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Membrane Bound Pyrophosphatase and P-Type Adenosine Triphosphatase of *Leishmania donovani* as Possible Chemotherapeutic Targets: Similarities and Differences in Inhibitor Sensitivities

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Abstract—The activities of inorganic pyrophosphatase (PPase) and adenosine triphosphatase (ATPase) were studied in the plasma membrane of *Leishmania donovani* promastigotes and amastigotes. It was shown that the specific activity of PPase was greater than that of ATPase in the promastigote plasma membrane. We characterized H^+-PPase present in the plasma membrane of *L. donovani* and investigated its possible role in the survival of promastigote and amastigote. PPase activity was stimulated by K^+ and sodium orthovanadate and inhibited by pyrophosphate analogs (imidodiphosphate and alendronate), KF, N,N′-dicyclohexylcarbodiimide (DCCD), thiol reagents (*p*-chloromercuribenzenesulfonate (PCMBS), N-ethylmaleimide (NEM), and phenylarsine oxide (PAO)), the ABC superfamily transport modulator verapamil, and also by the F_1F_o-ATPase inhibitor quercetin. ATPase activity was stimulated by K^+ and verapamil, inhibited by DCCD, PCMBS, NEM, sodium azide, sodium orthovanadate, and quercetin, and was unaffected by PAO. We conclude that there are significant differences within promastigote, amastigote, and mammalian host in cytosolic pH homeostasis to merit the inclusion of PPase transporter as a putative target for rational drug design.

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Key words: *Leishmania donovani* promastigote and amastigote, pyrophosphatase, ATPase, plasma membrane, imidodiphosphate, verapamil

Protozoan parasites are responsible for important diseases that threaten the lives of nearly one-quarter of the human population worldwide. Among them, leishmaniasis has become the second cause of death, mainly due to the emergence of resistance of the parasite to conventional drugs [1]. *Leishmania donovani*, the causative agent of visceral leishmaniasis, encounters a wide range of pH values in its life cycle. The gut of the phlebotomized insect vector is extremely alkaline, whereas promastigotes of *L. donovani* invade host cells via acidic lysosomes [2]. Mechanisms to cope with this varied environmental pH and maintain cytosolic pH homeostasis might involve the use of proton pumps (H^+-ATPases and H^+-PPases) on both plasma membrane and internal membranes. These transport proteins specifically and actively mobilize ions, generating chemical gradients across a membrane. This movement of ions is vital for numerous cellular functions ranging from energy production, motility, nutrient uptake, ionic homeostasis, intracellular signaling, and differentiation, to name a few.

Membrane-bound proton-translocating inorganic pyrophosphatases (H^+-PPase; EC 3.6.1.1) belong to a new category of proton pumps, distinct from F-, P-,

Abbreviations: ATPase, adenosine triphosphatase; DCCD, N,N′-dicyclohexylcarbodiimide; IDP, imidodiphosphate; NEM, N-ethylmaleimide; PAO, phenylarsine oxide; PCMBS, *p*-chloromercuribenzenesulfonate; PPase, pyrophosphatase; PP_i, pyrophosphate.

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and V-ATPases, which utilize pyrophosphate (PP_i) hydrolysis as the driving force for H^+ movement across biological membranes [3]. The membrane-bound H^+-PPase was first described [4] in chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum*. Under physiological conditions, this enzyme can both synthesize and hydrolyze PP_i. It was also demonstrated [5, 6] that PP_i could drive a number of energy requiring reactions in chromatophores including ATP synthesis in the dark [7]. H^+-PP_i synthase is the only known alternative to the well-known H^+-ATP synthase in biological electron transport phosphorylation [8]. The H^+-PPase gene has been reported to occur in *Leishmania* parasites [9]. Eukaryotic cells also possess soluble inorganic pyrophosphatases. PP_i is formed as a byproduct in several metabolic reactions, for example, DNA and RNA synthesis. It has to be hydrolyzed in order not to stop these reactions. This is a major function of the soluble, cytoplasmic PPases. Membrane-bound PPases do not exist in plasma membrane from mammals [10], thus, H^+-PPases from *Leishmania* plasma membrane might be a potential target in rational chemotherapy of the diseases caused by *Leishmania* parasites [11].

In the present work, we demonstrate that *L. donovani* promastigote plasma membrane possesses ATPase and PPase activity, whereas amastigote plasma membrane possesses only PPase activity with features in common with the trypanosomatid and plant enzymes [4]. Our results also indicate that PP_i analogs inhibit the PPase of *Leishmania* parasite without affecting ATPase.

MATERIALS AND METHODS

Materials. All chemicals unless otherwise mentioned were from Sigma-Aldrich (USA). Panmede was purchased from Paines and Byrne (UK).

Promastigote culture method. *Leishmania donovani* promastigote strain MHOM/IN/1978/UR6, a clinical isolate from a confirmed kala azar patient [12], was grown at 24°C on blood agar medium, pH 7.5 [13]. The cells were washed at 500*g* twice in cold Tris-sucrose-salt solution (250 mM sucrose, 50 mM NaCl, 20 mM KCl, 1 mM EDTA, 20 mM Tris, pH 7.2) and kept at 4°C until use. Viability of harvested cells was monitored microscopically by trypan blue exclusion [14].

Amastigote culture method. *Leishmania donovani* amastigote strain MHOM/IN/1978/UR6 was grown and maintained as described [15]. Axenically grown amastigotes of *L. donovani* were maintained at 37°C with 5% CO_2 by weekly subpassages in MAA/20 (medium for axenically grown amastigotes) at pH 5.5 in Petri dishes (diameter 15 cm) [16]. Under these conditions, promastigotes (extracellular flagellate form) differentiated to amastigotes (cellular non-flagellate form) within 120 h. Cultures were maintained by 3-fold dilution once a week. The axenic amastigotes remained stable in culture for a long time. Axenic amastigotes were routinely recycled every 10 weeks by differentiation back to promastigotes, and in parallel, initiating a new line of promastigotes. Transformation of amastigotes to promastigotes was performed by centrifugation of amastigotes (1000*g* at room temperature for 10 min), suspension in promastigote medium [17], and incubation at 25°C. Under these conditions, amastigotes differentiate to promastigotes within 72 h.

Assay of ATPase and PPase activity. PPase and ATPase activity, in terms of P_i release, was assayed according to the method of Katewa and Katyare [18]. The reaction mixture (1 ml) contained buffer B (300 mM sucrose, 50 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.0 (adjusted with hydrochloric acid)), 0.5 mM $MgCl_2$, 0.25 mM ATP (disodium salt) (for ATPase) or PP_i (for PPase). The reaction was initiated by addition of *L. donovani* plasma membrane preparation (25 μg protein) or digitonin permeabilized cells (100 μg protein). The mixture was incubated at 25°C for 15 min, and the reaction was terminated by adding 0.14 ml of 2.5 M $HClO_4$. In control experiments, the reaction mixture was incubated, and the protein solution was added after reaction termination. The amount of enzyme catalyzing hydrolysis of 1 μmol of ATP or PP_i per minute was taken as the unit. The phosphatase assay was calibrated with a phosphate standard solution. For enzyme inhibition study, inhibitor was added to the reaction mixture containing enzyme but lacking ATP or pyrophosphate. The mixture was preincubated for 10 min at 25°C before starting the reaction with ATP or pyrophosphate. Activity values shown below represent means ± S.E. of three independent experiments.

Plasma membrane preparation. Plasma membranes from *L. donovani* promastigotes were prepared according to the method described before [19]. The parasites were harvested at a concentration of 10^8 cells/ml (5000*g*, 10 min, 4°C), washed twice in PBS (140 mM NaCl, 5 mM KCl, 0.08 mM Na_2HPO_4, 0.15 mM KH_2PO_4, pH 7.5) and pooled with ^{125}I cells. All subsequent steps were performed on ice or in refrigerated centrifuges. The cell pellet was suspended to $2 \cdot 10^7$ cells/ml in PBS buffer, pH 7.2, that contained 10 mM $MgCl_2$ and rapidly mixed with an equal volume of 1 mg/ml concanavalin A in the same buffer. Cell aggregation was apparent within 1 min. After 5 min, cells were gently spun at 1000*g* for 1 min to remove excess concanavalin A. The supernatant was discarded, and the cell pellet was resuspended in 12 ml of 10 mM Tris-HCl, pH 7.5, that contained 10 μg/ml leupeptin and 1 mM $MgCl_2$. After swelling for 10 min in that hypotonic buffer, the cells were homogenized by 18-20 strokes in a tightly fitting Dounce-type homogenizer. Cell lysis and formation of membrane sheets were verified by phase-contrast microscopy. The homogenate was layered over a two-step gradient consisting of 8 ml of 0.5 M mannitol over 4 ml 0.58 M sucrose, both in 10 mM Tris-HCl,

pH 7.5, and spun at 1000*g* for 20 min. For analysis, material remaining at the top of the 0.5 M mannitol was saved. Large crude plasma membrane fragments were separated as a tight pellet at the bottom of the gradient. This pellet was resuspended in 10 mM Tris-HCl, pH 7.5, that contained 1 M α-methylmannoside and left on ice for 40 min with occasional mixing. This plasma membrane, free from bulk concanavalin A, was diluted into three volumes of 10 mM Tris-HCl, pH 7.5, and homogenized by 80 strokes with a glass Dounce-type homogenizer. This second homogenate was layered on a single-step gradient that consisted of 20% sucrose in 10 mM Tris-HCl, pH 7.5, and spun for 30 min at 500*g*. Scrolls and large plasma membrane sheets above the 20% (w/v) sucrose layer were collected by centrifugation at 40,000*g* for 1 h. The pellet containing the enriched plasma membranes was resuspended in 10 mM Tris-HCl, pH 7.5. All samples were either assayed immediately or frozen at −20°C for further use.

Preparation of digitonin permeabilized *Leishmania* cells. *Leishmania donovani* promastigote and/or amastigote cells were collected, washed once by buffer A (140 mM NaCl, 20 mM KCl, 20 mM Tris, 1 mM EDTA, pH 7.5), and resuspended in isolation buffer (20 mM MOPS-NaOH, 0.3% BSA, 350 mM sucrose, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, pH 7.0). Cells were permeabilized in a separate tube with digitonin (50 μg/mg protein) and incubated on ice for 10 min. After incubation, the cells were centrifuged at 6000*g* for 7 min. Pellets were resuspended in assay buffer.

Protein estimation. Total cell protein was determined by the biuret method in the presence of 0.2% deoxycholate [20]. One milligram of protein corresponds to $1.75 \cdot 10^8$ promastigote cells and $1.14 \cdot 10^8$ amastigote cells. Cytosolic, mitochondrial, and plasma membrane protein were determined by a modified method of Lowry et al. [21].

Statistical analysis. All experiments were performed in triplicate, with similar results obtained in at least three separate experiments. Statistical significance was determined by Student's *t*-test. Significance was considered as $P < 0.05$.

RESULTS AND DISCUSSION

Isolation of plasma membranes. Development of a method based on cell disruption by osmotic swelling in the presence of a plasma membrane specific lectin, concanavalin A, and differential centrifugation proved to be ideal for obtaining a plasma membrane fraction from *L. donovani* promastigote [19]. This membrane fraction consists of open membrane sheets with microtubules attached to the membranes [22]. The utility of the method was assessed by assaying marker enzymes. The activity of tartrate-resistant acid phosphatase, a plasma membrane marker of *L. donovani* [23], was enriched in this fraction. An enrichment of 28-fold was obtained when cell surface plasma membrane was labeled with ^{125}I. The plasma membrane fraction was devoid of acidocalcisomes as examined by direct transmission electron microscopy (data not shown).

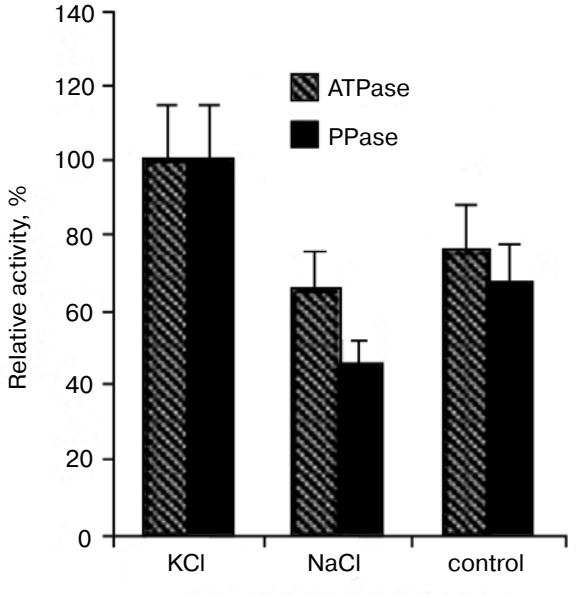

Fig. 1. Effect of salts on plasma membrane PPase and ATPase activity. Plasma membranes were incubated for 15 min at 25°C in a reaction medium containing 300 mM sucrose, 50 mM KCl (or 50 mM NaCl), 50 mM Tris, 2 mM EGTA, pH 7.0, and 0.25 mM PP_i (for PPase) or ATP (for ATPase).

PPase and ATPase activities in plasma membranes. In the present study, we identified and characterized PPase and ATPase activities in the plasma membrane of *L.*

Substrate specificity of phosphatase in *L. donovani* promastigote membranes*

Substrate	Enzyme activity, nmol P_i/min per mg protein		
	DPC	LDC	PM
PP_i	47 ± 6	10.0 ± 0.8	117 ± 18
ATP	27 ± 3	0	44 ± 5
AMP	9.0 ± 0.8	0	0
ADP	8.0 ± 0.6	0	0

* DPC (digitonin-permeabilized *Leishmania donovani* cells), LDC (normal *Leishmania donovani* cells), or PM (plasma membranes) were incubated for 15 min at 25°C in a reaction medium containing 300 mM sucrose, 50 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.0, and a substrate at concentration of 0.25 mM (PP_i, ATP, AMP, or ADP).

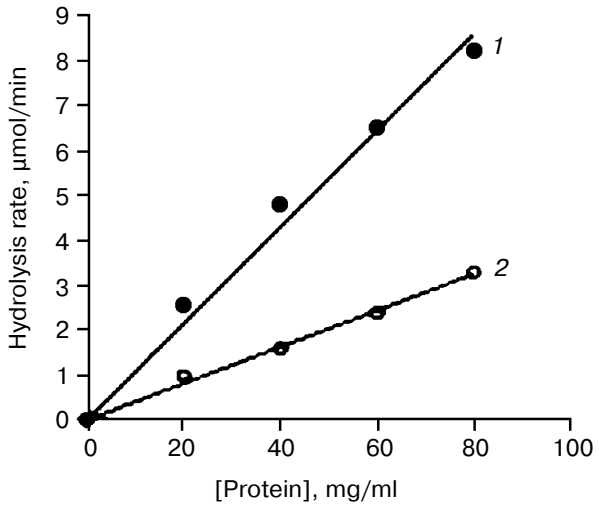

Fig. 2. Effect of plasma membrane protein concentration on the rate of PP_i (*1*) and ATP (*2*) hydrolysis by PPase and ATPase, respectively.

donovani promastigote. This is the first report demonstrating biochemically the presence of a PPase in the plasma membrane of *L. donovani* promastigote. Activity of PPase and ATPase was maximal at pH 6.8 and 7.0, respectively (data not shown). KCl greatly stimulated PPase and ATPase activities (Fig. 1). Replacing 50 mM KCl with 50 mM NaCl or 50 mM choline chloride in the buffer resulted in a substantial loss of PPase and ATPase activities (Fig. 1). These results suggest that K^+ is necessary for PPase and ATPase activity, but substantial amount of K^+- and Na^+-independent PPase and ATPase was also present in the plasma membrane. Genomic analysis also revealed the presence of K^+- and Na^+-ATPases [23], and their possible function has been suggested to be K^+/H^+ and Na^+/H^+ exchange [24].

The table shows that activity of PPase and ATPase in plasma membranes is located at the endoplasmic face of the plasma membrane. Permeabilized *L. donovani* cells showed substantial PPase and ATPase activity, whereas non-permeabilized cells did not. However, very little ecto-PPase and ATPase activity was observed at exoplasmic face of the plasma membrane. It is also evident from the table that very little ADPase and AMPase activities are present in the digitonin-permeabilized cells. On the other hand, ADPase and AMPase activities were eliminated from the purified plasma membranes (table). Figure 2 shows that the relationship between PPase and ATPase activities and the amount of plasma membrane proteins added was linear. Linearity of PPase and ATPase activity was also observed for digitonin-permeabilized cells (Fig. 3). It is also evident from Fig. 3 that in digitonin permeabilized amastigote cells, PPase activity was less than in promastigote cells. In contrast, ATPase activity in digitonin-permeabilized amastigotes was completely abolished.

Specific inhibitors and stimulators of PPase and ATPase activities. PP_i hydrolysis in plasma membranes and digitonin permeabilized *L. donovani* promastigote cells was inhibited by KF in a dose-dependent manner (Fig. 4). In contrast, ATPase activity of plasma membrane was not inhibited by KF.

ATPase activity was not substantially inhibited by PP_i analogs imidodiphosphate (IDP) and alendronate (Fig. 5a), whereas PP_i hydrolysis was inhibited by IDP and alendronate (Fig. 6b). Alendronate is used clinically in the treatment of bone resorption disorders [25].

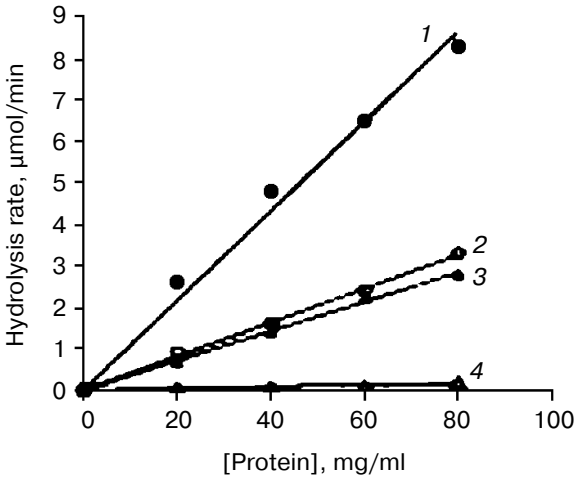

Fig. 3. Effect of digitonin-permeabilized *Leishmania* cell protein concentration on the rate of PP_i and ATP hydrolysis by promastigote (Pro) PPase (*1*) and ATPase (*2*) and amastigote (Am) PPase (*3*) and ATPase (*4*).

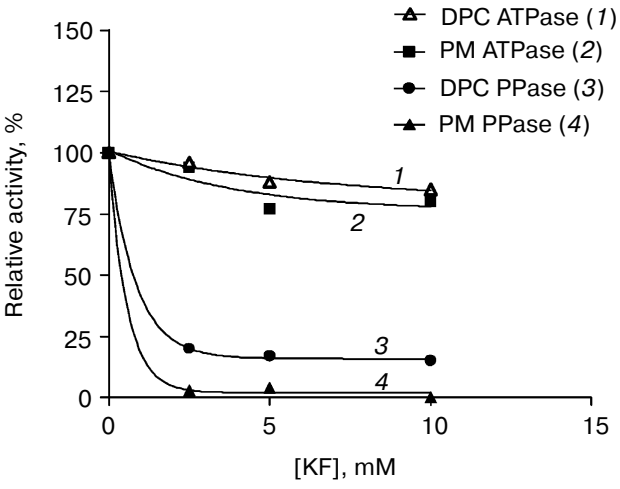

Fig. 4. Effect of KF concentration on the rate of PP_i and ATP hydrolysis by plasma membrane (PM) ATPase (*2*) and PPase (*4*) and digitonin-permeabilized *Leishmania donovani* cells (DPC) ATPase (*1*) and PPase (*3*).

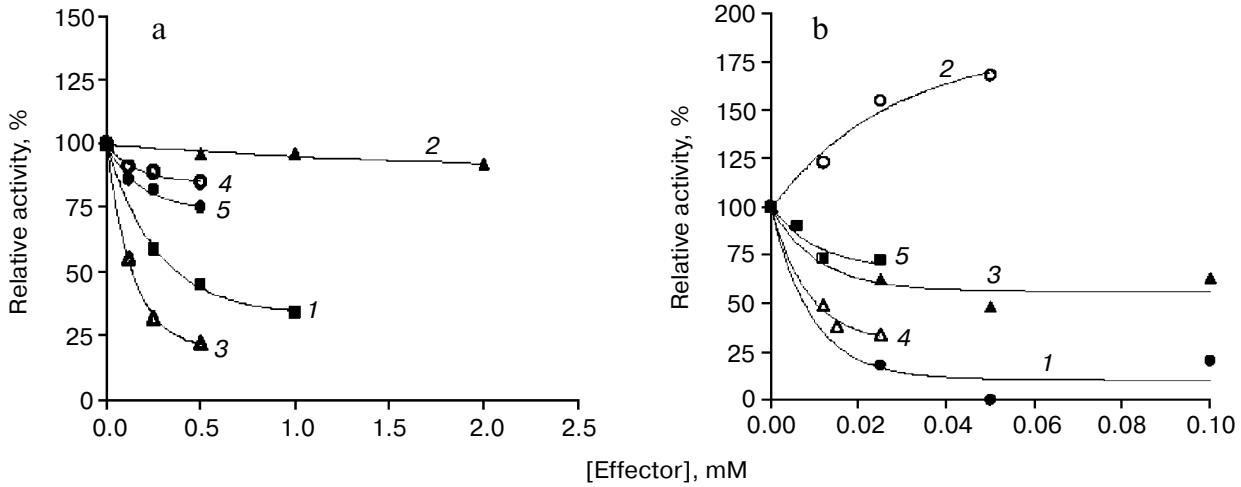

Fig. 5. Concentration response curve describing the effect of effectors on promastigote plasma membrane ATPase. a) PCMBS (*1*), PAO (*2*), NaN_3 (*3*), IDP (*4*), alendronate (*5*). b) Na_3VO_4 (*1*), verapamil (*2*), DCCD (*3*), NEM (*4*), quercetin (*5*). The effectors were added to the plasma membrane 10 min before the addition of ATP.

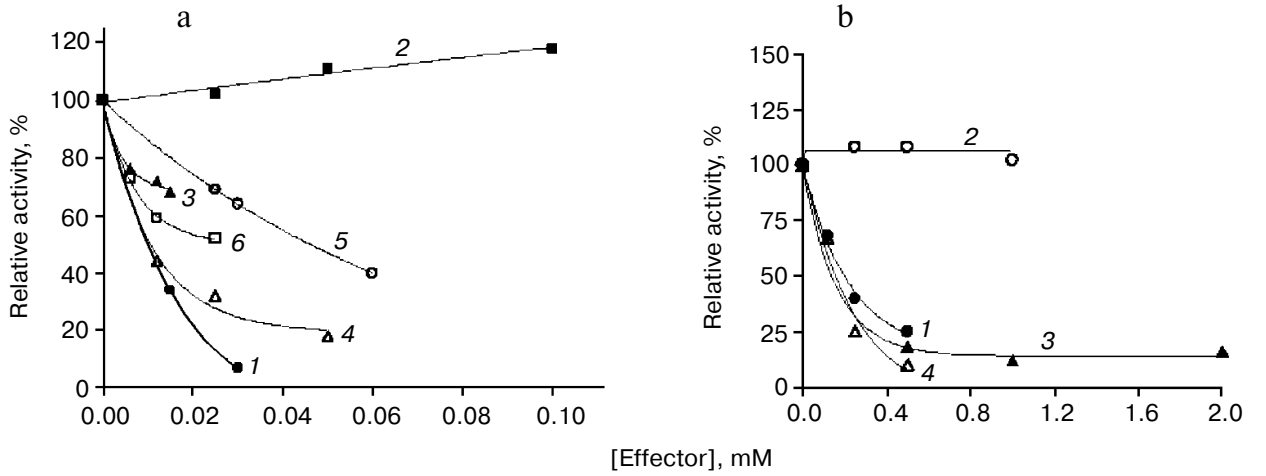

Fig. 6. Concentration response curve describing the effect of effectors on promastigote plasma membrane PPase. a) PCMBS (*1*), Na_3VO_4 (*2*), quercetin (*3*), verapamil (*4*), DCCD (*5*), NEM (*6*). b) Alendronate (*1*), NaN_3 (*2*), PAO (*3*), IDP (*4*). The effectors were added to the plasma membrane 10 min before the addition of PP_i.

The sulfhydryl group inhibitors—*p*-chloromercuribenzenesulfonate (PCMBS) and phenylarsine oxide (PAO)—showed potent inhibition on PPase activity (Fig. 6, a and b). ATPase activity was less potently inhibited by PCMBS, and no inhibition was observed with PAO (Fig. 5a). The chemical modification with the trivalent arsenical reagent PAO indicates the involvement in PPase activity of a vicinal sulfhydryl group [26]. Some compounds used to inhibit H^+-ATPase activity, such as N-ethylmaleimide (NEM), and N,N′-dicyclohexylcarbodiimide (DCCD) [27] are also able to inhibit the plasma membrane PPase and ATPase (Figs. 5b and 6a).

F_1-ATPase and F_1F_o-ATPase inhibitor sodium azide [28] inhibited only plasma membrane ATPase activity but not the PPase activity (Figs. 5a and 6b).

The bioflavonoid quercetin, which can inhibit F_1F_o-ATPase strongly at low concentration and F_1-ATPase moderately at high concentration [29], inhibited plasma membrane bound ATPase and PPase moderately at high concentration (Figs. 5b and 6a).

Sodium orthovanadate, an inhibitor of a P-type H^+-ATPase [22] and P-type ABC superfamily of transporters, such as P-glycoprotein involved in multidrug resistance [30], inhibited ATPase activity in a dose-dependent manner (Fig. 5b). In contrast, PPase activity was stimulated (Fig. 6a). The reason for PPase stimulation by orthovanadate is not known. Effects of stimulators and inhibitors on PPase in digitonin-permeabilized amastigotes were very similar to the effect on PPase of promastigote plasma membrane (data not shown).

Verapamil, a P-gp ATPase stimulator [31], stimulated ATPase activity (Fig. 5b), whereas PPase was inhibited (Fig. 6a).

Our results using permeabilized amastigote cells (Fig. 3) suggests that ATPase does not function in amastigote plasma membrane; instead, PPase fulfills the function of ATPase. The functional significance of PPase as an extrusion pump cannot be overruled. The function of PPase in the amastigote plasma membrane may be proton pumping to maintain neutral cytosolic pH [32] in an environment of acidic pH of phagolysosomes [33]. Data provided evidence that ΔpH formation in promastigote and amastigote was partially inhibited by the H^+-ATPase inhibitor DCCD. This fact suggested that mechanisms in addition to that inhibited by DCCD may also be involved in controlling homeostasis of pH_i [32]. It appears from our observations that PPase can also play an important role in the extrusion of protons from the cytosol.

In conclusion, this is the first report of a PPase in plasma membrane of an organism different from plants and bacteria [34]. Since membrane-bound PPases are apparently absent from vertebrates, analysis of the role of PPase in pH regulatory mechanisms of *L. donovani* amastigote might provide a potential target for putative therapeutic intervention.

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