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Trypanosoma brucei brucei: Biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities

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Abstract

In this work we describe the ability of living cells of *Trypanosoma brucei brucei* to hydrolyze extracellular ATP. In these intact parasites there was a low level of ATP hydrolysis in the absence of any divalent metal (4.72 ± 0.51 nmol Pi $\times 10^{-7}$ cells $\times h^{-1}$). The ATP hydrolysis was stimulated by $MgCl_2$ and the Mg-dependent ecto-ATPase activity was 27.15 ± 2.91 nmol Pi $\times 10^{-7}$ cells $\times h^{-1}$. This stimulatory activity was also observed when $MgCl_2$ was replaced by $MnCl_2$. $CaCl_2$ and $ZnCl_2$ were also able to stimulate the ATPase activity, although less than $MgCl_2$. The apparent K_m for ATP was 0.61 mM. This ecto-ATPase activity was insensitive to inhibitors of other ATPase and phosphatase activities. To confirm that this Mg-dependent ATPase activity is an ecto-ATPase activity, we used an impermeable inhibitor, DIDS (4, 4′-diisothiocyanostylbene 2′-2′-disulfonic acid), as well as suramin, an antagonist of P_2 purinoreceptors and inhibitor of some ecto-ATPases. These two reagents inhibited the Mg^{2+}-dependent ATPase activity in a dose-dependent manner. Living cells sequentially hydrolyzed the ATP molecule generating ADP, AMP and adenosine, and supplementation of the culture medium with ATP was able to sustain the proliferation of *T. brucei brucei* as well as adenosine supplementation. Furthermore, the E-NTPDase activity of *T. brucei brucei* is modulated by the availability of purines in the medium. These results indicate that this surface enzyme may play a role in the salvage of purines from the extracellular medium in *T. brucei brucei*.

Index Descriptors and Abbreviations: *Trypanosoma brucei brucei*; Ecto-ATPase; Adenosine

1. Introduction

Parasitic protozoa of the *Trypanosoma brucei* subgroup are the causative agents of African sleeping sickness in man and the related livestock disease, nagana. These parasites have a digenetic life cycle, with two main stages: the bloodstream form that lives in the bloodstream of its mammalian host and the procyclic form that lives in the insect vector (tsetse fly) with changes in metabolism, morphology and membrane composition (Vickerman, 1986; Clayton and Michels, 1996). The plasma membrane of cells may contain enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using intact cells (Fernandes et al., 1997; Meyer-Fernandes, 2002; Pinheiro et al., 2006). Knowledge about interactions between components of the external surface of the *Trypanosoma brucei brucei* and the cellular elements of the host is of obvious importance for the understanding of the complex pathology of nagana disease.

Cell membrane ecto-ATPases are integral membrane glycoproteins that are millimolar divalent cation-dependent, low specificity enzymes that hydrolyze all nucleoside

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triphosphates (Plesner, 1995; Kirley, 1997; Meyer-Fernandes et al., 1997). The identity and the function of ecto-ATPases have been reviewed and the nomenclature of "E-type ATPases" was proposed to describe these enzymes (Plesner, 1995). More recently, these enzymes were grouped into ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family (Zimmermann, 2001). Their physiological role is still unknown. Nevertheless, several hypotheses have been suggested, such as (i) protection from cytolytic effects of extracellular ATP (Filippini et al., 1990; Zanovello et al., 1990; Steinberg and Di Virgilio, 1991), (ii) regulation of ectokinase substrate concentration (Plesner, 1995), (iii) termination of purinergic signaling (Weisman et al., 1996; Westfall et al., 1997), (iv) involvement in signal transduction (Margolis et al., 1990; Dubyak and El-Moatassim, 1993; Yagi et al., 1994), and (v) involvement in cellular adhesion (Cheung et al., 1993; Dzhandzhugazyan and Bock, 1993; Stout et al., 1995; Kirley, 1997).

Here, we show the presence of a Mg^{2+}-dependent E-NTPDase activity on the cell surface of living *Trypanosoma brucei* and characterize the properties of this enzyme. Additionally, we investigated the sequential hydrolysis of the ATP molecule (ATP → ADP → AMP → adenosine) by living *T. brucei brucei*. Finally, we also demonstrated that this E-NTPDase activity decreased along the 144 h of *T. brucei brucei* grown *in vitro* and respond to purine starvation indicating that this activity may play a role in the salvage of purines from the extracellular medium.

2. Materials and methods

2.1. Culture methods

Trypanosoma brucei brucei procyclic forms (Iltar 1 procyclics) were grown at 28 °C in SDM-79 medium (Cunningham, 1977) supplemented with hemin (7.5 mg/mL) and 10% fetal bovine serum. For growth under conditions with controlled purine concentrations, procyclic cells were grown in purine free trypanosome medium (PFTM) supplemented with 10% (w:v) dialysed fetal bovine serum (Gibco) and adenosine or ATP as required. As previously described by De Koning et al. (2000), one litre of PFTM consisted of 130 mL RPMI 1640, 7 g S-MEM, 8 mL MEM (50×) amino acids w:o glutamine, 6 mL MEM non-essential amino acids, 265 μM $CaCl_2$ 5.5 mM glucose, 8 g Hepes, 5 g Mops, 23.8 mM $NaHCO_3$, 0.9 mM sodium pyruvate, 2.2 mM L-alanine, 475 μM L-arginine, 2.0 mM L-glutamine, 470 mM L-methionine, 480 mM L-phenylalanine, 5.2 mM L-proline, 570 μM L-serine, 2.9 mM L-threonine, 550 μM L-tyrosine, 230 μM glucosamine HCl, 9.1 μM folic acid, 15 μM *p*-aminobenzoic acid, 0.8 μM biotin, 80 μM sodium acetate, and 11.5 μM hemin (pH 7.3 (NaOH)). Two to three days after inoculations, cells were collected by centrifugation, washed twice and kept in 50 mM Tris–HCl, pH 7.2, 20 mM KCl, and 100 mM sucrose. Cellular viability was assessed, before and after incubations, by motility and Trypan blue dye exclusion. For Trypan staining the cells were incubated in the presence of 0.01% Trypan blue for 10 min in the buffer used in each experiment. The viability was not affected under the conditions employed here.

2.2. Ecto-ATPase activity measurements

Intact cells were incubated for 1 h at 30 °C in 0.5 mL of a mixture containing, unless otherwise specified, 50 mM Tris–HCl, pH7.2, 20 mM KCl, 100 mM sucrose, 5.0 mM ATP, and 10^8 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 [γ-^{32}P]ATP (10^4 Bq/nmol ATP) (Saad-Nehme et al., 2000). The experiments were started by the addition of living cells and terminated by the addition of 1.0 mL of a cold mixture containing 25% charcoal in 0.1 M HCl. The tubes were then centrifuged at 1500*g* for 10 min at 4 °C. Aliquots (0.5 mL) of the supernatants containing the released ^{32}Pi were transferred to scintillation vials containing 9.0 mL of scintillation fluid. The ATPase activity was calculated by subtracting the non-specific ATP hydrolysis measured in the absence of cells. The ATP hydrolysis was linear with time under the assay conditions used and was proportional to the cell number. In the experiments where other nucleotides were used, the hydrolytic activities measured under the same conditions described above were assayed spectrophotometrically by measuring the release of Pi from the nucleotides (Lowry and Lopes, 1946). The values obtained for ATPase activities measured using both methods (spectrophotometric and radioactive) were exactly the same. In the experiments where high concentrations of Mn^{2+}, Zn^{2+}, Ca^{2+}, and Sr^{2+} were tested, possible precipitates formed were checked as previously described (Meyer-Fernandes and Vieyra, 1988). Under the conditions employed, in the reaction medium containing 50 mM Tris–HCl, pH 7.2, 20 mM KCl, 100 mM sucrose and 5.0 mM ATP, no phosphate precipitates were observed in the presence of these cations.

2.3. Reverse-phase HPLC analysis

The HPLC system consisted of LC-10At pump, FCV-10AL solvent mixer, DGU-14A degasser, SPD-M10A diodearray detector, and a CLASS-LC10A (version 1.41) computing integrator, all of Shimadzu (Kyoto, Japan). The flow rate was maintained at 2 mL/min. The separation of the nucleotides and nucleosides was achieved by ion-pair reversed-phase chromatography on an analytical Supelcosil LC-18 (46 × 250 mm, 5 μm particle diameter; Supelco, St. Louis, USA) equipped with a guard column Supelguard (4 × 20 mm, 5 μm, Supelco). The eluents, 50 mM KH_2PO_4, 50 mM K_2HPO_4, 4 mM TBAB, and 10% methanol adjusted to pH 6.0 with H_3PO_4 were prepared in the day of use and filtered through a 0.22-μm filter (Millipore). The methodology used was modified of the original protocol proposed by

Kawamoto et al. (1998) for the best separation and repeatability under ours conditions. The adenine nucleotides of interest were separated (retention times, min: adenosine, 4.52 ± 0.10; AMP, 3.61 ± 0.07; ADP, 5.30 ± 0.18; ATP, 7.47 ± 0.24) and detected by UV spectroscopy at 254 nm. For calibration graphs, five replicate determinations at each concentrations of a standard mixture were assessed. The calibration graphs were constructed by plotting the peak area ratios against amounts inject. The hydrolysis of ATP and generations of ADP and AMP was determinate incubating 10^8 cells/mL in 0.5 mL of a mixture containing 50 mM Tris–HCl, pH 7.2, 20 mM KCl, 100 mM sucrose, 5 mM $MgCl_2$ and 100 mM ATP. After 5, 15, 30, 45, 60, and 120 min, aliquots of 200 µl was taken and load in the system for the separation. The amount of nucleotides was calculated using the peak area ratio in the calibration graph.

2.4. Statistical analysis

All experiments were performed in triplicates, with similar results obtained in at least three separate cell suspensions. Apparent K_m and V_{max} values were calculated using a nonlinear regression analysis of the data to the Michaelis–Menten equation. Statistical significance was determined by Student's t test. Significance was considered as $P < 0.05$. Data from the pH curve were analyzed by means of ANOVA One Way followed by the Tukey test using the Prism computer software (Graphpad Software Inc., San Diego, CA, USA).

2.5. Chemicals

All reagents were purchased from E. Merck (D-6100 Darmstadt, Germany) or Sigma–Aldrich (Sigma Co. St Louis, MO). [γ^{32}P]ATP was prepared as described by Glynn and Chappell (1964). Distilled water was deionized using a MilliQ system of resins (Millipore Corp., Bedford, MA) and was used in the preparation of all solutions.

3. Results

Trypanosoma brucei brucei procyclic forms, whose viability was assessed before and after the reactions by motility and by Trypan blue dye exclusion, presented low ATP hydrolysis (4.72 ± 0.51 nmol Pi $\times 10^{-7}$ cells $\times$ h^{-1}) 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 *T. brucei brucei* 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 total (measured in the presence of 5 mM $MgCl_2$) minus 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 $\times 10^8$ cells (Fig. 1). In order to check the possibility that the observed ATP hydrolysis was the result of secreted soluble enzymes, as observed for other parasites (Smith et al., 1997), we prepared a reaction mixture with cells that were incubated in the absence of ATP. Subsequently, the suspension was centrifuged to remove cells and the supernatant was checked for ATPase activity. This supernatant failed to show ATP hydrolysis either in the absence or in the presence of $MgCl_2$ (data not shown). These results also rule out the possibility that the ATPase activity here described could be from lysed *T. brucei brucei* cells.

The influence of other divalent cations on the *T. brucei brucei* ecto-ATPase activity was evaluated by the determination of the rate of ATP hydrolysis in the presence of

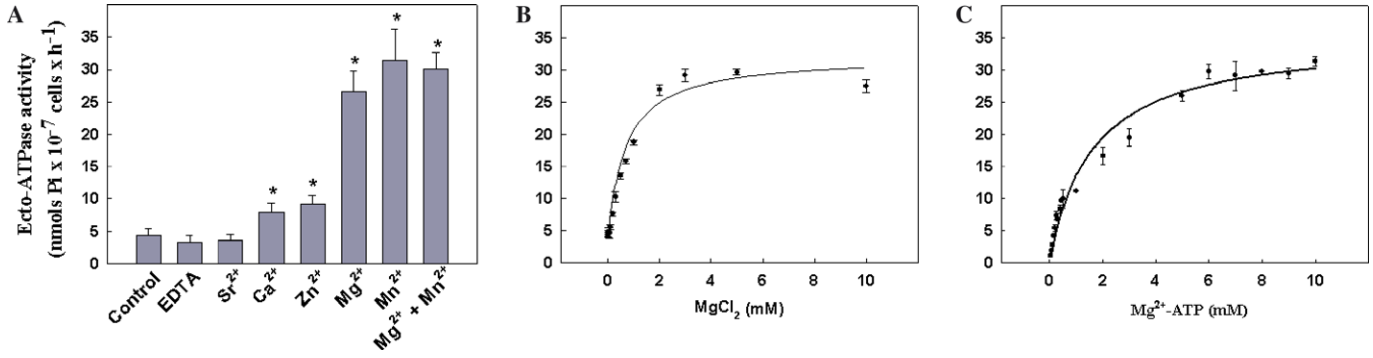

Fig. 1. Stimulation of the ecto-ATPase activity in *T. brucei brucei* by divalent cations. (A) Influence of different divalent cations on the ecto-ATPase activities in intact cells of *T. brucei brucei*. Cells were incubated for 1 h at 30 °C in a reaction medium (control) containing 50 mM Tris–HCl buffer, pH 7.2, 100 mM sucrose, 20 mM KCl, 10^8 cells/mL, and 5 mM ATP [γ-^{32}P]ATP (specific activity = 10^4 Bq/nmol ATP). Chloride salts of the ions described in the figure were added at a final concentration of 5 mM. Alternatively, the medium was supplemented with EDTA, in order to avoid interference. Asterisks denote significant differences ($P < 0.05$) after comparison with the enzyme activity in the presence of EDTA. (B) $MgCl_2$ enhanced enzyme activity in a dose-dependent pattern, as determined after incubation of living cells under the conditions described in (A). (C) K_m determination. Experiments were performed under the conditions described in (A), in reaction media supplemented with varying concentrations of ATP. The ATPase activity was measured at different periods of time and ATP hydrolysis did not exceed 10%. The curve represents the fit of experimental data by nonlinear regression using the Michaelis–Menten equation. Data are means ± standard errors of three determinations with different cell suspensions.

5 mM $CaCl_2$, $MnCl_2$, $ZnCl_2$, and $SrCl_2$. As shown in Fig. 1A, Ca^{2+}, Zn^{2+}, and Mn^{2+} stimulated the surface ATPase activity, while Sr^{2+} did not. The addition of Mg^{2+} and Mn^{2+} together did not increase the rate of ATP hydrolysis observed in the presence of Mg^{2+} or Mn^{2+} only. Mg^{2+} positively modulated the enzyme activity in a dose-dependent manner (Fig. 1B). At 5 mM ATP, half maximal stimulation of ATP hydrolysis was obtained in the presence of 0.60 mM $MgCl_2$ and an apparent K_m for ATP corresponding to 1.57 mM was determined (Fig. 1C).

The time course of ATP hydrolysis (Fig. 2A) by the *T. brucei brucei* Mg^{2+}-dependent ecto-ATPase was linear for at least 60 min ($r^2 = 0.9882$). Similarly, in assays to determine the influence of cell density (Fig. 2B), the Mg^{2+}-dependent activity measured over 60 min was linear over a nearly five-fold range of cell density ($r^2 = 0.9977$). 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 (Knowles, 1988; Barbacci et al., 1996) are inhibited by impermeable inhibitors. To confirm this hypothesis, ATP hydrolysis by *T. brucei brucei* was performed in the presence of an extracellular impermeant inhibitor 4,4′-diisothiocyanostylbene 2,2′-disulfonic acid (DIDS) (Knowles, 1988; Barbacci et al., 1996; Meyer-Fernandes et al., 1997) and a trypanocidal pharmaceutical suramin (Docampo and Moreno, 2003), which is also an ecto-ATPase inhibitor (Ziganshin et al., 1995; Meyer-Fernandes et al., 2004). As shown in Fig. 3 (open circles), the Mg^{2+}-dependent ATPase activity of *T. brucei brucei* was completely inhibited by DIDS in a dose-dependent manner, indicating its ecto-enzymatic nature. On the other hand, suramin only inhibited 50% of the ATPase activity (Fig. 3, closed circles). This result suggests that at least two enzymes could be involved with the ATP hydrolysis on the external surface of *T. brucei brucei*.

Trypanosoma brucei brucei procyclic forms express surface acid phosphatases (Fernandes et al., 1997, 2003), which could contribute to ATP hydrolysis. To evaluate whether phosphate release from ATP was influenced by phosphatase activities, different experimental approaches were

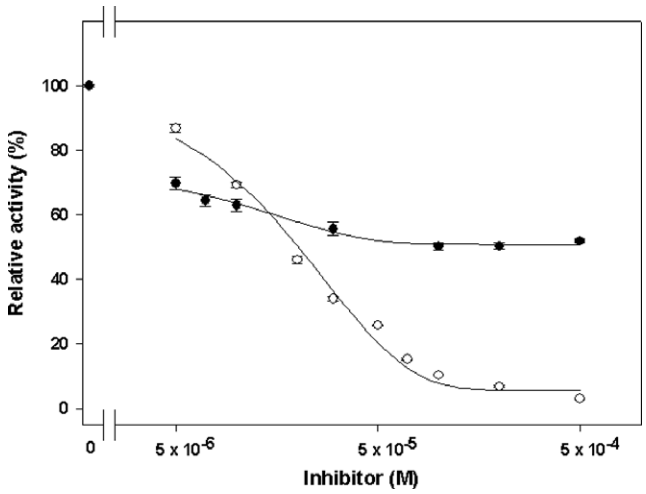

Fig. 3. Effect of increasing concentrations of DIDS and suramin on the Mg^{2+}-dependent ecto-ATPase activity of intact cells of *T. brucei brucei*. Cells were incubated for 1 h at 30 °C in the same reaction medium (Final volume 0.5 mL) as that described in the legend of Fig. 1, in the absence or in the presence of 5 mM $MgCl_2$ with increasing concentrations of DIDS (open circles) or suramin (closed circles). 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 $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

followed, as presented in Fig. 4 and Table 1. We showed that the increase of pH inhibited the phosphatase activity present on the external surface of *T. brucei brucei* (Fernandes et al., 1997, 2003). On the other hand the Mg^{2+}-dependent ATPase activity was not modified by the increased of the 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. Table 1 shows that sodium fluoride (NaF), tartrate and ammonium molybdate, potent inhibitors of acid phosphatase (Fernandes et al., 1997; Gomes et al., 2006), had no effect on ATPase activity. Levamizole, a specific inhibitor of alkaline phosphatases (Van Belle, 1976), also failed to inhibit the ATP hydrolysis catalyzed by intact *T. brucei brucei*. In addition, the lack of response to *p*-nitrophenylphosphate (*p*-NPP), a substrate for phosphatase

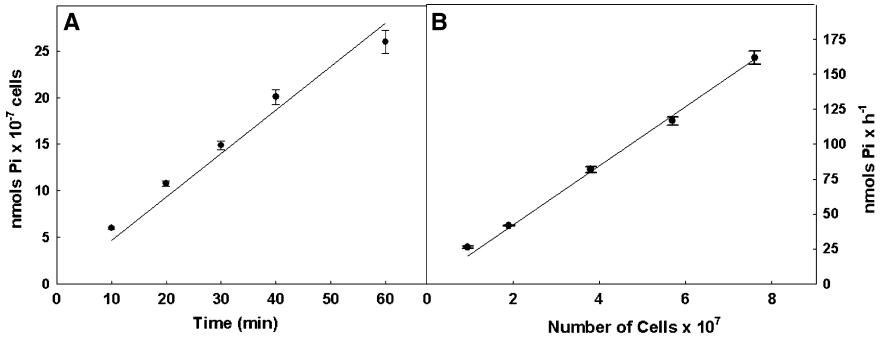

Fig. 2. Time course (A) and cell density dependence (B) of the Mg^{2+}-dependent ecto-ATPase activity of intact cells of *T. brucei brucei*. Cells were incubated for different periods of time (A) or for 1 h (B) at 30 °C, in a reaction medium containing 50 mM Tris–HCl buffer, pH 7.2, in the absence or in the presence of 5 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 $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

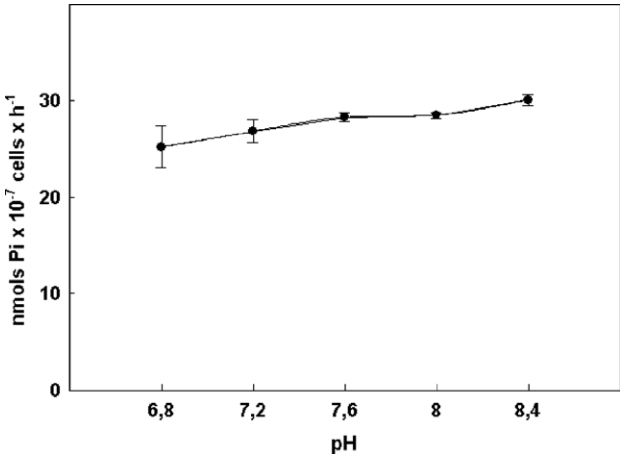

Fig. 4. Effect of pH on the ecto-ATPase activity of intact cells of *T. brucei brucei*. Cells were incubated for 1 h at 30 °C in a reaction medium containing 100 mM sucrose, 20 mM KCl, 10^8 cells/mL, 5 mM ATP [^{32}P]ATP (specific activity = 10^4 Bq/nmol ATP), and 50 mM Tris–HCl buffer, adjusted to pH values between 6.8 and 8.4 with HCl and Tris, in the absence or in the presence of 5 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 $MgCl_2$. In this pH range the cells were viable throughout the course of the experiments. There was no statistically significant differences in ATPase activity between pH 6.8 and 8.4. Data are means ± SE of three determinations with different cell suspensions.

Table 1
Influence of various agents on ATP hydrolysis by intact cells of *T. brucei brucei*

Additions	Relative activity(%)
Control	100.0 ± 4.6
Levamizole (1.0 mM)	110.0 ± 1.6
Molybdate (1.0 mM)	107.4 ± 5.7
Naf (1.0 mM)	108.1 ± 1.6
Tartrate (10.0 mM)	114.0 ± 1.0
Vanadate (1.0 mM)	84.9 ± 0.4
p-NPP (5.0 mM)	85.2 ± 0.9
Ouabain (1.0 mM)	113.1 ± 1.2
Furosemide (1.0 mM)	80.1 ± 0.3
Bafilomycin (1.0 μM)	113.0 ± 1.6
Oligomicyn (2.0 μM)	105.0 ± 1.6
5′-AMP (5.0 mM)	88.7 ± 0.5
Dipiridamole (10.0 μM)	89.1 ± 2.7
ADP (5.0 mM)	50.1 ± 0.6*
NaN_3 (10.0 mM)	41.7 ± 0.8*

Note. ATPase activity was measured at pH 7.2 in the standard assay described under in Section 2 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 $\times 10^{-7}$ cells $\times$ h^{-1}) activity was taken as 100%. Data are means ± SE of triplicate of three determinations with different cell suspensions.

* $p < 0.05$ values significantly different from control by unpaired student *t* test.

activity (Table 1), indicated that this enzyme did not contribute to the observed ATP hydrolysis. The Mg-dependent ATPase activity was insensitive to oligomycin, one inhibitor of mitochondrial Mg-ATPase (Meyer-Fernandes et al., 1997); bafilomycin, a V-ATPase inhibitor (Bowman et al., 1988); ouabain, a Na^+/K^+-ATPase inhibitor (Caruso-Neves et al., 1998a); furosemide, a Na^+-ATPase inhibitor (Caruso-Neves et al., 1998b); as well as to vanadate, which is a potent inhibitor of P-ATPases (Sodre et al., 2000). Dipyridamole, a nucleoside transporter antagonist (Lemmens et al., 1996) also failed to inhibit the ATPase activity (Table 1). A possible explanation for the ATP hydrolysis was that 5′ nucleotidase, another enzyme present on the external surface of *T. brucei brucei* (Fig. 5) could be responsible for this hydrolysis. However, the lack of response to molybdate (Table 1), a potent inhibitor of 5′-nucleotidase (Gottlieb and Dwyer, 1983) 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 ecto-nucleoside triphosphate diphosphohydrolase.

The ability of the *T. brucei brucei* ecto-enzyme to hydrolyze other nucleotides was also evaluated. Table 2 shows that ITP, ATP, CTP, GTP, and UTP were the preferred substrates for the surface enzyme, although it also efficiently hydrolyzed TTP and ADP. The ATP:ADP hydrolysis ratio was 1:0.7, a ratio very similar to that observed to E-NTPDase type 1, a plasma membrane-associated ecto-enzyme (Zimmermann, 2001). The observation that molybdate, a potent inhibitor of 5′-nucleotidase (Gottlieb and Dwyer, 1983), did not inhibit ATP hydrolysis (Table 1) discard that the AMP hydrolysis by *T. brucei brucei* (Fig. 5) was catalyzed by the same enzyme. These data confirm that a 5′ nucleotidase activity also present on the surface of *T. brucei brucei* (Fig. 5) together with the nucleoside triphosphate diphosphohydrolase characterized here might sequentially dephosphorylate ATP to adenosine: ATP → ADP → AMP → adenosine, making adenosine available to *T. brucei brucei* from nucleotides which,

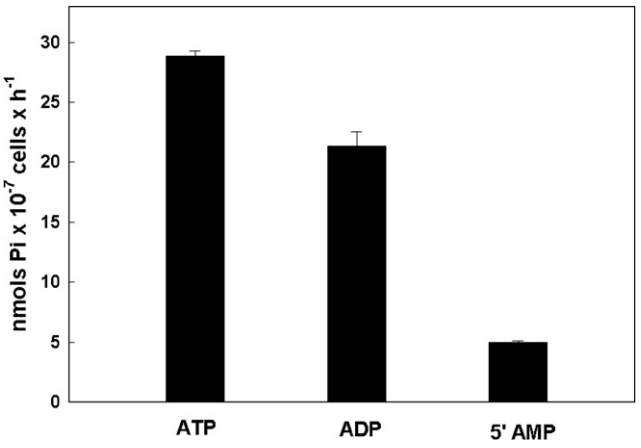

Fig. 5. Ecto-phosphohydrolase activities of intact cells of *T. brucei brucei*. Cells were incubated for 1 h at 30 °C in the same reaction medium (final volume: 0.5 mL) as that described in the legend of Fig. 1, in the presence of 5 mM of either ATP, ADP, or 5′-AMP. Bars: total activity measured in the presence of 5 mM $MgCl_2$. In these experiments ATP hydrolysis were measured using the same spectrophotometric assay (described in Section 2) for Pi release as that used for the other nucleotides. Data are means ± SE of three determinations with different cell suspensions.

Table 2
Substrate specificity of Mg-dependent ecto-ATPase activity

Nucleotides	Relative activity (%)
ATP	100.0 ± 8.5
CTP	94.7 ± 4.0
GTP	97.8 ± 8.6
ITP	128.2 ± 4.2
UTP	94.1 ± 7.2
TTP	31.7 ± 0.6
ADP	72.2 ± 7.6

Note. The ecto-nucleotidase activity was measured in medium containing the nucleotides listed (5 mM), 50 mM Hepes, pH 7.2, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, and 1.0×10^7 cells/mL in the absence or in the presence of 5 mM $MgCl_2$. The Mg^{2+}-dependent ATP hydrolysis was taken as 100% (23.98 ± 2.3 nmol Pi $\times 10^{-7}$ cells $\times h^{-1}$). The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and were converted to a percentage of the control value. In this experiments release of Pi from all nucleotides, including ATP, was measures using a spectrophotometrical assay as described in Section 2.

because of their charge, are not permeable to the plasma membrane.

Therefore, using ion-pair reversed-phase HPLC, we identified and quantified the nucleotides sequentially generated from the ATP degradation by the surface-located enzymes in *T. brucei brucei* (Fig. 6). The concentration of ATP decreased by approximately 60% in 120 min of reaction, generating 58.8 ± 8.4 μM of ADP. During the course of 120 min, the highest AMP concentration (5.3 ± 0.4 μM) was accompanied by its hydrolysis yielding adenosine. A simple possible role for the ecto-ATPase of *T. brucei brucei* could be the degradation of nucleotides in the surroundings of the parasite, since this parasite lack the ability to synthesize purines de novo, and their growth and survival is dependent upon the scavenging of these essential nutrients and their derivatives from the external medium (De Koning et al., 1998, 2000). We also inferred that adenosine molecules could be transported into the parasite, probably via an adenosine transporter (De Koning et al., 1998, 2000).

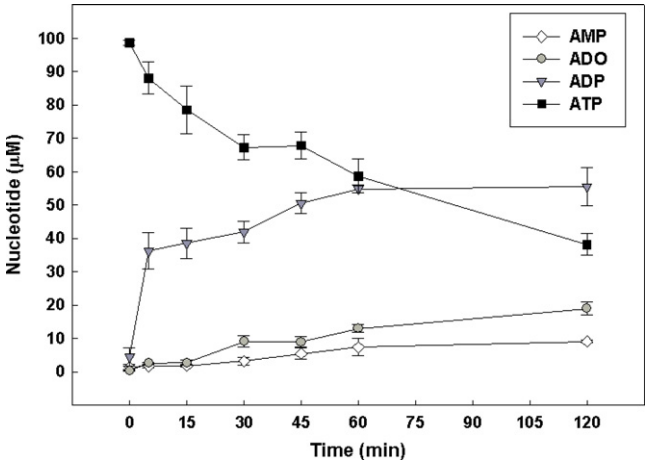

Fig. 6. Analysis of ATP hydrolysis by procyclic form of *T. brucei brucei*. The parasites (10^8 cells/mL) were incubated for each indicated period of time at 30 °C in the presence of 100 μM ATP. The amount of nucleotides (ATP, ADP, and AMP) and nucleoside (adenosine) was determined by HPLC, as described in Section 2. Data are means ± SE of three independent determinations.

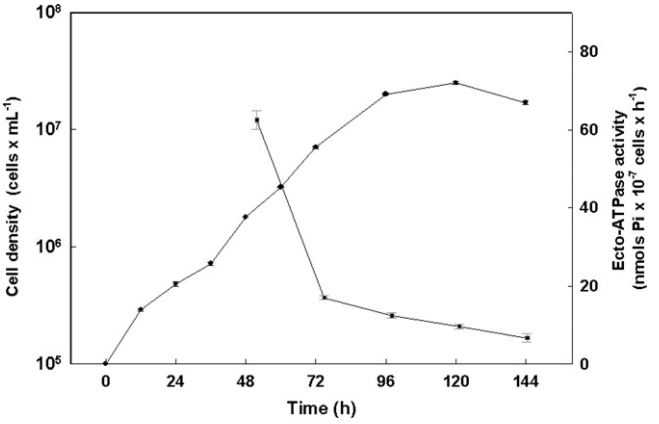

Fig. 7. Regulation of Mg^{2+}-dependent ecto-ATPase activity in *T. brucei brucei* during growth cycle. Cells were taken from a culture of *T. brucei brucei* at near mid-log phase were harvested, washed twice and seeded into fresh medium and grown for the indicated times before being harvested and assayed for ecto-ATPase activity. At each time point, cells were incubated for 1 h at 30 °C in the same reaction medium (final volume 0.5 mL) as that described in the legend of Fig. 1, in the absence or in the presence of 5 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 $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions. Cell densities at the time of assay are indicated (●).

The physiological role of the ecto-ATPases in protozoa parasites is still unknown, but a possible involvement in parasite proliferation has been proposed (Meyer-Fernandes, 2002; Meyer-Fernandes et al., 2004; Fonseca et al., 2006). Fig. 7 shows that the Mg^{2+}-dependent ATPase activity decreased during the time course of cell growth. The Mg^{2+}-dependent ATPase activity is ninefold higher on the second day than on the sixth day. This data could be indicating that this enzyme would be important for the development of *T. brucei brucei*. Accordingly, Fig. 8 shows that ATP supplementation to the medium was able to replace adenosine supplementation on the stimulation of the cell growth indicating that the sequential hydrolysis of ATP to adenosine: ATP → ADP → AMP → adenosine, would make adenosine available to *T. brucei brucei*. The levels of ecto-ATPase activity from parasites grown in a medium supplemented with 100 μM ATP were compared to the *T. brucei brucei* grown in a control medium depleted of purine. As shown in Fig. 9, parasites grown in control medium showed a twofold increase in ecto-ATPase activity when compared to parasites grown in a medium supplemented with 100 μM ATP.

4. Discussion

This paper reports the presence of Mg-dependent ecto-ATPase on the external surface of *T. brucei brucei*. Cellular integrity and viability were assessed, before and after the reactions, by mobility and by Trypan blue dye exclusion. The integrity of the cells was not affected by any of the

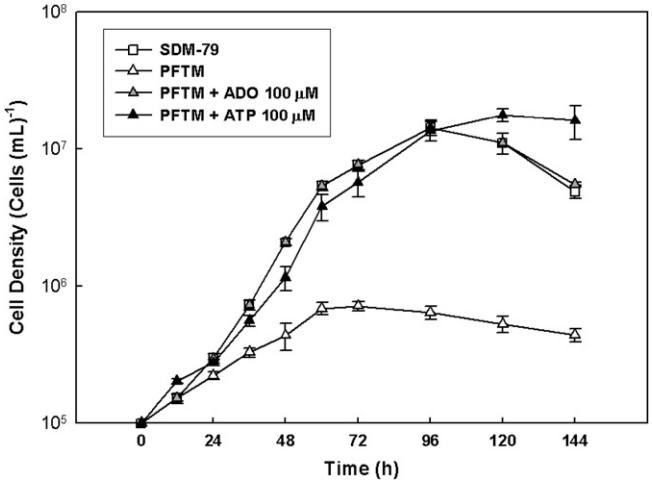

Fig. 8. Growth of procyclic *T. brucei brucei* in the presence of adenosine or ATP. Procyclic cells growing in mid-log phase were harvested, washed twice in Purine free trypanosome medium (PFTM) without Purine supplement and seeded at 10^5 cells/mL in various medium as indicated. Data are means ± SE of three determinations in triplicate with different cell suspensions. Samples were taken at regular intervals and cell density determined in triplicate, using a hemocytometer.

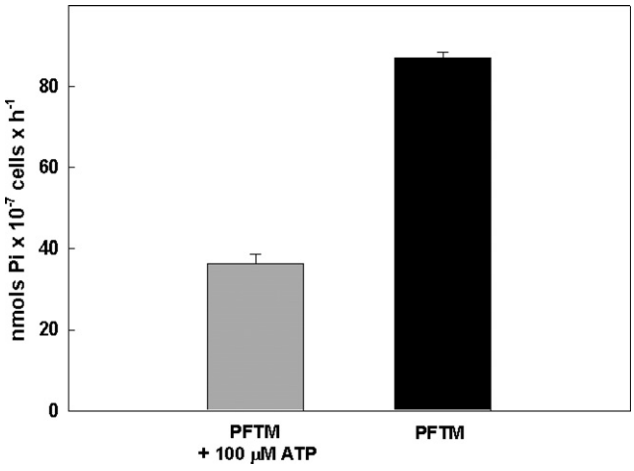

Fig. 9. Mg^{2+}-dependent ecto-ATPase activity in *T. brucei brucei* procyclics after 72 h in purine free trypanosome medium (PFTM) or in PFTM supplemented with ATP during growth cycle. Parasites were seeded into PFTM (gray bars) or in PFTM supplemented with 100 μM ATP (black bars) and grown for 72 h. Cells were harvested and incubated for 1 h at 30 °C in the same reaction medium (final volume 0.5 mL) as that described in the legend of Fig. 1, in the absence or in the presence of 5 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 $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

conditions used in the assays. The external location of the ATP-hydrolyzing site is supported by its sensitivity to the impermeable inhibitor DIDS (Fig. 4, panel A) (Knowles, 1988; Barbacci et al., 1996; Meyer-Fernandes et al., 1997). Moreover, a battery of inhibitors for other ATPases that have intracellular ATP binding sites showed no effect on the ecto-ATPase activity (Table 1). The Mg-dependent ATPase activity reported in this work could not be attributed to a mitochondrial ATPase, since this activity was insensitive to oligomycin (Table 1), a known F-type ATPase inhibitor (Meyer-Fernandes et al., 1997). Since the Mg-dependent ecto-ATPase activity was also insensitive to vanadate (Table 1), the possibility that this activity was due to a P-ATPase present on the surface of the plasma membrane was discarded. For these reasons, we assign an ecto-localization for the Mg-dependent ATPase activity described here. ATP hydrolysis could not be due to a phosphatase activity present on the external surface of *T. brucei brucei* membrane (Fernandes et al., 1997, 2003), because as shown in Table 1 potent inhibitors for phosphatase activities were not capable of 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, a potent inhibitor of 5′ nucleotidase (Gottlieb and Dwyer, 1983) (Table 1).

Most of the ecto-ATPases are Mg^{2+} or Ca^{2+} stimulated (Plesner, 1995). The addition of $CaCl_2$, $ZnCl_2$, $MgCl_2$, and $MnCl_2$ to the extracellular medium stimulated the ecto-ATPase activity (Fig. 1A). The ecto-ATPase activity present in *T. brucei brucei* hydrolyses ATP, ITP, GTP, CTP, and UTP at high rates (Table 2) as also observed with the ecto-ATPase present on the surface of *Trypanosoma cruzi* (Meyer-Fernandes et al., 2004) and *Trypanosoma rangeli* (Fonseca et al., 2006). ADP was also recognized as substrate, indicating that this enzyme is an authentic nucleoside triphosphate diphosphohydrolase as described in other cells (Wang and Guidotti, 1996; Barros et al., 2000; Fonseca et al., 2006).

Trypanosoma brucei brucei, as well as *Leishmania amazonensis*, are pathogens which cannot synthesize purines de novo (De Koning et al., 2000; Berredo-Pinho et al., 2001). It has been postulated that these ecto-ATPases in protozoa parasites could play a role in the salvage of purines from the host cells (Berredo-Pinho et al., 2001; Meyer-Fernandes, 2002). The ability of *T. brucei brucei* to hydrolyze ATP, ADP, and AMP (Fig. 5) might sequentially dephosphorylate ATP to adenosine: ATP → ADP → AMP → adenosine, indicating that this enzyme in *T. brucei brucei* might play a role in the salvage of purines from extracellular medium.

The recently identified family of ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase family) contains multiple members that differ in their substrate specificities and cellular locations (Zimmermann, 1999). It has been demonstrated that mammalian membrane-associated ecto-ATPDase was homologous to human CD39 (or E-NTPDase1), a lymphoid cell activation antigen (Handa and Guidotti, 1996). Wang and Guidotti discovered that CD39 has sequence homology with a potato apyrase and that CD39 has apyrase activity (Wang and Guidotti, 1996). Their work led to the identification of a family of ecto-ATPases that are related in sequence but vary in their membrane topology and tissue distribution (Plesner, 1995; Zimmermann, 1999; Goding, 2000). Further characterization of cloned members of proteins related to CD39 allowed the suggestion of a unifying nomenclature.

All members of the CD39-ATP diphosphohydrolase family belong to the E-NTPDase family (Lemmens et al., 2000; Zimmermann, 2000).

Recently, *T. brucei* genome was totally sequenced (Berriman et al., 2005). We could identify two genes, Tb927.7.1930 and Tb927.8.3800, that encode hypothetical proteins with significant similarity to human E-NTPDases 1, 2 and 3 (Accession Nos. AAB32152, NP_982293, and O75355, respectively). The presence of two putative enzymes in *T. brucei* may explain the existence of two subpopulations of E-NTPDases activities: one sensitive and another insensitive to suramin inhibition (Fig. 3B). Elucidation of the primary sequence of the enzymes responsible for these two *T. brucei brucei* E-NTPDase activities described here will be required to positively identify these enzymes as members of this family.

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