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Na^+ ATPase activities in chela muscle of the euryhaline crab *Neohelice granulata*: Differential response to environmental salinity

S.A. Pinoni, A.A. López Mañanes *

Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina
CONICET, Argentina

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ABSTRACT

The occurrence and characteristics of ouabain-insensitive Na^+ ATPase activity and the response to environmental salinity of the coexistent Na^+–K^+ ATPase and ouabain-insensitive Na^+ ATPase activities were studied in chela muscle of the euryhaline crab *Neohelice* (*Chasmagnathus*) *granulata* from Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina). Chela muscle exhibited two ouabain-insensitive Na^+ ATPase activities (a furosemide-insensitive and a furosemide-sensitive activity). I_{50} for ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity was about 1.4 mM. Both ouabain-insensitive, furosemide-insensitive and furosemide-sensitive Na^+ ATPase activities were weakly affected by pH and showed Michaelis–Menten kinetics ($K_m = 0.021$ and 0.224 mM, respectively). These characteristics appeared to be quite different from those previously described for Na^+–K^+ ATPase activity in chela muscle of this crab. Na^+–K^+ ATPase and ouabain-insensitive, furosemide-sensitive Na^+ ATPase activities appeared to be sensitive to environmental salinity. In crabs acclimated to low salinity (10‰), a salinity at which *N. granulata* exhibits a strong hyperregulatory capacity, Na^+–K^+ ATPase activity was higher (117 ± 26 nmol Pi min^{-1} mg $prot^{-1}$) than in 35‰ salinity (23 ± 6 nmol Pi min^{-1} mg $prot^{-1}$) (a salinity at which this crab is osmoionoconforming). On the contrary, ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity was higher in 35‰ salinity (108 ± 15 nmol Pi min^{-1} mg $prot^{-1}$) than in crabs acclimated to 10‰ salinity (36 ± 11 nmol Pi min^{-1} mg $prot^{-1}$). Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity was not affected by acclimation of crabs to low salinity. The response to low salinity suggests that Na^+–K^+ ATPase could be a component of muscle regulatory mechanisms at the biochemical level secondary to hyperregulation whereas ouabain-insensitive, furosemide-sensitive activity appeared to be predominant upon osmoconforming conditions. The possible differential functional roles of Na^+–K^+ ATPase and ouabain-insensitive Na^+ ATPase activities in muscle are discussed.

1. Introduction

Crabs inhabiting coastal waters, tide areas or estuaries have to cope with a variety of challenges such as frequent and abrupt changes in environmental salinity. Fluctuations in environmental salinity can trigger adjustments at different levels (i.e. biochemical, physiological) for controlling movements of water and ions between the individuals and their medium (reviewed by Kirschner, 1991, 2004). In low salinities, hyperregulating crabs maintain the hemolymph osmotic concentrations above those of the external medium by absorbing both sodium and chloride from the environment. Posterior gills appeared to be the main site of the biochemical adaptations involved in ion transport processes upon hyperregulation (reviewed by Lucu and Towle, 2003; Kirschner, 2004). In the euryhaline crab *Cyrtograpsus angulatus*, a hyperregulatory role for the anterior gills has also been suggested (López Mañanes et al., 2002). Adaptive increases in branchial Na^+–K^+ ATPase point out this activity as a central component of the ionorregulatory process at the biochemical level in euryhaline crabs (reviewed by Towle, 1997; Lucu and Towle, 2003) (Lovett et al., 2006; Tsai and Lin, 2007; Lucu et al., 2008). In comparison, the responses at the biochemical level in other organs or tissues (i.e. muscle) of euryhaline crabs to environmental salinity have received little attention. In *Cancer irroratus* a cell volume regulation of muscle fibers occurs under hyposmotic stress by a coordinated use of inorganic ions and free aminoacids (Moran and Pierce, 1984). Hyposmotic stress led to adjustments associated with acid–base regulation in leg muscle of *Eriocheir sinensis*, (Whiteley et al., 2001) and to an increase of arginine kinase flux in muscle of *Callinectes sapidus* (Holt and Kinsey, 2002). Little is known about the responses of key enzymes probably involved in these processes. We have recently demonstrated the occurrence of alkaline phosphatase (AP) and Na^+–K^+ ATPase activities in chela muscle of the euryhaline crab *C. angulatus*

* Corresponding author: Postal address: Funes 3250 (7600) Mar del Plata, Argentina. Tel.: +54 223 4753554.
E-mail address: mananes@mdp.edu.ar (A.A. López Mañanes).

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whose responses to low salinity suggest the role of muscle in biochemical adaptations to environmental salinity of this crab (Pinoni and López Mañanes, 2004, 2008).

It is well-known that in mammalian cells, Na^+–K^+ ATPase (the classical sodium pump) plays a key role by maintaining the Na^+ gradient necessary for the activity of Na^+ coupled transport processes involved in essential functions (i.e. cell volume and pH regulation, uptake of solutes, excitability and contractility) (Mobasheri et al., 2000; Scheiner-Bobis, 2002; Jørgensen et al., 2003). Apart from Na^+–K^+ ATPase, a coexistent Na^+ ATPase activity (so called the second sodium pump) has been found in several vertebrate tissues (del Castillo and Robinson, 1985b; Proverbio et al., 1986; Moretti et al., 1991; Ventrella et al., 1992, 2001; Camejo et al., 1995; Caruso-Neves et al., 1997; Dópido et al., 2004; Romero et al., 2005; Wengert et al., 2005). Na^+ ATPase participates in the efflux of Na^+ from the cell by transporting this ion against an electrochemical gradient (del Castillo et al., 1982; del Castillo and Robinson, 1985a; Proverbio et al., 1991; Caruso-Neves et al., 1997). Na^+–ATPase, which has been identified as a new member of the P-type ATPase exhibits quite distinct biochemical differences from the classical sodium pump (Thomas et al.; 2003; Romero et al., 2005). Unlike Na^+–K^+ ATPase, vertebrate Na^+ ATPase does not require K^+, and is refractory to ouabain but is strongly inhibited by furosemide (del Castillo and Robinson, 1985b; Proverbio et al., 1986; Moretti et al., 1991; Ventrella et al., 1992; Camejo et al., 1995; Caruso-Neves et al., 2002; Thomas et al., 2003; Beltowski et al., 2004). The differential sensitivity to inhibitors has been commonly used to distinguish between both Na^+–K^+ ATPase and Na^+ ATPase activities in animal tissues. In mammals, ouabain-insenstitive Na^+ ATPase has been suggested to participate in several essential functions (Caruso-Neves et al., 1997, 2002; Beltowski et al., 2004; Wengert et al., 2005). In gills of the rainbow trout *Oncorhynchus mykiss*, ouabain-insensitive Na^+ ATPase activity would have a role in the active salt uptake from the environment upon hyposmotic conditions (Ventrella et al., 1992, 2001). In enterocytes of *Sparus aurata* ouabain-insensitive Na^+ ATPase activity would support the functioning of secondary active transport processes and osmoionorregulatory mechanisms (Dópido et al., 2004). In comparison, so much less is known about ouabain-insensitive Na^+ ATPase activity in invertebrates. Besides the classical sodium pump, a coexistent ouabain-insensitive Na^+–ATPase activity has been identified in the Malpighian tubules of the bloodsucking insect *Rhodnius sp*. (reviewed by Caruso-Neves and Lopes, 2000) and in gills and mantle of the mussel *Mytilus galloprovincialis* and of the clam *Tapes philippinarum* (Pagliarani et al., 1996). Like vertebrate Na^+ ATPase activity, the activity found in this invertebrate species is Mg^{2+} dependent, does not require K^+ and is refractory to ouabain. Furthermore, in the Malpighian tubules of *Rhodnius prolixus* Na^+–ATPase activity has been shown to be fully inhibited by 2 mM furosemide (reviewed by Caruso-Neves and Lopes, 2000). In bivalve mollusks, ouabain-insensitive Na^+ ATPase has been suggested to participate along with Na^+–K^+ ATPase in the regulation of intracellular Na^+ concentration by pumping Na^+ outside the cell and even replacing it under environmental conditions that differentially inhibit the classical sodium pump (Pagliarani et al., 1996, 2006, 2008). Studies on the occurrence and characteristics of ouabain-insensitive Na^+ ATPase in crustaceans, particularly in euryhaline crabs, as well as its possible role as a component of the biochemical adaptations to environmental salinity are lacking.

Neohelice (*Chasmagnathus*) *granulata* is a semiterrestrial euryhaline crab which is found from southern Brazil to Patagonia (Argentina) (Boschi, 1964; Botto and Irigoyen, 1979). In Mar Chiquita coastal lagoon, it is one of the dominant crabs in the outer parts where it is exposed to a highly and abruptly variable environmental salinity ranging from 4 to 36‰ (Anger et al., 1994; Spivak et al., 1994). Environmental salinity affects several aspects of *N. granulata* biology (reviewed by Bianchini et al., 2008). Previous work in our laboratory demonstrated that *N. granulata* from the outer parts of Mar Chiquita coastal lagoon exhibits a strong hyperregulatory capacity in low salinity (López Mañanes et al., 2000, 2002; Schleich et al., 2001; Pinoni et al., 2005). The short- and long-term responses of Na^+–K^+ ATPase activity in anterior and posterior gills to low salinity indicate that this enzyme is a component of the branchial ionoregulatory mechanisms at the biochemical level suggesting its differential role in individual gills in ion transport process of this crab (Schleich et al., 2001; López Mañanes et al., 2002; Elhalem and López Mañanes, 2003). Biochemical responses to environmental salinity in other tissues of *N. granulata* have been less investigated. The mobilization of lipids from muscle increases upon acclimation to low salinity (Luvizotto-Santos et al., 2003). In jaw muscle, an enhancement in gene expression of phosphoenolpyruvate carboxykinase (PEPCK), gluconeogenic and PEPCK activities, and uptake of aminoacids occurs upon hyperosmotic stress (Schein et al., 2004, 2005). We have previously shown the occurrence of a salinity dependent AP activity in chela muscle of *N. granulata* suggesting a role for this tissue in biochemical adaptations to environmental salinity in this crab (Pinoni et al., 2005).

As part of our integrative studies on the identification of enzyme activities involved in biochemical adaptations to environmental conditions in estuarine crabs, the aims of this work were to determine the occurrence and characteristics of ouabain-insensitive Na^+ ATPase activity in chela muscle of *N. granulata* from Mar Chiquita coastal lagoon and the response of the coexistent Na^+–K^+ ATPase and ouabain-insensitive Na^+ ATPase activities to low salinity. To our knowledge this is the first attempt to determine the occurrence of ouabain-insensitive Na^+ATPase and the response of the coexistent Na^+ATPase activities to environmental factors in a euryhaline crab.

2. Materials and methods

2.1. Chemicals

Na_2ATP (adenosine 5' triphosphate, vanadium-free), Tris-(hydroxymethylamino-methane) (Tris), ethylenglicol *N*,*N*',*N*'-tetraacetic acid (EGTA), imidazole, bovine serum albumin, G-Strofantin (ouabain) and furosemide were from Sigma (St. Louis, MO, USA); sodium azide, sucrose and sodium chloride were obtained from Merck (Darmstadt, Germany); magnesium chloride was from ICN (Ohio, USA); potassium chloride and Coomassie Blue G250 were from Fluka (Germany). All solutions were prepared in glass-distilled water.

2.2. Animal collection and maintenance

Crabs were caught from a single area of Mar Chiquita lagoon which exhibited high and abrupt variations in salinity ranging from 4 to 35‰. Only adult male crabs with a carapace width greater than 2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. Crabs were maintained in natural seawater (35‰ salinity) or dilute seawater (10‰ salinity) for at least 10 days prior to use. Diluted seawater was obtained by dilution of natural seawater with distilled water. The aquaria contained 36 l of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 22 ± 2 °C. Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Cichlind T.E.N., Wardley, USA) (about 0.07 g/individual) but they were starved 48 h prior to experiments.

2.3. Preparation of enzyme muscle extract

The crabs were cryoanesthesized by putting them on ice for about 20 min. After removing the chelae, the muscle was immediately excised, mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA-Tris, pH 7.4; 8 ml g^{-1} of muscle tissue) and homogenised (CAT

homogeniser ×120, tool T10) on ice. The muscles from both chelae of one individual were pooled and used for each preparation of enzyme extract. The homogenate was fractionated into 500 µl aliquots on ice and immediately used for enzyme activities assays.

2.4. Assay of ouabain-insensitive Na^+ ATPase activity

In the standard assay ouabain-insensitive Na^+–ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 130 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA, 1 mM ouabain and 1 mM sodium azide (to inhibit F-ATPases) in 20 mM imidazole buffer (pH 7.4) in the absence (total ouabain-insensitive Na^+ATPase activity) and in the presence of 2 mM furosemide (ouabain-insensitive, furosemide-insensitive Na^+ATPase activity). Ouabain-insensitive, furosemide-sensitive Na^+ATPase activity was estimated as the difference between the two assays. In all cases, residual Mg^{2+}–ATPase activity (assayed in the standard assay but without NaCl) was subtracted. When corresponding, choline chloride was used to replace NaCl and keep ionic strength of the assay mixture.

An aliquot of the corresponding sample (linearity zone on activity vs. protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 30 °C. The reaction was initiated by the addition of ATP (final concentration 3 mM). Incubation was carried out at 30 °C for 15 min. The reaction was stopped by addition of 2 ml of cooled Bonting's reagent (560 mM sulphuric acid, 8.1 mM ammonium molybdate and 176 mM ferrous sulphate). After 20 min at room temperature, the amount of released Pi was determined by reading the absorbance at 700 nm of the reduced phosphomolybdate complex (Bonting, 1970). To study the effect of ATP concentration and pH on ouabain-insensitive Na^+ ATPase activities, the procedure was the same as described above except that the activities were determined in the presence of varying ATP concentrations or at different pH levels of the reaction mixture, respectively.

2.5. Assay of Na^+–K^+ ATPase activity

Total (Mg^{2+}–Na^+–K^+) ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EGTA in 20 mM imidazole buffer (pH 7.4). Residual (Mg^{2+}–Na^+) ATPase activity was assayed in the same medium but without KCl and in the presence of 1 mM ouabain. Na^+–K^+ATPase activity was determined as the difference between the two assays. An aliquot of the corresponding sample (linearity zone on activity vs. protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 30 °C. The reaction was initiated by the addition of ATP (final concentration 13 mM). Incubation was carried out at 30 °C for 15 min. The reaction was stopped by addition of 2 ml of cooled Bonting's reagent as described above for ouabain-insensitive Na^+ATPase activity.

2.6. Protein analysis

Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard.

2.7. Measurement of hemolymph osmolality and ionic concentration

Hemolymph (about 500 µl) was sampled from the infrabranchial sinus by mean of a syringe at the base of the cheliped, and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10,000 ×*g* (Beckman, Microfuge, B) for 30 s. Osmolality was measured with a microosmometer (Radiometer, Copenhagen, 3MO). Na^+ and K^+ were determined by flame photometry. Cl^- was determined by a colorimetric method (Randox Commercial Kit) based on the formation of a blue Fe-2,4,6-tri-(2-pyridyl)-1.3.5-triazine–ferrous sulphate complex.

2.8. Statistical analysis

Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A parametric (one-way ANOVA or repeated measures ANOVA) or non-parametric (Kruskal–Wallis) analysis of variance was used. A posteriori ANOVA test using the Holm–Sidak method was used to identify differences and $p<0.05$ was considered significant. Results of effect of varying concentrations of Na^+ and ATP on Na^+ATPase activities were analyzed by means of non-linear regression analysis (GraphPad Prism 2.01 software). The corresponding curves shown are those which best fit the experimental data. $K_{0,5}$(Na^+ concentration that produced 50% of stimulation of Na^+–ATPase activity) and K_m values (Michaelis–Menten constant) were estimated by analysis of these curves (GraphPad Prism 2.01 software). I_{50} (furosemide concentration at which ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity was 50% inhibited) was calculated from the inhibition curve (GraphPad Prism 2.01 software).

3. Results

3.1. Ouabain-insensitive Na^+ ATPase activities of chela muscle of *N. granulata*: effect of Na^+ and furosemide

Initially, the occurrence of ouabain-insensitive Na^+ATPase activity in chela muscle of *N. granulata* was determined by studying the effect of varying Na^+ concentrations (20–150 mM) on ATPase activity under conditions of total inhibition of Na^+–K^+ ATPase. The stimulation by Na^+ under these conditions showed the occurrence in chela muscle of ouabain-insensitive Na^+ ATPase activity (Fig. 1). Ouabain-insensitive Na^+–ATPase activity was dose-dependently stimulated by Na^+ being maximal at 130–150 mM Na^+. $K_{0.5}$(the concentration of Na^+which produced half of maximal stimulation) was 28.8 ± 13.4 mM (Fig. 1). The effect of furosemide concentrations on total ouabain-insensitive Na^+ ATPase activity in chela muscle is shown in Fig. 2. Ouabain-insensitive Na^+ ATPase activity was dose-dependently inhibited by furosemide. The inhibition of total ouabain-insensitive Na^+ ATPase activity by furosemide revealed the presence in chela muscle of *N. granulata* of two ouabain-insensitive Na^+ ATPase activities (a furosemide-insensitive and a furosemide-sensitive Na^+ ATPase activity). Since, in the presence of 2 mM furosemide, inhibition of total ouabain-insensitive Na^+ ATPase activity was maximal, this concentration of inhibitor was used for further experiments described below. I_{50}(the concentration of furosemide that produced 50% ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity inhibition) was about 1.4 mM.

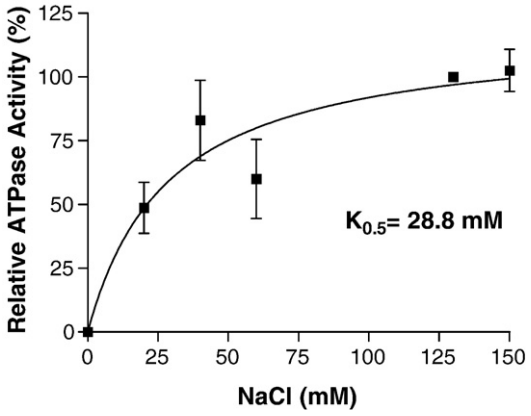

Fig. 1. Effect of Na^+ on ouabain-insensitive ATPase activity in chela muscle of *N. granulata*. The values of Na^+ ATPase activity are expressed as relation to the activity at 130 mM Na^+ (100%). The curve is the one which best fits the experimental data. Data are the mean ± S.E. for three to four individuals.

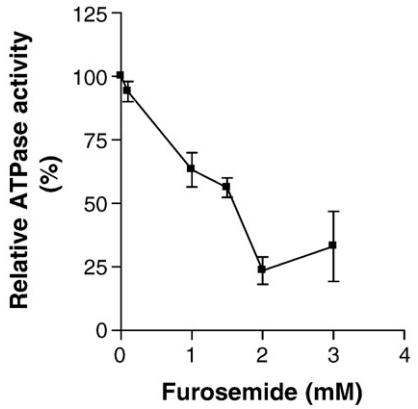

Fig. 2. Dose-dependent inhibition of ouabain-insensitive Na^+ ATPase activity by furosemide in chela muscle of *N. granulata*. The values of ouabain-insensitive Na^+ ATPase are expressed as relation to the activity in the absence of inhibitor (100%). I_{50}: furosemide concentration that produced 50% of inhibition, and was calculated by GraphPad Prism 2.01. Data are the mean ± S.E. for three to four individuals.

3.2. Effect of pH on ouabain-insensitive Na^+ ATPase activities in chela muscle of N. granulata

Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity tended to decrease from pH 6.2 to 7.0. At pH 7.4 this activity increased, being similar to that found at pH 6.2. At pH 7.8 ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity was about 60% of the activity at pH 7.4 (Fig. 3). Ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity increased upon enhancement of pH from 6.2 to 6.6 being maximal up to pH 7.4. At pH 7.8 this activity decreased by about 40% (Fig. 3B).

3.3. Effect of ATP on ouabain-insensitive Na^+ ATPase activities in chela muscle of N. granulata

The effect of ATP concentrations on ouabain-insensitive Na^+ ATPase activitiesof chela muscle of *N. granulata* is shown in Fig. 4. Both ouabain-insensitive, furosemide-insensitive and ouabain-insensitive, furosemide-sensitive Na^+ ATPase activities exhibited Michaelis–Menten kinetics ($K_m = 0.023$ and 0.213 mM, respectively).

3.4. Effect of environmental salinity on Na^+–K^+ ATPase and on ouabain-insensitive Na^+ ATPase activities in muscle of N. granulata

To determine the effect of environmental salinity on Na^+–K^+ ATPase activity and ouabain-insensitive Na^+ ATPase activities in chela muscle of *N. granulata*, specimens were acclimated to 35 and 10‰ salinity, salinities at which this crab osmoconforms and hyperregulates, respectively (Table 1). Na^+–K^+ ATPase and ouabain-insensitive Na^+ATPase activities in chela muscle were differentially affected by salinity.

In crabs acclimated to 35‰ salinity, chela muscle exhibited a low Na^+–K^+ ATPase activity (23 ± 6 nmol Pi min^{-1} mg $prot^{-1}$). In individuals acclimated to low salinity (10‰), Na^+–K^+ ATPase activity was about 5 fold higher (117 ± 26 nmol Pi min^{-1} mg $prot^{-1}$) than the activity at 35‰ salinity (Fig. 5).

In individuals acclimated to 35‰ salinity, chela muscle exhibited a high ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity (108 ± 15 nmol Pi min^{-1} mg $prot^{-1}$). In 10‰ salinity ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity was lower (about 3 fold) than the activity in crabs acclimated to 35‰ salinity (36 ± 11 nmol Pi min^{-1} mg $prot^{-1}$).

Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity of chela muscle of *N. granulata* was 15 ± 7 nmol Pi min^{-1} mg $prot^{-1}$ in individuals acclimated to 35‰ salinity. This activity was not affected by acclimation of crabs to reduced salinity (10‰) (Fig. 5).

4. Discussion

Our results show the occurrence of two ouabain-insensitive Na^+ ATPase activities (a furosemide-insensitive and a furosemide-sensitive Na^+ ATPase activity) in chela muscle of the euryhaline crab *N. granulata* from Mar Chiquita coastal lagoon. To our knowledge this is the first work to demonstrate the occurrence of ouabain-insensitive Na^+ ATPase activity in a euryhaline crab. These activities appeared to be coexistent with the Na^+–K^+ ATPase activity previously found in chela muscle of *N. granulata* (Pinoni and López Mañanes, 2003).

The insensitivity to ouabain (a well-known inhibitor of Na^+–K^+ ATPase) and the inhibition by furosemide (unlike Na^+–K^+ ATPase) have allowed to identify the coexistent Na^+ ATPases in several vertebrate tissues such as kidney, intestine, heart and gills (del Castillo et al., 1982; del Castillo and Robinson, 1985b; Proverbio et al., 1986, 1991; Moretti et al., 1991; Camejo et al., 1995; Caruso-Neves et al., 1999). In chela muscle of *N. granulata*, maximal inhibition by furosemide of

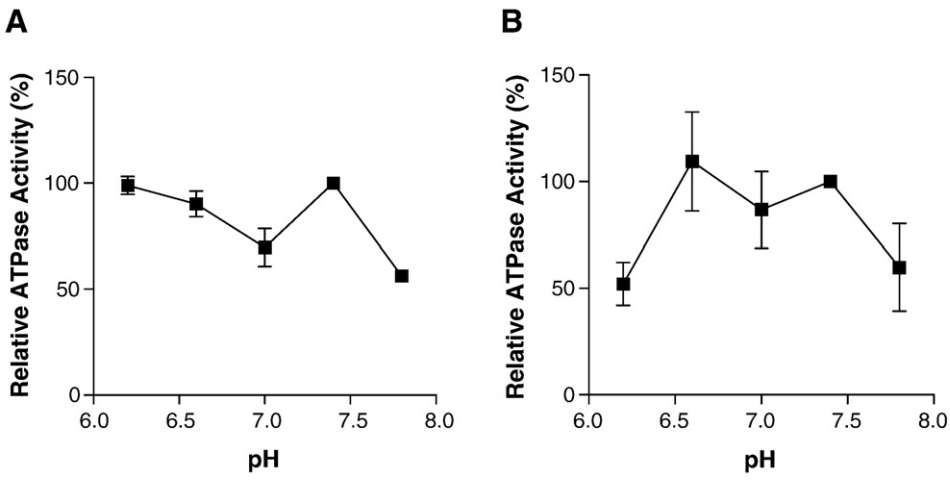

Fig. 3. Effect of pH (6.2–7.8) on ouabain-insensitive Na^+ ATPase activities in chela muscle of *N. granulata*. The values of Na^+ ATPase activity are expressed as relation to the corresponding activity at pH 7.4 (100%). In some cases, error bars were smaller than symbols used. Data are the mean ± S.E. for three to four individuals. (A) Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity. (B) Ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity.

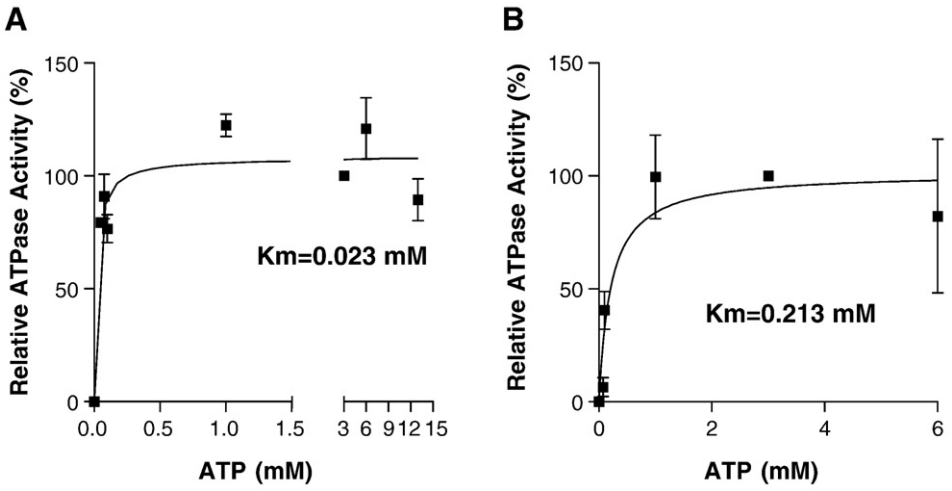

Fig. 4. Effect of ATP on ouabain-insensitive Na^+ ATPase activities in chela muscle of *N. granulata*. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The values of activity are expressed as relation to the corresponding activity in the presence of 3 mM ATP (100%). In some cases, error bars were smaller than symbols used. Data are the mean ± S.E. for three to four individuals. (A) Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity. (B) Ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity.

ouabain-insensitive Na^+ ATPase activity was reached at 2 mM (Fig. 2), a concentration of the inhibitor that fully inhibits ouabain-insensitive Na^+ ATPase activity in mammals and insects (del Castillo and Robinson, 1985b; Moretti et al., 1991; Camejo et al., 1995; Caruso-Neves and Lopes, 2000; Caruso-Neves et al., 2002; Beltowski et al., 2004) and in the protozoa *Leishmania amazonensis* (de Almeida-Amaral et al., 2008). Unlike mammals and insects, an ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity appeared also to occur in chela muscle of *N. granulata*. In several animal tissues, ouabain-insensitive Na^+–ATPase activity exhibits distinct biochemical characteristics (i.e. cation requirements, optimal pH and response to inhibitors) to those of Na^+–K^+ ATPase (del Castillo and Robinson, 1985b; Proverbio et al., 1986; Ventrella et al., 1990; Moretti et al., 1991; Beltowski et al., 2004). This appeared to be the case for both ouabain-insensitive, furosemide-sensitive and ouabain-insensitive, furosemide-insensitive Na^+ ATPase activities in chela muscle of *N. granulata*. Unlike the coexistent Na^+–K^+ ATPase activity in chela muscle of this crab (Pinoni and López Mañanes, 2003), ouabain-insensitive, furosemide-sensitive and furosemide-insensitive Na^+ ATPase activities were weakly affected by pH (Fig. 3) and exhibited Michaelis–Menten kinetics in response to varying ATP concentrations (Fig. 4). The Michaelis–Menten kinetics of ouabain-insensitive Na^+ ATPase activities of chela muscle of *N. granulata* are in agreement with those previously described for ouabain-insensitive Na^+ATPase of rainbow trout gills (Ventrella et al., 1992), pig kidney (Caruso-Neves et al., 2002) and of *L. amazonensis* (de Almeida-Amaral et al., 2008).

The ability of hyperregulating crabs to adapt to varying environmental salinities may imply responses from the molecular level to the organism. In low salinity, hyperregulating crabs absorb both sodium and chloride from the external medium via the gills, thus regulating their concentrations in the hemolymph and compensating for salt losses. In posterior gills of several hyperregulating crabs, adaptive increases of Na^+–K^+ ATPase activity occur both upon acclimation and after transfer to reduced salinity. Thus, this enzyme plays a central role in the biochemical adaptation of euryhaline crabs to reduced salinity (reviewed by Towle, 1997; Lucu and Towle, 2003). We have previously shown the occurrence of differential changes in Na^+–K^+ATPase activity of individual anterior and posterior gills of *N. granulata* from the Mar Chiquita coastal lagoon suggesting that the branchial enzyme is a component of the biochemical adaptations of this crab to low salinity (Schleich et al., 2001). The higher Na^+–K^+ ATPase activity of chela muscle of *N. granulata*at 10‰ (Fig. 5), a salinity at which this crab exhibits a strong hyperegulatory capacity (Table 1), along with the fact that under osmoionoconforming conditions (35‰ salinity) Na^+–K^+ATPase activity was low (Fig. 5), suggests the role of this enzyme in regulatory mechanisms at the biochemical level secondary to hyperregulation. In mammal skeletal muscle, Na^+–K^+ ATPase activity is involved in the response to various stressful conditions and the maintenance of resting membrane potential and osmotic balance. The Na^+ gradient created by the Na^+–K^+ ATPase would constitute the driving force for the maintenance of Na^+-dependent secondary processes such as transport of nutrients and Ca^{2+} (Clausen, 1996, 2003; McCarter et al., 2001). In frog muscle, Na^+–K^+ATPase has a role

Table 1
Concentration of ions (mEq l^{-1}) and osmolality (mOsm kg^{-1}) in external medium and in *N. granulata* hemolymph.

	35‰ salinity[a]		10‰ salinity[a]	
	Medium	Hemolymph	Medium	Hemolymph
Na^+	427.9 ± 19.9	419 ± 14.9	148.2 ± 8.1	341 ± 13.1*
K^+	10.1 ± 0.5	11.3 ± 0.5	3.4 ± 0.1	9.2 ± 0.4*
Cl^-	492 ± 13.5	427 ± 26.5	167 ± 7.7	384.7 ± 30,2*
Osmolality	902.9 ± 52.2	868.8 ± 14.0	311 ± 5.6	689.7 ± 48.3*

*Significantly different from the corresponding concentration of the external medium (ANOVA, $p<0.05$). Data are the mean ± S.E., $n=4$–9.
[a] Hemolymph of crabs acclimated in either 35‰ or 10‰ salinity.

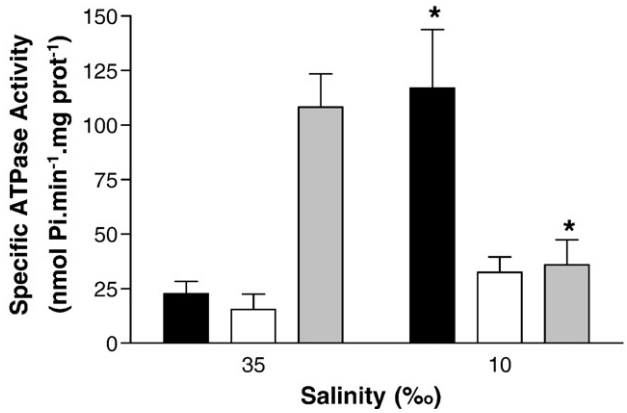

Fig. 5. Effect of acclimation to reduced salinity on Na^+–K^+ ATPase activity and ouabain-insensitive Na^+ ATPase activities in chela muscle of *N. granulata*. *Significantly different from the corresponding activity of individuals acclimated to 35‰ salinity ($p<0.05$). Data are the mean ± S.E. for five to nine individuals. Black bars: Na^+–K^+ ATPase activity. Open bars: Ouabain-insensitive, furosemide-insensitive Na^+ ATPase activity. Grey bars: Ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity.

in mechanisms of adjustment to hyposmotic stress (Venosa, 1991, 2003). Muscle of euryhaline crabs has been involved in cellular volume regulation (Lang and Gainer, 1969; Moran and Pierce, 1984) and acid–base balance (Whiteley et al., 2001) under osmotic stress. In *N. granulata*, muscle appears to provide an energy source through mobilization of lipids under hyposmotic stress (Luvizotto-Santos et al., 2003). The enhanced Na^+–K^+ ATPase activity in chela muscle of individuals acclimated to low salinity (Fig. 5) could support the ion concentrations and electrochemical gradients necessary for functioning of the transport systems probably involved in these physiological processes secondary to osmoionoregulation.

Ouabain-insensitive, furosemide-sensitive Na^+ATPase activity in chela muscle of *N. granulata* was differentially affected by environmental salinity (Fig. 5). Unlike Na^+–K^+ ATPase activity, ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity was high in individuals acclimated to 35‰ salinity, thus being predominant upon osmoconforming conditions (Fig. 5). The different responses of these activities to salinity could be associated with their differential physiological roles under osmoconforming and hyperregulation conditions in *N. granulata*. Ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity could have a house-keeping role by generating a Na^+ gradient for supporting the activity of transport systems involved in the maintenance of cellular essential functions (i.e. intracellular pH and volume regulation) in chela muscle. In mammals, Na^+ ATPase has been suggested to participate in several functions related to the efflux of Na^+ by the cells (Proverbio et al., 1991; Camejo et al., 1995; reviewed by Caruso-Neves et al., 2001; Beltowski et al., 2004; del Castillo et al., 2005). Under hyperosmotic stress an ouabain-insensitive Na^+ dependent uptake of aminoacids occurs in the jaw muscle of *N. granulata* (Schein et al., 2005). Whether Na^+ ATPase ouabain-insensitive, furosemide-sensitive activity of chela muscle of this crab is involved in this process remains to be investigated. In gills of the water mussel *Mytilus edulis* Na^+ ATPase activity is higher than that of Na^+–K^+ATPase (Howland and Faus, 1985). In low salinity, when ouabain-insensitive Na^+ATPase activity is decreased, the several fold enhanced Na^+–K^+ATPase activity could play the main role in maintaining the Na^+ gradient to support biochemical and physiological adjustments probably occurring in chela muscle of *N. granulata* upon hyperregulation. A distinct response of the coexistent Na^+–K^+ATPase and ouabain-insensitive Na^+ATPase activities to environmental conditions, exogenous chemicals and intrinsic factors has been described in some mammals, insects and bivalve tissues (reviewed by Caruso-Neves and Lopes, 2000; Ventrella et al., 2001; Caruso-Neves et al., 2004; Beltowski et al., 2004; Wengert et al., 2005; Pagliarani et al., 2006, 2008). In gills of the rainbow trout *Oncorhynchus mykiss*, Na^+–K^+ ATPase and ouabain-insensitive Na^+ATPase activities are differentially affected by environmental salinity (Ventrella et al., 1992, 2001). The lower ouabain-insensitive, furosemide-sensitive Na^+–ATPase activity and the enhanced Na^+–K^+ATPase activity in low salinity suggest that a coordinated regulation (down and up regulation, respectively) of these activities in chela muscle could constitute one of the responses underlying hyperregulation in *N. granulata*. Whether the differential modulation of ouabain-insensitive, furosemide-sensitive Na^+–ATPase activity and Na^+–K^+ ATPase activity is due to either a differential activation/deactivation of preexistent enzymes (i.e. by endogenous factors involved in the regulation of osmoionoregulatory processes) or synthesis/degradation process requires further investigation.

In summary, our results show the existence of two distinct ouabain-insensitive Na^+ ATPase activities in chela muscle of *N. granulata* and the differential response of the coexistent Na^+–K^+ ATPase and ouabain-insensitive Na^+ ATPase activities to low salinity, suggesting the differential participation of these activities in responses at the biochemical level of this crab to reduced environmental salinity. Whether these activities are involved in physiological processes secondary to osmotic balance and ionoregulation (i.e. cell volume regulation, acid–base equilibrium, mobilization of substrates, regulation of intracellular osmolytes concentrations) remains to be investigated. Although, ouabain-insensitive, furosemide-insensitive Na^+ ATPase activities exhibited quite distinct characteristics from the furosemide-sensitive activity (i.e. response to pH, ATP and salinity) further characterization is needed to establish the occurrence of different isoforms in chela muscle of *N. granulata*. Future studies must be focused on establishing the exact physiological role of these muscle activities in the integrative responses of this crab to varying environmental conditions thus allowing a better understanding of the complex mechanisms underlying biochemical–physiological adaptations in euryhaline crabs.

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