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Tiana Tasca · Carla D. Bonan · Geraldo A. De Carli
João J. F. Sarkis

Trichomonas vaginalis: cytochemical localization of a NTPDase1 and an ecto-5′-nucleotidase and effects of adenine nucleotides on cellular viability

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Abstract Nucleoside triphosphate diphosphohydrolase 1 (NTPDase1), which hydrolyzes extracellular ATP and ADP, and ecto-5′-nucleotidase, which hydrolyzes AMP, are characterized for *Trichomonas vaginalis*. Ultrastructural cytochemical microscopy showed NTPDase1 and ecto-5′-nucleotidase activities on the surface of the parasites. High levels of extracellular adenine nucleotides and adenosine did not exert cytolytic effects in intact cells of *T. vaginalis*. Our results suggest that these enzymes are relevant for the survival of the parasite during exposure to extracellular nucleotides. Since the ecto-localization of these enzymes is essential for the maintenance of adenosine extracellular levels, this nucleoside could be important for the purine salvage pathway in the parasite.

Purine nucleosides and nucleotides are mostly released from cells which are stressed or anoxic, injured and metabolically active (reviewed in Chow et al. 1997). There is evidence that purines have cytotoxic properties (Steinberg and Di Virgilio 1991). Signaling actions induced by extracellular ATP are correlated with the activity of a group of ecto-enzymes, the ectonucleotidases, which hydrolyze ATP to adenosine. This group includes NTPDase1 (CD39, ecto-apyrase, EC 3.6.1.5) and the ecto-5′-nucleotidase (CD73, EC 3.1.3.5), which were previously characterized by our laboratory for *Trichomonas vaginalis* (Matos et al. 2001; Tasca et al. 2003a). Trichomonosis is the most common non-viral sexually transmitted disease in the world and it is associated with serious health consequences (Lehker and Alderete 2000).

Recently, ecto-phosphatase activity and its cytochemical localization were characterized in *T. vaginalis* (De Jesus et al. 2002). However, the cytochemical localization of ectonucleotidases has been shown in various parasites, but not in *T. vaginalis*. The distribution of NTPDase1 has been demonstrated in *Schistosoma mansoni* (Vasconcelos et al. 1993, 1996) and in *Leishmania amazonensis* promastigotes (Coimbra et al. 2002). A study on the cytochemical localization of ecto-5′-nucleotidase was made in *Toxoplasma gondii*, which is deficient in the activity of this enzyme (Ngô et al. 2000). To our knowledge, this is the first report of the cytochemical localization of NTPDase1 and ecto-5′-nucleotidase activities in intact cells of *T. vaginalis*. In parallel, we evaluated cellular viability in the presence of increasing concentrations of adenine nucleotides.

The 30236 isolate of *T. vaginalis*, from the American Type Culture Collection, was cultivated in trypticase-yeast extract-maltose medium (Diamond 1957) with 10% heat-inactivated bovine serum, as previously described (Tasca et al. 2003a). For enzyme cytochemistry and viability assays, trichomonads were counted with a hemocytometer and adjusted to a concentration of 1.5×10^6 cells/ml (0.3–0.7 mg/ml of protein). Protein was measured by the Coomassie blue method (Bradford 1976). The NTPDase1 and ecto-5′-nucleotidase activities were measured as previously described (Tasca et al. 2003b). All samples were run in triplicate, with similar results achieved in at least four different cell suspensions. In cytochemistry assays, these enzyme activities were

T. Tasca · J. J. F. Sarkis (✉)
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, Brazil
E-mail: jjsarkis@plug-in.com.br
Tel.: +55-51-33165554
Fax: +55-51-33165535

T. Tasca · G. A. De Carli
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, Brazil

C. D. Bonan
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, Brazil

T. Tasca
Department of Microbiology, University of Texas Health Science Center, MC7758, 7703 Floyd Curl Drive, San Antonio,
TX 78229-3900, USA

detected by an electron-dense precipitate of lead phosphate that is formed by the release of Pi after ATP, ADP (for NTPDase1) and AMP hydrolysis (for ecto-5′-nucleotidase) (Caldas and Wasserman 1995). Intact cells were incubated in a reaction medium containing 25 mM TRIS-maleate buffer (pH 7.2), 5.0 mM $CaCl_2$ (for NTPDase 1), 5.0 mM $MgCl_2$ (for 5′-nucleotidase) and 2.0 mM $Pb(NO_3)_2$. To avoid the influence of other enzymes, inhibitors (1.0 mM levamisole, 0.1 mM vanadate, 0.1 mM *N*-ethyl maleimide and 1.0 mM ouabain) were added to both reaction media. The reaction was initiated by the addition of 3.0 mM ATP or ADP and AMP. The incubation for both enzyme activities was performed at 37°C for 1 h and the reaction was stopped by immersion of the samples in ice-water for 5 min followed by centrifugation. Controls were performed in which the incubations were conducted in the above media without substrates. After centrifugation, the pellets were washed three times with 0.1 mM cacodylate buffer, pH 7.2 and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mM cacodylate buffer, pH 7.2, for 1 h. Post-fixation was performed in 1% osmium tetroxide in 0.1 mM cacodylate buffer, pH 7.2, for 30 min. Dehydration was carried out in acetone and samples were included in araldite resin. Ultrathin sections (100 nm) were cut with an ultramicrotome (Leica UTC 2.0), and samples were stained with uranyl acetate and lead citrate. Stained and unstained sections were observed with a Jeol (JEM 1200 EXII) electron microscope. For the cellular viability assays, the cells were

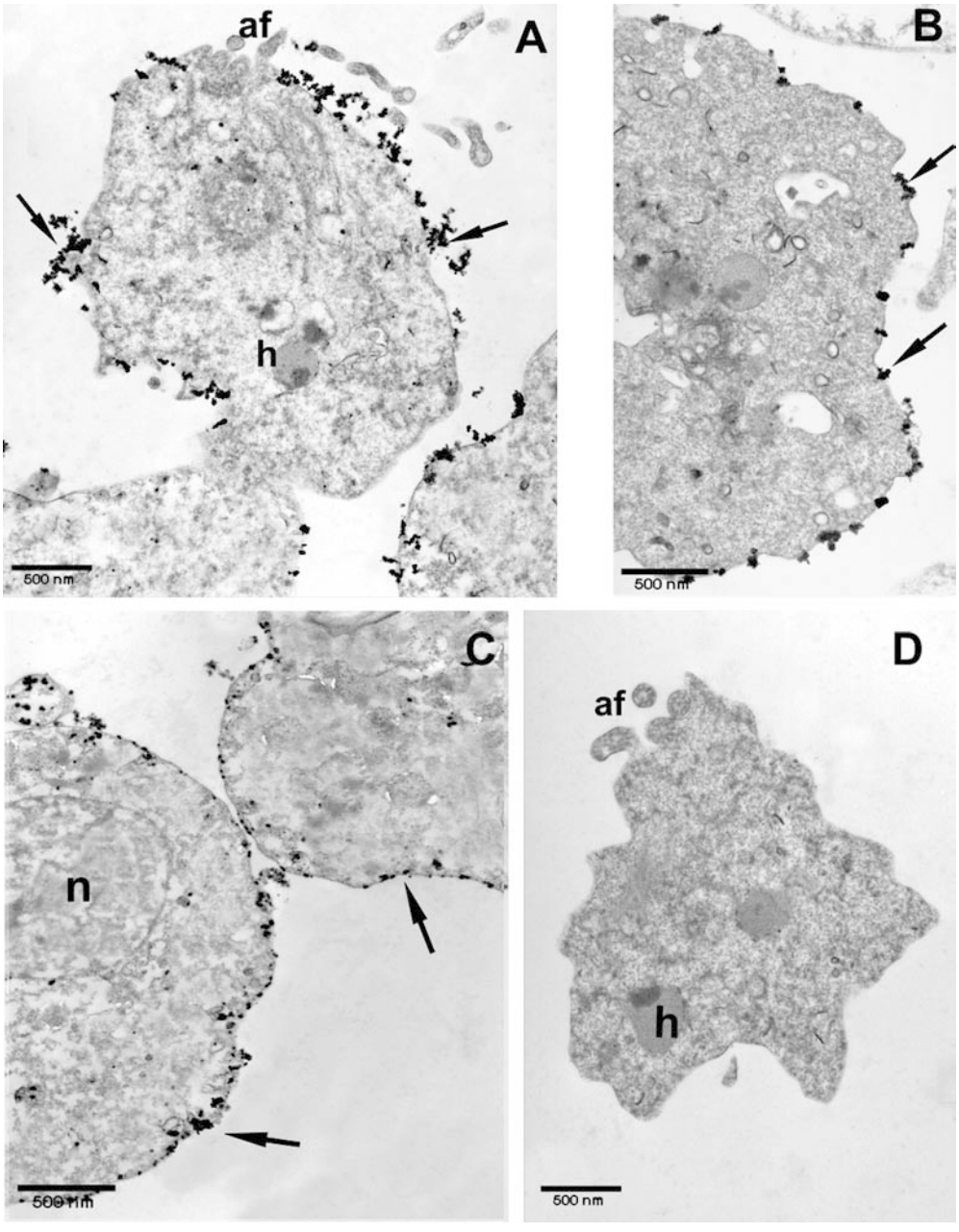

Fig. 1A–D Ultrastructural localization of NTPDase1 and ecto-5′-nucleotidase activities in intact cells of *Trichomonas vaginalis*. *Arrows* show electron-dense lead phosphate deposits originating from: **A** ATP, **B** ADP, and **C** AMP hydrolysis distributed at the surface of the plasma membrane of the parasites. **D** Control without substrates as described in the text. *af* Anterior flagella, *h* hydrogenosomes, *n* nucleus

incubated at 37°C in the presence of 5.0, 10 or 15 mM ATP, ADP, AMP or adenosine. To evaluate cellular viability, a 10-µl aliquot of cells was collected from the assays at 2, 4 and 8 h of incubation and mixed with 10 µl 0.4% trypan blue dye and counted. In parallel, cell viability was evaluated by lactate dehydrogenase activity, as a percentage, in comparison with the lysed cells (100%), after immersion in liquid nitrogen (−196°C) for 60 s.

We previously characterized NTPDase1 and ecto-5′-nucleotidase activities in *T. vaginalis* (Matos et al. 2001; Tasca et al. 2003a). The 30236 isolate showed NTPDase1 activity, 0.50 ± 0.06 nmol Pi/min/1.5×10^6 cells for ATP and 0.43 ± 0.08 nmol Pi/min/1.5×10^6 cells for ADP (mean ± SD, $n = 12$). The ecto-5′-nucleotidase activity shown by this isolate was 0.32 ± 0.05 nmol Pi/min/1.5×10^6 cells ($n = 9$).

The cytochemical localization of NTPDase1 and ecto-5′-nucleotidase activities was detected by the formation of electron-dense precipitates of lead phosphate, deposited in the vicinity of the enzymes, as observed at the plasma membrane surface, along the trophozoites (Fig. 1). Figure 1A and B shows the ecto-localization of NTPDase1, which appeared to be distributed at the external surface of the parasite even when different substrates, ATP (Fig. 1A) or ADP (Fig. 1B), were used. The external localization of ecto-5′-nucleotidase is shown in Fig. 1C, with the lead phosphate deposits formed at the plasma membrane surface, demonstrating AMP hydrolysis. In the absence of nucleotides, no electron-dense deposits were seen (Fig. 1D). The evaluation of cellular viability using trypan blue exclusion and lactate dehydrogenase activity showed that none of the tested concentrations of adenine nucleotides and adenosine produced cytotoxicity to *T. vaginalis* (data not shown).

Several studies have shown that extracellular ATP may act as a signaling compound in cytolytic mechanisms (Steinberg and Di Virgilio 1991). The concentration of free purine nucleotides encountered in the human vagina, the natural site of *T. vaginalis*, originating from the discharged and lysed epithelial cells during the infection, can reach 10 mM, of which 90% are adenine nucleotides (Munagala and Wang 2003). Considering the high levels of extracellular nucleotides, the presence of enzymes performing ATP, ADP and AMP hydrolysis is essential for parasite survival in a hostile changing environment by modulating the nucleotide concentration in the extracellular space and by protecting from the cytolytic effects of extracellular ATP. Our data show that high levels of extracellular adenine nucleotides and adenosine did not exert cytolytic effects on intact cells of *T. vaginalis*, being hydrolyzed by NTPDase1 and ecto-5′-nucleotidase. Experiments using antibodies against the enzymes and the search for the corresponding genes in *T. vaginalis* are in progress.

Modulation of nucleotide concentration in the extracellular space, protection from the cytolytic effects of extracellular ATP, and participation in the salvage pathway of nucleosides are some aspects of the physiology of *T. vaginalis* involving the breakdown of ATP. *T. vaginalis* lacks the ability to synthesize purines and pyrimidines de novo, and its growth and survival is dependent on salvage pathways to generate nucleotides (Heyworth et al. 1982). Taking into account the high levels of purine nucleotides and nucleosides in the host site occupied by *T. vaginalis*, the lack of cytolytic effects on the parasites and the ecto-localization of the enzymes involved in the hydrolysis of the nucleotides, we suggest that the NTPDase1 and the ecto-5′-nucleotidase modulate the levels of extracellular ATP, ADP and AMP. The final product of these reactions, the nucleoside adenosine, is taken up by the parasite (Harris et al. 1988). Recently, Munagala and Wang (2003) showed that adenosine is the primary precursor of the entire purine nucleotide pool in *T. vaginalis*, and they identified adenosine deaminase, IMP dehydrogenase and GMP synthetase activities in the parasite lysate, suggesting a pathway capable of converting adenine to GMP via adenosine. Considering the importance of adenosine in the purine salvage in *T. vaginalis*, NTPDase1 and ecto-5′-nucleotidase are essential for the survival strategies of the parasite during exposure to the extracellular nucleotides.

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