Characterization and cytochemical localization of an ATP diphosphohydrolase from *Leishmania amazonensis* promastigotes

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SUMMARY

An ATP diphosphohydrolase was identified in the plasma membranes isolated from promastigote forms of *Leishmania amazonensis*. Both ATP and ADP were hydrolysed at similar rates by the enzyme. Other nucleotides such as UTP, GTP and CTP were also degraded, revealing a broad substrate specificity. Adding ATP and ADP simultaneously, the amount of hydrolysis achieved was compatible with the presence of a single enzyme. ATPase activity was not affected by addition of vanadate, ouabain, thapsigargin, dicyclohexylcarbodiimide, oligomycin and bafilomycin A, thus excluding involvement of P-, F- and V-type ATPases. The effects of pH in the range 6·5–8·5 were examined using ATP or p-NPP as substrate. At pH 7·4, the phosphatase activity decreased, and did not show a significant contribution to ATP hydrolysis. In addition, the enzyme was not inhibited by levamisole and ammonium molybdate, excluding alkaline phosphatase and nucleotidase activities, respectively. Sodium azide (5–10 mM) caused inhibition of the ATP and ADP hydrolysis in a dose-dependent manner. Calcium was the best activating metal ion for both ATPase and ADPase activities. Ultrastructural cytochemical microscopy showed ATP diphosphohydrolase on the surface and flagellar pocket of the parasite. We have proposed that *L. amazonensis* ATP diphosphohydrolase may participate in the salvage pathway of nucleosides.

Key words: ATP diphosphohydrolase, apyrase, *Leishmania amazonensis*, promastigote.

INTRODUCTION

Leishmania are digenetic protozoan parasites which live as promastigotes in the digestive tract of sandflies and as amastigotes in the phagolysosomes of mammalian macrophages. Leishmaniasis results in a broad spectrum of diseases in which clinical manifestations are dependent on both parasite species and immune responses of the host (Brandão-Filho & Shaw, 1994). The increase in the incidence of the disease, associated with higher morbidity rates, the spread of some of the forms of leishmaniasis to new geographical areas and *Leishmania*–HIV co-infection, has become an important public health problem in the world (Brandão-Filho & Shaw, 1994; Wolday *et al*. 1999).

In leishmaniasis, the identification, location and characterization of proteins in parasites are essentials to the understanding of the host–parasite relationship and/or for the development of improved therapies. In several *Leishmania* species, the presence of phosphohydrolases has been characterized, such as 3′-nucleotidase, 5′-nucleotidase, phosphatases, Ca^{2+}-ATPase, H^+-ATPase, Na^+/K^+-ATPase and Mg^{2+}-ecto-ATPase (Schneider, Bordier & Etges, 1992; Corte-Real, Porrozi & Meirelles, 1993; Felibertt *et al*. 1995; Meyer-Fernandes *et al*. 1997). However, few descriptions of the biochemical properties of individual *Leishmania amazonensis* membrane proteins are available in the literature.

ATP diphosphohydrolases have been characterized in plants, mammals and parasites, including *Schistosoma mansoni*, *Toxoplasma gondii* and *Entamoeba histolytica* (Vasconcelos *et al*. 1993; Bermudes *et al*. 1994; Plesner, 1995; Barros *et al*. 2000). The family of ATP diphosphohydrolases, E-ATPases, was recently described. These enzymes share several common features, such as ability to hydrolyse di- and triphosphate nucleosides and activation by bivalent cations (Vasconcelos *et al*. 1996; Torres *et al*. 1998; Zimmermann, 1999). In this work, we characterize a novel ATP diphosphohydrolase, or

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apyrase (EC 3.6.1.5), present on the *L. amazonensis* promastigote. It is the first demonstration of an ecto-ATP diphosphohydrolase in the *Leishmania* genus.

MATERIALS AND METHODS

Chemicals

Nucleotides, ouabain, sodium azide, sodium orthovanadate, dicyclohexylcarbodiimide, oligomycin, thapsigargin, bafilomycin A, and protease inhibitors were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of the highest analytical grade available.

Animals

Female Balb/c mice aged 4 to 8 weeks were used. These mice were originally obtained from Jackson Laboratories, Bar Harbor, Maine (USA) and have been propagated in the Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

Parasites

The MHOM/Br/76/Ma strain of *L. amazonensis* isolated from a patient with diffuse cutaneous leishmaniasis (Schottelius & Gonçalves da Costa, 1982) has been maintained *in vivo* by serial passages in Balb/c mice. Purified suspensions were obtained from non-ulcerated histiocytoma 4 months after subcutaneous injection of the *L. amazonensis* strain in female Balb/c mice. Parasites were harvested and purified after the rupture of the tissue nodules in a Potter-Elvehjem homogenizer with a Teflon pestle in phosphate-buffered saline. Cells were then filtered through gauze using a 10 ml syringe. The filtered suspension was generally free from intact host cells. Evaluation of the percentage of damaged amastigotes cells was made using erythrosin B strain, as described elsewhere (Hodgkinson, Herman & Semprevivo, 1980). This suspension was then put in LIBHIT medium (Gonçalves da Costa & Lagrange, 1981) in a concentration of 10^4 cells.

Isolation of the plasma membrane fraction

A plasma membrane fraction from promastigote forms was obtained as previously described by Pereira *et al*. (1978). Isolated plasma membrane was stored at −70 °C until use, in the presence of 5 mM Tris–HCl, pH 7·4, 8% sucrose plus the protease inhibitors leupeptin (0·5 μg/ml), pepstatin (0·07 μg/ml), soybean trypsin inhibitor (50 ng/ml), and phenylmethylsulfonyl fluoride (2 μg/ml). Protein determination was performed by Lowry's method (Lowry *et al*. 1951).

Activity measurements

Activity measurements were performed in standard reaction medium containing 50 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer, pH 7·4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0·01–0·05 mg protein/ml and 4 mM of either ADP or ATP, unless otherwise specified. The reaction was initiated by addition of substrate, allowed to proceed for 30 min at 37 °C and the amount of inorganic phosphate (Pi) liberated was determined according to the method described by Taussky & Shorr (1953). Incubation times were chosen to ensure the linearity of the reaction with substrate and protein content. The *p*-nitrophenylphosphatase activity was determined in the same medium described above, using 4 mM *p*-nitrophenylphosphate as substrate. The reaction was arrested by addition of 0·5 M NaOH. The released *p*-nitrophenol was determined spectrophotometrically at 400 nm using an extinction coefficient of $18{\cdot}8 \times 10^{-3}$/M/cm.

Electron microscopy

For cytochemical detection of ATP diphosphohydrolase activity, the *L. amazonensis* promastigote forms were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0·1 M Na-cacodylate buffer, pH 7·4, for 30 min. Subsequently, the cells were washed with the cacodylate buffer and incubated in the cytochemical medium, which contained 50 mM MOPS, pH 7·4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM vanadate, 1 mM ouabain, 100 μM of the ammonium molybdate, 10 mM oligomycin, 1 mM levamisole, 2 mM $Pb(NO_3)_2$ and 3 mM of either ATP or ADP for 1 h at 37 °C. The specificity of the reaction was checked by controls run in parallel in which the incubations were performed in the above medium without substrates. Post-fixation was done in 1% OsO_4 in Na-cacodylate buffer for 1 h at 4 °C. Dehydration was done in acetone and inclusion in Epon. Stained and unstained thin sections were observed in an EM 10C Zeiss electron microscope.

RESULTS

The presence of phosphohydrolytic activity was assayed in samples of plasma membrane fractions from *L. amazonensis* promastigotes using different nucleotide substrates. Table 1 shows that the plasma membrane fraction contained a phosphohydrolytic activity that was equally effective at hydrolysis of either ATP or ADP, in the range of 137 ± 35 nmol Pi/mg/min or 143 ± 29 nmol Pi/mg/min (mean ± S.D.), respectively. In the presence of ADP (4 mM) and ATP (4 mM) simultaneously in the assay medium, the phosphohydrolytic activity was close to the values obtained in the presence of each nucleotide

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Table 1. Substrate specificity of *Leishmania amazonensis* ATP diphosphohydrolase

(Enzyme activity was measured in the standard reaction medium in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ with 4 mM substrate as described in the Materials and Methods section. Values represent averages ± S.D. and the numbers of determinations are shown in parentheses, using different plasma membrane preparations. *p*-NPP, *p*-nitrophenylphosphate; PPi: inorganic pyrophosphate.)

Substrate	Specific activity (nmol Pi/mg/min)	Relative activity
ATP	137 ± 35 (20)	1·00
ADP	143 ± 29 (11)	1·04
ATP + ADP*	169 ± 21 (4)	1·23
UTP	210 ± 10 (2)	1·50
GTP	83 ± 15 (2)	0·60
CTP	31 ± 20 (2)	0·23
5′-AMP	32 ± 14 (6)	0·23
PPi	0 (2)	0·0
p-NPP	13 ± 2 (2)	0·09

* In this case 4 mM each of ADP and ATP were used.

Table 2. Effects of inhibitors on ATP hydrolysis

(ATPase activity is expressed as a percentage of the control that was measured in the standard reaction medium, in the absence of the compound indicated, with or without 2·5 % (by vol.) dimethyl sulfoxide or ethanol, which is the solvent used for preparing dicyclohexylcarbodiimide (DCCD), bafilomycin or thapsigargin. Results are the mean ± S.D. for the number of determinations shown in parentheses, using different preparations of plasma membrane fractions.)

Compounds	Relative activity (%)
Molybdate (0·1 mM)	115 ± 31 (3)
Vanadate (0·1 mM)	112 ± 11 (4)
Ouabain (1 mM)	101 ± 2·6 (4)
DCCD (0·1 mM)	109 ± 8·8 (8)
Thapsigargin (1·5 μM)	122 ± 10 (2)
Bafilomycin A (10 μM)	103 ± 4 (2)
All inhibitors*	109 ± 20 (8)

* In this experiment, ATP hydrolysis was measured in the presence of 1 mM ouabain, 100 μM vanadate, 100 μM molybdate and 1 mM azide, simultaneously.

independently, rather than the sum of the activities with each nucleotide (Table 1). In addition, broad substrate specificity was observed under our experimental conditions. Table 1 shows that UTP (210 ± 10 nmol Pi/mg/min), GTP (83 ± 15 nmol Pi/mg/min) and CTP (31 ± 20 nmol Pi/mg/min) were also degraded by the plasma membrane preparations, suggesting that the presence of an ATP diphosphohydrolase in *L. amazonensis* promastigote was responsible by the hydrolysis of di- and triphosphate nucleosides analysed. Hydrolysis of 5′-AMP could also be detected (Table 1) to a small extent (32 ± 14 nmol Pi/mg/min), and was probably related to the presence of a 5′-nucleotidase activity previously described (Corte-Real *et al*. 1993). However, this hydrolytic activity did not contribute to an overestimation of ATPase or ADPase activity, since hydrolysis of ATP was not significantly affected by the addition of 100 μM of ammonium molybdate (Table 2), which has been described as an inhibitor of 5′-nucleotidases (Gottlieb & Dwyer, 1983).

Possible enzyme combinations that might lead to an apparent ATP diphosphohydrolase activity as an artifact were excluded (Tables 1 and 2). The possibility of the combined action of an ATP pyrophosphohydrolase and an inorganic pyrophosphatase was discarded because no Pi was released when PPi (pyrophosphate) was used as substrate (Table 1). To verify the possible relation of the ATPase activity observed in this work with classical well-known ATPases, several inhibitors were tested (Table 2). ATPase activity was insensitive to 100 μM vanadate (Table 2), a specific inhibitor of P-type cation transport ATPases (Carafoli, 1991). In addition, ouabain (1 mM), an inhibitor of Na^+/K^+-ATPase (Felibertt *et al*. 1995), and thapsigargin (1·5 μM), an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Treiman, Caspersen & Christensen, 1998), did not affect ATPase activity (Table 2). Hydrolysis was also insensitive to 10 μM bafilomycin A, a macrolide antibiotic inhibitor of vacuolar ATPases (Bowman, Siebers & Altendorf, 1988), and to 100 μM dicyclohexylcarbodiimide (DCCD), a mitochondrial ATPase inhibitor (Weber & Senior, 1998) (Table 2).

Low concentrations of sodium azide (1 mM), but far higher than necessary to inhibit mitochondrial ATPase (100 μM) (Knowles & Nagy, 1999), did not inhibit the ATP or ADP hydrolysis significantly (approximately 15 %, Fig. 1). However, it is interesting to note that when sodium azide was tested at higher concentrations (5–10 mM), under our experimental conditions, a significant and parallel ATP and ADP hydrolysis inhibition occurred (Fig. 1). With 10 mM azide, inhibition of ATP and ADP hydrolysis was approximately 50 %. The azide concentration dependence was similar for inhibition of either ATPase or ADPase activities, supporting the idea that a single enzyme degraded ATP and ADP.

In order to exclude phosphatase activities, the effects of pH in the range 6·5–8·5 were examined using ATP or p-nitrophenylphosphate (*p*-NPP) as substrate (Fig. 2). It can be observed that at pH 7·5 the ATPase activity from plasma membrane was approximately 140 nmol Pi/mg/min and increased concomitantly as the pH became higher (Fig. 2). In contrast, the p-NPPase activity began to decline until it reached the basal level, corresponding to < 10 % of ATPase activity at pH 8·5 (Fig. 2). Therefore, at pH 7·5, the phosphatase activity does

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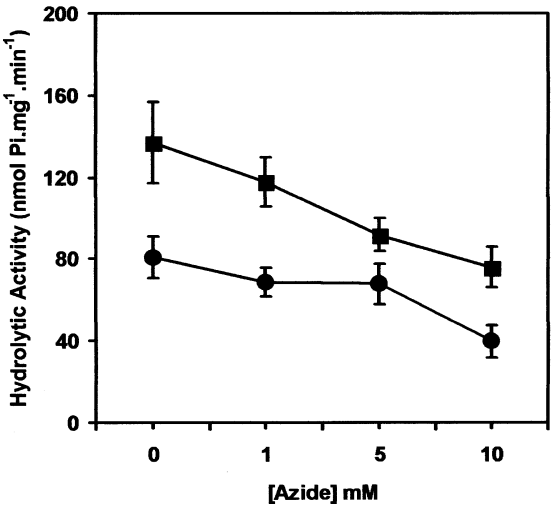

Fig. 1. Effect of azide on ATP diphosphohydrolase activity of plasma membrane fractions from *Leishmania amazonensis* promastigotes. ATPase (■) and ADPase (●) activities were measured in the standard assay which contained 50 mM MOPS, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0–10 mM NaN_3 and 0·03 mg of protein/ml, as described in the Materials and Methods section. Values represent averages ± S.D. for 8 determinations, using different preparations of plasma membranes.

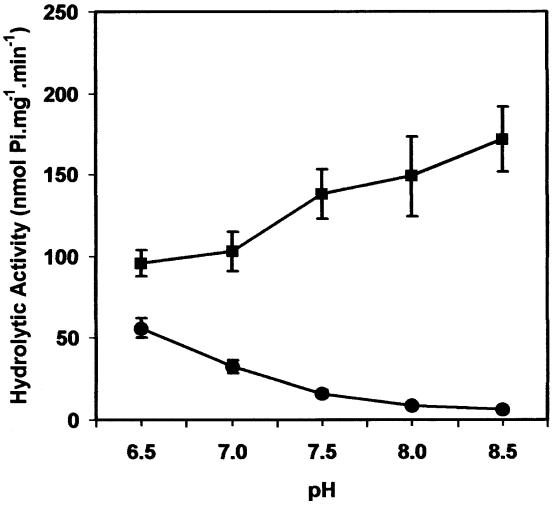

Fig. 2. Effect of pH on the ATPase and *p*-nitrophenylphosphatase activities of plasma membrane fractions from *Leishmania amazonensis* promastigotes. ATPase and *p*-NPPase activities were determined as described in the Materials and Methods section in a medium containing 4 mM of ATP or *p*-NPP. Aliquots of plasma membranes (0·03 mg of protein/ml) and 50 mM MOPS–Tris buffer, adjusted to pH values between 6·5 and 8·5 with HCl and Tris. ATPase activity (■); *p*-NPPase activity (●). Values represent averages ± S.D. for 3 determinations, using different preparations of plasma membranes.

not show a significant contribution to ATP hydrolysis. Furthermore, vanadate that inhibited acid phosphatase activity (Meyer-Fernandes *et al*. 1997), did not affect ATPase activity (Table 2). In addition, the hydrolysis of ATP was not affected by 1 mM levamisole (data not shown), an alkaline phosphatase inhibitor (Van Belle, 1972), and no significant hydrolysis was observed when *p*-NPP was used as a substrate in standard reaction medium (Table 1).

Table 3. Cation dependence on the ATPase and ADPase activities

(Cation concentration was 1 mM while ATP or ADP concentration was 4 mM. Each value is the mean of 3 experiments ± S.D. In the absence of added bivalent cations, ATPase activity was 38 ± 13 (%) and ADPase activity was 37 ± 0·7 (%) of the total measurable activity in the presence of 1 mM of Ca^{2+}.)

Cation	ATPase activity (%)	ADPase activity (%)
Ca^{2+}	100	100
Mg^{2+}	61 ± 24	83 ± 3
Zn^{2+}	70 ± 6	26 ± 5·6
Co^{2+}	73 ± 4	40 ± 3·5
Mn^{2+}	65 ± 6	52 ± 2

Hydrolysis of ATP or ADP catalysed by the ATP diphosphohydrolase from *L. amazonensis* was measured in the presence of different bivalent metal ions (Table 3). On comparison of the effects of cations on ATPase and ADPase activities, different patterns were observed. Ca^{2+} was the best activating metal ion for the ATPase activity. Other bivalent cations, such as Mg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+}, also activated the ATP hydrolysis but were much less effective than Ca^{2+} (Table 3). The hydrolysis of ADP was also more stimulated in the presence of Ca^{2+} (1 mM), but Zn^{2+} and Co^{2+} had no effect (Table 3). In the absence of added bivalent cations, a residual ATPase or ADPase activity was observed, equivalent to approximately 37 % of the total measurable activity in the presence of 1 mM of Ca^{2+} (Table 3). This endogenous activity could be removed completely by the addition of 1 mM EDTA (result not shown), indicating the presence of ion contaminants in the plasma membrane preparation.

Ecto-localization of ATP diphosphohydrolase in *L. amazonensis* promastigotes was obtained by ultrastructural cytochemical techniques (Fig. 3A–D). Enzyme activity was found distributed as an electro-dense lead phosphate deposit at the plasma membrane surface, flagellar pocket, and flagellar membrane of *L. amazonensis* promastigote forms when they were incubated in the cytochemical complete medium containing ATPases, nucleotidase and alkaline phosphatase inhibitors plus ADP (Fig. 3A) or ATP (Fig. 3B) as substrate. The enzyme activity appeared to be homogeneously and identically distributed at the external surface of the parasite when different substrates were used (Fig. 3C and D). In the absence of nucleotides, controls showed no electron-dense deposits (data not shown).

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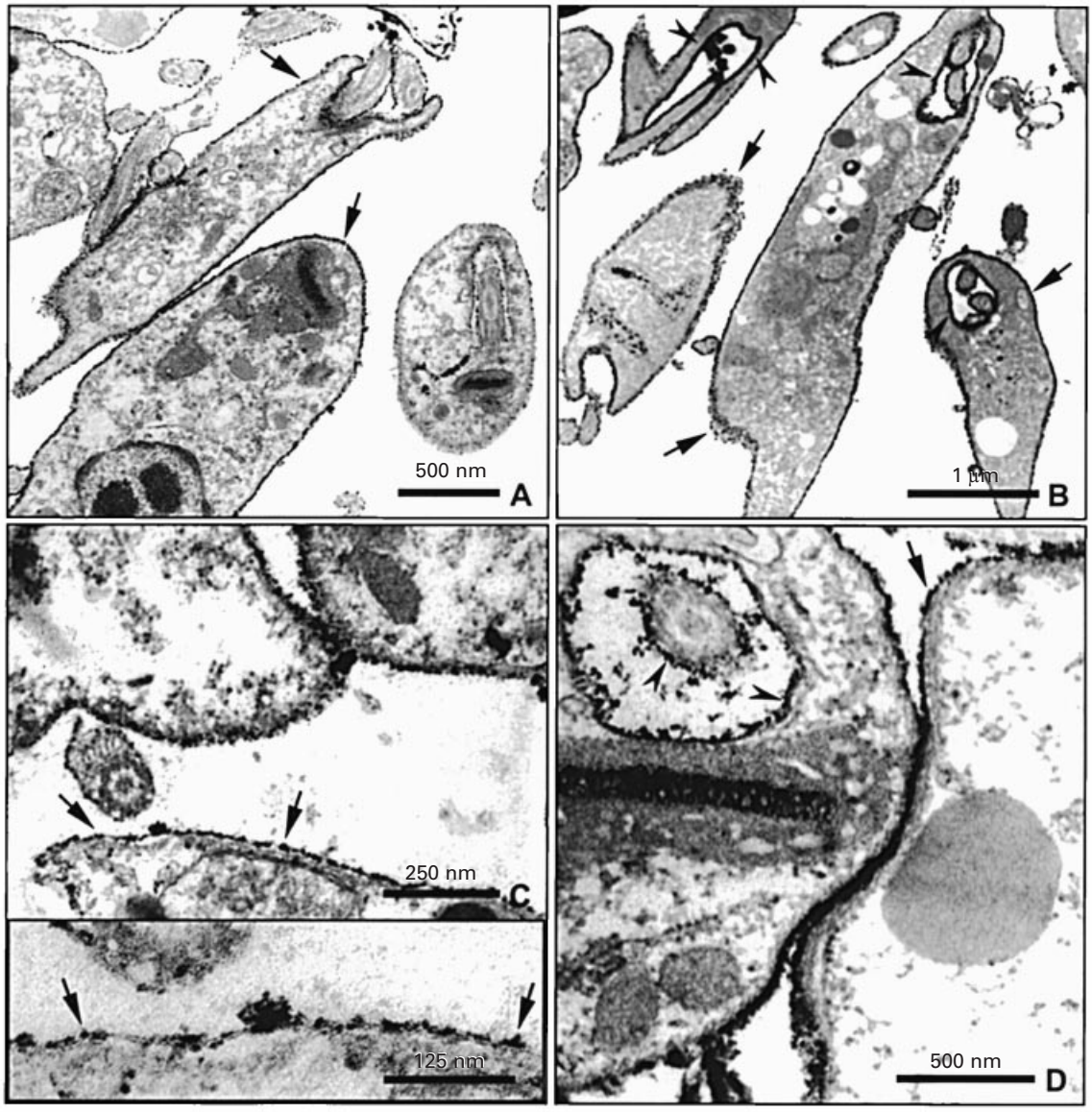

Fig. 3. Ultrastructural localization of the enzymatic activity (A, B). General aspect of *Leishmania amazonensis* promastigotes, showing homogeneously distributed electron-dense lead phosphate deposits originating from ADP (A) and ATP (B) hydrolysis at the surface of the plasma membrane of the parasites (arrows) and at flagellar pocket membrane and flagellum (arrowheads). (C) ADPase activity can be seen as an electron-dense product associated at the external surface of the plasma membrane (arrows). The *inset* shows the enlargement of a region of the plasma membrane. (D) ATPase activity is seen at the external surface of the plasma membrane (arrow) and at flagellar pocket membrane and flagellum (arrowheads).

DISCUSSION

This work shows the presence of ecto-ATP diphosphohydrolase on *L. amazonensis* promastigotes. The first evidence that a single enzyme present in the plasma membrane preparation degraded ADP and ATP was obtained by a classical procedure for characterization of an enzyme acting on 2 substrates (Dixon & Webb, 1979), shown in Table 1. The protocol of the mixed substrate experiment does not exclude the possibility that a nucleotide might inhibit the hydrolysis of each other. That might occur, for example, if 2 distinct enzymes are present in the preparation. However, our data of broad substrate specificity for di- and triphosphate nucleosides, activation by bivalent cations, insensitivity to specific inhibitors of phosphatases, 5′-nucleotidase and the F-, P-, and V-type ATPases, common features found in ATP diphosphohydrolases of several origins (Plesner, 1995), exclude other phosphohydrolases and, taken together, suggest strongly that a true ATP diphosphohydrolase is present in *L. amazonensis* promastigotes.

Sodium azide (10 mM) caused inhibition of ATP and ADP hydrolysis corresponding to about 50 % of the total activities. Sensitivity to azide inhibition has been generally considered as an important criterion in distinguishing the ecto-ATP diphosphohydrolase,

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described in this work, from the ecto-ATPase, which appear not to hydrolyse diphosphate nucleosides nor to be inhibited by azide (Knowles & Nagy, 1999). These enzymes constitute subfamilies of the E-ATPases, a new family recently described (Vasconcelos *et al*. 1996; Zimmermann, 1999).

We showed that *L. amazonensis* ATP diphosphohydrolase was activated by bivalent ion metals. Ca^{2+} was the best activating metal ion for both ATPase and ADPase activities. Recently ecto-ATPase activity was described in *L. tropica* (Meyer-Fernandes *et al*. 1997). ATP was the best substrate for this enzyme and other nucleotides such as ADP, GTP and UTP produced lower reaction rates. Furthermore, it is stimulated by $MgCl_2$ but not by $CaCl_2$ (Meyer-Fernandes *et al*. 1997), and thus cannot be related to the ATP diphosphohydrolase described here.

Ecto-localization of *L. amazonensis* ATP diphosphohydrolase confirms that this parasite possesses mechanisms capable of hydrolysing nucleoside di- and triphosphates, and that the expression of ATP diphosphohydrolase is associated with the outer surface of the plasma membrane of parasite. A possible role for the ATP diphosphohydrolase of *L. amazonensis* could be the degradation of nucleotides in the surroundings of the parasite. All parasitic protozoa so far examined lack the ability to synthesize purines *de novo*, and their growth and survival is dependent upon the scavenging of purines and their derivatives from the host (Bermudes *et al*. 1994). Then, we hypothesize that the ATP diphosphohydrolase, together with a 5′-nucleotidase, also present in the *L. amazonensis* plasma membrane (Corte-Real *et al*. 1993), participate in the acquisition of these essential nutrients. Furthermore, saliva from *Lutzomyia longipalpis* the sand-fly that transmits the *Leishmania* protozoan, contains significant levels of endogenous ATP diphosphohydrolase. This enzyme prevents platelet aggregation by degradation of pro-aggregatory ADP and, together with a 5′-nucleotidase, permits accumulation of adenosine, a potent vasodilator, by degradation of AMP (Charlab *et al*. 1999). Thus, we hypothesize that the ecto-ATP diphosphohydrolase and the 5′-nucleotidase from *Leishmania* promastigotes, the infective form of the parasite, and the vector saliva may, together, facilitate blood-feeding and transmission of leishmaniasis.

It has been demonstrated that most published data concerning kinetic parameters of ATP diphosphohydrolases were obtained with preparations often containing ATP diphosphohydrolase isoforms and it was necessary to analyse the different catalytic properties in purified isozymes (Bermudes *et al*. 1994; Vasconcelos *et al*. 1996). The purification of the ATP diphosphohydrolase from *L. amazonensis* is being carried out in our laboratory and should enable us to obtain information about individual kinetic characteristics, function, structural aspects and similarities with ATP diphosphohydrolases from different origins.

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REFERENCES

BARROS, F. S., DE MENEZES, L. F., PINHEIRO, A. A. S., SILVA, E., LOPES, A. H. C. S., DE SOUZA, W. & MEYER-FERNANDES, J. R. (2000). Ectonucleotide diphosphohydrolase activities in *Entamoeba histolytica*. *Archives of Biochemistry and Biophysics* **375**, 304–314.

BERMUDES, D., PECK, K. R., AFIFI, M. A., BECKERS, C. J. M. & JOINER, K. A. (1994). Tandemly repeated genes encode nucleoside triphosphate hydrolase isoforms secreted into the parasitophorous vacuole of *Toxoplasma gondii*. *Journal of Biological Chemistry* **269**, 29252–29260.

BOWMAN, E. M., SIEBERS, A. & ALTENDORF, K. (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proceedings of theNational Academy of Sciences, USA* **85**, 7972–7976.

BRANDÃO-FILHO, S. & SHAW, J. (1994). Leishmaniasis in Brazil. *Parasitology Today* **10**, 329–330.

CARAFOLI, E. (1991). Calcium pump of the plasma membrane. *Physiology Reviews* **71**, 129–153.

CHARLAB, R., VALENZUELA, J. G., ROWTON, E. D. & RIBEIRO, I. M. (1999). Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*. *Proceedings of the National Academy of Sciences, USA* **21**, 15155–15160.

CORTE-REAL, S., PORROZI, R. & MEIRELLES, M. N. L. (1993). Immunogold labeling and cerium cytochemistry of the enzyme ecto-5′-nucleotidase in promastigote forms of *Leishmania* species. *Memórias do Instituto Oswaldo Cruz* **88**, 407–412.

DIXON, M. & WEBB, E. C. (1979). *Enzymes*. 3rd Edn. Academic Press, New York.

FELIBERTT, P., BERMÚDEZ, R., CERVINO, V., DAWIDOWICZ, K., DAGGER, F., PROVERBIO, T., MARÍN, R. & BENAIM, G. (1995). Ouabain-sensitive N^+,K^+-ATPase in the plasma membrane of *Leishmania mexicana*. *Molecular and Biochemical Parasitology* **74**, 179–187.

GONÇALVES DA COSTA, S. C & LAGRANGE, P. H. (1981). Development of cell mediated immunity to flagellar antigens and acquired resistance to infection by *Trypanosoma cruzi* in mice. *Memórias do Instituto Oswaldo Cruz* **76**, 367–381.

GOTTLIEB, M. & DWYER, D. M. (1983). Evidence for distinct 5′- and 3′-nucleotidase activities in the surface membrane fraction of *Leishmania donovani* promastigotes. *Molecular and Biochemical Parasitology* **7**, 303–317.

HODGKINSON, V. H., HERMAN, R. & SEMPREVIVO, L. (1980). *Leishmania donovani* correlation among assays of amastigotes viability. *Experimental Parasitology* **50**, 397–408.

KNOWLES, A. F. & NAGY, A. K. (1999). Inhibition of an ecto-ATP-diphosphohydrolase by azide. *European Journal of Biochemistry* **262**, 349–357.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MEYER-FERNANDES, J. R., DUTRA, P. M. L., RODRIGUES, C. O., SAAD-NEHME, J. & LOPES, A. H. C. S. (1997). Mg-dependent ecto-ATPase activity in *Leishmania tropica*. *Archives of Biochemistry and Biophysics* **341**, 40–46.

PEREIRA, N. N., TIMM, S. L., GONÇALVES DA COSTA, S. C., REBELLO, M. A. & DE SOUZA, W. (1978). *Trypanosoma cruzi*: isolation and characterization of membrane and flagellar fractions. *Experimental Parasitology* **46**, 225–234.

PLESNER, L. (1995). Eco-ATPases: identities and functions. *International Review of Cytology* **158**, 141–214.

SCHNEIDER, P., BORDIER, C. & ETGES, R. (1992). Membrane proteins and enzymes of *Leishmania*. In *Subcellular Biochemistry* (ed. Avila, J. L. & Harris, J. R.), pp. 39–72. Plenum Press, New York.

SCHOTTELIUS, J. & GONÇALVES DA COSTA, S. C. (1982). Studies on the relationship between lectin binding carbohydrates and different strains of *Leishmania* from the New World. *Memórias do Instituto Oswaldo Cruz* **77**, 19–27.

TAUSSKY, H. M. & SHORR, E. (1953). A microcolorimetric method for the determination of inorganic phosphorus. *Journal of Biological Chemistry* **202**, 675–685.

TORRES, C. R., VASCONCELOS, E. G., FERREIRA, S. T. & VERJOVSKI-ALMEIDA, S. (1998). Divalent cation dependence and inhibition of *Schistosoma mansoni* ATP diphosphohydrolase by fluorosulfonylbenzoyl adenosine. *European Journal of Biochemistry* **251**, 516–521.

TREIMAN, M., CASPERSEN, C. & CHRISTENSEN, S.B. (1998). A tool coming age: thapsigargin as an inhibitor of sarcro-endoplasmic reticulum Ca^{2+}-ATPases. *Trends in Pharmacological Sciences* **19**, 131–135.

VAN BELLE, H. (1972). Kinetics and inhibition of alkaline phosphatases from canine tissues. *Biochimica et Biophysica Acta* **289**, 158–168.

VASCONCELOS, E. G., FERREIRA, S. T., CARVALHO, T. M. U., DE SOUZA, W., KETTLUN, A. M., MANCILLA, M., VALENZUELA, M. A. & VEJOVSKI-ALMEIDA, S. (1996). Partial purification and immunohistochemical localization of an ATP diphosphohydrolase from *Schistosoma mansoni*. Immunological cross reactivities with potato apyrase and *Toxoplasma gondii* nucleoside triphosphate hydrolase. *Journal of Biological Chemistry* **271**, 22139–22145.

VASCONCELOS, E. G., NASCIMENTO, P. S., MEIRELLES, M. N. L., VERJOVSKI-ALMEIDA, S. & FERREIRA, S. T. (1993). Characterization and localization of an ATP-diphosphohydrolase on the external surface of the tegument of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **58**, 205–214.

WEBER, J. & SENIOR, A. E. (1998). Effects of the inhibitors azide, dicyclohexylcarbodiimide, and aurovertin on nucleoside binding to the three F1-ATPase catalytic sites measured using specific tryptophan probes. *Journal of Biological Chemistry* **273**, 33210–33215.

WOLDAY, D., BERHE, N., AKUFFO, H. & BRITTON, S. (1999). *Leishmania*–HIV interaction: immunopathogenic mechanisms. *Parasitology Today* **15**, 182–187.

ZIMMERMANN, H. (1999). Two novel families of ectonucleotidases: molecular structures, catalytic properties and search for function. *Trends in Pharmacological Sciences* **20**, 231–236.