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Magnesium-Dependent Ecto-ATP Diphosphohydrolase Activity in *Leishmania donovani*

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Abstract In this work, we have described the expression of ecto-ATPDase on the external surface of *Leishmania donovani*. This enzyme has the ability to hydrolyze extracellular ATP. There is a low level of ATP hydrolysis in the absence of divalent cation 2.5 ± 0.51 nM Pi 10^7 cells/h which shows the divalent cation-dependent activity of this enzyme in the intact parasite. However, $MgCl_2$ stimulated the ATP hydrolysis to a greater extent compared with $CaCl_2$ and $ZnCl_2$. This activity was also observed when replaced by $MnCl_2$. The Mg-dependent ecto-ATPase activity was 46.58 ± 6.248 nM Pi 10^7 cells/h. The apparent K_m for ATP was 5.76 mM. Since *Leishmania* also possesses acid phosphatase activity and to discard the possibility that the observed ATP hydrolysis was due to acid phosphatase, the effect of pH was examined. In the pH range 6.0–9.0, in which the cells were viable, the phosphatase activity decreased while ATPase activity increased. To show that the observed ATP hydrolysis was not due to phosphatase or nucleotidase activity, certain inhibitors for these enzymes were tested. Vandate and NaF inhibited the phosphatase activity; Ammonium molybdate inhibited 5′-nucleotidase activity, but these inhibitors did not inhibit the observed ATP hydrolysis. However, when ADP was used as a substrate, there was no inhibition of ATP hydrolysis showing the possibility of ATP diphosphohydrolase activity. To confirm that this Mg-dependent ATPase activity is an ecto-ATPase activity, we used an impermeable inhibitor, 4,4′-diisothiocyanostilbene 2,-2′-disulfonic acid, as well as suramin, an antagonist of P2-purinoceptors and inhibitor of some ecto-ATPases. These two reagents inhibited the Mg^{2+}-dependent ATPase activity in a dose-dependent manner. The presence of *L. donovani* E-NTPDase activity was demonstrated using antibodies against NTPDase by Western blotting and flow cytometry. The presence of Mg^{2+}-dependent ATP diphosphohydrolase activity on the surface of *L. donovani* modulates the nucleotide concentration and protects the parasite from the lytic effects of the nucleotides mainly ATP. Ecto-ATPDase from *L. donovani* may be further characterized as a good antigen and as a target for immunodiagnosis and drug development, respectively.

Keywords *Leishmania donovani* · Ecto-ATPDase activity · Adenosine · Magnesium

Introduction

Visceral leishmaniasis (VL, commonly known as kala-azar) is a parasitic protozoan disease. Many people are affected by it throughout the World. This disease has emerged as a major health problem as it is spreading rapidly to large urban centers in the endemic areas, and AIDS patients are more susceptible to it [9]. There are few anti-leishmanial drugs to treat VL patients, and few of them are toxic. Hence, the development of new drugs and other strategies to control and prevent the disease is required [3, 37]. *Leishmania* and other parasites depend on

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purine salvage pathway for the synthesis of nucleic acid and other biomolecules [44]. Extracellular nucleoside tri- and diphosphates are hydrolyzed by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) in the purine salvage pathway and produce nucleoside monophosphates. These nucleoside monophosphates are then converted to nucleosides by ecto-5′-nucleotidases and enter into the cells for intracellular purine nucleotide synthesis [6, 53]. Purine salvage pathway enzymes may function as target for drug development in those pathogens which depend on this pathway [26]. One of the studies conducted on *Trypanosoma cruzi, Toxoplasma gondii*, and species of *Leishmania* revealed that E-NTPDases plays a major role in its infectivity, virulence, and purine acquisition [5–7, 11, 12, 46]. These suggest the critical role of E-NTPDases in parasitic infections through nucleotide signaling pathway. There exist possible binding sites for NTPDase in Macrophages which may facilitate adherence and infection. Antibodies against the NTPDase when added to macrophages before *Leishmania infantum chagasi* infection reduced adherence and infection by the parasites [49].

Cell membrane ecto-NTPDase are integral membrane glycoprotein, millimolar divalent cation-dependent, low specificity enzymes and hydrolyze all nucleoside triphosphates [24, 27, 30]. These enzymes were grouped into ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family [18]. These enzymes show many physiological functions such as (i) protection from lytic effects of extracellular ATP [41, 45, 50], (ii) regulation of ectokinase substrate concentration [14], (iii) termination of purinergic signaling [28, 29], (iv) involvement in signal transduction [1, 31, 33], and (v) involvement in cellular adhesion [15, 24, 40].

Leishmania donovani promastigotes and amastigotes possess 5′-nucleotidase and three different phosphomonoesterases located on the external surface of the plasma membrane [4, 10, 23, 36, 48, 52]. These ecto-phosphomonoesterases are not able to promote high levels of ATP hydrolysis. Extracellular ATP causes plasma membrane depolarization, calcium influx, and cell death except those cells that express a high level of ATP-breakdown activity. Hence, in this study, we show the presence of magnesium-dependent ecto-ATPDase activities in this species and characterize the following properties of this enzyme: its divalent cation dependence, pH activation profile, specificity to Suramin, an inhibitor of NTPDase and 4,4′-diisothiocyanostilbene 2,-2′-disulfonic acid (DIDS), and an antagonist of P2 purine receptor. Further, the presence of ecto-ATPDase on the surface of *L. donovani* was shown by flow cytometry and western blotting.

Materials and Methods

Culture Methods

The AG83 strain of *L. donovani* promastigotes was cultured in RPMI 1640 medium (Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco®, USA), 100 U/ml penicillin G potassium (USB Corporation®, Cleveland, OH, USA), 100 µg/ml streptomycin, and 10 µg/ml gentamycin, pH 7.4, at 25 °C. Cellular viability was assessed, before and after incubations, by motility and trypan blue dye exclusion. The viability was not affected under the conditions employed here.

Ecto-ATPase Activity Measurements

Intact cells were incubated for 1 h at 30 °C in 0.5 ml of a mixture containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50.0 mM Hepes-Tris buffer, pH 7.2, 5.0 mM ATP, and 3.0×10^7 cells/ml, in the absence or in the presence of 5.0 mM $MgCl_2$. The Mg^{2+}-dependent ecto-ATPase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$, minus the basal activity, measured in the absence of $MgCl_2$. The ATPase activity was determined by measuring the hydrolysis of ATP using spectrophotometric method and recording the absorbance at 655 nm. ATP hydrolysis was terminated after 20 min by the addition of 30 µl of stop solution consisting of 13 % SDS and 120 mM EDTA. In the control experimental set up, stop solution was added prior to the addition of ATP, in order to correct for any nonenzymatic hydrolysis of ATP. Color development was initiated with the addition of 200 µl of color reagent warmed to 30 °C.

Color Reagent Consists of the Following Composition

Twenty milliliter of 10 % ascorbic acid (pH 5.0) was mixed with 5 ml of 35 mM ammonium molybdate (prepared in 15 mM zinc acetate). The experiments were started by the addition of living cells and terminated by the addition of 13 % SDS and 120 mM EDTA. The tubes were then centrifuged at $1500 \times g$ for 10 min at 4 °C. To the 60 µl of the supernatants containing the released Pi, 200 µl of the coloring solution was added, and the reaction was incubated for 20 min at 30 °C. Absorbance was recorded at 655 nm. The ATPase activity was calculated by subtracting the nonspecific ATP hydrolysis measured in the absence of cells.

Phosphatase Measurements

In addition to the measurements of ecto-ATPase activity, the ecto-p-nitrophenylphosphate activity was determined in the same medium as that for ATP hydrolysis except that ATP was replaced 5.0 mM *p*-nitrophenylphosphate (*p*-NPP).The reaction was determined spectrophotometrically at 425.0 nm.

Flow Cytometry Analysis

Metacyclic parasites (5.0 × 106 cells) were isolated from a mixed population of *L. donovani* using Ficoll density gradient centrifugation at 700×*g* for 30 min. The cells were collected from the interphase layer. These cells maintained their morphological integrity, as verified by light microscopic observation. These cells were incubated for 30 min with 5 μg anti-E-NTPDase antibody: mouse monoclonal anti-human CD39-PE labeled (eBioscience, Inc., San Franciso, CA) .The cells were then washed with stain buffer and were then analyzed in fluorescence activated cell sorter. IgG-PE was used as an Isotype control. The mapped population (*n* 10,000) was then analyzed for log red fluorescence by using a single-parameter histogram. The analysis was done in triplicate set of cells [47].

Isolation of Plasma Membrane Protein

Promastigotes (3.0 × 109 cells) at late log phase of growth were harvested by centrifugation and washed three times in cold PBS. Plasma membrane was obtained as following. Parasites were incubated for 1 hour in Tris–Cl (5 mM) at 4 °C (pH 7.2). The cells were then freeze-thawed for 6 cycles and then sonicated for 3 cycles of 20 s each. The suspension was then centrifuged at 500×*g* for 10 min at 4°C. The supernatant was extracted and further ultracentrifuged (Himac CP 100WXTM, Hitachi, Japan) at 10,0000×*g* for 1 h at 4 °C. The supernatant was removed and the pellet was extracted with PBS-containing 2 % β-octylglucopyronoside by incubation overnight at 4 °C. After centrifugation at 10,0000×*g* for 1 h at 4 °C, the plasma membrane was obtained and kept at −80 °C, for further analysis. Protein concentration of each fraction was determined by Pierce BCA Protein Assay Kit (Thermo Scientific®, USA).

SDS-PAGE and Western Blot Analysis

Cell lysate and Plasma Membrane Protein were resolved in 12 % SDS-PAGE and the separated polypeptides were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5 % non-fat dried milk in TBS (150 mM NaCl; 10 mM Tris, pH 7.4) containing 0.1 % Tween 20 (TBS/Tween), overnight at 4 °C. Then, membranes were washed with TBS/Tween-20 and incubated with anti-E-NTPDase antibody (Human CD39/ENTP1antibody, Mouse Monoclonal, R&D Systems), at 1:1000 dilution for 2 h. The secondary antibody used was HRP-conjugated anti-mouse at 1:1000 dilution for 1 h. Blots were then detected using chromogenic secondary antibody substrate 0.001 % 3′, 3′-diaminobenzidine (DAB) and H_2O_2 [47].

Statistical Analysis

All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Apparent K_m and V_{max} values were calculated using a computerized nonlinear regression analysis of the data to the Michaelis-Menten equation. Data were analyzed using the Prism computer software (Graphpad Software Inc., San Diego, CA, USA).

Results

L. donovani promastigotes show viability before and after the reactions by motility and by Trypan blue dye exclusion method. *L. donovani* presented low ATP hydrolysis (2.50 ± 0.51 nmol Pi 10^7 cells/h) in the absence of any divalent metal (1 mM EDTA).

Ecto-ATPases are usually activated by divalent cations, such as Ca^{2+} and Mg^{2+}. Therefore, we evaluated whether the ecto-ATPase activity observed in *L. donovani* was influenced by the addition of such ionic components. At pH 7.2, the addition of 5 mM $MgCl_2$ stimulated the ATP hydrolysis, and the Mg^{2+}-dependent ecto-ATPase activity difference between the total (measured in the presence of 5 mM $MgCl_2$) minus the basal ecto-ATPase activity (measured in the presence of 1 mM EDTA) present in these parasites hydrolyzed ATP at 27.15 ± 2.91 nmol Pi/h 10^8 cells (Fig. 1).

To check the possibility that the observed ATP hydrolysis was the result of secreted soluble enzymes, as observed for other parasites [16], a reaction mixture was prepared with cells that were incubated in the absence of ATP. The suspension was centrifuged to remove the cells, and the supernatant was checked for ATPase activity. This supernatant failed to show ATP hydrolysis both in the absence and in the presence of $MgCl_2$ (data not shown). These results disprove the possibility that the ATPase activity observed here could be from lysed *L. donovani* cells.

To observe the influence of other divalent cations on the *L. donovani* ecto-ATPase activity, the rate of ATP hydrolysis was shown in the presence of 5 mM $CaCl_2$, $MnCl_2$, $ZnCl_2$, and $SrCl_2$. As shown in Fig. 1a, Mg^{2+} and

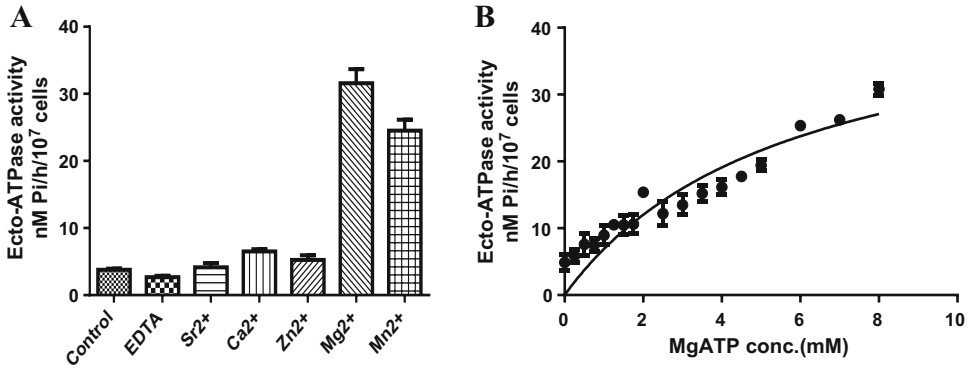

Fig. 1 **a** Stimulation of the ecto-ATPase activity in *L. donovani* by divalent cations: Influence of different divalent cations on the Ecto-ATPase activities in intact cells of *L. donovani* showing high rate of ATP hydrolysis in the presence of Mg^{2+} and Mn^{2+} when compared with EDTA, Sr^{2+}, Ca^{2+}, and Zn^{2+}. **b** K_m determination The ATPase activity was measured using increasing concentration of substrate $MgATP^{2-}$. The *curve* represents the fit of experimental data by nonlinear regression using the Michaelis–Menten equation. K_m value for $MgATP^{2-}$ is 5.76 mM. Data are means ± SE of three determinations with different cell suspension

Mn^{2+} stimulated the surface ATPase activity, while Ca^{2+}, Zn^{2+}, and Sr^{2+} did not. Mg^{2+} positively modulated the enzyme activity in a dose-dependent manner (Fig. 1a).

The time course of ATP hydrolysis (Fig. 2a) by the *L. donovani* Mg^{2+}-dependent Ecto-ATPase was linear for at least 60 min. Similarly, in assays to determine the influence of cell density (Fig. 2b), the Mg^{2+}-dependent activity measured over 60 min was linear over the range of cell density.

All these experiments were performed with intact cells, suggesting that the described Mg^{2+}-dependent ATPase is an ecto-enzyme. This hypothesis was investigated following previous definitions of ecto-enzymes which, according to several authors [32, 39], are inhibited by impermeable inhibitors. To confirm this hypothesis, ATP hydrolysis by *L. donovani* was performed in the presence of an extracellular impermeant inhibitor DIDS [27, 32, 39] and a pharmaceutical suramin [37], which is also an ecto-ATPase inhibitor [14, 34]. As shown in Fig. 3a, the Mg^{2+}-dependent ATPase activity of *L. donovani* was completely inhibited by Suramin in a dose-dependent manner, indicating its ecto-enzymatic nature. On the other hand, DIDS inhibited up to 80 % of the ATPase activity (Fig. 3b).

Leishmania donovani promastigote forms express surface acid phosphatases which may contribute to ATP hydrolysis. To observe whether phosphate release from ATP was influenced by phosphatase activities, different experimental approaches were followed, as presented in Fig. 4 and Table 1. We showed that the increase in pH inhibited the phosphatase activity present on the external surface of *L. donovani* [8]. On the other hand, the Mg^{2+}-dependent ATPase activity was not influenced by the increase in pH (Fig. 4).

Several inhibitors of phosphatases and other classes of ATPases were tested in order to exclude the possibility that the ATP hydrolysis was due to the mentioned enzymes. From Table 1, it can be observed that sodium fluoride (NaF), tartrate, and ammonium molybdate, which are the potent inhibitors of acid phosphatase [27, 35], had no effect on ATPase activity. In addition, the lack of response to *p*-

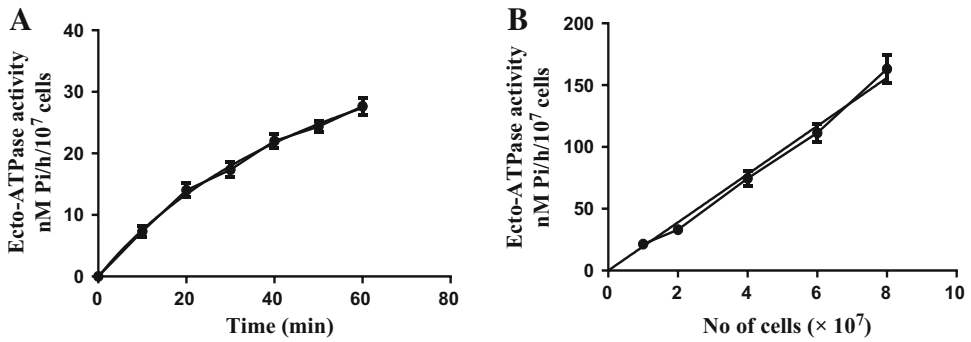

Fig. 2 Time course (**a**) and cell density dependence (**b**) of the Mg^{2+}-dependent ecto-ATPase activity in intact cells of *L. donovani*. Cells were incubated for different periods of time (**a**) or for 1 h (**b**) at 30 °C, in the reaction medium in the absence or in the presence of 5 mM $MgCl_2$ showing linear increase in ATP hydrolysis with increasing time period (**a**) and number of cells (**b**). Data are means ± SE of three determinations with different cell suspensions

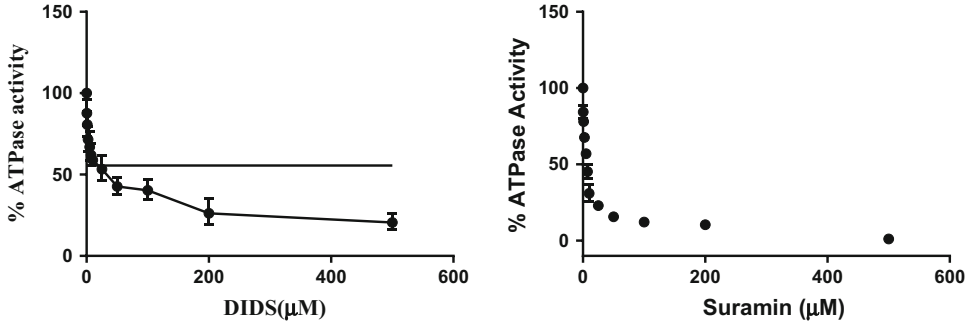

Fig. 3 Effect of increasing concentrations of DIDS and Suramin on the Mg^{2+}-dependent ecto-ATPase activity in *L. donovani*. Cells were incubated for 1 h at 30 °C in the reaction medium with increasing concentrations of DIDS or Suramin. There was up to 80 % inhibition in ATP hydrolysis with increasing concentrations of DIDS and complete inhibition in increasing concentrations of Suramin. Data are means ± SE of three determinations with different cell suspensions

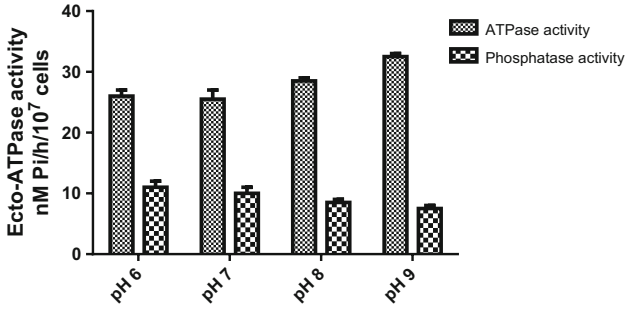

Fig. 4 Effect of pH on the ecto-ATPase and phosphatase activities of intact cells of *L. donovani*. Cells were incubated for 1 h at 30 °C in a reaction medium adjusted to pH values between 6.0 and 9.0 showing ATP hydrolysis not due to phosphatase activity. Data are means ± SE of three determinations with different cell suspensions

nitro-phenylphosphate (*p*-NPP), a substrate for phosphatase activity (Table 1), indicated that this enzyme did not contribute to the observed ATP hydrolysis. The Mg-dependent ATPase activity was insensitive to oligomycin, an inhibitor of mitochondrial Mg-ATPase [27], as well as to vanadate, which is a potent inhibitor of P-ATPases [42] (Table 1).

ATP hydrolysis may be due to 5′-nucleotidase, an enzyme present on the external surface of *L. donovani* (Fig. 5). However, the lack of response to molybdate (Table 1), a potent inhibitor of 5′-nucleotidase, [52] and AMP (Table 1), the substrate for this enzyme, indicated that a 5′-nucleotidase did not contribute for the observed ATP hydrolysis. On the other hand, sodium azide, an inhibitor of some ecto-ATPDases, and ADP inhibited the ATP hydrolysis, suggesting that the ATP hydrolysis would be catalyzed by an authentic E-NTPDase. These data confirm that a 5′-nucleotidase activity also present on the surface of *L. donovani* (Fig. 5) together with the nucleoside triphosphate diphosphohydrolase characterized here might sequentially dephosphorylate ATP to adenosine: ATP → ADP → AMP → adenosine, making adenosine available to *L. donovani* from nucleotides.

We tested the antibody NTPDase CD39-PE to verify the expression of this enzyme in *L. donovani*, and the results

Table 1 Influence of various agents on the ecto-ATPDase and ecto-phosphatase activity of *L. donovani*

Modulator	p-NPPase % of control	ATPase % of control
None (control)	100.0 ± 4.5	100.0 ± 6.5
Ammonium molybdate (100 μM)	5.6 ± 2.5	89.60 ± 10.25
Sodium azide (1 mM)	106.53 ± 2.5	82.90 ± 7.5
Sodium fluoride (10 mM)	5.01 ± 0.1	98.25 ± 8.2
Sodium orthovanadate (200 μM)	5.0 ± 0.6	89.74 ± 9.6
Sodium tartrate (1 mM)	90.5 ± 1.2	118.1 ± 12.4
p-NPP (5.0 mM)	92 ± 5.6	80.67 ± 9.6
5′-AMP (5.0 mM)	89.52 ± 10.5	84.98 ± 12.5
ADP (5.0 mM)	100.56 ± 9.5	40.67 ± 10.56
DIDS (0.5 mM)	90.0 ± 10.2	16.9 ± 4.5
Suramin (0.5 mM)	92.0 ± 9.8	18.2 ± 5.2

ATPase activity was measured at pH 7.2 in the standard assay with 5 mM ATP. The ATPase activity is expressed as the percentage of that measured under control conditions, i.e., without other additions. The Mg^{2+}-dependent ATPase (28.8 ± 1.2 nmol Pi/10^7 cells h^{-1}) activity was taken as 100 %. Data are means ± SE of triplicate of three determinations with different cell suspensions

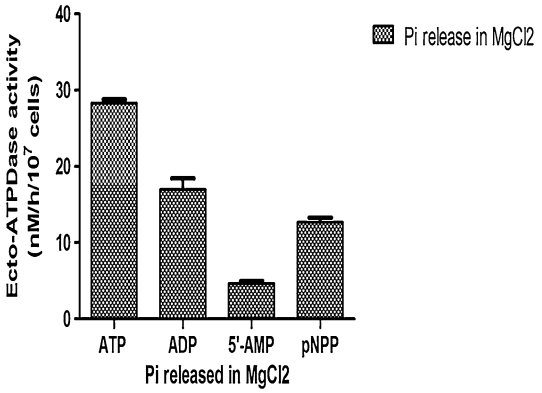

Fig. 5 Ecto-ATPDase activities of intact cells of *L. donovani*. Cells were incubated in the presence of 5 mM of either ATP, ADP, or 5′-AMP showing increased ecto-ATPDase activity in the presence of ATP when compared with ADP, AMP, and *p*NPP. Data are means ± SE of three determinations with different cell suspensions

were analyzed by flow cytometry. A total of 10,000 cells were analyzed for its expression (Fig. 6a). The anti-human CD39 antibody showed expression on the cell surface of *L. donovani* which also shows sequence homology between human CD39/ENTP1 and *L. donovani* E-NTPDase.

We obtained cell lysate and plasma membrane-rich fraction through differential centrifugation to characterize the ecto-NTPDase expressed in promastigotes of *L. donovani*. Western blotting utilizing the anti-human CD39 antibody showed reaction with the plasma membrane fraction as well as cell lysate. The polypeptide content of the membrane fraction was applied on 12 % SDS-PAGE, and polypeptide bands were observed with apparent molecular masses varying from 12 to 225 kDa (Fig. 6b). Western blotting analysis showed the E-NTPDase antibody to be a polypeptide of 45 kDa (Fig. 6c).

Discussion

This paper reports the presence of Mg-dependent ecto-ATPase on the external surface of *L. donovani*. Cell viability was observed, before and after the reactions, by motility and by trypan blue dye exclusion. The integrity of the cells was not affected by any of the conditions used in the assays.

The ATP-hydrolyzing enzyme is present on the external location of *L. donovani* supported by its sensitivity to the impermeable inhibitor DIDS (Fig. 4) [27, 32, 39]. Inhibitors for other ATPases showed no effect on the ecto-ATPase activity (Table 1). The Mg-dependent ecto-ATPase activity was insensitive to vanadate (Table 1),

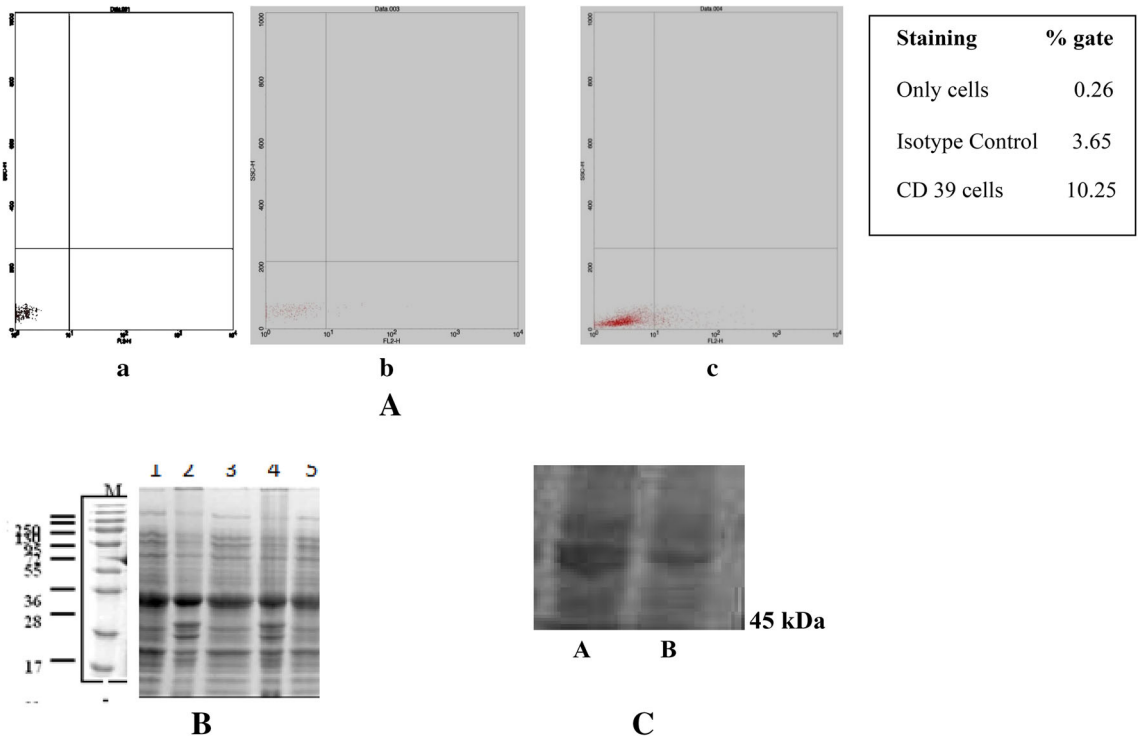

Fig. 6 Expression of ecto-NTPDase in *L. donovani* membrane. A flow cytometry analysis of *L. donovani* using the anti-human CD39-PE-conjugated antibody. 10,000 cells are analyzed for ecto-NTPDase expression. **a** Only cells. **b** Isotype control (IgG-PE). **c** CD39-PE stained cells. B SDS-PAGE showing protein bands of 15 to 225 kDa in cell lysate of *Leishmania donovani*. *Lane M*-marker, *Lanes-1–5* cell lysate. **c** Western blot analysis using purified mouse anti-human CD39 E-NTPDase against cell lysate (**a**) and total membrane protein (**b**) of *L. donovani* (showing a specific band of molecular weight 45 kDa)

which discarded the possibility that this activity was due to a P-ATPase. ATP hydrolysis could not be due to phosphatase activity present on the external surface of *L. donovani* membrane [8], because as shown in Table 1, potent inhibitors for phosphatase activities were not capable to modify the Mg-dependent ecto-ATPase activity. The Mg-dependent ecto-ATPase activity described here could not be attributed to a 5′-nucleotidase, since the ATP hydrolysis was not inhibited by ammonium molybdate, an inhibitor of 5′-nucleotidase [8] (Table 1). The addition of $CaCl_2$, $ZnCl_2$, and $SrCl_2$ to the extracellular medium did not stimulate the ecto-ATPase activity (Fig. 1a). ADP was also recognized as a substrate, indicating that this enzyme is an authentic nucleoside triphosphate diphosphohydrolase as described in other cells [2, 13, 20]. *Trypanosoma brucei brucei* and *Leishmania amazonensis* are pathogens which cannot synthesize purines de novo [6, 51]; hence, it has been postulated that these ecto-ATPases in protozoa parasites play a role in the salvage of purines from the host cells [6, 43] *L. donovani* might sequentially dephosphorylate ATP to adenosine: ATP → ADP → AMP → adenosine (Fig. 5), indicating that this enzyme plays a role in the salvage of purines from extracellular medium.

It has been studied in *Leishmania* parasites that an increase in ectonucleotidase activity causes the increased production of adenosine which establishes infection by immunosuppressive mechanisms. Infective *L. amazonensis* promastigotes possess higher ATPase activity when compared with noninfective promastigotes [6]. Amastigotes form of parasites hydrolyzes ATP at higher rates than promastigotes, hence showing an increased ectonucleotidase activity [47]. *L. amazonensis* showing a higher ectonucleotidase activity are more effective in establishing infection [12]. Comparison of ectonucleotidase activities between *L. amazonensis*, *Leishmania braziliensis*, and *L. major* showed that the more virulent parasite causes nonhealing lesions in mice (e.g., *L. amazonensis*) and hydrolyzes higher amounts of ATP, ADP, and 5′-AMP [21]. Adenosine treatment at the time of *L. braziliensis* inoculation delays lesion resolution and induces increased parasite burdens. Inhibition of adenosine receptor A2B led to decreased lesion size and lower parasite burden [10]. Ecto-ATPase activity is involved in virulence, and *L. amazonensis* strain isolated from VL human case possess higher ecto-ATPase activity than strains isolated from CL cases [22].

Leishmania parasites also express a bifunctional enzyme called 3′-nucleotidase/nuclease (3′-NT/NU) in the plasma membrane with a high capacity to hydrolyze 3′ ribonucleotides and nucleic acids [10, 25]. It was first identified in *L. donovani* and later found in *L. chagasi* [19], *L. major* [38], *L. mexicana* [17], and *L. amazonensis*. The 3′-nucleotidase activity of *L. chagasi* has been characterized and correlated with parasite virulence.

The VL-causing species *L. chagasi* and *L. donovani* had higher 3′-nucleotidase activity compared to the New World and Old World CL-causing species, i.e., *L. amazonensis, L. major, L. tropica*, and *L. braziliensis*. *L. chagasi* metacyclics (infective promastigote stage) had higher 3′-nucleotidase activity compared to *L. chagasi* nonmetacyclics (noninfective promastigote stage) [19]. Infective *L. amazonensis* promastigotes possess higher 3′-nucleotidase activity compared to nonvirulent promastigotes.

The composition of the cell surface changes during the life cycle of *Leishmania*. The *L. amazonensis* ecto-NTPDase activities were highest when cells were in the logarithmic growth phase (24–48 h) and reduced expression of ecto-ATPDase activity during 96 h of growth in culture medium, suggesting that this increase occurs as the cells prepare for cell division. The amastigotes expressed on their surface an increase in ecto-ATPDase activities when compared with promastigotes in *L. amazonensis* [38]. The infective stages of *T. cruzi* had much higher Mg^{2+}-dependent ecto-ATPase activity than the noninfective epimastigotes, suggesting this ecto-enzyme as a virulence marker [36].

One of the protein from *Leishmania infantum chagasi* named E-NTPDase-2 was expressed, purified, and characterized leading to in vitro infection, and its blockade leads to lower rate of infection of macrophages. It can be established as a good antigen for immunodiagnosis of canine visceral leishmaniasis and a target for drug development [49]. However, ecto-ATPDase from *L. donovani* may be further characterized as a good antigen for immunodiagnosis and as a target for drug development.

Our present study revealed that magnesium-dependent ecto-ATPDase present on the external surface of *L. donovani* protects the parasite from the extracellular ATP concentration, and its characterization leads to better understanding about the mechanisms and the substrate specificity of the enzyme. This makes an easier approach to target this enzyme as drug and diagnosis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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