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

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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*

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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