Switching of Na^+ , K^+ -ATPase isoforms by salinity and prolactin in the gill of a cichlid fish

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Abstract

We identified and investigated the changes in expression of two gill Na^+ , K^+ -ATPase α -subunit isoforms (α -1a and α -1b) in relationship with salinity acclimation in a cichlid fish, Mozambique tilapia. Transfer of freshwater (FW)-acclimated fish to seawater (SW) resulted in a marked reduction in α -1a expression within 24 h and a significant increase in α -1b expression with maximum levels attained 7 days after the transfer. In contrast, transfer of SW-acclimated fish to FW induced a marked increase in α -1a expression within 2 days, while α -1b expression decreased significantly after 14 days. Hypophysectomy resulted in a virtual shutdown of α -1a mRNA expression in both FW- and SW-acclimated fish, whereas no significant effect was observed in α -1b expression. Replacement therapy by ovine prolactin (oPrl) fully restored α -1a expression

in FW-acclimated fish, while cortisol had a modest, but significant, stimulatory effect on α -1a expression. In hypophysectomized fish in SW, replacement therapy with oPrl alone or in combination with cortisol resulted in a marked increase in α -1a mRNA to levels far exceeding those observed in sham-operated fish. Expression of α -1b mRNA was unaffected by hormone treatment either in FW-acclimated fish or in SW-acclimated fish. The mRNA expression of *fxyd-11*, a regulatory Na^+ , K^+ -ATPase subunit, was transiently enhanced during both FW and SW acclimation. In hypophysectomized fish in FW, oPrl and cortisol stimulated *fxyd-11* expression in a synergistic manner. The clear Prl dependence of gill α -1a expression may partially explain the importance of this hormone to hyperosmoregulation in this species.

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Introduction

The gill epithelium of euryhaline teleosts has an unusual functional flexibility inasmuch as it shifts between ion uptake in freshwater (FW) and ion excretion in seawater (SW). In both environments, mitochondrion-rich cells (MRCs) and adjacent accessory and pavement cells are fundamental to compensatory ion transport, for which the driving force is delivered by the activity of the Na^+ , K^+ -ATPase ion pump, possibly complemented by an apical V-type H^+ -ATPase in FW (Evans *et al.* 2005, Hwang & Lee 2007). The discovery of two Na^+ , K^+ -ATPase α -1 isoforms in the gill of salmonids, α -1a, expressed mainly in FW, and α -1b, expressed mainly in SW, provided important insight into transepithelial ion transport in the gill. In every salmonid species examined to date, salinity acclimation induces a reciprocal switch between α -1a and α -1b isoforms, α -1a being prominent in FW and α -1b in SW (Richards *et al.* 2003, Mackie *et al.* 2005, Bystrinsky *et al.* 2006, Madsen *et al.* 2009, McCormick *et al.* 2009), suggesting that α -1a is an FW isoform involved specifically in ion uptake, whereas α -1b is an SW isoform associated with ion excretion. Whether this phenomenon is

restricted to the early salmonid branch of the teleost clade or is a more general prerequisite for euryhalinity is unknown.

In fish, a suite of endocrine factors coordinate the physiological changes necessary to respond to changes in environmental salinity, and among these, the pituitary hormones prolactin (Prl) and GH along with cortisol are of critical importance (Sakamoto & McCormick 2006). The importance of Prl in osmoregulation was first demonstrated by the finding that hypophysectomized killifish require replacement therapy with ovine prolactin (oPrl) to survive in FW (Pickford & Phillips 1959). Prl treatment is associated with a marked reduction in extrarenal ion loss (Potts & Evans 1967). The effects of Prl on active ion uptake are somewhat unclear since both inhibition (e.g. killifish: Pickford *et al.* 1970; rainbow trout: Madsen & Bern 1992; tilapia: Sakamoto *et al.* 1997; seabream: Mancera *et al.* 2002) and the lack of an effect (tilapia: Herndon *et al.* 1991; brown trout: Madsen *et al.* 1995) on gill Na^+ , K^+ -ATPase activity have been reported.

Independent of its effect on somatic growth, GH has the ability to facilitate osmoregulation in SW. This ability is supported by increased gill Na^+ , K^+ -ATPase activity along with increased MRC size and quantity following GH

treatment, an effect observed in several species (e.g. brown trout: Madsen 1990; Atlantic salmon: McCormick 1996; tilapia: Sakamoto *et al.* 1997; killifish: Mancera & McCormick 1998). The corticosteroid, cortisol, is involved in both FW and SW osmoregulation (Sakamoto & McCormick 2006). The role of cortisol in SW acclimation has been documented in a variety of fish species, in which the steroid simultaneously stimulates gill Na^+ , K^+ -ATPase activity and SW tolerance (e.g. Sakamoto *et al.* 2001). In the hypophysectomized FW eel, cortisol treatment restores normal plasma osmolality (Chan *et al.* 1968), indicating a role for the steroid in FW osmoregulation. Moreover, following hypophysectomy, catfish require both Prl and cortisol to osmoregulate in FW (Fortner & Pickford 1982, Eckert *et al.* 2001).

This study investigated the presence of gill Na^+ , K^+ -ATPase isoforms in a euryhaline cichlid, the Mozambique tilapia which belongs to the more recently radiated group of perciform fish. Key regulators of Na^+ , K^+ -ATPase activity include regulatory subunits, termed Fxyd proteins (Garty & Karlish 2006), that were recently described in Atlantic salmon (Tipismark 2008, Tipismark *et al.* 2010b) and green pufferfish (Wang *et al.* 2008). We identified Na^+ , K^+ -ATPase catalytic (α subunits) and regulatory (Fxyd proteins) isoforms in the expressed sequence tag (EST) database for tilapia using the NCBI web resource. We first examined changes in these isoforms following the transfer from FW to SW and vice versa. Then, hypophysectomy and replacement therapy trials were conducted to assess the relative contribution of environmental salinity and endocrine signaling to the expression of Na^+ , K^+ -ATPase and Fxyd isoforms.

Materials and Methods

Fish

Male tilapia (*Oreochromis mossambicus*) were selected from an FW population maintained at the Hawaii Institute of Marine Biology, University of Hawaii. Fish were maintained outdoors with a continuous flow of FW (tap water: 0.98 mM Na^+ , 0.25 mM Ca^{2+} , 0.01 mM Mg^{2+} , and 0.04 mM K^+) under natural photoperiod and fed a commercial diet (Silver Cup Trout Chow, Nelson & Sons, Murray, UT, USA) at ~5% of their body weight per day. Some fish were acclimated to SW (Kaneohe Bay, Hawaii; 34 ppt) for 4 weeks before the start of the FW transfer experiment. In all experiments, water temperature was maintained at 24–26 °C. The Institutional Animal Care and Use Committee of the University of Hawaii approved all surgical and experimental protocols.

Experiments

Experiment 1: SW challenge Fish (160–170 g) were held in 700 l tanks, and salinity was changed from FW to 70% SW (23 ppt) by opening a SW valve and adjusting the flow of incoming FW; 70% SW was reached after about 15 min.

After 24 h, the FW valve was turned off to gradually raise the salinity to full-strength SW. This mode of SW acclimation was adopted to reduce handling stress and allow adaptive processes to be activated (Breves *et al.* 2010a). Control groups were maintained in FW for the entire experiment. Fish were fasted for the duration of the experiment. Fish were sampled ($n=8$) at 0, 1, 7, and 14 d after transition to SW. At the time of sampling, fish were netted and anesthetized with a lethal dose of 2-phenoxyethanol (2 ml/l). They were rapidly decapitated, and 15–20 filaments from the first gill arch were frozen in liquid nitrogen and stored at -80°C until RNA isolation and analyses of gene expression.

Experiment 2: FW challenge Fish (75–180 g) were held in 700 l tanks and acclimated to SW under natural photoperiod for more than 4 weeks. At the start of the experiment, tank water was changed to FW with minimal disturbance by switching inflow from an SW valve to an FW valve as described previously (Breves *et al.* 2011). FW conditions were reached after 60 min. Control groups were maintained continuously in SW. Fish were fasted for the duration of the experiment. Fish were sampled at 0, 0.25, 1, 2, and 14 d after transition to FW ($n=8$).

Experiment 3: hypophysectomy and FW or SW transfer

Hypophysectomy and sham operation of FW-acclimated fish were performed by the transorbital technique (Nishioka 1994). Following the operation, hypophysectomized, sham-operated, and intact fish were transferred to recirculating aquaria containing brackish water (12 ppt). Following a postoperative recovery period in brackish water for 5–7 days, hypophysectomized, sham-operated, and intact fish ($n=6-8$) were exposed to FW or SW for 3 days by adding FW or SW directly to the recirculating aquaria. Tank water reached FW conditions within 90 min. Fish were transferred to SW by gradually adding SW to the aquaria to raise the salinity to 23 ppt after 90 min. After 24 h, SW was added again to raise the salinity to full-strength SW. Completeness of hypophysectomy was confirmed by postmortem inspection of the hypothalamic region.

Experiment 4: hypophysectomy and replacement therapy in FW or SW

Hypophysectomized fish, held in brackish water for 5–7 days ($n=6-8$), were injected i.p. with oPrl (5 µg/g body weight; Sigma), oGH (5 µg/g; National Hormone and Peptide Program, Torrance, CA, USA) or cortisol (1 µg/g; Sigma), either alone or in combination, and were exposed to FW or SW as in Experiment 3. Hormones were delivered in saline vehicle (0.9% NaCl; 1.0 µl/g body weight). The first injection was administered just prior to transfer to either FW or SW. After 24 h, fish were given a second injection. Fish were then left undisturbed for 48 h, after which time they were sampled as described previously. Sham-operated fish were injected with saline vehicle only.

Analytical techniques

Extraction of RNA and cDNA synthesis Total RNA was extracted by the TRI Reagent procedure (MRC, Cincinnati, OH, USA) according to the manufacturer's recommendation. Total RNA concentrations were determined by measuring A_{260} in duplicate with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA was synthesized by reverse transcription carried out on 0.6 μg total RNA using qScript cDNA SuperMix (Quanta, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Sequences Non-redundant sequences of β -actin (*actb* Acc. No. AB037865), acidic ribosomal phosphoprotein p0 (*rplp0*; Acc. No. EF452495), and Na^+ , K^+ -ATPase α -1b (*atp1a1b*; Acc. No. TMU82549) were used. Na^+ , K^+ -ATPase α -1b has previously been characterized and annotated as an α -1 isoform in tilapia (Hwang *et al.* 1998). However, since it is similar to α -1b of the salmonid isoform, having all the specific conserved amino acid sequence (Jorgensen 2008), we used this nomenclature. Na^+ , K^+ -ATPase α -1a sequences (*atp1a1a*; Acc. No. GR645170 and GR644771) were identified in the Nile tilapia (*Oreochromis niloticus*) transcriptome (Lee *et al.* 2010) based on a specific amino acid substitution in the fifth transmembrane domain (lysine instead of asparagine) in the cation binding site. This substitution is observed in previously examined α -1a isoforms and distinguishes them from the α -1b isoforms (Jorgensen 2008). *fxyd-9* (Acc. No. GR611225, GR682782, GR684667, GR652932, GR654431, GR646538, GR629763, GR622096, GR618400, GR623531, GR614978, GR605558, GR604084, and GR644771) and *fxyd-11* (Acc. No. GR648095, GR628272, and GR624008) were both identified in the *O. niloticus* transcriptome by searching with tBLASTn at the NCBI web resource using the relevant sequences known from Atlantic salmon (Tipsmark 2008; *fxyd-9*: NP001117201; *fxyd-11*: NP001117198).

Primers Primers were designed using Primer3 software (Rozen & Skaletsky 2000) and checked using NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA). All primers were purchased from Applied Biosystems (Foster City, CA, USA). Primer sequences were tested for non-specific product amplification and primer-dimer formation by melting curve analysis; amplicon size was verified by agarose gel electrophoresis. Elongation factor 1a (*ef-1a*) primers have previously been reported (Breves *et al.* 2010b). Additional primers used were (5'-3'): β -actin (forward, CTCTTCCAGCCTTCCTTCCT; reverse, ACAGGTCCTTACG-GATGTCG; amplicon size = 100 nt), acidic ribosomal phosphoprotein p0 (*rplp0*; forward, CCCCTGTGATGT-GACAGTG; reverse, GTGATACCCAGAGCCTGGAA; amplicon size = 82 nt), Na^+ , K^+ -ATPase α -1a (forward, AACTGATTTGGTCCCTGCAA; reverse, ATGCATTTCTGGGCTGTCTC; amplicon size = 80 nt), Na^+ , K^+ -ATPase

α -1b (forward, GGAGCGTGTGCTTCATCACT; reverse, ATCCATGCTTTGTGGGGTTA; amplicon size = 90 nt), *fxyd-9* (forward, TCTTCGTCATTTGCTTGTCG; reverse, TGTTCTTCTGCGAACACCAG; amplicon size = 97 nt), and *fxyd-11* (forward, TGCTGTTCAGCCTTTTTGTG; reverse, GACCAGTCCTCCAATCCGTA; amplicon size = 80 nt).

Quantitative PCR Quantitative PCR (QPCR) assays were run on a StepOnePlus real-time PCR system (Applied Biosystems). Reactions were carried out with a cDNA amount equivalent to 15 ng total RNA, 200 nM forward and reverse primers, using Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 15 μL . Cycling conditions were 95 °C for 15 s and 60 °C for 60 s in 40 cycles. The geNorm (version 3.5) tool was applied to determine a normalization factor from the examined reference genes (β -actin, *ef-1a* and *rplp0*) and used to normalize the expression for the target genes (Vandesompele *et al.* 2002). geNorm establishes the individual stability of genes within a cluster and computes a geometric mean as a normalizing factor. Relative copy numbers of the target genes were calculated as $E_a^{-C_t}$, where C_t is the threshold cycle number and E_a is the amplification efficiency. Normalized units were obtained by dividing relative copy number of target genes with the determined normalization factor.

Statistical analysis

The salinity transfer experiments were analyzed by a two-factorial ANOVA followed by the Bonferroni-adjusted Fisher's LSD test. The tissue distribution and hypophysectomy experiments were analyzed with one-way ANOVA, and differences between group means were assessed by Tukey's HSD test. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances. All statistical analyses were performed using GraphPad Prism 4.0 software (San Diego, CA, USA), and significant differences were accepted when $P < 0.05$.

Results

Tissue distribution

The tissue distribution of Na^+ , K^+ -ATPase catalytic α -1a and α -1b isoforms and the putative regulatory subunit isoforms, *fxyd-9* and *fxyd-11*, was examined in FW-acclimated tilapia. The α -1a isoform was expressed in 2–4 orders of magnitude higher in the gill than in the other examined tissues (Fig. 1A). The levels of α -1b isoform were highest in the intestine and kidney (Fig. 1B). *fxyd-9* was expressed at similar levels in most tissues, with higher levels found in the brain and stomach (Fig. 1C). *fxyd-11* exhibited 3–4 orders of magnitude higher expression in the gill than in the other tissues (Fig. 1D).

Figure 1 Expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D) mRNA in the brain, gill, skin, esophagus, stomach, anterior intestine (ant. int.), middle intestine (mid. int.), posterior intestine (post. int.), kidney, skeletal muscle (muscle), liver, and urinary bladder (urin. bladder) in FW-acclimated tilapia. ND, no detection. Groups with no shared letters are statistically different. Means $\pm$ S.E.M. ($n=4-5$).

Transfer from FW to SW

When FW-acclimated tilapia were exposed to SW, α -1a levels rapidly declined after 1 day and remained low during the remainder of the experiment (Fig. 2A). Transfer to SW produced a doubling of the α -1b expression by day 1 and a tenfold rise after a week (Fig. 2B). *fxyd-9* expression doubled after 1 day in SW, but was not significantly different from FW controls at days 7 and 14 (Fig. 2C). *fxyd-11* was elevated threefold after 1 and 7 days, but no significant difference was observed at day 14 (Fig. 2D).

Transfer from SW to FW

When SW-acclimated tilapia were exposed to FW, α -1a mRNA increased by tenfold after 1 day and by 60-fold after

2 days, and remained high during the remainder of the experiment (Fig. 3A). There was no significant change in α -1b expression during the first 2 days; a significant reduction was observed only after 14 days (Fig. 3B). A significant increase in *fxyd-9* expression was observed in FW-challenged fish at day 14, while *fxyd-11* expression was elevated after 2 days (Fig. 3C and D).

Hypophysectomy and FW or SW transfer

In both FW- and SW-exposed fish, gill α -1a mRNA expression was markedly reduced in hypophysectomized fish compared with levels observed in intact and sham-operated controls (Fig. 4A). The expression of α -1b, *fxyd-9* and *fxyd-11* was not significantly altered by hypophysectomy in FW- or SW-acclimated fish (Fig. 4B-D).

Hypophysectomy and replacement therapy

In saline-injected, hypophysectomized tilapia, transfer from brackish water to FW for 3 days (controls) produced a virtual shutdown of gill α -1a mRNA expression compared with levels in sham-operated fish (Fig. 5A). Replacement therapy by oPrl alone or in combination with cortisol restored this drop-off in expression. Cortisol had a slight but significant stimulatory effect on α -1a expression. Gill α -1b and *fxyd-9* mRNA were unaffected by hypophysectomy and hormone treatment (Fig. 5B and C). On the other hand, pituitary removal depressed *fxyd-11* expression significantly. Both oPrl and cortisol restored *fxyd-11* expression in hypophysectomized fish (Fig. 5D). The effects of oPrl and cortisol on *fxyd-11* were synergistic and were not observed for α -1a.

Hypophysectomized tilapia transferred to SW also experienced an inhibitory effect of pituitary removal on gill α -1a

Figure 2 Effects of transfer from FW to SW on branchial mRNA expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D). FW-acclimated tilapia were transferred to SW (filled circle), while control fish were maintained in FW (open circle). Asterisks indicate significant effects compared with controls ($***P<0.001$). Values are means $\pm$ S.E.M. ($n=7-8$).

Figure 3 Effects of transfer from SW to FW on branchial mRNA expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D). SW-acclimated tilapia were transferred to FW (filled circle), while control fish were maintained in SW (open circle). Asterisks indicate significant effects compared with controls (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Values are means $\pm$ S.E.M. ($n = 7-8$).

mRNA expression. As in the FW-exposed fish, treatment with oPrl alone or in combination with cortisol induced significant increases in α -1a expression to levels significantly higher than those in the sham-operated fish (Fig. 6A). No significant change was observed in α -1b and *fxyd-9* expression following hormone injections (Fig. 6B and C). *fxyd-11* expression patterns were variable following hormone injections. Compared with sham-operated fish, hypophysectomized fish treated with either oPrl or oGH showed significantly higher expression of *fxyd-11*, although the levels were not significantly different from saline-injected hypophysectomized controls (Fig. 6D). Treatment with cortisol alone or in combination with oPrl or oGH had no significant effect compared with levels in sham-operated or saline-injected, hypophysectomized fish.

Discussion

This study identified two branchial Na^+ , K^+ -ATPase α isoforms in a cichlid fish and verified a strong relationship with environmental salinity in their expression, suggesting that α -1a is exclusively associated with branchial ion uptake in FW, while α -1b is involved in ion extrusion in SW. Hypophysectomy and hormone replacement studies showed that mRNA expression of the FW isoform (α -1a) is dependent on pituitary Prl. Conversely, the mRNA expression of the SW isoform (α -1b) showed no dependence on pituitary hormones. Concerning the regulatory *Fxyd* proteins, *fxyd-9* was not impacted by hormonal factors, while *fxyd-11* expression was stimulated by both oPrl and cortisol. The transient stimulation of *fxyd-11* during both FW and SW

acclimation suggests a key role for this regulatory subunit in net ion transport transitions in gill.

Recently, a series of studies has elucidated the sub-cellular components of FW-type MRCs in Mozambique tilapia (Hiroi *et al.* 2008, Inokuchi *et al.* 2008, Watanabe *et al.* 2008). These studies described the expression of either a sodium proton exchanger type 3 (*nhe3*) or a sodium chloride cotransporter (*ncc*) in two distinct FW-type MRCs. In accordance with their activity to support Cl^- uptake in hypoosmotic environments (Horng *et al.* 2009), Ncc-expressing MRCs are stimulated by oPrl injection (Breves *et al.* 2010b). The drop in Na^+ , K^+ -ATPase α -1a mRNA during SW acclimation observed in this study correlates well with decreased *ncc* and *nhe3* mRNA expression reported for larval and adult tilapia transferred to SW (Hiroi *et al.* 2008, Breves *et al.* 2010a). Our results showed that Na^+ , K^+ -ATPase α -1a expression is stimulated by both FW and Prl. Accordingly, future efforts should employ *in situ* hybridization and immunohistochemical methods to fully describe the gene and protein expression patterns of Na^+ , K^+ -ATPase α -subunits in the four distinct MRC subtypes described in tilapia by Hiroi *et al.* (2008).

The tissue distributions of Na^+ , K^+ -ATPase α subunits established in this study are largely in accordance with those reported in salmonids. In tilapia, the α -1a isoform appears to be gill specific, as in rainbow trout (Richards *et al.* 2003) and Atlantic salmon (Tipismark *et al.* 2010a). The tilapia α -1b isoform was highly expressed in the intestinal sections of FW specimens, with comparatively lower expression in the gill. While keeping in mind that branchial expression of α -1b in SW is more than tenfold higher than that in FW, this still contrasts somewhat with findings in salmonids. In rainbow trout and Atlantic salmon, the α -1b isoform is not expressed

Figure 4 Effects of hypophysectomy (Hx) on branchial mRNA expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D). At 5 days after the operation, sham-operated (lightly gray bars), hypophysectomized (dark gray bars), and intact (open bars) tilapia were transferred from brackish water (12 ppt) to FW or SW and sampled after 3 days. Within a given ambient salinity, groups with different letters are significantly different ($P < 0.05$). Values are means $\pm$ S.E.M. ($n = 6-8$).

Figure 5 Effects of hypophysectomy (Hx) and hormone replacement therapy with oPrI, oGH and cortisol on branchial mRNA expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D) in tilapia transferred to FW. Fish received two i.p. injections of saline (Sham and Hx only), oPrI (5 $\mu\text{g/g}$), or oGH (5 $\mu\text{g/g}$) alone or in combination with cortisol (1 $\mu\text{g/g}$). The first injection occurred immediately prior to transfer from brackish water (12 ppt) to FW, and the second occurred after 24 h. Fish were sampled 3 days after the initial transfer to FW. Groups with different letters are significantly different ($P < 0.05$). Values are means $\pm$ S.E.M. ($n = 6-8$).

substantially in the intestine (Richards *et al.* 2003, Tipsmark *et al.* 2010a). The distribution of Fxyd proteins is in accordance with previous studies. Thus, *fxyd-9* is expressed in a wide variety of tissues in tilapia (this study), salmon (Tipsmark 2008) and pufferfish (Wang *et al.* 2008). On the other hand, *fxyd-11* is robustly expressed in the tilapia gill, as observed in Atlantic salmon (Tipsmark 2008) and zebrafish (Saito *et al.* 2010). This would suggest that *fxyd-11* is expressed in a gill-specific cell type, such as the MRC. It has been shown that Fxyd-11 is co-localized with the Na^+ , K^+ -ATPase α subunits in zebrafish (Saito *et al.* 2010) and in both FW and SW salmon (Tipsmark *et al.* 2010b). This does not preclude involvement of the more generally expressed *fxyd-9* in the control of MRCs, at least in some species, since this isoform co-localized with Na^+ , K^+ -ATPase in pufferfish gill (Wang *et al.* 2008). It is to be noted that the exact functions of the Fxyd proteins, e.g. involvement in endocrine-paracrine regulation or a general modulation of kinetics, are still unknown. Future work should assess whether one or more Fxyd isoforms are co-localized with Na^+ , K^+ -ATPase α subunits in tilapia gill.

The changes in α -1a and α -1b isoforms observed in tilapia after FW and SW transition in this study largely mirror previous reports in rainbow trout (Richards *et al.* 2003), Arctic char (Bystrinsky *et al.* 2006) and Atlantic salmon (Madsen *et al.* 2009, McCormick *et al.* 2009). The marked elevation in α -1a expression after FW transition (60-fold) and α -1b expression after SW transition (tenfold) observed in this

study is of a greater magnitude than that reported in the salmonid fishes described earlier. The increase in α -1b mRNA after SW transition is similar to the previous reports by Hwang *et al.* (1998) and Lin *et al.* (2004) in tilapia, regarding the gill α -1. They classified their isoform as α -1; however, our sequence analyses indicate that it is a homolog to the salmonid α -1b. As described earlier in the Materials and Methods section, the classification of tilapia α -1a and α -1b is based on the criteria outlined by Jorgensen (2008), an interpretation that is also clearly supported by the regulatory patterns observed in this study. While the functional significance of isoform switching in teleost gill is an open question, Jorgensen (2008) suggested the α -1a subunit may operate with different Na^+ stoichiometry from the α -1b subunit, thereby making ion extrusion in FW thermodynamically favorable.

An interesting pattern observed in this study is that gene expression for α -1a, but not for α -1b, is under pituitary control in tilapia. The lack of effect of hypophysectomy on the SW-type Na^+ , K^+ -ATPase isoform (α -1b) may at first seem puzzling. Nonetheless, this pattern is in accordance with our previous finding that a Na^+ , K^+ , 2Cl^- cotransporter (NKCC), a hallmark of SW-type MRCs, is insensitive to hypophysectomy (Breves *et al.* 2010b). It has been shown that branchial Na^+ , K^+ -ATPase activity in Mozambique tilapia is stimulated by oGH and diminished by oPrI treatment (Sakamoto *et al.* 1997). It seems likely that these effects were mediated by the SW-type Na^+ , K^+ -ATPase (α -1b),

Figure 6 Effects of hypophysectomy (Hx) and replacement therapy with oPrI, oGH, and cortisol on branchial mRNA expression of Na^+ , K^+ -ATPase α -1a subunit (A), α -1b subunit (B), *fxyd-9* (C), and *fxyd-11* (D) in tilapia transferred to SW. Fish received two i.p. injections of saline (Sham and Hx only), oPrI (5 $\mu\text{g/g}$), or oGH (5 $\mu\text{g/g}$) alone or in combination with cortisol (1 $\mu\text{g/g}$). The first injection occurred immediately prior to transfer from brackish water (12 ppt) to SW, and the second occurred after 24 h. Fish were sampled 3 days after the initial transfer to SW. Groups with different letters are significantly different ($P < 0.05$). Values are means $\pm$ S.E.M. ($n = 6-8$).

probably accompanied by Fxyd-11, since oGH or oPrl were injected into hypophysectomized fish kept in a hyperosmotic environment (25 ppt). The results by Sakamoto *et al.* (1997) are in agreement with findings in Atlantic salmon, showing a synergistic effect of cortisol and homologous GH on branchial SW-type Na^+ , K^+ -ATPase (α -1b) gene expression (Tipsmark & Madsen 2009). Since we used a single dose of hormones and one time point for sampling, we cannot rule out that we may have missed actions of GH on factors that support hypoosmoregulatory strategies. Another explanation worth investigating is that *O. mossambicus* is a marine species, and that FW adaptability is a derived characteristic. Under this scenario, it would not be surprising that α -1b might be expressed constitutively upon exposure to a hyperosmotic environment.

On the other hand, FW-type MRCs that express Ncc show a complete dependence on pituitary Prl (Breves *et al.* 2010b). This may underlie, at least in part, the inhibitory effect of Prl on SW acclimation (Pisam *et al.* 1993), since oPrl stimulates plasma Na^+ through the recruitment of FW-type MRCs (Breves *et al.* 2010b). This interpretation is in agreement with the present findings that oPrl treatment in hypophysectomized tilapia in SW induced a higher level of α -1a expression, even above the level of sham-operated fish (see Fig. 6A). On the other hand, in SW-acclimated Atlantic salmon, treatment with salmon Prl inhibited SW-type MRC abundance as shown by an overall reduction in gill α -1b and *nkcc1a* mRNA levels (Tipsmark & Madsen 2009).

In summary, this study provides the first evidence for a switch in the catalytic isoform of the branchial Na^+ , K^+ -ATPase during acclimation to FW- and SW-environments in a non-salmonid teleost. Moreover, this study demonstrates complete dependence on pituitary-derived Prl for expression of the Na^+ , K^+ -ATPase α -1a (FW-type) catalytic subunit. Our data suggest that Fxyd-11 may be an essential component of both FW- and SW-type MRCs, and that Prl is involved in regulating the gene in FW. When the patterns that we observed in tilapia are considered with previous reports in salmonids regarding isoform switching, we propose that Na^+ , K^+ -ATPase α -1 isoform switching is an essential and general prerequisite in euryhaline fish for adaptation to changes in salinity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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