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Prolactin regulates transcription of the ion uptake Na^+/Cl^- cotransporter (*ncc*) gene in zebrafish gill

Jason P. Breves [a], Sandy B. Serizier [a], Vincent Goffin [b], Stephen D. McCormick [a,c], Rolf O. Karlstrom [a,*]

[a] Department of Biology, Center for Neuroendocrine Studies, University of Massachusetts, Amherst, MA 01003, USA
[b] Inserm, Unit 845, Research Center Growth and Signaling, Prolactin/GH Pathophysiology Laboratory, University Paris Descartes, Sorbonne Paris Cité, Faculty of Medicine, Necker site, Paris F-75015, France
[c] USGS, Conte Anadromous Fish Research Center, Turners Falls, MA 01376, USA

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ABSTRACT

Prolactin (PRL) is a well-known regulator of ion and water transport within osmoregulatory tissues across vertebrate species, yet how PRL acts on some of its target tissues remains poorly understood. Using zebrafish as a model, we show that ionocytes in the gill directly respond to systemic PRL to regulate mechanisms of ion uptake. Ion-poor conditions led to increases in the expression of PRL receptor (*prlra*), Na^+/Cl^- cotransporter (*ncc*; *slc12a10.2*), Na^+/H^+ exchanger (*nhe3b*; *slc9a3.2*), and epithelial Ca^{2+} channel (*ecac*; *trpv6*) transcripts within the gill. Intraperitoneal injection of ovine PRL (oPRL) increased *ncc* and *prlra* transcripts, but did not affect *nhe3b* or *ecac*. Consistent with direct PRL action in the gill, addition of oPRL to cultured gill filaments stimulated *ncc* in a concentration-dependent manner, an effect blocked by a pure human PRL receptor antagonist (Δ1-9-G129R-hPRL). These results suggest that PRL signaling through PRL receptors in the gill regulates the expression of *ncc*, thereby linking this pituitary hormone with an effector of Cl^- uptake in zebrafish for the first time.

1. Introduction

It has long been recognized that the endocrine system plays a central role in the homeostatic regulation of salt and water balance in vertebrates (McCormick and Bradshaw, 2006). Among the pituitary hormones, prolactin (PRL) has received considerable attention as an osmoregulatory hormone with conserved actions across vertebrate groups. In mammals, PRL influences solute and water transport across renal, intestinal, mammary and amniotic epithelial membranes (Bole-Feysot et al., 1998; Freeman et al., 2000). In teleost fishes, PRL is recognized as an important "freshwater-adapting hormone" regulating osmoregulatory functions within the gill, kidney and gastrointestinal tract by promoting ion conserving and water secreting processes (Hirano, 1986; Sakamoto and McCormick, 2006). The gill possesses a rich population of ionocytes that are capable of active Na^+, Cl^-, and Ca^{2+} uptake needed to support hydromineral balance in freshwater environments that are hyposmotic to body fluids (Kaneko et al., 2008). Varying and contending models have been proposed for the cellular mechanisms that regulate branchial ion-uptake in fresh water (Evans, 2011), and this incomplete understanding of the molecular mechanisms driving ion uptake in freshwater ionocytes has impeded progress in understanding how PRL promotes acclimation to freshwater environments.

The zebrafish (*Danio rerio*) has emerged as a new model organism for studying vertebrate physiology and is particularly well suited to detailed mechanistic and comparative studies of developmental endocrinology (McGonnell and Fowkes, 2006; Löhr and Hammerschmidt, 2011) and osmoregulation (Hwang, 2009). Zebrafish are regarded as being stenohaline and are naturally distributed in soft-water rivers and streams of the Indian subcontinent. Adult zebrafish can rapidly adapt to ion-poor conditions and can survive in deionized water for extended periods (Craig et al., 2007; Boisen et al., 2003). To persist in ion-poor waters, zebrafish have high capacity for Na^+ and Cl^- uptake (Boisen et al., 2003), despite strong opposing electrochemical gradients across gill epithelium. This capacity for ion-uptake, along with genetic and experimental accessibility, makes zebrafish particularly useful for studies aimed at elucidating how the endocrine system governs effectors of ion transport in vertebrates.

Zebrafish possess at least three distinct ionocyte sub-types characterized by the expression of specific integral membrane ion transporters/exchangers. Cells expressing the Na^+/Cl^- cotransporter (SLC12A10.2; NCC-cells) play a key role in Cl^- ion uptake, while H^+-ATPase-rich (HR-cells) and Na^+–K^+-ATPase-rich (NaR-cells) cells function in the uptake of Na^+ and Ca^{2+}, respectively (Pan et al., 2005; Esaki et al., 2007; Wang et al., 2009). NCC expression in the apical membrane of teleost ionocytes was first reported

* Corresponding author. Tel.: +1 413 577 3448; fax: +1 413 545 3243.
E-mail address: karlstrom@bio.umass.edu (R.O. Karlstrom).

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in Mozambique tilapia (*Oreochromis mossambicus*) (Hiroi et al., 2008); Horng et al. (2009) subsequently demonstrated that NCC-expressing cells actively absorb Cl^-. As in tilapia, NCC is also expressed in a subset of ionocytes in the zebrafish gill and is essential for Cl^- balance (Wang et al., 2009). In HR cells, a Na^+/H^+ exchanger (NHE3b; SLC9A3.2) provides the apical pathway for Na^+ uptake from the external environment to the ionocyte interior where it is then transported into circulation (Yan et al., 2007). NaR cells specifically express an epithelial Ca^{2+} channel (ECaC; TRPV6) that facilitates the active uptake of Ca^{2+} from the external environment (Pan et al., 2005; Lin et al., 2011). The ion-absorptive functions of these three distinct zebrafish ionocytes have been demonstrated in zebrafish yolk integument, and all three genes are expressed in the gill (Liao et al., 2009). This characterization of ion transporters and cell types helps establish zebrafish as a new teleost model to assess the environmental and hormonal control of ion uptake capacities and mechanisms (Tseng et al., 2009; Chou et al., 2011; Lin et al., 2011).

While considerable progress has been made in establishing the cellular machinery supporting the functions of distinct ionocyte sub-types, the hormonal mechanisms that directly regulate ionocyte function, and thus ionoregulation by the gill, remain a mystery. PRL is a likely regulator of ionocytes based on the expression of teleost PRL receptors in gill tissue (Edery et al., 1984; Sandra et al., 1995; Weng et al., 1997; Santos et al., 2001; Lee et al., 2006), and the important role PRL plays in the osmoregulation of teleosts inhabiting freshwater environments. PRL can directly regulate gene expression in responding cells by binding to transmembrane receptors that activate the JAK/STAT signaling pathway (Bole-Feysot et al., 1998). There is evidence that the two zebrafish PRL receptors (denoted PRLRa and PRLRb) can regulate the transcription of distinct target genes upon PRL binding, at least *in vitro* (Chen et al., 2011). In Mozambique tilapia and black porgy (*Acanthopagrus schlegeli*), the expression of *prlr1* and *prlr2* transcripts (orthologous to zebrafish *prlra* and *prlrb*, respectively) in the gill is highly plastic, and the two *prlrs* are differentially influenced by osmotic and endocrine stimuli (Huang et al., 2007; Fiol et al., 2009; Breves et al., 2011). Modulation of *prlr* expression may provide a mechanism to regulate the sensitivity of target tissues to endocrine signaling. In fact, dynamic *prlr* expression in the gill appears to be an important aspect of adaptive responses to osmoregulatory challenges in euryhaline teleosts (Fiol et al., 2009; Breves et al., 2011; Flores and Shrimpton, 2012).

Here we show that PRL acts on ionocytes in the zebrafish gill by regulating the transcription of the ion cotransporter *ncc*, as well as the expression of *prlra*. The coordinated up-regulation of *prlra* and *ncc* in the gill upon transfer to ion-poor water, as well as following acute PRL treatment both *in vivo* and *in vitro*, suggests that PRL may be the key hormonal regulator of Cl^- uptake mechanisms in zebrafish gill.

2. Materials and methods

2.1. Animals and rearing conditions

Sexually mature zebrafish (*Danio rerio*) were selected from stocks maintained at the University of Massachusetts, Amherst Zebrafish Facility. Fish were maintained in a recirculating system of dechlorinated reverse-osmosis municipal water (6.9 mM Na^+, 6.6 mM Cl^-, 0.12 mM Ca^{2+}; pH 6.2–6.6) maintained at 26–27 °C. Fish were fed a flake diet supplemented with brine shrimp and maintained under a photoperiod of 14 h light: 10 h dark. The Institutional Animal Care and Use Committee of the University of Massachusetts approved the housing and maintenance of animals, and all experimental protocols.

2.2. Tissue distribution of PRL receptors

Tissues were collected from 10 adult zebrafish (5 males, 5 females, 1–2 g) maintained in standard rearing conditions for >1 year. Fish were lethally anesthetized with buffered tricaine methanesulfonate (MS-222; 250 mg/l), and the following tissues were collected: whole brain (olfactory bulb, telencephalon, optic tectum, cerebellum, diencephalon, and medulla), pituitary, gill, liver, body kidney, esophagus, anterior intestine and posterior intestine. Tissues were homogenized immediately in Trizol Reagent (Invitrogen, Carlsbad, CA) and stored at −80 °C until RNA isolation.

2.3. Transfer to ion-poor (ddH_2O) water

Seven days prior to experimentation, adult zebrafish (1–2 g) maintained in standard rearing conditions for >1 year were distributed into six static aquaria (9 L; 8–10 fish/tank) maintained with filtration and aeration. At the time of transfer (0 h), fish from two aquaria were quickly netted and transferred directly to two additional aquaria containing ion-poor water (Millipore ddH_2O; 0.2 mM Na^+, 0.1 mM Cl^-, 0.04 mM Ca^{2+}; pH 7.0–7.2) with filtration and aeration. Control fish were netted and then returned to the same aquaria to control for potential handling effects. Fish were fed twice daily during the initial 7-day acclimation and then fasted for the duration of the transfer experiment. Water temperature was maintained at 26–28 °C. At the time of sampling, fish (n = 8–10) from one system water- and one ion-poor water-containing aquaria were netted and anesthetized with a lethal dose of MS-222. Fish were sampled at 0, 2 and 7 days after transfer. Fish were rapidly decapitated and filaments from all branchial arches were homogenized immediately in Trizol Reagent and stored at −80 °C until RNA isolation. White muscle was sampled from the caudal musculature and the water content was measured gravimetrically after drying overnight at 90 °C.

2.4. In vivo effects of oPRL

Purified ovine PRL (oPRL; NIDDK-oPRL-21) was obtained from the National Hormone and Peptide Program and delivered in saline vehicle (0.9% NaCl; 20 μl/g body weight injection volume). Adult zebrafish (1–2 g) were administered oPRL (5 or 50 μg/g body weight) by two intraperitoneal (IP) injections. Fish were lightly anaesthetized with MS-222 and given the first injection. Twenty-four hours later, fish were netted, anaesthetized, and given a second injection. Fish were then returned to aquaria and left undisturbed for 24 h, after which time gill tissue was sampled as described above. The doses of oPRL were selected based on previous studies employing IP-injection in teleosts (Herndon et al., 1991; Eckert et al., 2001; Jackson et al., 2005; Breves et al., 2010).

2.5. Gill culture conditions and in vitro effects of oPRL and Δ1-9-G129R-hPRL

Gill filaments were isolated from adult zebrafish (1–2 g) and cultured according to McCormick and Bern (1989) with modifications. Fish were lethally anesthetized and branchial arches were removed and rinsed in pre-incubation Dulbecco's Modified Eagle Medium (DMEM; high glucose, HEPES, no phenol red; 311 mOsm; Invitrogen) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). Gill filaments from a single fish were severed from the arches at the septum and placed in a single well (24-well cell culture plate; Corning Inc., Corning, NY) containing pre-incubation medium for 3 h. Each sample/well was designated as an individual fish. After the pre-incubation period, medium