RESEARCH LETTER

Characterization of nucleoside triphosphate diphosphohydrolase activity in *Trichomonas gallinae* and the influence of penicillin and streptomycin in extracellular nucleotide hydrolysis

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Abstract

Here we described an nucleoside triphosphate diphosphohydrolase (NTPDase) activity in living trophozoites of *Trichomonas gallinae*. The enzyme hydrolyzes a variety of purine and pyrimidine nucleoside di- and triphosphates in an optimum pH range of 6.0–8.0. This enzyme activity was activated by high concentrations of divalent cations, such as calcium and magnesium. Contaminant activities were ruled out because the enzyme was not inhibited by classical inhibitors of ATPases (ouabain, 5.0 mM sodium azide, oligomycin) and alkaline phosphatases (levamisole). A significant inhibition of ATP hydrolysis (38%) was observed in the presence of 20 mM sodium azide. Sodium orthovanadate inhibited ATP and ADP hydrolysis (24% and 78%), respectively. The apparent K_M (Michaelis constant) values were 667.62 ± 13 μM for ATP and 125 ± 5.3 μM for ADP. V_{max} (maximum velocity) values were 0.44 ± 0.007 nmol Pi min^{-1} per 10^6 trichomonads and 0.91 ± 0.12 nmol Pi min^{-1} per 10^6 trichomonads for ATP and ADP, respectively. Moreover, we showed a marked decrease in ATP, ADP and AMP hydrolysis when the parasites were grown in the presence of penicillin and streptomycin. The existence of an NTPDase activity in *T. gallinae* may be involved in pathogenicity, protecting the parasite from the cytolytic effects of the extracellular nucleotides.

Introduction

Trichomonas gallinae is a flagellated protozoan that 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). Other columbiform hosts have been found to arbor the parasite, as have also galliform birds, Java sparrows, raptors and sea gulls. This trichomonad species has caused significant economic losses, especially in turkeys as well as in chickens and pet birds (BonDurant & Honigberg, 1994).

The normal sites of *T. gallinae* are the mouth, pharynx, esophagus and crop, where they cause the formation of caseous lesions (Jessup, 1980). In pigeons, trichomonosis is mainly a disease of young birds, causing serious losses among these birds (Stabler, 1954). The protozoan is the causative agent of canker in pigeons, causing a variety of pathological manifestations depending on the parasite isolate and the infected bird species (Henderson *et al.*, 1988). The virulent isolates may cause lesions in the upper digestive tract of birds, which allow the pathogen to enter the circulatory system and gain access to the liver, lungs, heart and pancreas (Baker, 1986; Cooper & Petty, 1988).

Besides its energy function in the intracellular environment, ATP has several effects on many biological processes, and can be found in significant concentrations outside of the cells (Dombrowski *et al.*, 1998). The level of exogenous ATP may be increased in various inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial and blood vessel cells (Bodin &

Burnstock, 1996; Dubyak, 2000), modulating biological processes by binding to specific cell surface receptors. In addition, extracellular ATP may act as a signalling compound in cytolytic mechanisms (Filippini *et al.*, 1990; Steinberg & Di Virgilio, 1991), causing plasma membrane depolarization, Ca^{2+} influx and cell death.

ATP and other nucleotides can be inactivated through hydrolysis by ecto-nucleotidases. This group of enzymes includes the E-NTPDase family (ectonucleoside triphosphate diphosphohydrolase family), the E-NPP family (ectonucleotide pyrophosphatase/phosphodiesterase family), ecto-5′-nucleotidase and alkaline phosphatases (Zimmermann, 2001). Some of these enzymes are attached to the plasma membrane, with their active sites facing the extracellular side/environment. Nucleoside triphosphate diphosphohydrolase (NTPDases) are enzymes that hydrolyze ATP, ADP and other triphosphonucleosides and diphosphonucleosides to their equivalent monophosphonucleosides and inorganic phosphate (Zimmermann & Braun, 1999). Furthermore, they are activated by high concentrations of divalent cations and they are not inhibited by classical inhibitors of ATPases or alkaline phosphatase (Zimmermann, 1996). The eight members of this protein family described already differ in their cellular localization and preference for nucleoside 5′-diphosphates (Zimmermann, 2001; Bigonnesse *et al.*, 2004).

Recently, we have demonstrated an ecto-5′-nucleotidase activity in intact trophozoites of *T. gallinae* (Borges *et al.*, 2007). 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 to be a potent inhibitor of 5′-nucleotidase activity) completely inhibited the enzyme activity. 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.

The activities of the ecto-enzymes can be measured using living cells (Furuya *et al.*, 1998; Zimmermann, 2001). The presence of these enzymes has been demonstrated in the surface membranes in some parasites. Vasconcelos *et al.* (1993, 1996) characterized and purified an ATP diphosphohydrolase activity (apyrase, CD39, NTPDase1) on the external surface of *Schistosoma mansoni*. Turner & Lushbaugh (1991) identified three ATPases in the sedimentable fractions of *Trichomonas vaginalis*. An ecto-nucleotide diphosphohydrolase was described in intact cells of *Entamoeba histolytica* (Barros *et al.*, 2000). Recently, Jesus *et al.* (2002) characterized an ecto-ATPase in *Tritrichomonas foetus*. Furthermore, an ATP diphosphohydrolase and an ecto-5′-nucleotidase were described in *T. vaginalis* (Matos *et al.*, 2001; Tasca *et al.*, 2003).

The present study describes the properties of an NTPDase activity in intact trophozoites of *T. gallinae*, the etiologic agent of trichomonosis in birds. Moreover, Stabler *et al.* (1964) showed that common laboratory procedures, such as the addition of antibiotics in cultures used to avoid contamination, could negatively influence the biological features of the isolate, such as pathogenicity levels. Therefore, we tested the hypothesis that the presence of penicillin and streptomycin sulfate would exert some effect on extracellular ATP, ADP and AMP hydrolysis from *T. gallinae* trophozoites.

Materials and methods

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 medium (Diamond, 1957) without agar (pH 7.2) supplemented with 10% (v/v) inactivated bovine serum, without antibiotics (Stabler *et al.*, 1964; Tasca & De Carli, 1999), at 37 °C. 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 hemocytometer and adjusted to a density of 4×10^6 organisms mL^{-1}. All samples were run in triplicate, with results achieved in at least three different parasite suspensions. All organisms were viable based on motility, assessed before and after incubations. The viability was not affected by the incubation conditions.

Enzyme assays for biochemical characterization of an NTPDase activity

After preparing the parasite samples, the optimum conditions for nucleotide hydrolysis were determined. Intact trophozoites of *T. gallinae* (10^6 trichomonads mL^{-1}) were added to the reaction mixture containing 50 mM Tris buffer (pH 7.2) and 1.0 mM $CaCl_2$. The samples were preincubated for 5 min at 37 °C in the reaction mixture. The reaction was initiated by the addition of substrate ATP or ADP to a final concentration of 1.0 mM. After 15 min, the reaction was stopped by adding 200 μL 10% trichloroacetic acid (TCA). The samples were chilled on ice before assaying for the release of inorganic phosphate (Chan *et al.*, 1986), using malachite green as the colorimetric reagent and KH_2PO_4 as the standard. Incubation times and parasite density were chosen in order to ensure the linearity of the reactions. Controls included intact organisms added to the reaction mixtures containing TCA in order to correct nonenzymatic

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^{-1} per 10^6 trichomonads.

Ecto-5′-nucleotidase

The determination of ecto-5′-nucleotidase activity was performed as described before (Borges *et al.*, 2007).

Treatment with antibiotics

In order to investigate the effect of penicillin and streptomycin, antibiotics commonly used in *in vitro* cultures, parasites were cultivated in the presence of 5000 IU penicillin and 1.0 mg mL^{-1} streptomycin sulfate for 90 days (Stabler *et al.*, 1964). Then, the organisms were washed three times with 0.9% (w/v) NaCl solution and the NTPDase and ecto-5′-nucleotidase activities were determined as described above. Controls were parasites cultivated without antibiotics.

Statistical analysis

Statistical analysis was conducted by Student's *t*-test or one-way ANOVA. $P < 0.05$ was considered to represent statistical significance.

Results

Intact trophozoites of *T. gallinae* demonstrated the ability to hydrolyze ATP and ADP. The time course for ATP and ADP hydrolysis was linear up to 20 min in the presence of Ca^{2+} and the product formation increased as a function of parasite density in the range of $0.8–2.0 \times 10^6$ trichomonads mL^{-1} (data not shown).

Like other NTPDases, the enzyme was divalent cation activated and Ca^{2+} was considered to be the best activator of ATP and ADP hydrolysis (Fig. 1), although Mn^{2+} and Mg^{2+} were also able to promote a stimulatory effect on NTPDase activity (data not shown). Considering these results, a concentration of 1.0 mM Ca^{2+} was selected for subsequent enzyme assays. Cation activation was confirmed by a significant decrease in ATP and ADP hydrolysis in the absence of Ca^{2+} or in the presence of Ca^{2+} plus 10 mM EGTA, a cation chelator (Fig. 1). To evaluate the optimum pH of these enzyme activities, Ca^{2+}-ATP and Ca^{2+}-ADP were used as substrates in a medium containing 50 mM Tris-histidine (pH 6.0, 6.5, 7.2, 8.0, 9.0). The maximum rate of nucleotide hydrolysis was observed at pH 7.2 (data not shown).

Taking into account that NTPDases have been described as enzymes with broad substrate specificity, we also investigated the ability of the enzyme to hydrolyze other di- and triphosphate nucleosides. All nucleotides tested were hydrolyzed by intact trophozoites of *T. gallinae* (Table 1). The high

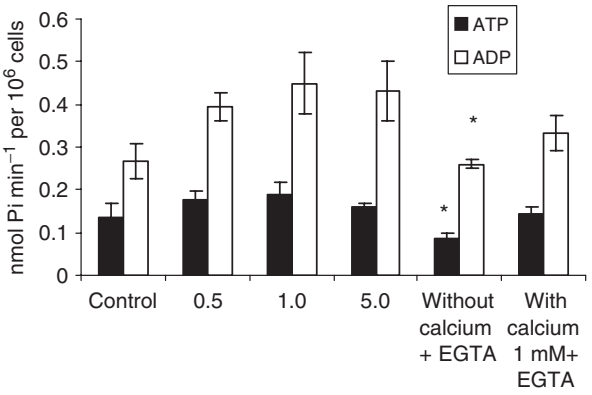

Fig. 1. Effect of $CaCl_2$ concentration on NTPDase activity in intact trophozoites of *Trichomonas gallinae*. Closed and open bars represent ATPase and ADPase activity in the presence of $CaCl_2$, respectively. Incubation conditions were described in 'Materials and methods.' The control group was assayed without the addition of a cation. Bars represent the means ± SD for three experiments, using different parasite suspensions. *Significant difference from activity in the presence of 1.0 mM $CaCl_2$ ($P < 0.05$).

rate of AMP hydrolysis was due to an ecto-5′-nucleotidase activity present in the parasite surface, which has already been characterized by our laboratory (Borges *et al.*, 2007). The low rate of pyrophosphate and cyclic AMP hydrolysis excludes the presence of nonspecific pyrophosphatases and phosphodiesterases as contaminant enzymes participating in ATP or ADP hydrolysis in *T. gallinae*.

To eliminate the possibility that the ATP hydrolysis was due to other possible contaminant enzymes, different inhibitors were tested (Table 2). No significant inhibition was observed with 1.0 mM ouabain (Na^+-K^+-ATPase inhibitor), 5.0 mM sodium azide or 2.0 μg mL^{-1} oligomycin (inhibitors of mitochondrial ATPase). Levamisole (1.0 mM), a specific alkaline phosphatase inhibitor, also did not inhibit the NTPDase activity. However, in the presence of 20 mM sodium azide, significant inhibition of ATP hydrolysis was observed (38%). When 0.1 mM sodium orthovanadate was tested, ATPase and ADPase activities were strongly inhibited (24% and 78%, respectively).

To show that a single active site is involved in ATP and ADP hydrolysis in the enzyme from intact trophozoites of *T. gallinae*, intact organisms were incubated in the standard reaction medium containing 1.0 mM ATP, 1.0 mM ADP or a mixture containing 1.0 mM ATP and ADP at the same time (Fig. 2). The activities obtained with each individual nucleotide were 0.14 ± 0.01 nmol Pi min^{-1} per 10^6 trichomonads and 0.49 ± 0.05 nmol Pi min^{-1} per 10^6 trichomonads for ATP and ADP, respectively. In the presence of a mixture containing both substrates at a final concentration of 1.0 mM, the specific activity was 0.39 ± 0.05 nmol Pi min^{-1} per 10^6 trichomonads. This value approximately corresponds to the arithmetic mean of the activities observed

with each individual nucleotide, indicating that a single enzyme is involved in ATP and ADP hydrolysis (Fig. 2).

ATP hydrolysis and ADP hydrolysis were determined at substrate concentrations in the range of 100–2000 μM (Fig. 3). The enzyme activity increased with increasing concentrations of the nucleotide in the presence of 1.0 mM Ca^{2+}. K_M and V_{max} values for ATP and ADP hydrolysis were estimated from the Lineweaver–Burk plots. The apparent K_M (Michaelis constant) values were 667.62 ± 13 μM for ATP and 125 ± 5.3 μM for ADP. V_{max} (maximum velocity) values were 0.44 ± 0.007 nmol Pi min^{-1} per 10^6 trichomonads and 0.91 ± 0.12 nmol Pi min^{-1} per 10^6 trichomonads for ATP and ADP, respectively.

Finally, we have shown the effect of penicillin and streptomycin sulfate on ATP, ADP and AMP hydrolysis (Fig. 4). Parasites cultivated in the presence of antibiotics showed a decrease in extracellular ATP, ADP and AMP hydrolysis (50%, 59% and 49%, respectively) when compared with control parasites (grown without antibiotics).

Table 1. Substrate specificity of NTPDase from intact trophozoites of *Trichomonas gallinae*

Substrate	Relative activity
ATP	100 ± 29
GTP	147 ± 29
GDP	135 ± 6
CTP	176 ± 23
UTP	194 ± 41
UDP	135 ± 29
ADP	280 ± 58
AMP	550 ± 100
PPi	53 ± 7
AMPc	11 ± 3

Data represents means ± SD of at least three experiments. The ATP hydrolysis (0.17 ± 0.05 nmol Pi min^{-1} per 10^6 trichomonads) was taken as 100%. The substrates were used at 1.0 mM (except AMP – 3.0 mM), with 1.0 mM $CaCl_2$.

Discussion

In this paper, we demonstrated that intact trophozoites of *T. gallinae* hydrolyze adenine nucleotides, such as ATP, ADP and other di- and triphosphate nucleotides, suggesting the presence of an NTPDase. The presence of enzymes performing ATP, ADP and other nucleotide hydrolyses has been described in some protozoa and has been associated with virulence and the evasion of parasites from the host defense mechanisms (Barros *et al.*, 2000; Berrêdo-Pinho *et al.*, 2001). Matos *et al.* (2001) reported the enzymatic properties of an apyrase in *T. vaginalis*. As observed previously in *T. vaginalis*, our results have shown that the ecto-enzyme from *T. gallinae* was inhibited by 20 mM sodium azide. Furthermore, orthovanadate, an inhibitor of mammalian NTPDase, at a low concentration (0.1 mM) inhibited ATP and ADP hydrolysis from *T. gallinae*. Jesus *et al.* (2002) characterized an ecto-ATPase in living trophozoites of *T. foetus*. ATP was the best substrate, but ADP was not hydrolyzed for this enzyme. In *T. gallinae*, we reported an enzyme able to hydrolyze di- and triphosphate nucleotides (ADP > UTP > CTP > GTP > UDP = GDP > ATP), but has a threefold preference for the hydrolysis of ADP over ATP. To exclude the possibility that the nucleotide hydrolysis was promoted by secreted soluble enzymes (Bermudes *et al.*, 1994; Smith *et al.*, 1997), the parasite suspension was centrifuged and the supernatant was assayed under the conditions described in 'Materials and methods.' However, neither ATP nor ADP hydrolysis was detected using this sample. Therefore, considering these findings and the viability of the intact organisms tested, we suggest an ecto-localization of the enzyme described here.

Tasca *et al.* (2005) have shown heterogeneity in the ATP : ADP hydrolysis ratio in fresh clinical isolates of *T. vaginalis*, as well as the absence of 5′-nucleotidase in some isolates. In contrast, the high rate of AMP hydrolysis observed here was due to a 5′-nucleotidase activity present in *T. gallinae* surface, which has already been described by

Table 2. Effect of inhibitors on NTPDase hydrolysis from intact trophozites of *Trichomonas gallinae*

		% Control enzyme activity	
Inhibitor	Concentration	ATP	ADP
Orthovanadate	0.1 mM	76 ± 10*	22 ± 3*
Azide	5 mM	85 ± 20	94 ± 3
	20 mM	62 ± 14*	89 ± 12
Levamisole	1 mM	123 ± 10	115 ± 2
Oligomicyn	2 μg mL^{-1}	81 ± 19	83 ± 7
Ouabain	1 mM	104 ± 24	87 ± 3

Results were expressed as percentage of control activity (100%). Control NTPDase activity was 0.21 ± 0.02 and 0.58 ± 0.12 nmol Pi min^{-1} per 10^6 trichomonads for ATP and ADP, respectively. ATP and ADP were used at 1.0 mM, in the presence of $CaCl_2$ 1.0 mM. Data represent the means ± SD for at least three determinations.

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

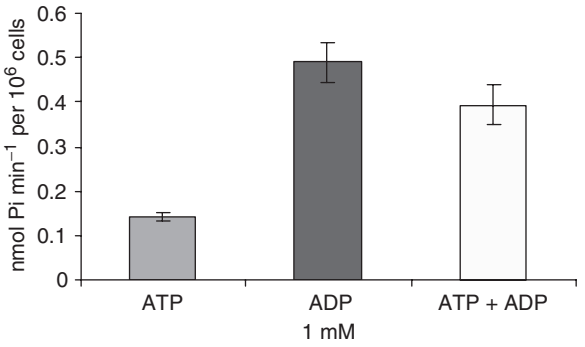

Fig. 2. Incubation of intact trophozoites of *Trichomonas gallinae* in the presence of 1.0 mM ATP, 1.0 mM ADP or a mixture of 1.0 mM ATP and ADP simultaneously. The specific activity was approximately the arithmetic mean of the activities obtained with each individual nucleotide. Bars represent the means ± SD for three experiments, using different parasite suspensions.

our laboratory (Borges *et al.*, 2007). This enzyme hydrolyzes nucleoside monophosphates at pH 7.2, is activated by divalent cations, such as magnesium, and is inhibited by 0.1 mM ammonium molybdate (considered to be a potent inhibitor of 5′-nucleotidase activity) (Borges *et al.*, 2007).

The presence of enzymes performing ATP, ADP and AMP hydrolysis in this parasite may be important for the modulation of nucleotide concentration in the extracellular medium. In some parasites, the level of ATP-breakdown activity on their surfaces is associated with virulence and evasion of parasites, escaping the host defense mechanisms (Barros *et al.*, 2000; Berrêdo-Pinho *et al.*, 2001). Considering that *T. gallinae* is a serious pathogen of birds causing a variety of pathological manifestations including lesions in various organs due to access into blood circulation, the presence of enzyme activity that inactivates extracellular ATP is a defense mechanism for the parasite to escape from cytolytic effects and to acquire success in parasitism. Furthermore, this study showed the effect of a common laboratory procedure, cultivation with antibiotics, on NTPDase and ecto-5′-nucleotidase activities. Both penicillin and streptomycin decreased ATP, ADP and AMP hydrolysis, yielding an accumulation of these nucleotides on extracellular milieu. Considering the natural site of the parasite, modulation of the nucleotide concentration may have an important role during inflammation, because extracellular ATP is an immune-modulatory molecule, involved in lymphocytes function regulation through stimulation of cytokines IL-2 and IFN-γ (Langston *et al.*, 2003). On the other hand, adenosine may act as an anti-inflammatory agent and, through interaction with specific receptors, this compound regulates the consequences of inflammation (Hasko & Cronstein, 2004). Therefore, in the presence of antibiotics, *T. gallinae* accumulates ATP, ADP and AMP, creating a disadvantageous environment, with the

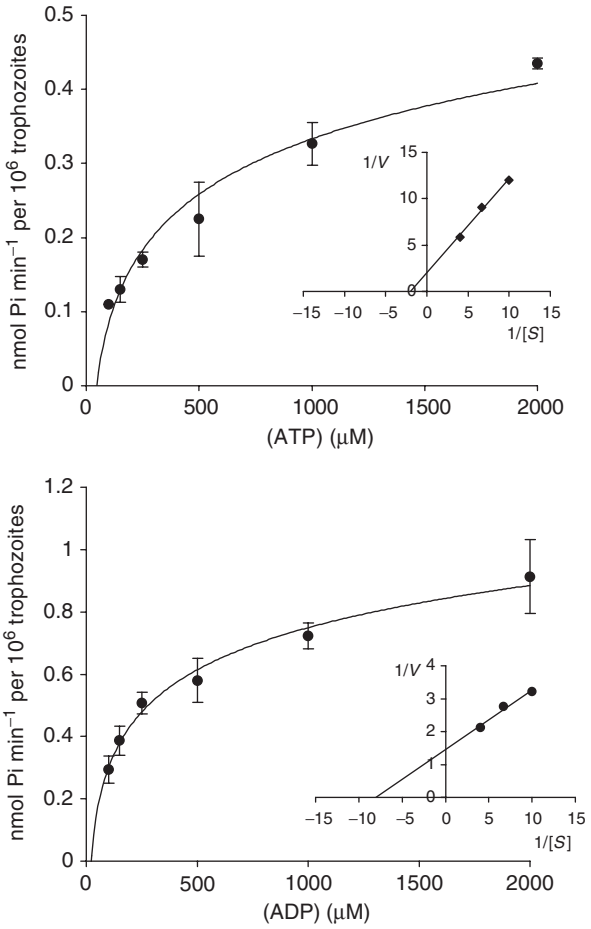

Fig. 3. Effect of different concentrations of substrate (100–2000 μM) on ATP and ADP hydrolyses in intact trophozoites of *Trichomonas gallinae*. All experiments used fixed 1.0 mM Ca^{2+} with variable concentrations of nucleotide. Data represent mean ± SD of three different experiments, each in triplicate.

presence of ATP (with cytolytic and proinflammatory properties) and absence of adenosine (anti-inflammatory molecule). These results are in agreement with those of Stabler *et al.* (1964), which showed that antibiotics could reduce the pathogenicity levels of *T. gallinae* isolates. Although the physiological function of NTPDase and ecto-5′-nucleotidase in the cell surface of trichomonads is still unknown, studies have been revealing the implication of NTPDase in the virulence of pathogens, including the protozoa *Trypanosoma cruzi* and the bacteria *Legionella pneumophila* (Fietto *et al.*, 2004; Sansom *et al.*, 2007).

Finally, trichomonads lack the ability to synthesize purines and pyrimidines *de novo* and their growth and survival depend on salvage pathways to generate nucleotides (Heyworth *et al.*, 1982, 1984). Then, it is strongly suggested that the presence of ecto-nucleotidases in trichomonads protects the parasite from the cytolytic effects of nucleotides and provides the adenosine required for parasite growth.

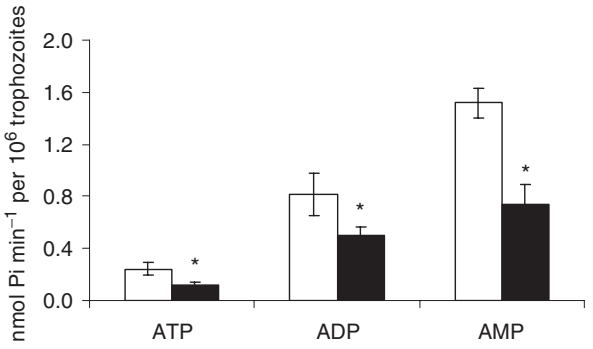

Fig. 4. Effect of penicillin and streptomycin sulfate on ATP, ADP and AMP hydrolyses. The enzyme activities are those incubated with no antibiotics (clear bars) and with antibiotics (solid bars). Data represent mean ± SD of three different experiments, each in triplicate. *Statistically significant enzyme activity differences when compared with control ($P < 0.05$).

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