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Characterization of an ecto-5′-nucleotidase (EC 3.1.3.5) activity in intact cells of *Trichomonas vaginalis*

Tiana Tasca,[a,b] Carla D. Bonan,[c] Geraldo A. De Carli,[b] Ana M.O. Battastini,[a] and João J.F. Sarkis[a,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600—Anexo, 90035-003 Porto Alegre, RS, Brazil*
[b] *Laboratório de Parasitologia Clínica, Departamento de Análises Clínicas, Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900, Porto Alegre, RS, Brazil*
[c] *Laboratório de Pesquisa Bioquímica, Departamento de Ciências Fisiológicas, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900, Porto Alegre, RS, Brazil*

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Abstract

The enzymatic properties of an ecto-5′-nucleotidase were described in *Trichomonas vaginalis*. The enzyme hydrolyzes nucleoside monophosphates in optimum pH values of 7.5 and 6.5 for the 30236 strain and for the 30238 strain, respectively. Mg^{2+} and Ca^{2+} were activators of AMP hydrolysis in both strains. The apparent K_m (Michaelis constant) values for Mg^{2+}-AMP were 111.4 ± 28.1 μM (mean ± SD, $n = 3$) for 30236 strain and 420.2 ± 35.7 μM (mean ± SD, $n = 3$) for 30238 strain. The ecto-5′-nucleotidase activity was insensitive to levamisole and tetramisole, inhibitors of alkaline phosphatases, whereas α,β-methylene-ADP inhibited the enzymatic activity of both strains. Our results showed that the AMP hydrolysis presents differences in some kinetic parameters between the two strains investigated. An analysis of the enzymatic chain involved in the ATP hydrolysis to adenosine will contribute to understanding the biochemical aspects of the parasite and the mechanisms related to host–parasite interactions.

Index Descriptors and Abbreviations: Trichomonas vaginalis; Parasitic protozoan; Intact cells; Ecto-5′-nucleotidase; Adenosine; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; NTPDase1, nucleoside triphosphate diphosphohydrolase 1 (EC 3.6.1.5); NTPDase2, nucleoside triphosphate diphosphohydrolase 2 (EC 3.6.1.3); Ecto-5′-nucleotidase (EC 3.1.3.5); CMP, cytidine 5′-monophosphate; UMP, uridine 5′-monophosphate; IMP, inosine 5′-monophosphate; GMP, guanosine 5′-monophosphate, K_m, Michaelis constant; V_{max}, maximum velocity; 3′,5′-cAMP, adenosine 3′, 5′-cyclic monophosphate; NAD^+, nicotinamide adenine dinucleotide; Ap_nA, diadenosine polyphosphates; AMPCP, α,β-methylene adenosine diphosphate.

1. Introduction

The parasitic protozoan *Trichomonas vaginalis* lives in the human urogenital tract. The parasite is a common cause of infection of the female tract and the annual incidence of trichomoniasis is more than 170 million cases worldwide (WHO, 1995). Trichomoniasis is a sexually transmitted disease (STD), and its clinical presentation ranges from a totally asymptomatic infection to a severe vaginitis. The clinical features of trichomoniasis in women are therefore limited to the urogenital tract. In men, the infection is mostly asymptomatic, but in some cases it can lead to a mild urethritis, which usually resolves spontaneously within two weeks.

Although *T. vaginalis* is the most intensely studied trichomonad and is the world's most common cause of nonviral STDs, the exact mechanism of its pathogenesis has not been clearly elucidated (Brasseur and Savel, 1982; Petrin et al., 1998; Roussel et al., 1991). The host–parasite relationship is very complex, and the broad range of clinical symptoms cannot be easily attributed to a single pathogenic mechanism.

The proteins and glycoproteins on the cell surface of trichomonads plays a major role in cytoadhesion, host-parasite interaction, nutrient acquisition and in the

* Corresponding author. Fax: +55-51-3316-5530.
E-mail address: jjsarkis@plug-in.com.br (J.J.F. Sarkis).

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protection from the cytolytic effects (Petrin et al., 1998). Extracellular ATP may act as a signaling compound in cytolytic mechanisms (Filippini et al., 1990; Steinberg and Di Virgilio, 1991) and it is hydrolyzed to adenosine by a group of enzymes: NTPDase1 (apyrase, ecto-ATP diphosphohydrolase, CD39, EC 3.6.1.5) and NTPDase2 (ecto-ATPase, CD39L1, EC 3.6.1.3), which hydrolyze nucleoside di- and tri-phosphates, and the ecto-5′-nucleotidase (EC 3.1.3.5), which hydrolyzes nucleoside monophosphates (Sarkis et al., 1995; Zimmermann, 1996b, 2001).

5′-Nucleotidase activity has been described for bacteria and plant cells and the enzyme is also widely distributed in vertebrate tissues (Zimmermann, 1992). This enzyme catalyzes the hydrolysis of a variety of nucleoside 5′-monophosphates such as AMP, CMP, UMP, IMP, and GMP. Generally, AMP is the most effectively hydrolyzed nucleotide, presenting K_m values in the lower micromolar range (Zimmermann, 1996b). 5′-Nucleotidase activity is found in a soluble form and also in a membrane-bound, ecto-surface-located form, which is one of the contributors to the cascade that completely hydrolyzes extracellular ATP to adenosine, and thus of major pharmacological interest. Ecto-5′-nucleotidase forms dimers and the apparent molecular weight of the monomer ranges from 62 to 74 kDa (Zimmermann, 2001). Beyond its enzymatic properties, 5′-nucleotidase is a lymphocyte surface protein CD73 and may be involved in cell adhesion (Zimmermann, 1992, 1996a).

Several studies have reported the presence of ecto-nucleotidases on the surface of parasites. Recently, we characterized an ATP diphosphohydrolase activity in *T. vaginalis* (Matos et al., 2001). Previous studies have demonstrated the presence of an ATP diphosphohydrolase on the external surface of the *Schistosoma mansoni* tegument (Vasconcelos et al., 1993, 1996). An ectonucleotide diphosphohydrolase was described in intact cells of *Entamoeba histolytica* (Barros et al., 2000) and a Mg-dependent ecto-ATPase activity has been described on the external surface of *Leishmania tropica* (Meyer-Fernandes et al., 1997) and in promastigotes of *Leishmania amazonensis* (Berrêdo-Pinho et al., 2001). More recently, an ATP diphosphohydrolase was characterized in promastigotes of *L. amazonensis* (Coimbra et al., 2002). However, 5′-nucleotidase has not been demonstrated in trichomonads. The present study describes the properties of an ecto-5′-nucleotidase activity in intact cells of two strains of *T. vaginalis.*

2. Materials and methods

2.1. Parasites and culture conditions

The 30236 and 30238 *T. vaginalis* strains from the American Type Culture Collection were used in this study. Both strains were cultivated axenically in trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957) without agar (pH 6.0) supplemented with 10% (v/v) heat-inactivated cold serum, penicillin (1000 IU/ml), and streptomycin sulphate (1 mg/ml) in aerobiosis at 37 °C (±0.5). Isolates were subcultured every 48 h in TYM medium. Trichomonads in the logaritimic phase of growth and within 48 h of subculture (exhibiting more than 95% mobility and normal morphology) were harvested and washed with sterile saline solution (NaCl 0.85%) (750 g for 5 min) three times. Parasites were ressuspended in saline solution and counted with a haemocytometer and adjusted to a concentration of 1.5×10^6 cells/ml, corresponding to 0.3–0.7 mg/ml protein. All experiments were performed using intact cells and cellular viability was assessed, before and after incubations, by mobility. The viability was not affected by incubation conditions.

2.2. Enzyme assays

Intact cells of 30236 and 30238 *T. vaginalis* strains were added to the reaction mixture containing 50 mM Tris buffer (pH 7.5, for 30236 strain and pH 6.5, for 30238 strain) and 3 mM $MgCl_2$ or $CaCl_2$. The samples were preincubated for 5 min at 37 °C in 200 µl of the reaction mixture. The reaction was initiated by the addition of substrate (AMP or other as indicated) to a final concentration of 3 mM and stopped by adding 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) (Chan et al., 1986). Incubation times and cellular density were chosen in order to ensure the linearity of the reactions. Controls with the addition of the intact cells after mixing trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol Pi released/h/1.5×10^6 cells. All samples were run in triplicate, with similar results achieved in at least three different cell suspensions.

2.3. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin as standard.

2.4. Statistical analysis

Statistical analysis was conducted by Student's *t* test, considering a level of significance of 5%.

3. Results

An enzyme with characteristics of an ecto-5′-nucleotidase was detected in intact cells of two *T. vaginalis*

strains. The time course for AMP hydrolysis occuring in both strains investigated was linear up to 90 min in the presence of Mg^{2+} or Ca^{2+}. Mg^{2+}-AMP hydrolysis increased as a function of cell density. The product formation was linear in the range of $0.8–2.0 \times 10^6$ cells/ml in the incubation medium (data not shown).

The 5′-nucleotidase activity was divalent cation-dependent and its sensitivity to Mg^{2+} and Ca^{2+} is demonstrated in Fig. 1. Mg^{2+} and Ca^{2+} were activators of AMP hydrolysis in both *T. vaginalis* strains (Figs. 1A,B). The activity was increased in the 30236 strain with magnesium concentrations of 5 and 8 mM (Fig. 1A), whereas, in these concentrations, a plateau was observed in the 30238 strain (Fig. 1B). Taking these results into account, the concentration of 3 mM magnesium, which is a classical activator of 5′-nucleotidase, was chosen for subsequent enzyme assays for both strains.

The AMP hydrolysis was evaluated by measuring pH dependence on activity in both strains. In the pH range of 5.5–8.5, where the cells were alive throughout the time course of reaction, the 5′-nucleotidase activity progressively increased to reach a maximal level at pH 6.5, for the 30238 strain, and pH 7.5, for the 30236 strain (Fig. 2).

Mg^{2+}-AMP hydrolysis was determined at substrate concentrations in the range of 100–3000 μM. The enzyme activity increased with increasing concentrations of the nucleotide (Mg^{2+} fixed at 3 mM with variable concentrations of AMP). K_m (Michaelis constant) and V_{max} (maximum velocity) values of intact cells samples of both *T. vaginalis* strains were estimated from the Eadie–Hofstee plot with three different enzyme preparations (Figs. 3A,B). The apparent K_m values for Mg^{2+}-AMP were 111.4 ± 28.1 μM (mean ± SD, $n = 3$) for the 30236 strain and 420.2 ± 35.7 μM (mean ± SD, $n = 3$) for the 30238 strain. The V_{max} values for Mg^{2+}-AMP were 6.5 ± 2.4 and 12.9 ± 2.3 nmol Pi/h/1.5×10^6 cells (mean ± SD, $n = 3$) for the 30236 and the 30238 strains, respectively.

Ecto-5′-nucleotidase has been described as an enzyme with a broad substrate monophosphate specificity and a preference for AMP (Zimmermann, 1996a). All nucleoside monophosphates tested were hydrolysed at a lower rate than AMP by intact cells of both strains, except GMP, which was hydrolyzed at a higher rate than AMP by 30238 the strain (Table 1).

To discard the influence of other enzymes, such as alkaline phosphatase, in the AMP hydrolysis, inhibitors of this enzyme were tested. Levamisole and tetramisole, specific alkaline phosphatase inhibitors, had no effect upon AMP hydrolysis in both strains of *T. vaginalis* (Table 2). Conversely, the known 5′-nucleotidase inhibitor, adenosine 5′-[α,β-methylene]diphosphate (AMPCP)

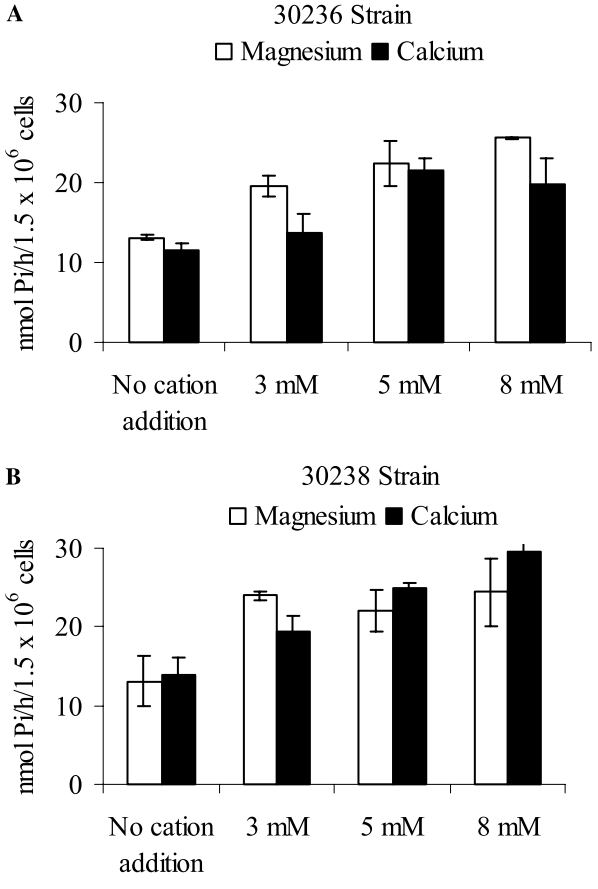

Fig. 1. Effect of $MgCl_2$ and $CaCl_2$ concentration on 5′-nucleotidase activity in intact cells of 30236 strain (A) and 30238 strain (B). Open bars represent 5′-nucleotidase activity in presence of $MgCl_2$ and closed bars, in presence of $CaCl_2$. Incubation conditions are described in Section 2. The control group was incubated without the addition of cation. Bars represent the mean ± SD of three different determinations using different cells suspensions, each in triplicate.

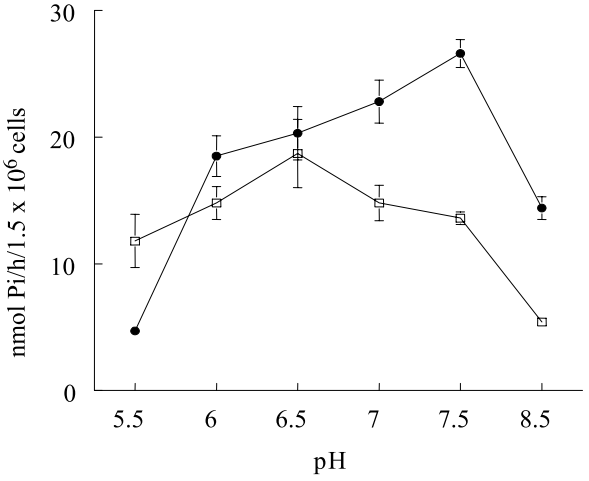

Fig. 2. Effect of pH on 5′-nucleotidase activity in intact cells of 30236 strain (●) and 30238 strain (□). Incubation conditions are described in Section 2, in presence of $MgCl_2$ 3 mM. In this pH range (5.5–8.5), the cells were viable throughout the course of the experiments. Data are means ± SD of three determinations with different cell suspensions.

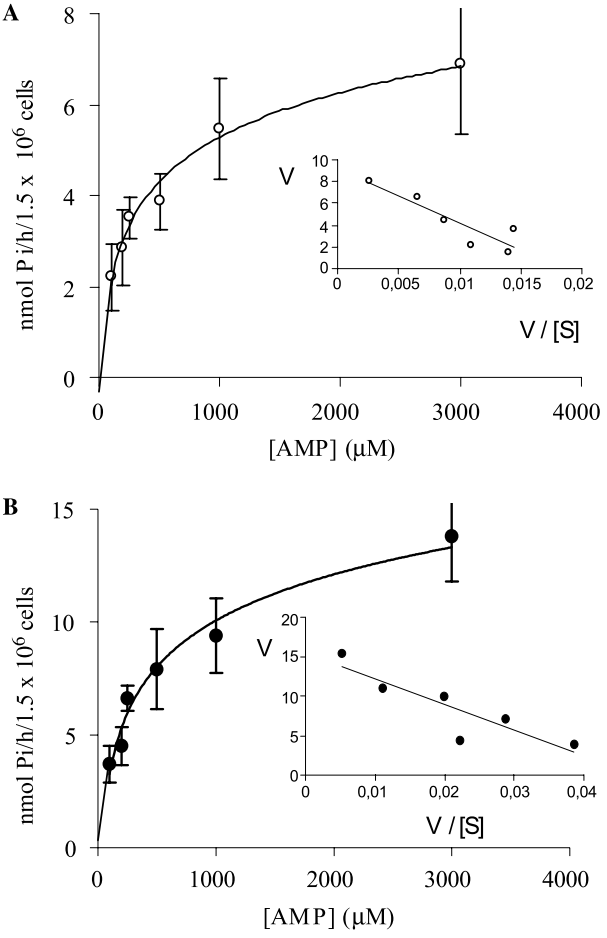

Fig. 3. Dependence of 5′-nucleotidase activity on AMP concentrations in intact cells of 30236 strain (A) and 30238 strain (B). Incubation conditions are described in Section 2, in presence of $MgCl_2$ 3 mM. Data are means ± SD of three determinations with different cell suspensions. Insets: Eadie–Hofstee plots for AMP in both strains. V, velocity of 5′-nucleotidase activity, expressed in nmol Pi/h/1.5×10^6 cells; V/[S], velocity/substrate concentration (AMP).

Table 1
Substrate specificity of 5′-nucleotidase of *T. vaginalis*[a]

Substrate	Relative activity	
	30,236 strain	30,238 strain
AMP	1.00 ± 0.15	1.00 ± 0.11
CMP	0.09 ± 0.02	0.50 ± 0.09
GMP	0.67 ± 0.18	1.40 ± 0.13
UMP	0.03 ± 0.002	0.50 ± 0.04

[a] Results are expressed as the mean ± SD for at least four experiments. All substrates were used at 3 mM (3 mM Mg^{2+}). Control 5′-nucleotidase activity was 8.8 and 16.9 nmol Pi/h/1.5×10^6 cells for the 30236 and 30238 strains, respectively.

was also tested and inhibited the enzyme activity in both strains (Table 2).

The possibility that the AMP hydrolyzed by 5′-nucleotidase could be originated from a phosphodiesterase must be ruled out since the released Pi should be a superestimated amount. Thus, to avoid the influence of a phosphodiesterase, cyclic AMP, substrate for this enzyme, was incubated with the intact cells and the activity was tested. The control activity was 6.0 ± 1.7 and 14.9 ± 0.7 nmol Pi/h/1.5×10^6 cells (mean ± SD, $n = 3$) for the 30236 and 30238 strains, respectively, whereas the activity in the presence of cyclic AMP (3 mM) was 5.5 ± 1.3 and 14.4 ± 2.0 nmol Pi/h/1.5×10^6 cells (mean ± SD, $n = 3$) for the 30236 and 30238 strains, respectively. Hence, the interference of a phosphodiesterase could be excluded in both *T. vaginalis* strains, given that the activity in the presence of cyclic AMP was lower than the control activity, showing no AMP production from phosphodiesterase activity. The kinetic data strongly suggest that the AMP hydrolysis characterized here is due to a true ecto-5′-nucleotidase activity in both *T. vaginalis* strains.

Table 2
Effects of inhibitors on AMP hydrolysis by *T. vaginalis*[a]

Inhibitor	Concentration (mM)	% Control enzyme activity	
		30,236 strain	30,238 strain
Levamisole	1	140 ± 11*	109 ± 25
Tetramisole	1	110 ± 21	102 ± 20
AMPCP	0.2	53 ± 9 *	69 ± 7*

*Significant difference from control activity (100%) by Student's *t* test ($P < 0.05$).

[a] Control 5′-nucleotidase activity was 7.9 ± 0.4 and 19.9 ± 1.0 nmol Pi/h/1.5×10^6 cells for 30236 and 30238 strains, respectively. Results are expressed as percentage of control activity (100%). AMP was used at 3 mM (3 mM Mg^{2+}). Data represent the means ± SD for at least four experiments and were analysed statistically by Student's *t* test.

4. Discussion

In this study, we report the properties of an ecto-5′-nucleotidase acitivity present on the external surface of intact cells of *T. vaginalis.* Cellular integrity and viability were assessed, before and after the reactions, by the mobility of the trophozoites. The integrity of the cells was not affected by any of the conditions used in the assays. Enzyme activity does not depend on added divalent cations, but it can be increased by the addition of millimolar concentrations of Mg^{2+} and Ca^{2+} (Zimmermann, 1992). In the ecto-5′-nucleotidase activity of *T. vaginalis*, Mg^{2+} and Ca^{2+} were activators (Figs. 1A,B). The K_m values vary to some extent between cell type and preparation, generally they are in the lower micromolar range (Zimmermann, 1996a). For both *T. vaginalis* strains investigated, the apparent K_m values for Mg^{2+}-AMP were 111.4 ± 28.1 μM (mean ± SD, $n = 3$) for 30236 strain and 420.2 ± 35.7 μM (mean ± SD, $n = 3$) for 30238 strain. The characteristic broad substrate monophosphates specificity of ecto-5′-nucleotidase was also observed in both strains studied, with a preference

for AMP in the 30236 strain and for GMP in the 30238 strain (Table 1).

Nucleoside 5′-monophosphates are subject to hydrolysis by alkaline phosphatases in addition to ecto-5′-nucleotidase and presumably also by some members of the ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family (Zimmermann, 2001). The influence of contaminating alkaline phosphatases on AMP hydrolysis in our assay conditions was excluded since 1 mM levamisole and tetramisole, classical alkaline phosphatases inhibitors, did not affect the AMP hydrolysis in both *T. vaginalis* strains investigated (Table 2). The members of the E-NPP family reveal alkaline phosphodiesterase as well as nucleotide pyrophosphatase activity, which are properties of the same enzyme molecule. They have comparable catalytic properties and are capable of hydrolyzing 3′,5′-cAMP to AMP, ATP to AMP and PPi, ADP to AMP, and Pi, NAD^+ to AMP and nicotinamide mononucleotide, and the diadenosine polyphosphates Ap_nA (Zimmermann, 2001). The possibility of a phosphodiesterase interference in our experiments can be ruled out since we incubated cyclic AMP with the intact cells and the activities of the 30236 and 30238 strains were not higher than control activity. These results indicate that the hydrolysis observed correspond to the AMP added to the incubation medium, and not to AMP derivated from a phosphodiesterase reaction. The ecto-5′-nucleotidase of vertebrates is competitively inhibited by the nucleotide analogue adenosine 5′-[α,β-methylene]diphosphate (AMPCP). Our results showed that the inhibition was observed also in both *T. vaginalis* strains (Table 2).

Nucleotides are released from dying or destroyed cells under physiological or pathological conditions after massive injury (Ralevic and Burnstock, 1998). ATP is known to be an important extracellular signaling molecule which is supposed to increase the plasma membrane permeability and has important effects on cell membrane properties (Steinberg and Di Virgilio, 1991). Extracellular ATP may be one of the signaling molecules involved in cell-mediated cytotoxicity (Filippini et al., 1990). It has been demonstrated that extracellular ATP has profound effects on cellular functions, causing plasma membrane depolarization, Ca^{2+} influx, and cell death (Filippini et al., 1990; Steinberg and Di Virgilio, 1991). Filippini et al. (1990) have shown that ATP can kill different cells, with the exception of cells that express a high level of ATP-breakdown activity on their surface. Extracellular ATP is hydrolyzed to adenosine by a group of enzymes: the NTPDase1, that hydrolyzes nucleoside di- and triphosphates, and the 5′-nucleotidase, able to hydrolyze nucleoside monophosphates. Taking into account the fact that the NTPDase1 was discussed in *T. vaginalis* (Matos et al., 2001), the ecto-5′-nucleotidase described here would thus represent the final step of the enzymatic chain which leads from the extracellular trinucleotide to the nucleoside. Reuptake of the nucleoside would then serve the salvage of released nucleotides (Zimmermann, 1992).

In animals, the ecto-5′-nucleotidase is present in pratically all tissues, but not on all cell types. The cell- or tissue-specific physiological consequences of ATP-elimination and/or adenosine production vary, since both ATP and adenosine induce receptor-mediated physiological functions (Zimmermann, 1992). Extracellular ATP has several effects on many biological processes, including immune response, inflammation, platelet aggregation and pain (Ding et al., 2000; Ralevic and Burnstock, 1998; Sitkovsky, 1998; Sneddon et al., 1999). Adenosine induces vasodilation, a decrease in glomerular filtration rate, inhibition of neurotransmitter release, inhibition of the immune and inflammatory response or lipolysis. In more primitive organisms, the bacterial 5′-nucleotidase has an ecological function in nutrient recycling in aqueous habitats (Zimmermann, 1992).

The presence of enzymes performing ATP, ADP (Matos et al., 2001), and AMP hydrolysis in trichomonads may be important for the modulation of nucleotide concentration in the extracellular space, protecting the parasite from the cytolytic effects of the nucleotides, mainly ATP. In addition, *T. vaginalis* lacks the ability to synthesize purines and pyrimidines de novo and its growth and survival is dependent of salvage pathways to generate nucleotides (Heyworth et al., 1982, 1984). Purine salvage is mediated by nucleoside phosphorylases and kinases (Miller and Lindstead, 1983), whereas phosphoribosyltransferases and nucleoside kinases are able to recover pyrimidines (Wang and Cheng, 1984). Thus, these enzymes can be regarded as potential targets for anti-*T. vaginalis* chemoterapy (Wang, 1990).

The enzymatic chain present in *T. vaginalis* which hydrolyzes extracellular nucleotides may contribute to escape mechanisms of the parasite by breaking down ATP and providing the adenosine required for parasite growth. Furthermore, the 30236 and 30238 strains investigated are virulent and present differences in some kinetic parameters of 5′-nucleotidase activity. The 30238 strain presented higher ecto-5′-nucleotidase activity when compared with the 30236 strain. Many studies have found strain variability in *T. vaginalis* expressed in variability of virulence, antigenic properties, geographic distribution, and isoenzyme analysis (Alderete, 1983, 1985; Christian et al., 1963; Farris and Honigberg, 1970; Hogue, 1943; Kotcher and Hoogasian, 1957; Kott and Alder, 1961; Krieger et al., 1985; Kulda, 1967; Teras, 1966; Torian et al., 1984). The clear identification of the physiological significance of these enzymes in the intracellular and extracellular environment of *T. vaginalis* could contribute to understanding biochemical aspects of the parasite and the mechanisms involved in specific host-parasite interactions. In addition, the

ecto-5′-nucleotidase could be considered a potential drug target for anti-trichomonad chemotherapy.

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