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A Mg^{2+}-dependent ecto-phosphatase activity on the external surface of *Trypanosoma rangeli* modulated by exogenous inorganic phosphate

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This work is dedicated to Leopoldo De Meis on his 70th birthday.

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ABSTRACT

In this work, we characterized a Mg^{2+}-dependent ecto-phosphatase activity present in live *Trypanosoma rangeli* epimastigotes. This enzyme showed capacity to hydrolyze the artificial substrate for phosphatases, *p*-nitrophenylphosphate (*p*-NPP). At saturating concentration of *p*-NPP, half-maximal *p*-NPP hydrolysis was obtained with 0.23 mM Mg^{2+}. Ca^{2+} had no effect on the basal phosphatase activity, could not substitute Mg^{2+} as an activator and in contrast inhibited the *p*-NPP hydrolysis stimulated by Mg^{2+}. The dependence on *p*-NPP concentration showed a normal Michaelis–Menten kinetics for this phosphatase activity with values of V_{max} of 8.94 ± 0.36 nmol *p*-NP $\times$ h^{-1} $\times$ 10^{-7} cells and apparent K_m of 1.04 ± 0.16 mM *p*-NPP. Mg^{2+}-dependent ecto-phosphatase activity was stimulated by the alkaline pH range. Experiments using inhibitors, such as, sodium fluoride, sodium orthovanadate and ammonium molybdate, inhibited the Mg^{2+}-dependent ecto-phosphatase activity. Inorganic phosphate (Pi), a product of phosphatases, inhibited reversibly in 50% this activity. Okadaic acid and microcystin-LR, specific phosphoserine/threonine phosphatase inhibitors, inhibited significantly the Mg^{2+}-dependent ecto-phosphatase activity. In addition, this phosphatase activity was able to recognize as substrates only *o*-phosphoserine and *o*-phosphothreonine, while *o*-phosphotyrosine was not a good substrate for this phosphatase. Epimastigote forms of *T. rangeli* exhibit a typical growth curve, achieving the stationary phase around fifth or sixth day and the Mg^{2+}-dependent ecto-phosphatase activity decreased around 10-fold with the cell growth progression. Cells maintained at Pi-deprived medium (2 mM Pi) present Mg^{2+}-dependent ecto-phosphatase activity approximately threefold higher than that maintained at Pi-supplemented medium (50 mM Pi).

1. Introduction

Trypanosomatids are a group of protozoa that parasitize a large number of eukaryotic organisms (D'Alessandro, 1976). In the Trypanosomatidae family, the genus *Trypanosoma* comprises digenetic flagellates that usually have insects as vectors and infect human beings and other animals as hosts (D'Alessandro, 1976; Garcia and Azambuja, 1991). *Trypanosoma rangeli* is a South American trypanosome able to infect mammals, but apparently unable to elicit pathology in them, although it is detrimental to the insect vector (D'Alessandro, 1976). This parasite co-exists with *T. cruzi*, the etiologic agent of Chagas' disease, in the Northern of South America, posing some problems for diagnosis (Martínez et al., 1993), occurring a high immunological cross-reactivity between these two parasites (Labriola and Cazzulo, 1995).

The life cycle of *T. rangeli* in the vertebrate host is poorly known. Some evidence suggests that proliferation occurs within monocytes (Osorio et al., 1995). In the invertebrate host, its life cycle is better characterized. After being ingested as trypomastigotes, *T. rangeli* multiply as epimastigotes in the midgut, invade the hemolymph and hemocytes to continue their growth, and complete their development in the salivary gland where metacyclogenesis takes place (Hoare, 1972).

The plasma membrane of cells may contain enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using intact cells (Gomes et al., 2006; Kiffer-Moreira et al., 2007; Meyer-Fernandes et al., 1997; Meyer-Fernandes, 2002). Knowledge about interactions between components of the external surface of the cells and the cellular elements of the host is of obvious importance for the understanding of the complex life cycle of *T. rangeli*. Ecto-phosphatases and ecto-kinases have been

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detected in several microorganisms, including protozoa (De Jesus et al., 2002; Dos Passos Lemos et al., 2002; Fernandes et al., 1997; Meyer-Fernandes et al., 1999; Remaley et al., 1984; Sacerdoti-Sierra and Jaffe, 1997), bacteria (Bliska et al., 1991, 1993; Braibant and Content, 2001; Madec et al., 2002) and fungi (Arnold et al., 1986; Bernard et al., 2002; Collopy-Junior et al., 2006; Kiffer-Moreira et al., 2007; Kneipp et al., 2003, 2004). Several biological roles for ecto-phosphatases have been proposed. In fungi these ecto-phosphatases are involved with the infection of the epithelial cells (Collopy-Junior et al., 2006; Kiffer-Moreira et al., 2007; Kneipp et al., 2004). In addition, the regulation of the complex interactions required for differentiation, proliferation (Bakalara et al., 1995,2000; Meyer-Fernandes et al., 1999) and infection of host cells (Furuya et al., 1998; Martiny et al., 1999; Zhong et al., 1998) is mediated in part by protein phosphorylation/dephosphorylation in higher eukaryotes as well as in trypanosomes.

These enzymes could also provide microbial cells with a source of inorganic phosphate (Pi) by hydrolyzing phosphomonoester metabolites (Bozzo et al., 2004; Braibant and Content, 2001; Kneipp et al., 2004; Li et al., 2002) and protect them upon entering the macrophage by suppressing the respiratory burst (Remaley et al., 1984, 1985).

Since ecto-phosphatases may provide cells as a source of Pi, the modulation of expression of these enzymes by exogenous phosphate content is well known in fungi (Bernard et al., 2002; Braibant and Content, 2001; Kneipp et al., 2004) and plant (Bozzo et al., 2004, 2006; Hur et al., 2007; Wang et al., 2008) cells. In this context, the kind of enzyme expressed in medium containing high or low phosphate content can be the same or different and the differences correspond to substrate affinity, hydrolytic ability, inhibitors sensitivity, among others (Kneipp et al., 2004). However, the modulation of ecto-phosphatases promoted by Pi content in culture medium in protozoa has not been investigated.

In this work we have characterized a Mg^{2+}-dependent phosphatase activity present on the cell surface of *T. rangeli*, verifying its substrate specificity, optimum pH and its response to Mg^{2+}, Ca^{2+} and to inhibitors. In addition, we verified the modulation of this activity by exogenous phosphate content. This activity was detected in intact epimastigote forms of the parasite and will contribute to the understanding of the physiology and biochemistry of this cell, as well as the event of the interaction parasite–host cell.

2. Materials and methods

2.1. Material

All reagents were purchased from E. Merck (D-6100 Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO). Distilled water was deionized using a MilliQ system of resins (Millipore Corp., Bedford, MA) and was used in the preparation of all solutions.

2.2. Parasites and growth conditions

Macias strain of *T. rangeli* (supplied by Dr. Maria Auxiliadora Sousa, from Fiocruz) was maintained at liver infusion tryptose (LIT) medium supplemented with 20% fetal calf serum (Gibco) at $28 \pm 2\,^{\circ}C$ and subcultivated at 5-day intervals where the parasites achieve the stationary phase of growth. For the experiments, the parasites were harvested from the culture medium by centrifugation at $1500 \times g$ at $4\,^{\circ}C$ for 10 min and washed twice in a cold buffer solution containing 100 mM sucrose, 20 mM KCl and 50 mM Tris, pH 7.2. Cellular viability was assessed, before and after incubations, by motility and Trypan blue dye exclusion (Leite et al., 2007). For Trypan staining the cells were incubated in the presence of 0.01% Trypan blue for 10 min in the buffer used in each experiment. The viability was not affected under the conditions employed here.

2.3. Cell proliferation

In the fifth day of culture, parasites were harvested in sterile tubes, centrifuged at $1500 \times g$ at $4\,^{\circ}C$ during 10 min and washed twice with the same cold buffer solution as described before. The cell density was estimated in a haemocytometric chamber and the growth curve was initiated with 1.0×10^6 cells mL^{-1}. The cell proliferation was verified every day by the counting of the cell number in a haemocytometric chamber.

2.4. Ecto-phosphatase activity measurements

Phosphatase activity was determined spectrophotometrically measuring the rate of *p*-nitrophenol (*p*-NP) production. Intact cells (3×10^7 cells/mL) of *T. rangeli* were incubated at $25\,^{\circ}C$ for 60 min in a reaction mixture (0.5 mL) containing, unless otherwise stated in legends of the figures, 50 mM Tris buffer, pH 7.2, 100 mM sucrose, 20 mM KCl and 5 mM *p*-NPP, as substrate. The Mg^{2+}-dependent phosphatase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$ minus the basal activity measured in the absence of $MgCl_2$. The experiments were started by the addition of living cells and terminated by the addition of 1.0 mL of 1.0N NaOH. After the end of the reaction the same volume of $MgCl_2$ or water were added to the correspondent tubes in the absence or in the presence of $MgCl_2$, respectively. The tubes were then centrifuged at $1500 \times g$ for 15 min at $4\,^{\circ}C$. The phosphatase activity was calculated by subtracting the nonspecific *p*-NPP hydrolysis measured in the absence of cells. The concentration of *p*-NP produced in the reaction was measured spectrophotometrically at 405 nm, using a standard curve of *p*-NP for comparison. In the experiments where other phosphatase substrates (β-glycerophosphate or phosphoaminoacids) were used, the hydrolytic activities measured under the same conditions described above were assayed spectrophotometrically by measuring the release of Pi from these substrates, following the Fiske and Subbarow (1925) method. The values obtained for *p*-NPP hydrolysis measured using both methods were exactly the same (Kiffer-Moreira et al., 2007).

2.5. Statistical analysis

All experiments were performed in triplicates, with similar results obtained from at least three separate cell suspensions. Apparent K_m and V_{max} values were calculated using a computerized nonlinear regression analysis of the data to the Michaelis–Menten equation (Saad-Nehme et al., 1997). Data were analyzed statistically using the Student's *t*-test. Statistical significance was considered as $p < 0.05$.

3. Results

3.1. Ecto-phosphatase activity

The measurement of the ecto-phosphatase activity present on the external surface of *T. rangeli* cells (Macias strain) was carried out in living epimastigote forms, maintained at physiological pH (pH 7.2) using *p*-nitrophenylphosphate (*p*-NPP) as substrate. Cellular viability was assessed before and after incubation by motility and Trypan blue dye exclusion. The viability was not affected by the conditions employed here (>97%). The time course of *p*-NPP hydrolysis by the phosphatase was linear for at least 60 min ($r^2 = 0.9939$).