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Characterization of an ecto-5′-nucleotidase activity present on the cell surface of *Tritrichomonas foetus*

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ABSTRACT

Tritrichomonas foetus is the causative agent of sexually transmitted trichomoniasis in cattle. In females, the infection can be associated with infertility, vaginitis, endometritis, abortion or pyometra, leading to significant economic losses in cattle raising. *T. foetus* is devoid of the ability to synthesize purine nucleotides *de novo*, depending instead on salvaging purines from the host environment. Ecto-5′-nucleotidase catalyzes the final step of extracellular nucleotide degradation, the hydrolysis of nucleoside 5′-monophosphates to the corresponding nucleosides and Pi. In this work we show that living, intact cells of *T. foetus* were able to hydrolyze 5′AMP at a rate of 12.57 ± 1.23 nmol Pi $\times$ h^{-1} $\times$ 10^{-7} cells at pH 7.2 and the 5′AMP hydrolysis is due to a plasma membrane-bound ecto-enzyme activity. The apparent K_m for 5′AMP was 0.49 ± 0.06 mM. In addition to 5′AMP, the enzyme hydrolyzed all substrate monophosphates tested except 3′AMP. No divalent metals or metal chelators were able to modulate enzyme activity. Phosphatase inhibitors did not have an effect on ecto-5′-nucleotidase activity while ammonium molybdate did inhibit the activity in a dose dependent manner. The presence of adenosine in the culture medium negatively modulated the enzyme. These results indicate the existence of an ecto-5′-nucleotidase that may play a role in the salvage of purines.

1. Introduction

Tritrichomonas foetus is the causative agent of cattle sexually transmitted trichomoniasis. The infection can persist in males in an asymptomatic carrier state while in females it can be associated with infertility, vaginitis, endometritis, abortion or pyometra, leading to significant economic losses in cattle raising (Corbeil et al., 1989; Aydintug et al., 1993; Singh et al., 1999). The life cycles of most trichomonads are simple, involving only monomorphic flagellate stages, with four to six flagella (Honigberg, 1963). The pathogenesis of bovine trichomoniasis may be linked to the ability of the parasite to adhere and colonize bovine vaginal epithelial cells.

More recently, *T. foetus* has been associated with an emerging infectious diarrheal disease in cats and sometimes in dogs (Payne and Artzer, 2009). Little is known about the infection and colonization process in the intestine by these parasites.

Similar to other protozoan such as *Leishmania* species (Meyer-Fernandes et al., 1997; Berrêdo-Pinho et al., 2001;

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de Almeida Marques-da-Silva et al., 2008), including *Trypanosoma cruzi* (Bisaggio et al., 2003; Meyer-Fernandes et al., 2004), *Trypanosoma brucei* (de Koning et al., 2000; Leite et al., 2007), *Giardia duodenalis* (Pinheiro et al., 2008) and *Trichomonas vaginalis* (Tasca et al., 2003), *T. foetus* is devoid of a *de novo* synthesis pathway for purine nucleotides (Wang et al., 1983), depending instead on salvaging purines from the host environment. In addition to their nutritional importance, extracellular nucleotides may participate in signaling functions through purine receptors located on the cell membrane.

Surface membrane interactions between parasites and their host cells are of critical importance for the survival of the parasite, from both immunological and physiological viewpoints. In this context, the presence of enzymes with catalytic sites facing the extracellular medium, such as ecto-ATPases and ecto-5′-nucleotidases, seem to be extremely important to the living parasite (Meyer-Fernandes, 2002; Meyer-Fernandes et al., 2010).

Ecto-5′-nucleotidase (CD73; E.C. 3.1.3.5) catalyzes the final step of extracellular nucleotide degradation, the hydrolysis of nucleoside 5′-monophosphates to the corresponding nucleosides and Pi (Zimmermann, 2000). Together with ecto-ATPases, ecto-5′-nucleotidase is responsible for the conversion of ATP to adenosine (Yegutkin, 2008; Sträter, 2006). Furthermore, these enzymes are shown to be implicated in T-cell activation and cell–cell adhesion (Sträter, 2006; Zimmermann, 2000; Hunsucker et al., 2005). Ecto-ATPase activity present on the surface of *T. foetus* has already been characterized by our group (Jesus et al., 2002). This enzyme can be stimulated by Mg^{2+} and D-galactose, a carbohydrate exposed on the surface of host cells that could be involved on *T. foetus* adhesion (Bonilha et al., 1995; Jesus et al., 2002).

Ecto-5′-nucleotidases have been described in some protozoan parasites, such as *G. duodenalis* (Russo-Abrahão et al., 2011), *T. vaginalis* (Tasca et al., 2003) and *T. gallinae* (Borges et al., 2007). Little is known about ecto-5′-nucleotidase in *T. foetus*. To investigate further the possible involvement of ecto-5′-nucleotide activity and the role of adenosine production in the survival of *T. foetus* parasite, we present herein the biochemical characterization of ecto-5′-nucleotidase activity on the surface of *T. foetus* living intact cells.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma (St. Louis, MO, USA) or Merck (São Paulo, Brazil).

2.2. Microorganisms

The long-term cultured microorganisms *T. foetus* (K strain) were analyzed throughout this study. The cells were axenically maintained by weekly passages in 10% heat-inactivated bovine serum-supplemented TYM medium (Diamond, 1957) at 36 °C. Parasites from cultures which reached the logarithmic phase of growth were collected by centrifugation at 1400 × *g* for 5 min at 4 °C, and washed three times with 50 mM Hepes-Tris buffer, pH 7.2, 116 mM NaCl, 5.4 mM KCl and 5.5 mM D-glucose. Cell growth was estimated by counting the number of parasites in a Neubauer chamber. Cellular viability was assessed before and after incubations by Trypan blue dye exclusion (Amazonas et al., 2009). The viability was not affected under the conditions employed here.

2.3. Subcellular fractionation

Parasites (1.0×10^9 cells) from 24 to 30 h cell culture were harvested by centrifugation and washed three times with 50 mM Hepes-Tris buffer, pH 7.2, 116 mM NaCl, 5.4 mM KCl and 5.5 mM D-glucose. Plasma membrane was obtained as reported by Cohen et al. (1986), with some modifications. Briefly, parasites were mixed with glass beads and disrupted by abrasion for 10 min on ice bath. After grinding, 25 mL of 10 mM Hepes-Tris buffer, pH 7.2, 400 mM mannitol, 10 mM KCl, 1 mM magnesium acetate, 1 mM PMSF, 10 μM E-64 and 1 μM pepstatin A were added to the mixture. Cell disruption was monitored by phase-contrast microcopy. The glass beads and unbroken cells were removed by centrifugation at 1400 × *g* for 15 min at 4 °C. The supernatant (total homogenate) was centrifuged at 5000 × *g* for 20 min, at 16,000 × *g* for 40 min and then at 105,000 × *g* for 60 min. The resulting supernatant (soluble fraction) was obtained. The resulting pellet (total membrane) was resuspended in 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT and 75 mM Hepes-Tris buffer, pH 7.2 and subsequently applied in a continuous density gradient of 18% Percoll in 250 mM sucrose and 12 mM Tris–HCl, pH 7.2. After centrifugation at 40,000 × *g* for 60 min, the plasma membrane was obtained. Protein concentration of each fraction was determined by Lowry et al. (1951), using BSA as standard.

2.4. Ecto-5′-nucleotidase activity measurements

Ecto-5′-nucleotidase activity was determined by the rate of inorganic phosphate (Pi) release. Intact cells (10^7 cells) were incubated for 1 h at 36 °C in 0.5 mL of reaction mixture containing, unless otherwise specified, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50 mM Hepes-Tris buffer, pH 7.2, and 5.0 mM 5′AMP as substrate. The reaction was initiated by the addition of cells and stopped by the addition of 1.0 mL of ice-cold 25% charcoal in 0.1 M HCl. This charcoal suspension was washed at least 20 times with 0.1 M HCl before use to avoid Pi contamination (Guilherme et al., 1991). This procedure reduce the values of blanks once that remove non-hydrolyzed 5′AMP from the sample that are spontaneously hydrolyzed in the presence of sulfuric acid present in Fiske–Subbarow reactive mixture (Fiske and Subbarow, 1925). Controls in which cells were added after interruption of the reaction were used as blanks. After the reaction, the tubes were centrifuged at 1500 × *g* for 15 min at 4 °C, and 0.5 mL of the supernatant was added to 0.5 mL of Fiske–Subbarow reactive mixture (Fiske and Subbarow, 1925). The ecto-5′-nucleotidase activity was calculated by subtracting the nonspecific 5′AMP hydrolysis measured in the absence of cells. The concentration of Pi released in the reaction was determined using a standard