparasitic agents.

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