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The Na^+, K^+, $2Cl^-$ cotransporter of estuarine pufferfishes (*Sphoeroides testudineus* and *S. greeleyi*) in hypo- and hyper-regulation of plasma osmolality ☆

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Abstract

The pufferfishes *Sphoeroides testudineus* and *Sphoeroides greeleyi* are estuarine species that osmoregulate efficiently, but *S. testudineus* tolerates seawater dilution to a much higher degree than *S. greeleyi*. This study aimed at testing whether NKCC is involved with their differential tolerance of seawater dilution, through the analysis of in vivo furosemide (NKCC inhibitor) injection both on hypo-regulation (in 35‰ salinity) and hyper-regulation (in 5‰ salinity). After exposure for 6 h or 5 days to both salinities, blood samples were obtained for determination of plasma osmolality, chloride, sodium and hematocrit, and muscle samples for determination of water content. Furosemide injection led to increased plasma osmolality and sodium in 35‰ and decreased osmolality and chloride in 5‰, when compared to saline-injected controls. Furosemide injection led to hematocrit reduction in both salinities, and muscle water content increase in 5‰ and decrease in 35‰ in *S. testudineus*. The results are compatible with NKCC working in branchial NaCl secretion in 35‰, in both species, and a higher role in cell volume regulation in blood and muscle cells of *S. testudineus*, in both salinities, which could partially explain the stronger capacity of *S. testudineus* to tolerate seawater dilution during low tide.

Keywords: Estuarine fish; Furosemide; Hematocrit; NKCC; Osmoregulation; Pufferfishes; Teleost fish

1. Introduction

Marine teleosts are hyposmotic to seawater, their gills acting in the maintenance of plasma homeostasis secreting the excess of salt taken up (Evans, 1993; Jobling, 1995). Mitochondria rich cells (chloride cells) of the branchial epithelium are mainly responsible for chloride secretion in seawater, along with adjacent accessory cells, both cells separated by a leaky paracellular pathway permeable to sodium. Chloride and accessory cells are filled with mitochondria associated to a dense membrane tubular system rich in ion transporting proteins, a continuation of the basolateral membrane (Philpott, 1980; Jobling, 1995; Perry, 1997).

The Na^+, K^+, and Cl^- cotransporter (NKCC) stands among the most relevant transport proteins of the fish gill, its secretory isoform NKCC1 being located in the basolateral membrane system of chloride cells in the teleost gill epithelium (Lytle and Forbush, 1992a,b; Lytle et al., 1995; Marshall et al., 2002a; Marshall, 2003; McCormick et al., 2003; Scott et al., 2004). NKCC is a secondary active transporter, found also in a wide variety of salt transporting epithelia and non-epithelial animal cells, working in the latter for cellular volume homeostasis (Russell, 2000). The absorptive isoform NKCC2 is apically located in epithelia. Both isoforms can be inhibited by the "loop diuretics" bumetanide and furosemide (Forbush et al., 1992; Lytle and Forbush, 1992a; Lytle et al., 1995; Mount et al., 1998; Russell, 2000). In teleosts in general, NKCC has been demonstrated not only in the branchial and opercular epithelia (Pelis et al., 2001; Pelis and McCormick, 2001; Cutler and Cramb, 2002; Marshall et al., 2002a, 2005; McCormick et al.,

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2003; Marshall et al., 2005; Scott et al., 2004), but also in the renal (Cutler and Cramb, 2002), and intestinal epithelia (O'Grady et al., 1987; Marshall et al., 2002b) as well.

In euryhaline marine teleosts NKCC1 has been demonstrated to have its expression down regulated when there is no further need of salt secretion in brackish water (McCormick et al., 2003; Scott et al., 2004), or when there is instead the need for salt absorption in freshwater or very dilute seawater (Pelis et al., 2001; Scott et al., 2004). In addition to down regulation of its expression, mainly short-term regulation of salt secretion by branchial or opercular epithelia has been shown to involve redistribution of NKCC from a basolateral distribution in seawater to eccentric distribution in fresh water (Marshall et al., 2002a), or even dephosphorylation by a protein phosphatase (Marshall, 2003). The same regulatory phenomenon applies to the chloride cell apical low conductance chloride channel CFTR (Marshall, 2003; McCormick et al., 2003; Scott et al., 2004). Both transporters are together responsible for the vectorial transcellular secretion of chloride in the gills of seawater teleosts (e.g., Marshall, 2003).

The pufferfish *Sphoeroides testudineus* Linnaeus, 1758 is a frequent and abundant species in bays and estuaries along the coast of Brazil. It occurs in waters of salinity ranging between 0‰ and 34‰ (Nardi, 1999; Figueiredo and Menezes, 2000). Its abundant congener *Sphoeroides greeleyi* Gilbert, 1900 is also estuarine, but restricted to areas of higher salinity than *S. testudineus* (~30‰), not remaining in the estuary during ebb tide (Figueiredo and Menezes, 2000; Vendel et al., 2002). Both pufferfishes are euryhaline, displaying similar capacities to regulate plasma osmolality and ions in seawater (Prodocimo and Freire, 2001, 2004). However, in very low salinities they behave differently, with *S. testudineus* being distinctively more tolerant when compared to *S. greeleyi*: *S. greeleyi* does not tolerate more than a week in 5‰ (Prodocimo and Freire, 2001). This pattern of tolerance is in accordance to their occurrence in nature (Figueiredo and Menezes, 2000; Vendel et al., 2002).

The present study was conducted to answer the question of whether NKCC plays any role in this differential tolerance of *S. testudineus* and *S. greeleyi* to very diluted seawater. The "signal" of the presence of NKCC in both pufferfishes was here evaluated using two different experimental techniques: in vivo furosemide injection, and gill immunocytochemistry. The effect of the injection of the inhibitor was evaluated in gills, as signaled by plasma osmotic, chloride, and sodium concentrations (NKCC action in osmoregulation), and in tissues, signaled by hematocrit and muscle water content (NKCC action in cell volume regulation).

2. Materials and methods

2.1. Animals

Adult *S. testudineus* (~15 cm body length, ~40 g body mass) and *S. greeleyi* (~10 cm of body length, ~20 g body mass) were obtained from Baguaçu river tidal creek (25°33′6.33″ S, 48°23′ 41.63″ W), at the south margin of Paranaguá Bay, State of Paraná, Brazil, from September, 2002 to May, 2004. Fish were obtained during flow tide using a fyke type net installed across the creek channel. The net has remained in place for 6 h, until the high tide peak, when the net was retrieved and the animals transferred to plastic gallons with aerated water from the collection site. Water salinity was verified (refractometer Shibuya S-28, Japan) immediately after fishing the animals, and varied between 29‰ and 30‰ in the collection dates. Fishes were then transferred to the laboratory, where they were acclimated for ~7 days in a 250 L tank of salinity 30‰, temperature of 21 ± 1 °C, constant aeration and biological filtration. Fish were daily fed ad libitum with fresh prawns and earthworms, both during their acclimation and 5-day experimental periods.

2.2. Experiments

S. testudineus and *S. greeleyi* specimens were then exposed for either 6 h or 5 days to full-strength seawater of salinity 35‰ and to diluted seawater (5‰ salinity). Full strength seawater of salinity 35‰ was prepared appropriately by mixing 30‰ seawater with first thawed concentrated seawater. Experimental fishes submitted to 35‰ salinity were directly transferred from the stock tank of salinity 30‰ to the experimental aquarium of salinity 35‰. Seawater was diluted down to 5‰ with filtered (cellulose and activated charcoal filters in series) dechlorinated tap water. Experiments were performed in 30 l aquaria with constant aeration and temperature of 21 ± 1 °C, and a maximum number of 3 fish per experimental aquarium. Experiments were performed at least in independent duplicates, until an adequate "*n*" was reached for each parameter. Seawater was diluted overnight from ~30‰ down to ~10–12‰ along 4 h (with flux of 0.7 ml/seg) to avoid osmotic shock to fishes exposed to 5‰. The fish remained 10–12 h in salinity 10–12‰, and were then transferred to 5‰. After spending either 6 h or 5 days in the experimental salinities (5‰ and 35‰), fishes were anaesthetized with benzocaine dissolved in ethanol in the aquarium water, to a final concentration of 80 mg/l. This dose of benzocaine was previously verified to allow full recovery. After complete anesthesia, experimental fishes were injected with 1 mL of furosemide (Aventis) 10 mg/mL, ~30 mM in 300 mM NaCl. The estimated doses of furosemide were: 0.75 µmol/g fish body mass for *S. testudineus* and 1.5 µmol/g fish body mass for *S. greeleyi*. This difference in the dose of furosemide applied will prevent direct comparisons of values between both species. All control fishes were injected with 1 mL of 300 mM NaCl, and all fishes received dorsolateral muscular injection. This dose of furosemide was the smallest dose found from preliminary tests, still able to induce significant changes in plasma osmolality, being lethal after 90–120 min post-injection. Fish were anaesthetized before injection to reduce the stress of holding and manual contention. After the injection, the experimental and control fish remained in an individual container for 1 h, when they were again anaesthetized and opened by ventral incision.

2.3. Assays

After ventral incision, blood samples were withdrawn through cardiac puncture using heparinized syringes. Samples