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Prolactin and cortisol mediate the maintenance of hyperosmoregulatory ionocytes in gills of Mozambique tilapia: Exploring with an improved gill incubation system

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ABSTRACT

Endocrine control of osmoregulation is essential for teleosts to adapt to various aquatic environments. Prolactin (PRL) is known as a fundamental endocrine factor for hyperosmoregulation in teleost fishes, acting on ionocytes in the gills to maintain ion concentrations of body fluid within narrow physiological ranges in freshwater conditions. Cortisol is also known as an osmoregulation-related steroid in teleosts; however, its precise function is still controversial. Here, we investigated more detailed effects of PRL and roles of cortisol on ionocytes of Mozambique tilapia (*Oreochromis mossambicus*) in freshwater, using an improved gill filament incubation system. This incubation system resulted in enhanced cell viability, as evaluated using the dead cell marker propidium iodide. PRL was shown to maintain the density of freshwater-type ionocytes in isolated gill filaments; this effect of PRL is not achieved by the activation of cell proliferation, but by the maintenance of existing ionocytes. Cortisol alone did not show any distinct effect on ionocyte density in isolated gill filaments. We also assessed effects of PRL and cortisol on relative mRNA levels of NCC2, NHE3, NKAa1a, and NKAa1b. PRL maintained relative NCC2 and NKAa1a mRNA abundance, and cortisol showed a stimulatory effect on relative NCC2 and NKAa1a mRNA levels in combination with PRL, though cortisol alone exerted no effect on these genes. An increase in NKAa1b mRNA abundance was detected in cortisol-treated groups. PRL treatment also maintained normal NCC2 localization at the apical membrane of the ionocytes. These results indicate that PRL maintains freshwater-type ionocytes, and that cortisol stimulates the function of ionocytes maintained by PRL.

1. Introduction

For vertebrates, body fluid homeostasis is fundamental to life. Osmoregulation in teleost fishes is achieved by integrated water and ion transport activities of osmoregulatory organs (Marshall and Grosell, 2006). Among these organs, the gills play a central role in ion regulation in aquatic environments: ionocytes (also referred to as chloride cells or mitochondrion-rich cells) located in gill epithelia are responsible for ion absorption and secretion in hyposmotic and hyperosmotic conditions, respectively. In the last decade, the molecular basis for ion transport mechanisms in the ionocytes has been well elucidated in some fish species (Hwang et al., 2011; Takei et al., 2014; Guh et al., 2015). In Mozambique tilapia (*Oreochromis mossambicus*) adapted to FW, two types of ionocytes have been identified; that is, Na^+, Cl^- cotransporter-2 (NCC2)-expressing cells and Na^+/H^+ exchanger-3 (NHE3)-expressing cells (Hiroi et al., 2008). Several studies have indicated that these transporters are localized at the apical membrane in different types of ionocytes, and are important for hyperosmoregulation (Hiroi et al., 2008; Inokuchi et al., 2008; Watanabe et al., 2008; Inokuchi et al., 2009).

It is widely accepted that these osmoregulatory functions are controlled by endocrine systems; however, the precise mechanisms and pathways of hormonal control for osmoregulation have remained unclear so far. Among proposed osmoregulatory endocrine factors in teleosts, prolactin (PRL) is one of the most studied hormones associated with freshwater (FW) adaptation (Manzon, 2002; Sakamoto and McCormick, 2006). The pioneer work by Pickford and Phillips (1959) showed that PRL restored hypophysectomy-induced malfunction of FW adaptation in killifish (*Fundulus heteroclitus*). Recently, Breves et al. (2010) showed that branchial NCC2 mRNA expression was suppressed in hypophysectomized Mozambique tilapia reared in FW, and that the decreased NCC2 mRNA expression was restored by PRL administration. Similarly, the mRNA expression of branchial Na^+, K^+

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ATPase (NKA) α1a (NKAa1a), which is one of NKAα1 isoforms specific for FW (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009; Tipsmark et al., 2011; McCormick et al., 2013), was also stimulated by PRL in hypophysectomized fish. On the other hand, the expression level of mRNA encoding NKAa1b gene, which is suggested to be involved in SW adaptation (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009; Tipsmark et al., 2011; McCormick et al., 2013), was not changed by hypophysectomy and the following hormonal treatments (Breves et al., 2010). The transcript level of each NKAa1 isoform was well linked with protein abundance in salmonid gills (McCormick et al., 2009), suggesting that expression levels of mRNAs for NKAa1 isoforms are one of the good indicators for branchial osmoregulatory functions. In addition, PRL is also likely to mediate the apical localization of NCC2 and the maintenance of proper density of ionocytes in FW tilapia (Breves et al., 2010). Similar effects of PRL on NCC2-expressing ionocytes were reported in zebrafish (*Danio rerio*) (Breves et al., 2013, 2014a), suggesting that PRL's function on branchial ionocytes is most likely to be conserved among teleost species. These studies indicate that PRL is involved in the enhancement of functionality, recruitment and/or maintenance of FW-type ionocytes; however, it is unknown whether PRL can directly mediate these proposed functions in ionocytes.

A number of *in vitro* studies have shown that isolated ionocytes cannot survive under simple dispersed cell culture conditions, suggesting that unknown endogenous factors are essential for the maintenance of ionocytes in teleost fishes (McCormick and Bern, 1989; Avella and Ehrenfeld, 1997; Wood and Pärt, 1997; Fletcher et al., 2000; Leguen et al., 2007). Several reports on a cell culture system, called double-seeded insert (DSI), have suggested that the cell-cell interaction is one of the most important factors for ionocyte maintenance in rainbow trout (*Oncorhynchus mykiss*), and that PRL has very limited effects on FW fish-derived branchial cell population (Fletcher et al., 2000; Zhou et al., 2003). The DSI is a widely accepted method as a model for branchial ion transport; however, isolated ionocytes have to pass through a dissociation process with extensive trypsinization. It is highly possible that the polarity of ionocytes is disrupted and membrane proteins, like receptors and transporters, on the cellular membrane of the ionocytes are damaged during cell preparation. To minimize these possible negative effects of dissociation, a gill filament incubation system is more suitable. McCormick and Bern (1989) are the first to establish a gill filament incubation method to examine the effects of cortisol on branchial epithelia. However, accessibility of supplements in the incubation medium, except for membrane-permeable compounds like cortisol, to the branchial cells is most likely limited due to the epithelial barrier function of the gills. Therefore, improvement of the gill filament incubation method that preserves the intact cellular arrangement with high permeability of supplements could enable better assessment of PRL's direct effects on ionocyte functions. In addition to PRL, cortisol is a strong candidate for the mediator of maintenance and/or proliferation of ionocytes (McCormick et al., 1991; Ayson et al., 1995; Dang et al., 2000; Zhou et al., 2003; Tipsmark et al., 2011), although the precise role of cortisol on ionocytes is still controversial because of its multi-functionality.

In the present study, we investigated possible effects of PRL and cortisol on FW-type ionocytes in Mozambique tilapia, using an improved gill filament incubation system. Compared to conventional methods, this new method improved the accessibility of additive factors to ionocytes without excessive disruption of the 3-dimensional epithelial structure. We employed microscopic analysis of ionocytes in incubated gill filaments by means of whole-mount immunohistochemistry, and mRNA quantification of NCC2, NHE3, NKAa1a and NKAa1b, all of which are ion transporters responsible for osmoregulation in branchial ionocytes.

2. Materials and methods

2.1. Fish

Mozambique tilapia were maintained and bred in tanks supplied with recirculating FW, and water temperature was maintained at 25 °C with a constant 14-h light/10-h dark cycle. Fish were fed on commercial carp pellets (Nihon Haigo Shiryo, Yokohama, Japan) once a day. Matured male fish (100–150 g) were anesthetized with a lethal dose of 2-phenoxyethanol (0.1%) and decapitated before removal of tissues. Experiments were conducted according to the principles and procedures approved by the Institutional Animal Care and Use Committee of The University of Tokyo.

2.2. Gill incubation

The blood was collected from the caudal vessels of anesthetized fish with a heparinized syringe and needle. Second gill arches were removed and washed with balanced salt solution (BSS: NaCl, 140 mM; KCl, 3 mM; $MgSO_4$, 1.25 mM; NaH_2PO_4, 0.4 mM; $NaHCO_3$, 2 mM; $CaCl_2$, 1.5 mM, Hepes, 10 mM; pH 7.5). Intact gill filaments were sampled prior to the preparation procedures to serve as initial controls. The dissected gills were perfused with Leibovitz's L-15 (Life technologies, Carlsbad, CA) via the opening of the efferent arch artery, and washed with 0.025% $KMnO_4$ in BSS for 1 min to remove mucus from the gill surface. Gill filaments (approximately 8-mm length) were removed and then cut into afferent and efferent halves by longitudinal incision at the center of the gill filament with a fine scalpel. Photographs of intact and split gill filaments are shown in Fig. 1A and B. The resulting pairs of split gill filaments were incubated in experimental media in sterile 12-well culture plates at 25 °C for up to 72 h under saturated oxygen (100% O_2) condition with gentle shake. Approximately 70% of the incubation medium was changed every 24 h. Glucose-supplemented (final conc. 5 mM) Leibovitz's L-15 with antibiotic-antimycotic solution (Life technologies) was used for basal incubation medium (310 mOsm/kg) in this experiment. The cell viability in incubated gill filaments was checked with vital staining of propidium iodide (Life technologies), a known dead cell marker.

The gill filaments were separated into four groups: control; PRL (7 μg/ml ovine prolactin from Sigma-Aldrich, St. Louis, MO); cortisol (0.5 μg/ml hydrocotisone from Wako, Osaka, Japan); and PRL + cortisol (7 μg/ml ovine prolactin + 0.5 μg/ml hydrocortisone). Cortisol dissolved in ethanol (1 mg/ml) was used as a 2000 times-concentrated stock. The same volume of ethanol as cortisol-treated groups was added to incubation media of experimental groups without cortisol. The gill filaments were sampled at 24 h, 48 h and 72 h after the onset of incubation. For whole-mount immunohistochemistry, the incubated gill filaments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at room temperature, and stored in 70% ethanol. Total RNA was extracted from the filaments with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. Morphology of the gill filament with a longitudinal incision was confirmed with a scanning electron microscope. Samples were processed and observed as previously reported (Inokuchi et al., 2008).

2.3. Double-color whole-mount immunofluorescence staining

For immunohistochemical detection of NKA-positive ionocytes, we used a rabbit polyclonal antiserum NAK121 (Uchida et al.,

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