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

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Abstract

This study describes the enzymatic properties of an ecto-5′-nucleotidase in *Trichomonas gallinae*. The enzyme hydrolyzes nucleoside monophosphates at pH 7.2 and is activated by divalent cations, such as magnesium. Ecto-5′-nucleotidase activity was insensitive to levamisole, tetramisole (alkaline phosphatase inhibitors), and AMPCP (adenosine 5′-[α,β-methylene]diphosphate), an ecto-5′-nucleotidase inhibitor, whereas 0.1 mM ammonium molybdate (considered a potent inhibitor of 5′-nucleotidase activity) completely inhibited the enzyme activity. The apparent K_M (Michaelis constant) and V_{max} (maximum velocity) values for Mg^{2+}-AMP were 466 ± 57 μM and 3.7 ± 0.59 nmolPi/min/10^6 trichomonads, respectively. Considering that trichomonads lack the ability to synthesize purines and pyrimidines *de novo*, the presence of an ecto-5′-nucleotidase in intact trophozoites of *T. gallinae* could be important in regulating the extracellular nucleotide levels and generating adenosine, essential for the survival strategies of the parasite.

Keywords: Adenosine; Ecto-5′-nucleotidase; Extracellular nucleotides; *Trichomonas gallinae*

1. Introduction

Purine nucleosides and nucleotides are mostly released from cells which are stressed or anoxic, injured and metabolically active (Chow et al., 1997). There is evidence that purines have cytotoxic properties (Steinberg and Di Virgilio, 1991). Extracellular ATP may act as a signaling compound in cytolytic mechanisms (Filippini et al., 1990) and it is hydrolyzed to adenosine by a group of ecto-enzymes named ecto-nucleotidases, which includes NTPDases (nucleoside triphosphate diphosphohydrolases) and ecto-5′-nucleotidase. NTPDase1 (CD39, apyrase, ATP diphosphohydrolase) dephosphorylates ATP to AMP, which is hydrolyzed by the ecto-5′-nucleotidase (EC 3.1.3.5), resulting in adenosine (Zimmermann, 1996, 2001).

Nucleoside monophosphate phosphohydrolase or 5′-nucleotidase, also known as CD73, is a glycosylated protein bound to the outer surface of the plasma

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membrane by a glycosylphosphatidylinositol anchor (Misumi et al., 1990). This enzyme acts on a variety of non-cyclic nucleoside monophosphates, such as AMP, CMP, UMP, IMP and GMP, inactivating them to the respective nucleosides and inorganic phosphate (Bianchi and Spychala, 2003). Although the 5′-nucleotidase has broad substrate specificity, AMP is considered to be the major physiological substrate with K_M values in the micromolar range (Zimmermann, 1992, 1996). The enzyme is variably expressed in a wide number of cells types under physiological and pathological conditions (Zimmermann, 1992).

The presence of ecto-nucleotidases has been reported on the surface of various parasites. Vasconcelos et al. (1993, 1996) demonstrated the presence of an ATP diphosphohydrolase in *Schistosoma mansoni*. A Mg-dependent ecto-ATPase activity was described in *Leishmania tropica* (Meyer-Fernandes et al., 1997) and *Leishmania amazonensis* (Berrêdo-Pinho et al., 2001). Furthermore, an ATP diphosphohydrolase and an ecto-5′-nucleotidase were described in *Trichomonas vaginalis* (Matos et al., 2001; Tasca et al., 2003). Ecto-5′-nucleotidase activity detected in intact trophozoites of two *T. vaginalis* isolates hydrolyzes nucleoside monophosphates and is activated by divalent cations, such as Mg^{2+} and Ca^{2+}. The presence of an enzyme that hydrolyzes AMP to adenosine provides the nucleoside required for parasite growth, due to the lack of *de novo* purine nucleotide synthesis among all trichomonad species (Heyworth et al., 1982, 1984; Wang, 1990; Munagala and Wang, 2003).

Trichomonas gallinae is a flagellated protozoan which parasitizes a variety of birds all over the world. The domestic pigeon, *Columba livia*, is the primary host of this parasite. This trichomonad occurs in the upper digestive tract and in various organs of different avian groups (Stabler, 1954; De Carli et al., 1979). The normal sites of *T. gallinae* are the mouth, pharynx, esophagus and crop, where they cause the formation of caseous lesions. In pigeons, trichomoniasis is mainly a disease of young birds, causing serious losses among these birds. The protozoan is the causative agent of canker in pigeons, causing a variety of pathologic manifestations depending on the parasite isolate and the infected bird species (Baker, 1986; Cooper and Petty, 1988). The virulent isolates may cause lesions in the upper digestive tract of birds, which allow the pathogen to enter the circulatory system, gain access to the liver, lungs, heart and pancreas, leading to host death. Indeed, avian trichomoniasis has been considered a significant economic loss in chickens and turkeys.

Taking into account that *T. gallinae* is a serious veterinary disease pathogen, it is important to investigate the biochemical aspects of this parasite that contribute to understanding features related to host–parasite interaction. The present study describes the properties of an ecto-5′-nucleotidase activity in intact trophozoites of *T. gallinae*.

2. Materials and methods

2.1. Parasite culture

The *T. gallinae* isolate, TG7, from the upper digestive tract of domestic pigeons, *C. livia*, was used in this study. Trichomonads were axenically cultured *in vitro* in trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957) without agar (pH 7.2) supplemented with 10% (v/v) inactivated bovine serum, without antibiotics (Stabler et al., 1964; Tasca and De Carli, 1999), at 37 °C. Viability of the isolates was maintained by storing them in liquid nitrogen (−196 °C) with 5% dimethyl sulfoxide (DMSO) (Honigberg et al., 1965). Trichomonads from the logarithmic phase of growth were collected by centrifugation at 750 × *g* for 5 min. The parasites were then washed three times with 0.9% (w/v) NaCl solution, counted with a haemocytometer and adjusted to a density of 4×10^6 organisms/mL. All samples were run in triplicate, with results achieved in at least three different parasites suspensions. All organisms were viable based on motility, assessed before and after incubations. The viability was not affected by incubation conditions.

2.2. Enzyme assays

After preparing the parasite samples, the optimum conditions for nucleotide hydrolysis were determined. Intact trophozoites of *T. gallinae* (10^6 trichomonads/mL) were added to the reaction mixture containing 50 mM Tris buffer (pH 7.2) and 3.0 mM $MgCl_2$. The samples were preincubated for 5 min at 37 °C in the reaction mixture. The reaction was initiated by the addition of substrate AMP to a final concentration of 3.0 mM. After 15 min, the reaction was stopped by adding 200 μL 10% trichloroacetic acid. The samples were chilled on ice before assaying for the release of inorganic phosphate (Chan et al., 1986). Incubation times and parasite 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 and the averages of control values were subtracted from the test samples. All enzyme assays were run in triplicate. Specific activity is expressed as nmol of Pi/min/10^6 trichomonads.

2.3. Statistical analysis

Statistical analysis was conducted by Student's *t* test or one-way analysis of variance (ANOVA), considering a level of significance of 5%.

3. Results

5′-Nucleotidase activity has been described in bacteria, plant cells and in various vertebrate tissues (Zimmermann, 1992). In this study, an enzyme with characteristics of an ecto-5′-nucleotidase was detected in intact trophozoites of *T. gallinae*. The time course for AMP hydrolysis was linear up to 15 min in the presence of Mg^{2+}. The product formation was linear in the range of 0.8–2.0 $\times$ 10^6 trichomonads/mL (data not shown).

AMP hydrolysis was activated in the presence of divalent cations (Fig. 1) at the concentrations of 3.0, 5.0 and 8.0 mM. In the presence of Mg^{2+} plus 5.0 mM EDTA, there was a significant decrease of AMP hydrolysis. The concentration of 3.0 mM magnesium, which is a classical activator of 5′-nucleotidase, was chosen for subsequent enzyme assays.

Ecto-5′-nucleotidase is an enzyme with a broad substrate specificity for nucleoside monophosphates, with preference for AMP (Zimmermann, 1996). Intact trophozoites of *T. gallinae* hydrolyzed all nucleoside monophosphates tested (CMP, GMP, UMP) at a lower rate than AMP (Table 1). To avoid the influence of pyrophosphatase, pyrophosphate, which is a substrate for this enzyme, was incubated with the intact organisms. The presence of pyrophosphatase can be excluded because there is no significant hydrolysis of pyrophosphate in these assay conditions (Table 1).

Table 1
Substrate specificity of ecto-5′-nucleotidase from intact cells of *T. gallinae*

Substrate	Relative activity
AMP	1.00 ± 0.02
CMP	0.87 ± 0.14
GMP	0.68 ± 0.10
UMP	0.60 ± 0.05
PPi	0.058 ± 0.01

Data represents means ± S.D. of at least three experiments. Control AMP hydrolysis was 1.99 ± 0.04 nmolPi/min/10^6 trophozoites. Results were expressed as percentage of control activity. The substrates were used at 3.0 mM, with $MgCl_2$ 3.0 mM.

AMP hydrolysis was determined at substrate concentrations in the range of 200–3000 μM. Enzyme activity increased with increasing concentrations of the nucleotide (Mg^{2+} fixed at 3.0 mM with different concentrations of AMP) (Fig. 2). K_M (Michaelis constant) and V_{max} (maximum velocity) values in intact trophozoites of *T. gallinae* were estimated from the Lineweaver–Burk plots with three different enzyme preparations (Fig. 2). The apparent K_M and V_{max} values for Mg^{2+}-AMP were 466 ± 57 μM (mean ± S.D.) and 3.7 ± 0.59 nmolPi/min/10^6 trichomonads (mean ± S.D.), respectively.

To discard the influence of alkaline phosphatase on AMP hydrolysis the classical inhibitors of this enzyme, levamisole and tetramisole, were tested. Both compounds, tested to a final concentration of 1.0 mM, had no effect upon AMP hydrolysis in intact trichomonads (Table 2). AMPCP (adenosine 5′-[α,β-methylene]diphosphate) and ammoniun molybdate, which are known 5′-nucleotidase inhibitors, were also tested. The enzyme activity was dramatically inhibited in the presence of

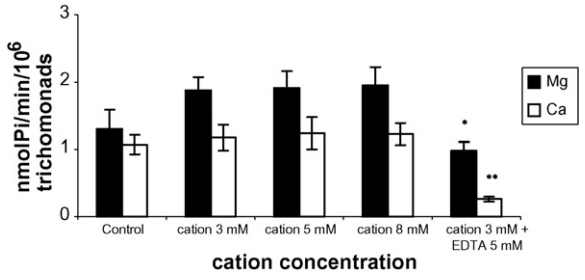

Fig. 1. Effect of $MgCl_2$ and $CaCl_2$ concentration on ecto-5′-nucleotidase activity in intact trophozoites of *Trichomonas gallinae*. Closed bars and open bars represent 5′-nucleotidase activity in presence of $MgCl_2$ and $CaCl_2$, respectively. Incubation conditions were described in Section 2. The control group was incubated without the addition of cation. Bars represent the means ± S.D. for three experiments, using different trophozoites suspensions. **Significant difference from activity in the presence of $MgCl_2$ 3.0 mM ($p < 0.01$). *Significant difference from activity in the presence of $CaCl_2$ 3.0 mM ($p < 0.05$).

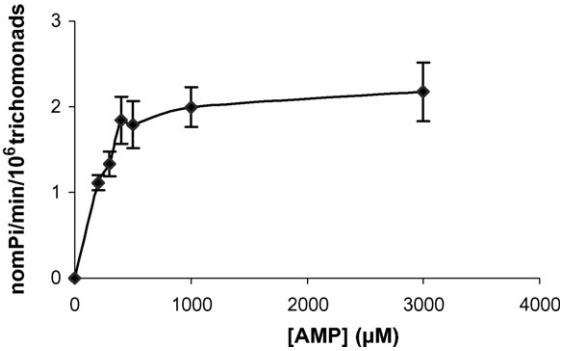

Fig. 2. Effect of different concentrations of substrate (200–3000 μM) on AMP hydrolysis in intact trophozoites of *T. gallinae*. All experiments used fixed 3.0 mM Mg^{2+} with variable concentrations of nucleotide. Data represents mean ± S.D. of three different experiments, each in triplicate.

Table 2
Effect of inhibitors on AMP hydrolysis from intact trophozoites of *T. gallinae*

Inhibitor	Concentration (mM)	% control enzyme activity
Levamisole	1.0	102.3 ± 7.6
Tetramisole	1.0	102 ± 4
AMPCP	0.1	106 ± 6
Molybdate	1.0	1.6 ± 0.05*

Results were expressed as percentage of control activity (100%). Control 5′-nucleotidase activity was 1.83 ± 0.14 nmolPi/min/10^6 trophozoites. AMP was used at 3.0 mM, in the presence of $MgCl_2$ 3.0 mM. Data represent the means ± S.D. for at least three determinations.

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

1.0 mM ammonium molybdate. However, AMPCP did not promote significant changes on AMP hydrolysis. These results suggest that AMP hydrolysis is due to an ecto-5′-nucleotidase in intact trophozoites of *T. gallinae*.

4. Discussion

The proteins on the cell surface of trichomonads play a major role in cytoadhesion, host–parasite interaction, nutrients acquisition and in the protection from the cytolytic effects (Petrin et al., 1998). The results of the present study demonstrate an ecto-5′-nucleotidase in intact trophozoites of *T. gallinae*. This enzyme was not dependent of divalent cations, but the activity was increased by the addition of millimolar concentrations of Mg^{2+} (Zimmermann, 1992). The broad substrate specificity for nucleoside monophosphates of ecto-5′-nucleotidase was also observed, with a preference for AMP. K_M values vary between cell type and preparation; generally they are in the lower micromolar range (Zimmermann, 1996). The influence of contaminating enzymes on AMP hydrolysis was discarded, since 1.0 mM levamisole or tetramisole did not affect the AMP hydrolysis. The possibility of a pyrophosphatase was excluded, since no significant enzyme activity was observed when 1.0 mM pyrophosphate (Ppi) was used as substrate instead AMP.

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the cell surface or may also be soluble in the interstitial medium (Zimmermann, 2001). Extracellular ATP can be hydrolyzed by NTPDase1, forming AMP, being the 5′-nucleotidase the final step of the enzymatic chain.

The ecto-5′-nucleotidase is present in various animals tissues, but not on all cell types. Seven human 5′-nucleotidases with different subcellular localization have been cloned (Bianchi and Spychala, 2003). This enzyme plays an important role in the formation of adenosine from extracellular AMP and the subsequent activation of P1 adenosine receptors (Zimmermann, 2001). Adenosine induces vasodilatation and inhibition of the immune and inflammatory response (Cronstein et al., 1992; Hasko and Cronstein, 2004). In addition, trichomonads lack the ability to synthesize purines and pyrimidines *de novo*. Consequently, the salvage pathways to generate nucleotides are essential for parasite survival (Heyworth et al., 1982, 1984). Munagala and Wang (2003) demonstrated incorporation of external adenine and guanine into the purine nucleotides of *T. vaginalis*. The purine salvage system in *T. vaginalis* consists of a single pathway of two enzymes: PNP (purine nucleoside phosphorylase) and PNK (purine nucleoside kinase). PNP catalyses interconversion between purine bases and purine nucleosides and PNK converts the nucleosides to nucleotides (Heyworth et al., 1982; Miller and Lindstead, 1983). The use of formycin A, a specific inhibitor of bacterial PNP, inhibited *T. vaginalis in vitro* growth. Furthermore, lysed epithelial cells during infection release a broad concentration of purine nucleotides (90% are adenine nucleotides) (Mandel, 1964). Adenosine is the primary precursor of the entire purine nucleotide pool in *T. vaginalis* and adenosine deaminase, IMP dehydrogenase, and GMP synthetase activities were identified in the parasite lysate, suggesting a pathway capable of converting adenine to GMP via adenosine (Munagala and Wang, 2003). Considering the close phylogeny between *T. vaginalis* and *T. gallinae*, it can be suggested that the hydrolysis of adenine nucleotides to nucleoside adenosine by a 5′-nucleotidase provides the primary precursor of the purine nucleotides in the parasites, cooperating to survival and success parasitism.

In addition, the enzymatic chain present in *T. gallinae* may contribute to escape mechanisms of the parasite by breaking down ATP and providing adenosine. The 5′-nucleotidase activity has been demonstrated in some protozoan. Tasca et al. (2003) described an ecto-5′-nucleotidase in two *T. vaginalis* isolates. These authors have shown that the enzyme hydrolyzes nucleoside monophosphates and it is activated by divalent cations, such Mg^{2+} and Ca^{2+}. The enzyme activities were insensitive to levamisole and tetramisole (inhibitors of alkaline phosphatases), whereas AMPCP inhibited the enzymatic activities in both isolates. Furthermore, Tasca et al. (2003) have shown different K_M values for both isolates (111 and 420 μM for 30,236 and 30,238 isolates, respectively). In the present report, the ecto-5′-nucleotidase characterized in intact trophozoites of *T. gallinae* was not inhibited by AMPCP, but presented a similar K_M

value when compared to 30,238 isolate of *T. vaginalis*. In contrast, the enzyme was strongly inhibited by ammonium molybdate, a 5′-nucleotidase inhibitor (Gottieb and Dwyer, 1983; Reilly and Calcutt, 2004; Barros et al., 2000). Gottieb and Dwyer (1983) described a 5′-nucleotidase activity in the surface membrane fraction of *Leishmania donovani* promastigotes. The enzyme was inhibited by ammonium molybdate, as observed in the present findings of this paper. Corte-Real et al. (1993) applied enzyme cytochemistry and immunological labeling techniques to characterize a 5′-nucleotidase in promastigote forms of four *Leishmania* species. Our results have shown an enzyme in intact trophozoites of *T. gallinae* that shares kinetic properties with an ecto-5′-nucleotidase.

The identification of the physiological significance of this enzyme could contribute to understanding biochemical aspects of *T. gallinae* and mechanisms involved in specific host–parasite interactions. Adenosine, a precursor of nucleotides in trichomonads, is a product of the 5′-nucleotidase activity. Thus, this enzyme could be considered a target for anti-trichomonad drugs in further studies.

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