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The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*

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Abstract

Chloride transport mechanisms in the gills of the estuarine spotted green pufferfish (*Tetraodon nigroviridis*) were investigated. Protein abundance of Na^+/K^+-ATPase (NKA) and the other four chloride transporters, i.e., $Na^+/K^+/2Cl^-$ cotransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR), Cl^-/HCO_3^- anion exchanger 1 (AE1), and chloride channel 3 (CLC-3) in gills of the seawater-(SW; 35‰) or freshwater (FW)-acclimatized fish were examined by immunoblot analysis. Appropriate negative controls were used to confirm the specificity of the antibodies to the target proteins. The relative protein abundance of NKA was higher (i.e., 2-fold) in gills of the SW group compared to the FW group. NKCC and CFTR were expressed in gills of the SW group but not in the FW group. In contrast, the levels of relative protein abundance of branchial AE1 and CLC-3 in the FW group were 23-fold and 2.7-fold higher, respectively, compared to those of the SW group. This study is first of its kind to provide direct *in vivo* evidence of the protein expression of CLC-3 in teleostean gills, as well as to examine the simultaneous protein expression of the Cl^- transporters, especially AE1 and CLC-3 of FW- and SW-acclimatized teleosts. The differential protein expression of NKA, chloride transporters in gills of the FW- and SW-acclimatized *T. nigroviridis* observed in the present study shows their close relationship to the physiological homeostasis (stable blood osmolality), as well as explains the impressive ionoregulatory ability of this euryhaline species in response to salinity challenges.

Keywords: Pufferfish; *Tetraodon nigroviridis*; Euryhaline teleost; CFTR; NKCC; Gill; Chloride transporter; Na^+/K^+-ATPase

1. Introduction

Maintaining a stable internal environment is important for vertebrate animals to survive in a variety of habitats. In response to changes of environmental conditions, the ion-transporting epithelia play the roles of modulating ion fluxes. Although ionoregulation in fish is mediated by a group of structures including the intestine and kidney, the gill is the major site for the balance of ion movement between gains and losses (Evans et al., 2005). In order to maintain the osmolality and ion balance, teleosts take up salts from fresh water (FW) through the gills and reabsorb salts in the kidney. In contrast, seawater (SW) teleosts excrete salts through the gills and absorb water in the kidney. The systems used by teleosts to adapt to FW or SW differ not only in the direction of ion and water movements but also in the molecular components of transporters. Euryhaline fish adapt to either FW or SW by switching these systems efficiently (Kato et al., 2005).

The fundamental transporters responsible for ion movement across gill epithelia have been reported in previous studies (see reviews of Perry, 1997; Hirose et al., 2003; Evans et al., 2005). Among the transporters, Na^+/K^+-ATPase (NKA) was thought to provide primary driving force for ion transport. NKA is a ubiquitous membrane-bound enzyme which is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex. The molecular mass of

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the catalytic α-subunit is about 100 kDa, while the smaller glycosylated β-subunit exists with a molecular mass of approximately 55 kDa (Blanco and Mercer, 1998). Most euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Lin et al., 2003). In gills of euryhaline teleosts, a basolaterally located NKA creates an electrochemical gradient to transport Na^+ and Cl^- actively across epithelia in both secretory (SW) and absorptive (FW) modes (Marshall, 2002; Hirose et al., 2003; Perry et al., 2003).

For secretion of chloride ions in gill epithelia of SW teleosts, Silva et al. (1977) proposed a model in which a basolaterally located Na^+/Cl^- exchanger that brought chloride ions into the cell. Then pharmacological evidence proposed that basolateral Na^+/Cl^- cotransport occurred through a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (NKCC; Degnan, 1984). NKCC included two isoforms, NKCC1 and NKCC2. Because of prominent expression of NKCC1 on basolateral confinement and it has been confirmed to operate in chloride-secreting epithelia, NKCC1 was considered to be the secretory isoform. A second form, NKCC2, was only found in the kidney (Lytle et al., 1995). Instead of being diffusive, Cl^- secretion at the apical membrane may use an anion channel with characteristics resembling that of the cystic fibrosis transmembrane conductance regulator (CFTR; Marshall et al., 1995). CFTR was reported to exist in gills of teleosts by electrophysiological, molecular biological, pharmacological, biochemical, and immunocytochemical studies (Marshall and Singer, 2002). On the other hand, for absorption of chloride ions in gills of FW teleosts, the model was not conclusive (Hirose et al., 2003). Chloride influx, however, was shown to be stimulated by infusion of bicarbonate with a good correlation between the rates of Cl^- absorption and base secretion (Kerstetter and Kirschner, 1972; de Renzis and Maetz, 1973). An epithelial Cl^-/HCO_3^- exchanger in fish gills was reported in a kinetic, pharmacological and correlative morphological study (Goss et al., 1995; Tresguerres et al., 2006). The studies of Wilson et al. (2000a, 2002) proved the distribution of Cl^-/HCO_3^- exchanger 1 (AE1) in fish gills. In addition, the chloride channel to absorb chloride from cell to blood in gills of FW teleosts is not clear (Evans et al., 2005; Tresguerres et al., 2006). A member of the CLC family was suggested to be the chloride channel in gills of FW teleosts (Hirose et al., 2003). Transporters of the CLC family, such as CLC-3, are important for transepithelial Cl^- transport in different organs of mammals (Kawasaki et al., 1994; Sasaki et al., 1994; Weylandt et al., 2001). In teleosts, the mRNA of CLC-3 was demonstrated to be abundant in the gills (Miyazaki et al., 1999).

The spotted green pufferfish (*Tetraodon nigroviridis*) is an advanced tetraodontid teleost whose native range covers the rivers and estuaries of Southeast Asia (Rainboth, 1996). Being a peripheral FW inhabitant (Helfman et al., 1997), this pufferfish was demonstrated to be an efficient osmoregulator in experimental conditions, as it can tolerate a direct transfer from FW to SW or vice versa (Lin et al., 2004; Lin and Lee, 2005). The present set of experiments was designed to identify the adaptive responses of protein expression of Cl^- transporters in gills of the spotted green pufferfish acclimated to salinities of a hyperosmotic environment (i.e., SW) or a hyposmotic environment (i.e., FW). This study, to our knowledge, is the first to provide direct *in vivo* evidence of CLC-3 protein expression in teleostean gills, as well as the first to compare simultaneous protein expression of the four Cl^- transporters of SW- and FW-acclimatized euryhaline teleost.

2. Materials and methods

2.1. Fish and experimental environments

The spotted green pufferfish (*T. nigroviridis*) were obtained from a local aquarium with the body weight of 5.6±0.4 g and the total length of 5.6±0.5 cm. Seawater (35‰; SW) used in this study was prepared from local tap water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The spotted green pufferfish were reared in either SW or FW at 27±1 °C with a daily 12 h photoperiod for at least 2 weeks before sampling (Lin et al., 2004). The water was continuously circulated through fabric-floss filters. The fish were fed with commercial arid shrimp daily.

2.2. Preparation of membrane fractions for NKA, AE1, CLC-3, NKCC and gill lysates for CFTR

Gill arches of the fish were excised and blotted dry. The gill epithelia were immediately scraped off from the underlying cartilage with a scalpel. All procedures were performed on ice. Gill scrapings were suspended in 300 μL of (a) buffer A (20 mmol L^{-1} Tris–base, 2 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 2 mmol L^{-1} EDTA, 0.5 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 250 mmol L^{-1} sucrose, proteinase inhibitor, pH 7.4) for NKA, AE1, and CLC-3, or (b) ice-cold SEI buffer (300 mmol L^{-1} sucrose, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} imidazole, pH 7.4) for NKCC and CFTR, respectively. Homogenization was performed in 2 mL tubes with the Polytron PT1200E (Lucerne, Switzerland) at maximal speed for 25 strokes. Membrane fractions of NKA, AE1, and CLC-3 were prepared according to the method modified from Stanwell et al. (1994). After homogenization, the homogenates for NKA, AE1, and CLC-3 were then centrifuged at 135,000 *g* for 1 h at 4 °C. Supernatants were the mixture of cytosol protein. The pellets were resuspended in 300 μL buffer B (20 mmol L^{-1} Tris–base, 2 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 5 mmol L^{-1} EDTA, 0.5 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 5 mmol L^{-1} NaF, 0.1% Triton X-100, proteinase inhibitor, pH 7.5) and vortexed every 10 min during a 1 h incubation period at 4 °C. Dissolved pellets were centrifuged again at 135,000 *g* for 1 h at 4 °C. Supernatants were the mixture of membrane fractions for NKA, AE1, and CLC-3. According to Tipsmark et al. (2004), the homogenates for NKCC was centrifuged at 1000 *g* for 20 min at 4 °C. The membrane fraction of NKCC was the pellet isolated from the supernatant by a second centrifugation at 50,000 *g* for 30 min at 4 °C. The homogenates for CFTR were carried out as described by Marshall et al. (2002b) with little modification. After centrifugation at 2000 *g* at 4 °C for 6 min, the pellet was