An Ectonucleotide ATP-diphosphohydrolase Activity in Trichomonas vaginalis Stimulated by Galactose and Its Possible Role in Virulence

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Trichomonas vaginalis, Ecto-ATPase, Ecto-phosphatase, Galactose

This work describes the ability of living Trichomonas vaginalis to hydrolyze extracellular ATP (164.0 ± 13.9 nmol Pi / h × 10^7 cells). This ecto-enzyme was stimulated by ZnCl_2, CaCl_2 and MgCl_2, was insensitive to several ATPase and phosphatase inhibitors and was able to hydrolyze several nucleotides besides ATP. The activity was linear with cell density and with time for at least 60 min. The optimum pH for the T. vaginalis ecto-ATPase lies in the alkaline range. D-galactose, known to be involved in adhesion of T. vaginalis to host cells, stimulated this enzyme by more than 90%. A comparison between two strains of T. vaginalis showed that the ecto-ATPase activity of a fresh isolate was twice as much as that of a strain axenically maintained in culture, through daily passages, for several years. The results suggest a possible role for this ecto-ATPase in adhesion of T. vaginalis to host cells and in its pathogenicity.

Introduction

The parasitic protozoan Trichomonas vaginalis causes human trichomoniasis, a common infection of the urogenital tract. This infection is globally considered one of the most frequent sexually transmitted diseases, with approximately 180 to 200 million cases annually (Petrin et al., 1998). This disease presents various degrees of severity in women, from asymptomatic (nearly 50% of the cases) to extremely acerbic infections (Catterall, 1974). Women who are infected during pregnancy are predisposed to premature rupture of the placental membrane, premature labor, and low-birth-weight infants (Petrin et al., 1998). Also linked to this disease are cervical cancer (Zhang and Begg, 1994), atypical pelvic inflammatory disease, infertility and enhanced HIV transmission (Sorvillo and Kerndt, 1998). T. vaginalis exerts its pathogenic effect when interacting with the surface of epithelial cells, although the mechanisms of the pathogenicity of T. vaginalis are not well defined (Alderete and Pearlman, 1984). Biochemical aspects on the surface membrane constituents of these parasites have been evaluated and may play an important role in the flagellate’s mobility and cytoadhesion (Arroyo et al., 1993).

Surface membrane interactions between parasites and their host cells are of critical importance for the survival of the parasite, from both the immunological and physiological viewpoints (Vanvier-Santos et al., 1995; Martiny et al., 1996, 1999). The plasma membrane of cells contains 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 living cells (Meyer-Fernandes et al., 1997; Furuya et al., 1998). Cell membrane ecto-ATPases are millimolar divalent cation-dependent, low specificity enzymes that hydrolyze all triphosphate nucleotides (Plesner, 1995; Zimmermann, 1999). 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). Their physiological role is until unknown, however, several hypotheses have been suggested, such as (i) protection from cytolysis effects of extracellular ATP.
Ecto-ATPases have been described in some protozoan parasites such as *Toxoplasma gondii* (Asai *et al.*, 1995; Bermudes *et al.*, 1994; Nakaar *et al.*, 1998), *Entamoeba histolytica* (Barros *et al.*, 2000), *Tetrahymena thermophila* (Smith *et al.*, 1998), *Leishmania* sp. (Meyer-Fernandes *et al.*, 1997; Berredo-Pinho *et al.*, 2001), *Trypanosoma cruzi* (Bernardes *et al.*, 2000) and *Tritrichomonas foetus* (Jesus *et al.*, 2002). Here we show the presence of an ecto-ATPase on the cell surface of intact living *T. vaginalis*. We characterized the properties of this enzyme and demonstrate the effects of d-galactose, a carbohydrate exposed on the surface of host cells, involved in adhesion. We also compared the ecto-ATPase activity of a strain maintained axenically for several years in culture with that of a fresh isolate of *T. vaginalis*.

**Materials and Methods**

**Microorganisms and growth conditions**

In this study we used two specimens of *Trichomonas vaginalis*, the JT strain, which has been maintained for several years in culture, as well as a fresh isolate, obtained from a clinically and pathologically confirmed case of human trichomoniasis. The parasites were axenically cultivated in TYM medium (Diamond, 1957), supplemented with 10% fetal calf serum, for 24 hours at 37 °C. The cells at late logarithmic phase of growth were collected by centrifugation at 1,400 × g for 5 min at 4 °C and washed three times with 0.5 mM HEPES pH 7.0, 5.5 mM D-glucose, 5.4 mM KCl and 116 mM NaCl. Cellular viability was assessed, before and after incubations, by mobility and the Trypan blue method (Dutra *et al.*, 1998). The viability of the cells was not affected under the conditions employed here.

**Ecto-ATPase activity measurements**

Intact living parasites were incubated for 1 h at 36 °C in 0.5 ml of a mixture containing, unless otherwise specified, 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50.0 mM HEPES (N-[2-hydroxyethyl]piperezine-N’-[2-ethanesulfonic acid]) – Tris (tris[hydroxymethyl]aminomethane) buffer, pH 7.2, 5.0 mM ATP and 3.0 × 10⁷ cells/ml. The ATPase activity was determined by measuring the hydrolysis of [γ-³²P]ATP (10⁴ Bq/nmol ATP) (Saad-Nehme *et al.*, 1997). The experiments were started by the addition of living cells and terminated by the addition of 1.0 ml of a cold mixture containing 0.2 g charcoal in 1 mM HCl. The tubes were then centrifuged at 1,500 × g for 10 min at 4 °C. Aliquots (0.5 ml) of the supernatant containing the released ³²Pi were transferred to scintillation vials. The ATPase activity was calculated by subtracting the nonspecific 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 activity measured under the same conditions described above was assayed spectrophotometrically by measuring the release of Pi from the nucleotides (Lowry and Lopez, 1946). The hydrolysis of other nucleotides was also calculated by subtracting the nonspecific nucleotide hydrolysis measured in the absence of parasites. The values obtained for the ATPase activities measured using both methods (spectrophotometric and radioactive) were exactly the same. All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions.

**Statistical analysis**

All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Apparent Kₐ and Vₐₘₙₙ values were calculated using a computerized nonlinear regression analysis of the data to the Michaelis-Menten equation (Guilherme *et al.*, 1991). Statistical significance was determined by Student’s *t* test. Significance was considered as *P* < 0.05.

**Chemicals**

All reagents were purchased from E. Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO). (γ³²P) ATP was prepared as described by Glynn and Chappel (1964).
Results and Discussion

In this paper we report the presence of an ecto-ATPase activity present on the external surface of *T. vaginalis*. Cellular integrity and viability were assessed, before and after the reactions, by motility and cell dye exclusion (Dutra *et al.*, 1998). The integrity of the cells was not affected by any conditions used in the assays. The time course of ATP hydrolysis by the ecto-ATPase present on the surface of *T. vaginalis* was linear for at least 60 min ($r^2 = 0.9984$). Similarly, in assays to determine the influence of cell density, the ATPase activity measured over 60 min was linear over a nearly 8-fold range of cell density ($r^2 = 0.9993$). The addition of ZnCl₂, CaCl₂ and MgCl₂, but not MnCl₂ or SrCl₂ stimulated the ATP hydrolysis (Fig. 1). To check the possibility that the observed ATP hydrolysis was the result of secreted soluble enzymes, as seen in other parasites (Bermudes *et al.*, 1994; Smith *et al.*, 1997), we prepared a reaction mixture with parasites 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 (data not shown). This data also rules out the possibility that the ATPase activity here described could be from disrupted *T. vaginalis* cells.

The optimum pH for the ecto-ATPase lies in the alkaline range. In the pH range from 6.4 to 8.0, in which the cells were alive throughout the time course of reaction, the activity increased with the pH, reaching a value 45% higher at pH 8.0 as compared to pH 6.4. Similar results were obtained for *Leishmania tropica* (Meyer-Fernandes *et al.*, 1997), *Leishmania amazonensis* (Berrêdo-Pinho *et al.*, 2001) and *Entamoeba histolytica* (Barros *et al.*, 2000) ecto-ATPases. To discard the possibility that the ATP hydrolysis was due to phosphatase or other type of ATPases with internal ATP binding sites, different inhibitors for those enzymes were tested. Table I shows that the ecto-ATPase activity was insensitive to oligomycin and sodium azide, two inhibitors of mitochondrial Mg-ATPase (Meyer-Fernandes *et al.*, 1997); bafilomycin A₁, a

![Graph showing influence of different divalent cations on the ecto-ATPase activity of intact living *T. vaginalis* parasites.](Image)

**Fig. 1.** Influence of different divalent cations on the ecto-ATPase activity of intact living *T. vaginalis* parasites. Cells were incubated for 1 h at 36°C, in a reaction medium (final volume: 0.5 ml) containing 50 mM Hepes-Tris buffer, pH 7.2, 116 mM NaCl, 5.4 mM KCl, 5.5 mM d-glucose, 3.0 x 10⁷ cells/ml, and 5 mM Tris-ATP (γ-³²P) ATP (specific activity = 10⁴ Bq/nmol ATP), with the addition of 5 mM of each divalent cation. Data are means ± SE of three determinations, performed in triplicate, with different cell suspensions. Data analyzed by Student’s *t* test. * Denotes statistically different from control parasites ($P < 0.05$).

<table>
<thead>
<tr>
<th>Additionsb</th>
<th>Relative activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.4 ± 12.7</td>
</tr>
<tr>
<td>Levamizolec (1.0 mM)</td>
<td>103.7 ± 10.6</td>
</tr>
<tr>
<td>Sodium orthovanadate (1.0 mM)</td>
<td>95.4 ± 10.1</td>
</tr>
<tr>
<td>Ammonium molybdate (0.1 mM)</td>
<td>100.5 ± 11.6</td>
</tr>
<tr>
<td>Sodium tartrate (1.0 mM)</td>
<td>90.4 ± 10.7</td>
</tr>
<tr>
<td>Sodium fluoride (1.0 mM)</td>
<td>108.9 ± 12.3</td>
</tr>
<tr>
<td>Ouabain (1.0 mM)</td>
<td>101.5 ± 9.7</td>
</tr>
<tr>
<td>Sodium azide (10.0 mM)</td>
<td>90.3 ± 11.9</td>
</tr>
<tr>
<td>Bafilomycin A₁ (1 µM)</td>
<td>105.5 ± 9.2</td>
</tr>
<tr>
<td>Oligomycin (1 µg/ml)</td>
<td>95.2 ± 9.7</td>
</tr>
<tr>
<td>Furosemide (1.0 mM)</td>
<td>91.6 ± 10.1</td>
</tr>
<tr>
<td>Dipyridamoled (10 µM)</td>
<td>92.1 ± 11.4</td>
</tr>
<tr>
<td>DIDS (1 mM)</td>
<td>37.4 ± 4.4</td>
</tr>
</tbody>
</table>

* The ectonucleotide ATP-diphosphohydrolase activity was measured in the standard assay described under Material and Methods section. ATPase activity is expressed as a percentage of that measured under control conditions, i.e., without other additions. The ectonucleotide ATP-diphosphohydrolase activity (157.4 ± 13.8 nmol Pi/h x 10⁷ cells) was taken as 100%. The standard errors were calculated from the absolute activity values of three experiments, performed in triplicate, with different cell suspensions and converted to percentage of the control value.

b The final concentrations of the different agents were the highest ones in which there was no alteration in the parasite integrity.

c Levamizole (L-2,3,5,6-tetrahydro 6-phenylimidazo [2,1-b] thiazole) is an inhibitor of alkaline phosphatase (Van Belle, 1979).

d Dipyridamole (2,6-bis (diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d] pyrimidine) is a nucleoside transporter antagonist (Lemmens *et al.*, 1996).
V-ATPase inhibitor (Browman et al., 1988); ouabain, a Na\(^+\)-K\(^+\)-ATPase inhibitor (Caruso-Neves et al., 1998a); furosemide, a Na\(^+\)-ATPase inhibitor (Caruso-Neves et al., 1998b); sodium fluoride and ammonium molybdate, two potent inhibitors of acid phosphatase activity (Dutra et al., 1998) and sodium orthovanadate, a potent inhibitor of P-AT-Pases and acid phosphatases (Fernandes et al., 1997; Dutra et al., 1998; Meyer-Fernandes et al., 1999). Levamizole, an inhibitor of alkaline phosphatase (Van Belle, 1976), and dipyridamole, a nucleoside transporter antagonist (Lemmens et al., 1996) also failed to inhibit the ATPase activity (Table I). Since we used intact cells for measuring the enzyme activity in all the experiments performed in this work, it is likely that the ATPase activity is an ectoenzyme. To confirm this, we applied the criterion that an authentic ectoenzyme should be inhibited by an added extracellular impermeant inhibitor such as 4, 4\(^{-}\)bis(2-hydroxyethyl)amino-2\(^{-}\)-methylimidazoline-N3-N3-1,2-diphenyldisulfonate (DIDS) (Barros et al., 2000; Meyer-Fernandes et al., 2000; Berre\textsuperscript{o}do-Pinho et al., 2001). Agreeably, this ATPase activity was inhibited by 63% in the presence of 1 mm DIDS (Table I). For these reasons we assign an ectolocalization of the ATPase activity described here (Plesner, 1995; Meyer-Fernandes et al., 1997, 2000; Berre\textsuperscript{o}do-Pinho et al., 2001). The dependence on ATP concentration shows a normal Michaelis-Menten kinetics for this ATPase activity and the values of \(V_{\text{max}}\) and apparent \(K_{m}\) for ATP were 182.5 \pm 2.63 nmol Pi/h \times 10^7 cells and 0.015 \pm 0.0013 mm, respectively (Fig. 2). It has been shown that the mechanism of nucleotide hydrolysis by ecto-ATPases is strongly dependent on the interaction of the transmembrane domains with the active site and solubilized ecto-ATPases have lower catalytic activity than membrane-bound ecto-ATPases (Wang et al., 1998).

The nucleoside triphosphate hydrolyse (NTPase) purified from \textit{T. gondii} was shown to be a mixture of two isozymes, termed NTPase I and NTPase II. A primary difference between these isozymes is that NTPase II hydrolyzes nucleoside triphosphate and diphosphate substrates at almost the same rate, whereas NPTase I was almost exclusively limited to nucleoside triphosphate hydrolysis (Asai et al., 1995). Recently it has been shown that avirulent \textit{T. gondii} strains express only NTPase II, whereas virulent strains express both NTPase I and NTPase II (Nakaar et al., 1998). We analyzed the specificity of this ecto-ATPase activity for other nucleotides. Table II shows that this ecto-ATPase hydrolyzed ATP, ADP, ITP, TTP, GTP, UTP and CTP at high rates, indicating that it is an ectonucleoside triphosphate diphosphohydrolase, described for other cells (Wang and Guidotti,

### Table II. Substrate specificity of the ectonucleotide ATP-diphosphohydrolase activity of \textit{T. vaginalis}.

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Relative activity (\times 10^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100.0 \pm 12.2</td>
</tr>
<tr>
<td>ITP</td>
<td>94.8 \pm 8.4</td>
</tr>
<tr>
<td>TTP</td>
<td>91.1 \pm 9.3</td>
</tr>
<tr>
<td>GTP</td>
<td>73.9 \pm 8.2</td>
</tr>
<tr>
<td>UTP</td>
<td>54.4 \pm 5.7</td>
</tr>
<tr>
<td>CTP</td>
<td>47.1 \pm 3.2</td>
</tr>
<tr>
<td>ADP</td>
<td>103.4 \pm 14.6</td>
</tr>
</tbody>
</table>

\(a\) The ectonucleotide ATP-diphosphohydrolase activity was measured in a standard assay described under Material and Methods section with the nucleotides listed (5 mm). The ATP hydrolysis (162.5 \pm 12.6 nmol Pi/h \times 10^7 cells) was taken as 100%. The standard errors were calculated from the absolute activity values of three experiments, performed in triplicate, with different cell suspensions and converted to percentage of the control value. In these experiments, ATP hydrolysis was measured using the same calorimetric assay of Pi release from other nucleotides as that described under Material and Methods section.
Carbohydrates exposed on the surface of mammalian cells play an important role in the interaction of those cells with *T. vaginalis* (Bonilha *et al.*, 1995). Adhesins with lectin properties comparable to those reported for *E. histolytica* and *Giardia lamblia* have also been implicated in the cytoadherence of *Trichomonas mobilensis* to mammalian cells (Demes *et al.*, 1989). We have previously shown that D-galactose stimulates a Mg\(^{2+}\)-dependent ecto-ATPase activity of *E. histolytica* (Barros *et al.*, 2000). Accordingly, the ecto-ATPase of *T. vaginalis* was stimulated by more than 90% by 50 mM D-galactose (Fig. 3). On the other hand, 50 mM D-mannose and 50 mM D-glucose did not significantly stimulate this ATPase activity. Recently, we have shown that the invasive amoebae *E. histolytica* presents a much higher ecto-ATP diphosphohydrolase activity than both the non-invasive amoebae *E. histolytica* and the free-living amoebae *E. moshkovskii* (Barros *et al.*, 2000). D-galactose, a sugar moiety known to be an important adhesion molecule between mammalian host cells and protozoan parasites (Ravdin *et al.*, 1989), promoted a twofold stimulation of this E-type ATPase in *E. histolytica* (Barros *et al.*, 2000). Adhesion of *T. vaginalis* has also been related to the presence of D-galactose exposed on the surface of its host cells (Bonilha *et al.*, 1995). The ectonucleotide ATP-diphosphohydrolase of *E. histolytica* (Barros *et al.*, 2000) and that here described for *T. vaginalis* share several characteristics, such as the sensitivity to the impermeant inhibitor DIDS (Table I), as well as the similar responses to the pH variation and to the stimulatory effect of D-galactose (Fig. 3). These enzymes may have similar functions in those parasites and they could be considered pathogenesis markers for them. Accordingly, the ecto-ATPase activity of a fresh isolate of *T. vaginalis* was almost threefold higher that that of a strain axenically maintained in culture, through daily passages, for several years (data not shown), which suggests an involvement of this ectonucleotide ATP-diphosphohydrolase in the pathogenicity of *T. vaginalis*.

The physiological role of ecto-ATPases is still unknown, but it has been suggested a possible involvement of these enzymes in cellular adhesion (Knowles, 1995; Kirley, 1997; Meyer-Fernandes *et al.*, 2000; Peres-Sampaio *et al.*, 2001). Ongoing studies in our group pursue further knowledge on the participation of the ectonucleotide ATP-diphosphohydrolase of *T. vaginalis* in the relationship between these parasites and mammalian epithelial cells.

Acknowledgments

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