An Ecto-ATPase Activity Present in *Leishmania tropica* Stimulated by Dextran Sulfate

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Z. Naturforsch. **56c,** 820–825 (2001); received April 11/May 30, 2001

Leishmania tropica, Ecto-ATPase, Sulfated Polysaccharides

In this study we report the effects of sulfated polysaccharides on the ecto-ATPase activity of intact cells of *Leishmania tropica*. Increasing concentrations of dextran sulfate stimulated progressively the ecto-ATPase activity, but did not modify other ecto-enzymes present on the surface of this parasite, such as 5′nucleotidase, 3′nucleotidase and a membrane-bound acid phosphatase activity. This stimulation was not observed when other sulfated polysaccharides such as chondroitin sulfates and heparin were tested. It depends on size and charge of the dextran sulfated molecule. When the cells were incubated in the presence of dextran sulfate *Mr* 8,000; 40,000 and 500,000 the stimulation of the ecto-ATPase activity was 11%; 23%; and 63%, respectively, and the stimulation was not observed when desulfated dextran (*Mr* 40,000) was used. The effects of dextran sulfate also depend on pH of the medium. At pH 7.5, the stimulation was over 60%, whereas at pH 8.5 only 25%. The effects of dextran sulfate 500,000 on the ecto-ATPase activity was totally abolished by spermidine and partially by putrescine, two polyamines synthesized and released by *Leishmania*.

Introduction

Infection with *Leishmania*, a parasitic protozoan, represents a major health problem in the tropics and subtropics, with ~380 000 cases annually and 367 million people at risk of infection (Ashford *et al.*, 1992). During their life cycle, the parasites undergo profound morphological changes. *Leishmania* parasites lead a digenetic life cycle, with the parasites migrating between insect vectors and mammalian hosts. The parasites exist in two forms, as extracellular flagellated promastigotes in the alimentary tract of the sand fly vector and as obligatory intracellular amastigote within the phagolysosome vacuoles of mammalian macrophages (Ashford *et al.*, 1992). Once inside the phagolysosome, promastigotes transform into non-motile, non-flagellated amastigote (Ashford *et al.*, 1992).

Cell-cell recognition and adherence are central processes to many fundamental areas of biology. Surface membrane interactions between parasites and their host cells are of critical importance for the parasite survival, from both immunological and physiological points of view (Martiny *et al.*, 1996, 1999). Ecto-ATPases are integral membrane glycoproteins that exhibit divalent cation-dependent ATPase activity on the extracellular side of the plasma membrane. The identities and functions of ecto-ATPases are the subject of a recent review in which the nomenclature of "E-type ATPases" was proposed to describe these enzymes (Plesner, 1995). Their physiological role is still unknown. However, several hypotheses have been suggested, such as regulation of ectokinase substrate concentration (Plesner, 1995), involvement in signal transduction (Dubyak and El-Motassim, 1993), and involvement in cellular adhesion (Dzhandzhugazyan and Bock, 1993; Kirley, 1997).

Recently, we have shown that in *Entamoeba histolytica,* galactose stimulated a Mg^{2+}-dependent ecto-ATPase involved in the virulence of this parasite (Barros *et al.*, 2000). In this regard, it has been shown that a 46-kDa lectin isolated from root extracts of legume *Doliclos biflorus* is a Nod factor binding protein as well as a nucleoside di- and tri-

Abbreviations: *p*-NP, *p*-nitrophenol; *p*-NPP, *p*-nitrophenylphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazoly)] benzene; Tris, Tris(hydroxymethyl)aminomethane.

0939–5075/2001/0900–0820 $ 06.00

phosphate hydrolase stimulated by carbohydrate ligands (Etzler *et al.*, 1999). Bacterial lipopolysaccharide (LPS) is a potent and pleiotropic stimulus of immune cells, both *in vitro* and *in vivo* (Morrison and Ryan, 1987; Lynn and Golenbock, 1992). In vertebrate blood cells the biological responses to LPS involve the increase of adhesion capacity (Lynn and Golenbock, 1992), whereas in endothelial cells, LPS treatment modifies pH- and cation-dependent ecto-ATPase activity, increasing the Mg-dependent ecto-ATPase activity (Kittel, 1992). Several studies have already demonstrated that sulfated polysaccharides interact with components of the plasma membrane of the parasite enhancing the process of invasion of the host cell. It has been shown the importance of glycosaminoglycans and glycosaminoglycan-binding proteins for the interactions between *Leishmania donovani* (Chakraborty and Das, 1988; Mukhopadhyay *et al.*, 1989; Butcher *et al.*, 1990, 1992) as well as *Trypanosoma cruzi* (Ortega-Barria and Pereira, 1991) and their mammalian target cells.

A previous report from our laboratory has shown a Mg^{2+}-dependent ecto-ATPase present on the external surface of *Leishmania tropica* (Meyer-Fernandes *et al.*, 1997). In this study we investigated the effects of sulfated polysaccharides on the ecto-ATPase activity of *L. tropica*.

Material and Methods

Growth of Microorganisms

L. tropica stock IOC-L 571, from a WHO collection, was provided by Dr. G. Grimaldi Jr. (Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil). The parasites were cultured as promastigotes in Schneider's *Drosophila* medium (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (GIBCO) at 24 °C (Lopes and McMahon-Pratt, 1989), and were harvested at the stationary phase of growth. Five days after inoculation, cells were collected by centrifugation, washed twice, and kept in 50 mM Tris-maleate, pH 7.5, 20 mM KCl, 100 mM sucrose, and 20 mM glucose. Cellular viability was assessed, before and after incubation, by mobility and Trypan blue dye exclusion (Dutra *et al.*, 2000). The viability was not affected under the conditions employed here. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Ecto-ATPase activity measurements

Intact cells were incubated for 1 h at 30 °C with gentle shacking (40 oscillations/min) in 0.5 ml of a mixture containing, unless otherwise specified, 50 mM Tris-maleate, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, and 1 mg/ml of protein which corresponds to 2.2×10^8 cells/ml. The ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P]-ATP (10^4 Bq/nmol ATP) (Bernardes *et al.*, 1997). The experiments were started by the addition of living cells and terminated with 1 ml of a cold mixture containing 0.2 g charcoal in 0.1 M HCl. The tubes were then centrifuged at $1500 \times g$ for 20 min at 4 °C, then 400 µl of the supernatant were added to 9 ml of scintillation liquid (2 g PPO, 1 g POPOP in 1 liter toluene) and counted in a liquid scintillation counter. The ATPase activity was calculated by subtracting the nonspecific ATP hydrolysis measured in the absence of cells. ATP hydrolysis was linear with time under the assay conditions used and was proportional to the cell density. In the experiments in which other nucleotides (5′AMP and 3′AMP) were used, the hydrolytic activity measured under the same conditions described above was assayed spectrophotometrically by measuring the release of P_i from the nucleotides (Lowry and Lopez, 1946). The values obtained for ATPase activities measured using both methods (colorimetric and radioactive) were exactly the same.

Phosphatase activity measurement

The ecto-*p*-nitropenylphosphatase activity was determined in the same medium for ATP hydrolysis except that ATP was replaced by 5 mM *p*-nitrophenylphosphatase (*p*-NPP). The reaction was terminated with 1 ml of 1 N NaOH. The released *p*-nitrofenol (*p*-NP) was determined spectrophotometrically at 425 nm using an extinction coefficient of 14.3×10^3 M^{-1}. cm^{-1} (Dutra *et al.*, 1998).

Reagents

All reagents were purchased from Merck S. A. (São Paulo, S. P.) or Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was prepared as described by Glynn and Chappel (1946). Distilled water deionized by the MilliQ system of resins

(Millipore Corp., Bedford, MA) was used in the preparation of all solutions.

Statistical analysis

All experiments were performed in triplicate, with similar results obtained at least three separate cell suspensions. Statistical significance was determined by Student's *t* test. Significance was considered as $P < 0.05$.

Results and Discussion

In invertebrate blood cells the biological responses to lipopolysacharide involve the increase of aggregation and phagocytosis (Lanz-Mendoza *et al.*, 1996), as well as the increase on ecto-ATPase activity (Meyer-Fernandes *et al.*, 2000). Sulfated polysaccharides are found at the extracellular space of different animal tissues, but their physiological role is poorly understood (Kjellèn and Lindahl, 1991; Hardingham and Fosang, 1992; Yanagishita, 1993). These molecules are able to bind at the surface of *Leishmania donovani* and *Trypanosoma cruzi* enhancing the invasion of the parasite to the host cells (Chakraborty and Das, 1988; Mukhopadhyay *et al.*, 1989; Butcher *et al.*, 1990, 1992; Ortega-Barria and Pereira, 1991). The objective of this work was to study the effect of sulfated polysaccharides on the Mg^{2+}-dependent ecto-ATPase of *Leishmania tropica.*

Fig. 1 shows that dextran sulfate *Mr* 500,000 stimulated the Mg^{2+}-dependent ecto-ATPase activity in a dose-dependent manner (open circles). This effect was not observed when dextran sulfate was replaced by other sulfated polysaccharides, such as heparin (closed circles), Condroitin 4-sulfate (open squares) and Condroitin 6-sulfate (closed squares). This effect is dependent on size and charge of the polysaccharide (Fig. 2A). Other sulfated dextrans with lower molecular weight and desulfated dextran were less efficient to promote stimulation of ecto-ATPase activity. When the cells were incubated at pH 7.5 in the presence of dextran sulfate *Mr* 500,000 (open circles); *Mr* 40,000 (closed circles) and *Mr* 8,000 (open squares) the stimulation of ecto-ATPase activity was 63%; 23%; and 11% respectively. This stimulation was not observed when desulfated dextran *Mr* 40,000 (Fig. 2A, closed squares) was added to the assay media. The degree of the stimulation

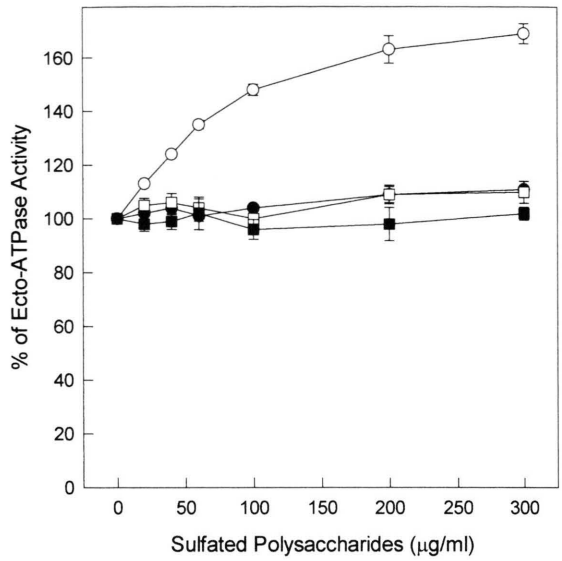

Fig. 1. Effects of increasing concentration of different sulfated polysaccharides on ecto-ATPase of living cells of *Leishmania tropica.* Cells were incubated for 1h at 30°C in a reaction medium (final volume 0.5 ml) containing 30 mM Tris-maleate buffer, pH 7.5, 5 mM Tris-ATP [γ^{32}P]ATP (specific activity about 10^4 Bq/nmol ATP), 5 mM $MgCl_2$, 1 mg/ml of protein which corresponds to 2.2 X 10^8 cells/ml with the concentrations of sulfated polysaccharides dextran sulfate *Mr* 500,000 (○), heparin (●), condroitin 4-sulfate (□) and chondroitin 6-sulfate (■) shown on the abscissa. The ATP hydrolysis (403.1 ± 31.3 nmol/mg protein × h) was taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

promoted by dextran sulfate also depended on the pH of the medium. As shown in Figure 2B, at pH 7.5, the stimulation was more than 65%, whereas at pH 8.5 the stimulation was only 25%. These results suggest that protonation or deprotonation of side-chain groups could be involved in the reversible binding of dextran sulfate to the ecto-ATPase.

Recent reports have shown that sulfated polysaccharides including dextran sulfate, modulate different intracellular ATPases. These polysaccharides are able to uncouple Ca^{+2}-ATPases from sarcoplasmic reticulum, blood platelets and brain microsomes (De Meis and Suzano,1994; Rocha *et al.*, 1996, 1998) , and these effects are antagonized by polyamines (De Meis and Suzano,1994). Polyamines, particularly spermidine, spermine and putrescine, are ubiquitous components of eukaryote

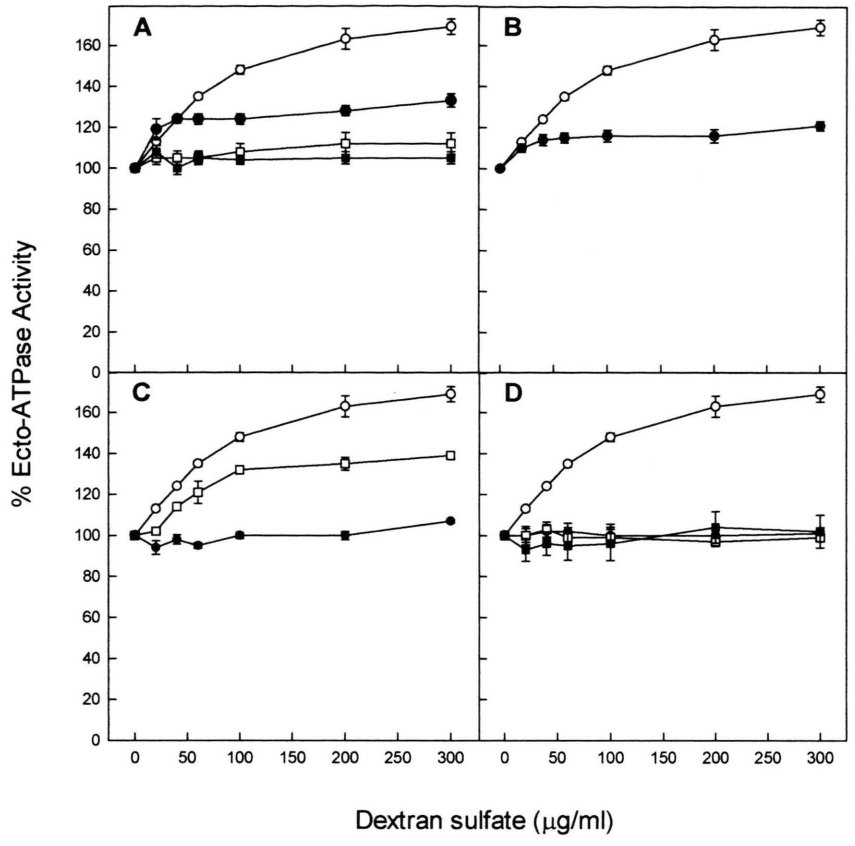

Fig. 2A. Effects of increasing concentrations of dextran sulfate on ecto-ATPase of living cells of *Leishmania tropica.* Cells were incubated for 1h at 30°C in the same reaction medium (final volume 0.5 ml) described in Fig. 1, with concentrations of dextran sulfate *Mr* 500,000 (○), *Mr* 40,000(•), *Mr* 8,000 (□) and dextran desulfated *Mr* 40,000 (■) indicated on the abscissa. The ATP hydrolysis (396.4 ± 21.3 nmol/mg protein × h) was taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

B. pH dependence of the stimulation of ecto-ATPase activity of living cells of *Leishmania tropica* promoted by dextran sulfate *Mr* 500,000. Cells were incubated for 1h at 30°C in the same reaction medium (final volume 0.5 ml) described in Figure 1 with the concentrations of dextran sulfate *Mr* 500,000 indicated on the abscissa at pH 7.5 (○) or pH 8.5 (•). 100% activity corresponds to 399.2 ± 28.3 nmol/mg protein × h at pH 7.5 and 723. 9 ± 54.7 nmol/mg protein × h at pH 8.5. The standard errors were calculated from the absolute activity values measured at pH 7.5 or pH 8.5 of three experiments with different cell suspensions and converted to percentage of the control value.

C. Antagonism effects of polyamines on the stimulation of ecto-ATPase activity of living cells of *Leishmania tropica* promoted by dextran sulfate *Mr* 500,000. Cells were incubated for 1h at 30 °C in the same reaction medium (final volume 0.5 ml) described in Fig. 1 with the concentrations of dextran sulfate *Mr* 500,000 indicated on the abscissa, in the abscense (○) or in the presence of 1 mM spermidine (•) or in the presence of 1 mM putrescine (□). The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

D. Effects of dextran sulfate *Mr* 500,000 on the ecto-enzymes (ATPase, 5′nucleotidase, 3′nucleotidase and phosphatase) activities of living cells of *Leishmania tropica.* Cells were incubated for 1h at 30 °C in the same reaction medium (final volume 0.5 ml) described in Figure 1, with 5 mM of each substrates ATP (○), 5′AMP (•), 3′AMP (□) and *p*NPP (■) with concentrations of dextran sulfate *Mr* 500,000 indicated on the abscissa. 100% activity corresponds to 423.9 ± 41.8 nmol/mg protein × h for ATPase activity; 123.6 ± 10.8 nmol/mg protein × h for phosphatase activity; 110.3 ± 5.53 nmol/mg protein × h for 3′nucleotidase activity and 12.25 ± 0.41 nmol/mg protein × h for 5′nucleotidase activity. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

cells, including *Leishmania* (Yarlett, 1988; Basselin *et al.*, 2000), and are essential for optimal cell proliferation and differentiation (Yarlett, 1988; Basselin *et al.*, 2000). In this regard, it has been shown that ATP and other nucleotides stimulate the proliferation of different cell types, while their metabolism is controlled mainly by enzymes like ecto-ATPase, ecto-5′nucleotidase and ecto-phosphatases, which are believed to be ubiquitously present on the surface of many cell types (Gordon, 1986; Lemmens *et al.*, 1996; Fernandes *et al.*, 1997; Meyer-Fernandes *et al.*, 1999). Figure 2C shows that the addition of 1 mM spermidine or putrescine did not have any effect on the ecto-ATPase activity, but was able to antagonize totally or partially, respectively, the stimulating effect promoted by dextran sulfate. These polyamines are synthesized and transported by *Leishmania* (Yarlett, 1988; Basselin *et al.*, 2000) and could modulate the effects of extracellular components on this ecto-ATPase.

Protozoa of genus *Leishmania* have been shown to possess three other distinct phosphomonoesterase activities (acid phosphatase, 5′nucleotidase and 3′nucleotidase) all of which are also localized on the external surface of the plasma membrane (Gottlieb and Dwyer, 1983; Dwyer and Gottlieb, 1984; Hassan and Coombs, 1987). Figure 2D, shows that 300 μg/ml dextran sulfate *Mr* 500,000 stimulated the ecto-ATPase more than 60% of activity, but had no effect on the ecto-phosphatase, 3′-nucleotidase or 5′-nucleotidase activities, showing that this effect promoted by dextran sulfate is specific for the ecto-ATPase present in this parasite. As it has been suggested that ecto-ATPases might be involved in cellular adhesion (Dzhandzhugazyan and Bock, 1993; Kirley, 1997; Barros *et al.*, 2000; Meyer-Fernandes *et al.*, 2000), it remains to be elucidated whether dextran sulfate may have an effect on the interaction between *Leishmania tropica* with their mammalian target cells.

Acknowledgments

We would like to acknowledge the excellent technical assistance of Fabiano Ferreira Esteves. This work was partially supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Programa de Núcleos de Excelência (PRONEX, grant 0885).

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