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Activity Determination of Na^+ K^+ - ATPase and Mg^{++} - ATPase Enzymes in the Gill of *Poecilia vivipara* (Osteichthyes, Cyprinodontiformes) in Different Salinities

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ABSTRACT

This work aimed to know the tolerance mechanisms through the salinity variation by $Na^+ K^+$ - ATPase and Mg^{++} - ATPase and enzymes encountered in the gills of Poecilia vivipara. *In field, the presence of this species was observed in salinities of 0 and 28‰. In laboratory, these fish were maintained in aquarium with mean salinity of 30‰ and positive responses were obtained. Some adult specimens, collected in a lagoon of the Coqueiros Beach, were utilized as matrixes. In the experiments the specimens used were those born in the test aquarium. For each salinity studied three replicates were made with three specimens for each one. The alevins were maintained in salinities of 5, 10, 15, 20, 25, 30 and 35‰ during a month for adaptation. Gills were extracted in appropriate buffer for isolation of plasma membrane and used for specific dosage of the total enzymatic activity of $Na^+ K^+$ - ATPase and Mg^{++} - ATPase. The relation of alevins to their adaptation towards the salinity variation was also studied. The activity of the two enzymes showed a different result. The major expression of $Na^+ K^+$ - ATPase was observed in 20‰ (35 µmoles Pi.mg protein.h^{-1}), the best salinity to cultivate* P. vivipara.

Key words: *Poecilia vivipara*, $Na^+ K^+$ ATPase, Mg^{++} ATPase, osmoregulation

INTRODUCTION

Poecilia vivipara (Bloch & Schneider, 1801) belongs to the family Poeciliidae and presents wide geographical distribution, being found in the Americas, from Illinois and New Jersey (EUA) to Argentina (Nascimento, 1984). They are commonly found in salt and brackish waters, mainly in the exits of ponds and river mouths (Suzuki, 1983). The individuals of this species are viviparous with internal fertilization. They present sex dimorphism, where the males possess an organ formed by the transformation of the anal fin in aid of the sexual intercourse (Nomura, 1984). The rays of the anal fin, particularly the third, fourth and fifth ones, are longer than the others. They are independent in the beginning of the development, coming unfastened later in the whole length,

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creating the gonopodium. The reproduction is continuous throughout the year. The females begin reproducing from 3 cm of total length, in continuous gestations. When they reach approximately 7 cm of length, they present an average of 51 embryos per gestation (Novaes & Andreata, 1994) The females have the ventral region permanently dilated by the continuous gestations. Thus, they are vulgarly called "Barrigudinho" (Pereira, 1979). These fish are omnivorous and they participate in the trophic chain, serving as food for carnivorous fish (Koblitz & Andreata, 1994; Novaes & Andreata, 1994).

P. vivipara was used in tests of thermal tolerance, lethal effects of cadmium and tests with derivatives of petroleum in Venezuela (Chunk, 1983; Chunk & Mendez, 1993) and in China (Xu *et al.*, 1991; Xu *et al.*, 1992). Damato (1995) used three species of fish, one of them belonging to *P. vivipara*, in toxicity tests with potassium dichromate. *P. vivipara* presented high sensitivity, suggesting its employment as biological indicator. The species *Poecilia reticula* and *P. sphenops* are cultivated for use in tests of aquatic effluents in Venezuela (Grau & Esclapés, 1995). Kraus *et al.* (1998) tested the sensitivity of *P. vivipara* to four reference substances (potassium dichromate, sodium dodecyl sulfate, copper and zinc).

In natural conditions *P. vivipara* is found in environments that differ from its geological and physiographical characteristics, such as salt concentrations and they are under different degrees of anthropogenetic impact (Caniçali & Lima, 1996). In the Coqueiros Beach, where the individuals were collected, the salinity varies from 0 to 28‰ (Amaral, 1997). The aim of this study was to dose Na^+ K^+ - ATPase and Mg^{++} - ATPase enzymes that are responsible for the individuals' osmoregulation.

MATERIAL AND METHODS

Adult specimens were captured with nets in a lagoon in Coqueiros Beach at Fundão Island, in contact with the waters of Guanabara Bay. The fish were transported to the laboratory in sea water and conditioned in an acclimatized aquarium. The proportion of two females for a male was maintained. After the birth, the alevins were placed in test aquariums with the capacity of 1,000 ml, being standardized in 300 ml of sea water for each individual with a salinity of 30‰. For seven consecutive days the salinity was gradually reduced and later they were maintained in 5, 10, 15, 20, 25, 30 and 35‰. For each salinity studied, three replicates were made with three specimens for each one during a month for total adaptation. The aquariums were aerated and maintained in an incubator with controlled temperature at 25±2°C and a 12-hour photoperiod. The alevins were fed twice a day with ration in flakes (Tetra Min) and nauplius of *Artemia* sp. "ad libitum".

For enzyme extraction, the gills were removed, dried with absorbent paper, and weighed in analytical balance. The membrane's cells of the gills of *P. vivipara* were isolated for dosage of the Na^+ K^+ - ATPase and Mg^{++} - ATPase activities (Bouef *et al.*, 1978; Harache *et al.*, 1980). Stages of the membrane isolation for determination of the ATPase activity: (1) Homogenization in the Ultra Turrax of 0.2 g of tissue in 0.5 ml of Tris buffer 5 mM, Sucrose solution 0.25 mM, EGTA (Ethylene-Glycol bis (β aminoethylether) N-N' Tetra-acetic Acid) 0.2 mM, pH 7.4. Time of homogenization 1.5 min per 7,000 rpm at 4°C. (2) Centrifugation at 8,714 xg for 10 min and removed the supernatant. (3) Centrifugation at 61,000 xg for 45 min. (4) Ressuspension in 1.5 ml of homogenization buffer in the Potter and preparation of a gradient 1:1 with the Tris buffer 5 mM, Sucrose solution 1.4 M, EGTA 0.2 mM pH 7.4 and centrifugation at 61,000 xg for 45 min. (5) Remotion of the membrane fraction and dilution of 5 times with Tris-EGTA buffer 0.2 mM pH 7.4 and centrifugation at 61,000 xg for 45 min. (6) Ressuspension in 1.5 ml with imidazole buffer 25 mM, Sucrose 0.25 mM, EGTA 0.2 mM pH 7.4. (7) Evaluation of the ATPase activity.

Dosage of Na^+, K^+ - ATPase and Mg^{++} - ATPase: The total enzymatic activity of the ATPase was determined by the incubation of the enzyme at 37°C, pH 7.5 in a solution (final volume equal to 0.5 ml) containing ATP.Na_2-TRIS 5 mM, NaCl 150 mM (final concentration of Na^+ 155 mM), KCl 15 mM, MgCl 15 mM in histidine HCl-

TRIS 30 mM. The dosage of Mg^{++} - ATPase was made under identical conditions. However, oubain was added in the final concentration of 1.5 mM. The Na^+, K^+ - ATPase activity is obtained by the difference between the total activity and the Mg^{++} - ATPase activity. For each sample, a reference solution was prepared in which the enzyme was added after the paralyzation of the reaction. The tubes containing the enzyme were pre-incubated for 5 min at 37°C. The reaction was started with the addition of ATP and paralyzed with the addition of 1 ml of reagent for dosage of inorganic phosphates (Pi) after 10 min of incubation at 37°C. The tubes rested for 15 to 20 min at room temperature and each one of the tubes (total ATPase, Mg^{++} - ATPase and "reference solution") was read at 700 nm by the modified method of Fiske & Subbarow (1925). Patterns of sodium phosphate were prepared concomitantly.
Na^+, K^+ - ATPase and Mg^{++} - ATPase activities were expressed in μmoles of Pi released per hour per milligram of protein (μmoles Pi.mg protein.h^{-1}) of the preparation adapted for Ortiz (1975).

Dosage of Protein: Protein concentration was measured by the modified method of Lowry *et al.* (1951). The band of protein was 1 to 100 μg, read at 700 nm for 1.1 ml (microassay) at room temperature after 5 min. Serum of bovine albumin was used as a pattern.

RESULTS AND DISCUSSION

The Na^+ K^+ - ATPase activity of *P. vivipara* was of 33.6 μmoles Pi per hour per mg of protein, presenting the same order of magnitude as Na^+ K^+ - ATPase of *Oncorhynchus kisutch* (salmon) (Harache *et al.*, 1980). In relation to this enzyme of *Tilapia rendalli*, the activity was about ten times higher (Arbex, 1983). This study demonstrated that Na^+ K^+ - ATPase activity of *P. vivipara* was very similar to fishes, which were adapted to salinity variations. The concentration effect of EGTA was tested in the homogenization buffer and it was found that the ATPase activity in the extract free from cell was sensitive from 0.2 mM onwards (Fig. 1).

The results for Na^+ K^+ - ATPase and Mg^{++} - ATPase demonstrated that they were enzymes totally different in relation to their kinetics parameters. These enzymes presented inhibition kinetics for substratum excess (type accompetitive) and Michaeliana, respectively (Fig. 2).
Their kinetic parameters were determined by non lineal programs in microcomputer by the algorithm of the Marquardt of Enzifit. The curves are theoretical and the points are experimental. The Vmax for enzyme Na^+K^+ATPase was 153.62±41.22 mM and the Km was 3.57±1.10 mM. This enzyme presented a Kis of 1.86±0.75 mM. The Vmax of enzyme Mg^{++}ATPase was 280.79±11.69 mM and the Km was 0.36±0.03 mM. The parameters determined presented quite different values from Na^+, K^+ - ATPase of the *Tilapia rendalli* in relation to the Km for ATP.Mg^{++} that is 0.53 mM. They presented a much higher value (3.618±0.3 mM) and this enzyme presented a Kis of 1.795 mM showing an inhibition for substract not reported for the enzyme of *Tilapia rendalli* (Arbex, 1983). The adjustment of the experimental data of this kinetics to the Michaeliana equation did not fit better than the equation for inhibition for substract.

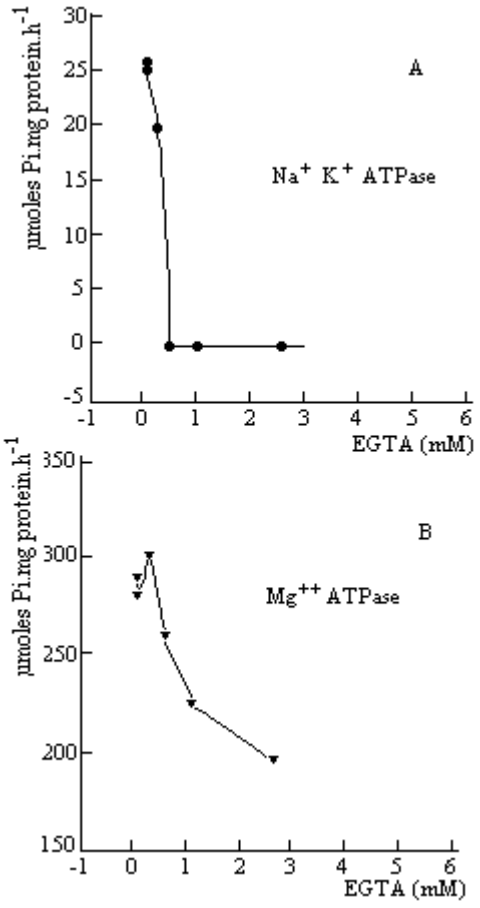

Figure 1 - EGTA effect in the ATPase activity in gills of *P. vivipara*.

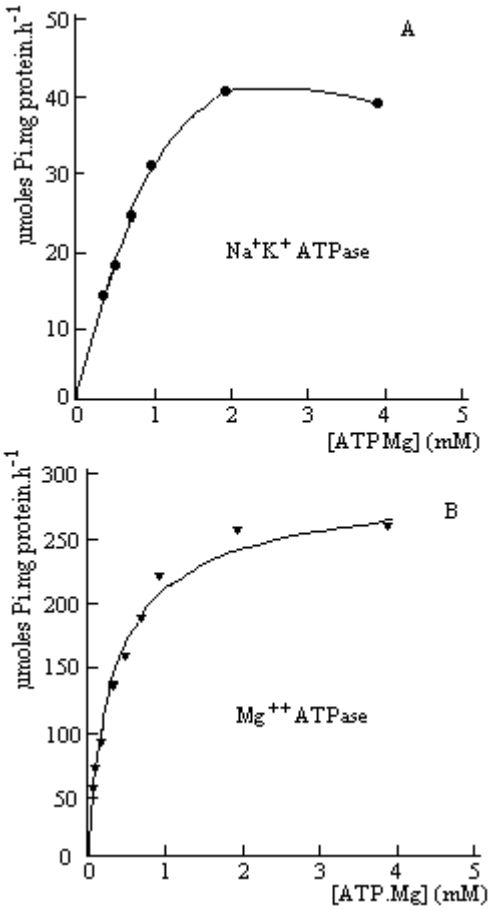

Figure 2 - ATP.Mg^{++} effect in the ATPase activity in gills of *P. vivipara*.

The value of Km for Mg^{++} - ATPase was in accordance with the values found for this enzyme in the literature. These values were for enzymes of gills of *P. vivipara* adapted to the salinity of 30‰. Na^+ K^+ - ATPase was an integral protein of membrane found in cells of superior eucaryotes and it was responsible for translocate ions of sodium and potassium through the membrane of cells using ATP as a driving force. For every three ions of sodium pumped off the cell, two potassium ions were pumped in. Such protein was specifically inhibited by cardiac glycosides, as, for example, ouabain and digoxin (Lingrel & Kuntzweiler, 1994).

Na^+ K^+ - ATPase presented inhibition for 1.5 mM of oubain and its activity for Na^+ and K^+, was optimum in the relationship of 150 mM and 15 mM, respectively. These concentrations were in accordance with the literature for *Tilapia rendalli* (Arbex, 1983) and *Oncorhynchus kisutch* (Harache *et al.*, 1980). The activity of Na^+ K^+ - ATPase presented a maximum of the expression of the enzyme in 20‰. Higher concentrations presented a decrease in the activity (Fig. 3). This result was not expected once it should have been around 35 µmoles Pi.mg protein.h^1. The difficulty of maintaining the high levels could be related to the stress accumulated from this value of 20‰ and linked to a factor that modified the activity Na^+, K^+ - ATPase "*in vivo*". Na^+ K^+ - ATPase increased its activity 7 times in the adaptation to the salinity. Its basal activity was of 5.4 µmoles Pi.mg

protein.h^{-1}. All the activities found in the salinity variation were in accordance with the expected for *O. kisutch* (Harache *et al.*, 1980). Mg^{++} - ATPase activity during the adaptation to the salinity was different from the one found for this fish, once it followed the Na^+ K^+ - ATPase profile, presenting a maximum in 20‰.

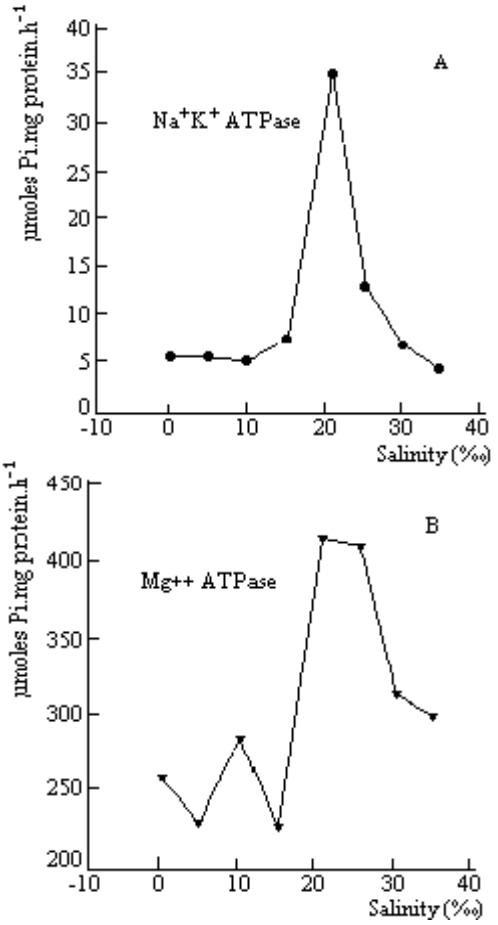

Figure 3 - Salinity effect in the ATPase activity in gills of *P. vivipara*.

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RESUMO

Este trabalho teve como objetivo conhecer os mecanismos de tolerância às variações de salinidade, pelas enzimas Mg^{++} - ATPase e Na^+ K^+ - ATPase, encontrada nas brânquias de *Poecilia vivipara*. No campo, foi observada a presença desta espécie em salinidades entre 0 e 28‰. No laboratório, os indivíduos foram mantidos em salinidade de 30‰ e responderam positivamente. Os indivíduos adultos, coletados em uma lagoa na praia dos Coqueiros, foram utilizados como matrizes. Nos experimentos foram usados alevinos que nasceram nos aquários testes. Para cada salinidade estudada foram feitas três réplicas com três espécimens em cada uma. Os alevinos foram mantidos em salinidades de 5, 10, 15, 20, 25, 30 e 35‰, durante um mês para total adaptação. As brânquias foram extraídas em tampão apropriado para isolar a membrana plasmática e usada dosagem específica para a atividade enzimática total de Na^+ K^+ ATPase e Mg^{++} - ATPase e sua relação a adaptação da variação da salinidade. A atividade das duas enzimas mostrou diferentes resultados. A maior expressão de Na^+ K^+ - ATPase foi observada em 20‰ (35 µmoles Pi/mg proteína.h), sendo a melhor salinidade para se manter o cultivo de *P. vivipara*.

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