***In vivo* and *in vitro* effects of high-K⁺ stress on branchial expression of ROMKα in seawater-acclimated Mozambique tilapia**

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Abstract

Recently, a teleost ortholog of renal outer medullary K⁺ channel (ROMK) expressed in gill ionocytes (ROMKa) has emerged as a primary K⁺-excreting pathway in fish. However, the mechanisms by which ROMKa expression is regulated in response to perturbations of plasma K⁺ levels are unknown. In this study, we aimed to identify potential links between the endocrine system and K⁺ regulation in a euryhaline fish. We assessed time-course changes in multiple endocrine parameters, including plasma cortisol and gene expression of branchial glucocorticoid and mineralocorticoid receptors (GR1, GR2, and MR) and pituitary hormones, in seawater (SW)-acclimated Mozambique tilapia (*Oreochromis mossambicus*) exposed to high-K⁺ (H-K) SW. Exposure to H-K SW elicited little effects on plasma cortisol or mRNA levels of GRs and pituitary hormones. Since plasma K⁺ and branchial ROMKa expression was increased within 6 h after H-K treatment *in vivo*, the effect of high K⁺ was subsequently tested in a gill filament incubation experiment using media with differing K⁺ concentrations. ROMKa mRNA levels were induced following incubation of filaments in H-K medium for 6 h. The present study is the first to demonstrate that the expression of ROMKa in teleost ionocytes can respond to high K⁺ conditions independent from systemic signaling.

Key Words:

potassium

ionocyte

ROMK

cortisol

pituitary

Oreochromis mossambicus

1. Introduction

Regulation of plasma K⁺ levels is crucial to all vertebrate animals. Plasma K⁺ regulatory mechanisms are extensively understood in mammals, where the kidney plays a key role in K⁺ homeostasis by modulating rates of K⁺ excretion in urine (Wang and Giebisch, 2009). In the kidney, renal outer medullary K⁺ channel (ROMK) is expressed in apical membrane of tubule cells from the thick ascending limb (TAL) through the outer medullary collecting duct (OMCD), and its expression mainly affects K⁺ excretion (Hebert et al., 2005). Historically, the mechanisms by which teleosts

regulate plasma K^+ have been largely unknown; however, we recently found that ROMK α expressed in gill ionocytes is the primary pathway for K^+ regulation in tilapia (Furukawa et al., 2012, 2014). Thus, in both mammals and teleosts it now appears that ROMK mediates K^+ homeostasis (Wang and Giebisch, 2009; Furukawa et al., 2012, 2014). Nonetheless, the question of how ROMK expression is regulated in teleosts remains to be addressed. In mammals, aldosterone, a mineralocorticoid hormone synthesized in the adrenal cortex, is the major regulator of ROMK by stimulating mRNA expression and cell surface expression of mature protein (Beesley et al., 1998; Yoo et al., 2003). Teleost fishes lack aldosterone, and the major corticosteroid is cortisol, a glucocorticoid produced by interrenal tissue located in head kidney (Bury and Sturm, 2007; Arterbery et al., 2011). Although 11-deoxycorticosterone (DOC), a recently proposed mineralocorticoid in teleosts, has been identified in some salmonid fish, its physiological significance is questioned based upon its extremely low plasma concentration (McCormick et al., 2008). Although fishes do not have a distinct mineralocorticoid, most species have two homologs of mammalian glucocorticoid receptors (GRs), GR1 and GR2, and one homolog of the mammalian mineralocorticoid receptor (MR). All of these receptors can bind cortisol and mediate downstream actions (Bury and Sturm, 2007; Arterbery et al., 2011).

In teleosts, although K^+ -regulatory hormones have not been reported, various studies have focused on endocrine control of body fluid regulation (Takei et al., 2014). Cortisol in teleosts is seemingly a multifunctional endocrine factor thought to be involved in various physiological events including osmoregulation. One of the proposed osmoregulatory roles of cortisol is as a modulator of salt secretion by the gills, acting in concert with the growth hormone (GH)/insulin-like growth-factor axis, although the degree of these interactions may vary across species (for review see Mancera and McCormick, 1998; McCormick and Bradshaw, 2006). Among the pituitary hormones, GH is known as a hyposmoregulatory hormone in teleost fishes (see the review by Sakamoto and McCormick, 2006), whereas prolactin (PRL) is a potent hyperosmoregulatory factor (reviewed by Breves et al., 2014). The physiological role(s) of somatolactin (SL) are still not clear (Kaneko, 1996; Fukamachi et al., 2005; Fukamachi and Meyer, 2007), but several studies have implied a possible link with acid-base regulation (Kakizawa et al., 1996; Kakizawa et al., 1997; Furukawa et al., 2010). Proopiomelanocortin (POMC) is the precursor of adrenocorticotrophic hormone (ACTH), the primary regulator of cortisol release from interrenal tissue (Mommsen et al., 1999).

In the present study, we first sought to identify possible K^+ regulatory hormones

in a model teleost. We exposed seawater (SW)-acclimated tilapia to a high- K^+ (H-K) environment and measured changes in plasma cortisol concentrations along with mRNA levels of its putative receptors in the gill. In addition, we assessed changes in mRNA levels of GH, PRL, SL, and POMC in the pituitary. Subsequently, we assessed the effects of high K^+ stress independent of systemic endocrine factors by incubating separated gill filaments in media with moderate or high K^+ concentrations. As a consequence of these *in vivo* and *in vitro* studies, we propose that ROMKa in gill ionocytes may be regulated in response to extracellular K^+ concentration.

2. Materials and Methods

2.1 Experimental animals

cDNA cloning and gill incubation experiments were conducted at the University of Tokyo, Japan. Mozambique tilapia (*Oreochromis mossambicus*) were maintained in tanks supplied with re-circulating SW (in mM: 470 Na^+ , 541 Cl^- , 7.95 K^+ , 9.90 Ca^{2+} , 52.3 Mg^{2+} , pH=7.7) at 25°C. The fish were fed a commercial carp chow (Nihon Haigou Shiryo, Kanagawa, Japan) once a day. The fish (100-250 g) were anesthetized with 0.1% 2-phenoxyethanol before blood sampling or removal of tissues. Experiments were conducted according to principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

We conducted an H-K SW exposure experiment at the Hawaii Institute of Marine Biology (HIMB), University of Hawaii. Tilapia were reared in 700-L outdoor flow-through tanks containing SW from Kaneohe Bay (in mM: 496 Na^+ , 529 Cl^- , 7.31 K^+ , 10.5 Ca^{2+} , 53.9 Mg^{2+} , pH=7.8) at 25-28°C. Fish were fed daily with Silver Cup Trout Chow (Nelson and Sons, Murray, UT, USA). These experiments were conducted according to principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

2.2 cDNA cloning of GR1, GR2 and MR

Gill filaments were removed from tilapia, and total RNA was extracted from the filaments with Isogen (Nippon Gene, Tokyo, Japan). After digestion of genomic DNA with DNase I (Life Technologies, Carlsbad, CA, USA), first-strand cDNA was synthesized from the total RNA with SuperScript III (Life Technologies). The cDNA fragments of GR1, GR2 and MR were amplified by degenerate PCR with a cDNA template obtained from tilapia gills. We designed degenerate primers corresponding to the conserved amino acid residues of known GRs and MRs (Table 1). The PCR

products were ligated into pGEM T-Easy vector (Promega, Madison, WI, USA) and sequenced using an ABI Prism 310 sequencer (Life Technologies). 5'- and 3'-RACE was conducted using CapFishingTM kit (Seegene, Seoul, Korea).

2.3 Exposure of SW tilapia to H-K SW

SW-acclimated tilapia (10-40 g) were kept in 5-L glass tanks filled with SW, each tank contained 8 fish. The fish were fasted 2 days prior to the experiment, and then concentrated KCl solution was added to the H-K treatment tanks. The ion concentrations and pH of the two experimental tanks were as follows: control SW (in mM), 483 Na⁺, 7.47 K⁺, 545 Cl⁻, 10.7 Ca²⁺, 52.7 Mg²⁺, pH=7.8; H-K SW (in mM), 483 Na⁺, 17 K⁺, 551 Cl⁻, 10.8 Ca²⁺, 52.7 Mg²⁺, pH=7.7. Sampling was conducted immediately before (0 h) and at 6, 12, 24, and 72 h after the addition of KCl. To avoid possible effects on plasma cortisol associated with the repeated sampling of single tanks, a tank was prepared for every sampling group/time point (9 tanks), and sampling was initiated by adding 2-phenoxyethanol directly to the tanks to reach final concentration of 0.1%. After anesthetizing the fish, blood was collected with a heparinized syringe and needle, and plasma was obtained after centrifugation at 5000 g for 10 min. Plasma levels of K⁺ and Na⁺ were determined with an ion analyzer (IA-200, TOA-DKK, Tokyo, Japan), and Cl⁻ with a chloridometer (Labconco, Kansas, MO, USA). The gills were removed and total RNA was extracted with Tri Reagent RT (Molecular Research Center, Cincinnati, OH, USA). Pituitaries were removed and stored in RNAlater RNA Stabilization Reagent (Life Technologies), and total RNA was extracted with Isogen (Nippon Gene). After digestion of genomic DNA with DNase I (Life Technologies), first-strand cDNA was synthesized with ReverTra Ace - α - reverse transcription kit (Toyobo, Osaka, Japan).

2.4 Radioimmunoassay (RIA)

Plasma cortisol levels were measured by RIA according to Witt et al. (2009). Plasma samples (5 μ l) were diluted with 95 μ l assay buffer (0.01 M PBS, 1% BSA, pH 7.3), and mixed with 150 μ l ³H-labelled cortisol (9,000 cpm) and anti-cortisol antibody (Fitzgerald Industries, North Acton, MA, USA) at 1:5000 dilution. The sample mixtures were incubated at room temperature for 2 h. To remove unbound cortisol, 400 μ l dextran-coated charcoal (Sigma-Aldrich, St. Louis, MO) was added to samples prior to incubation on ice for 15 min. Finally, the sample tubes were centrifuged at 3000 rpm for 20 min, and 450 μ l of supernatant was transferred into 4 ml of scintillation fluid (Scinti Safe Econo 1, Fisher Scientific, Pittsburgh, PA, USA) and counted by a

scintillation counter (1600CA Tri-Carb Liquid Scintillation Analyzer; Packard, Prospect, CT, USA). Plasma cortisol concentrations were calculated with reference to a standard curve with known concentrations of cortisol.

2.5 *qPCR*

In the H-K SW exposure experiment, mRNAs of GR1 (Genbank accession no., [AB771724](#)), GR2 ([AB771725](#)), MR ([AB771726](#)) in the gills, and GH ([AF033806](#)), PRL (PRL₁₈₈; [X92380](#)), SL ([AB442015](#)), and POMC ([AF116240](#)) in the pituitaries were quantified. The functional markers of gill ionocytes in SW-acclimated tilapia (Hiroi et al., 2008; Tipsmark et al., 2011; Furukawa et al., 2012), ROMKa ([AB771746](#)), Na⁺/K⁺/Cl⁻ cotransporter 1a (NKCC1a; [AY513737](#)), cystic fibrosis transmembrane conductance regulator (CFTR; [AB601825](#)), and Na⁺/K⁺-ATPase α 1b (NKA α 1b; [U82549](#)) were also assessed in mRNA level. In the gill incubation experiment using filaments collected from SW-acclimated fish, we measured mRNA levels of ROMKa, NKCC1a, CFTR, and NKA α 1b. Primer sequences are provided in Table 1. The copy numbers of target transcripts were calculated with reference to parallel amplifications of known concentrations of the respective cloned PCR fragments, and the specificity of each PCR was confirmed by melting curve analysis. The data were normalized to 18S rRNA levels in each sample.

2.6 *Incubation of isolated gill filaments*

To investigate the effects of H-K stress on branchial expression of ROMKa, we incubated isolated gill filaments in media containing moderate or high K⁺ (H-K) concentrations, according to a method established in our laboratory (Watanabe et al., submitted). Briefly, the gills were removed from SW-acclimated tilapia and washed in 0.025% KMnO₄ in balanced salt solution (BSS, in mM; 140 Na⁺, 146 Cl⁻, 2.95 K⁺, 1.5 Ca²⁺, 1.29 Mg²⁺, 5 HEPES; pH 7.5) for 1 min. After washing in BSS, gill filaments were separated from the gill arch, and cut with a fine scalpel into afferent and efferent halves. The preincubation filaments were sampled, and the rest of the filaments were incubated in 1 ml of modified L-15 (Life Technologies), containing 10% charcoal/dextran-treated fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA), 5 μ g cortisol (Sigma-Aldrich), and 1 $\times$ antibiotic-antimycotic solution (Life Technologies), with either normal (control; 5.4 mM K⁺) or high K⁺ concentrations (H-K; 10.4 mM K⁺) at 25°C. To maintain activities of SW-type ionocytes (Marshall et al., 2000; Hoffmann et al., 2002), osmolality of media was increased to 430 mOsm/kg H₂O by addition of appropriate amounts of NaCl and KCl. After 6 h of incubation, the

filaments were fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4), and then stored in 70% ethanol for immunofluorescence staining. Total RNA was extracted from other filaments with Isogen (Nippon Gene), and cDNA was synthesized with ReverTra Ace α - (Toyobo).

2.7 Triple-color immunofluorescence staining

To understand the effects of 6-h incubation in the control or H-K media on the morphology of the SW-type ionocytes in tilapia gills, filaments were subjected to triple-color immunofluorescence staining targeting ROMKa, NKA, and CFTR. The fixed gill filaments were washed in PBS containing 0.2% Triton X-100 (PBST), blocked in PBST containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin, 0.02% KLH, 0.01% sodium azide (NB-PBS) for 30 min, and then incubated overnight at 4°C in NB-PBS containing rabbit anti-NKA (NKA121; Ura et al., 1996) and mouse anti-human CFTR (MAB25031; R&D Systems, Boston, MA, USA), both antibodies were diluted 1:500. After washing in PBST, the filaments were incubated overnight at 4°C with goat anti-rabbit and anti-mouse IgGs labeled with Alexa Fluor 555 and 405 (Life Technologies), respectively, diluted 1:1000 in PBST. The filaments were washed in PBST, post-fixed in 4% PFA, and then incubated overnight at 4°C with rabbit anti-tilapia ROMKa labeled with Alexa Fluor 488 by using a Zenon Labeling Kit (Life Technologies). After washing in PBST and post-fixation, the filaments were observed with a confocal laser scanning microscope (C1; Nikon, Tokyo, Japan). The micrographs of the ionocytes were taken at relatively flat areas of the afferent side of gill filaments. Digital images of serial optical sections were obtained and overlaid to produce a panfocal image. The wavelengths of excitation and recorded emission for each dye were: Alexa Fluor 405, 405 nm and 432.5-467.5 nm; Alexa Fluor 488, 488 nm and 500-530 nm; Alexa Fluor 555, 543 nm and 57.2-637.5 nm.

2.8 Statistics

Data are expressed as means $\pm$ S.E.M. The significance of differences at $P < 0.05$ was examined by two-way analysis of variance (ANOVA) in the H-K SW exposure experiment, or by one-way ANOVA in the gill incubation experiment, followed by Tukey's HSD test. Significant differences between each sample are shown when a significant factor effect or interaction of the two factors (time and treatment) was detected (Figs. 1, 4)

3. Results

3.1 Experiment 1. Exposure of SW tilapia to H-K SW

3.1.1 Plasma ion concentration and osmolality

In the H-K exposure experiment, significant interaction (*int) of the two factors (time and H-K treatment) was detected in plasma levels of K^+ (Fig. 1A) and Na^+ (Fig. 1B), while plasma osmolality showed a significant main effect of time (*time; Fig. 1D). After exposure to H-K SW, plasma K^+ levels of H-K fish were increased within 6 h, and remained higher than those of control SW fish until the end of the experiment (Fig. 1A). Plasma Na^+ levels of H-K fish decreased slightly by 6 h after exposure, but rebounded within 24 h (Fig. 1B). Plasma Cl^- levels were not affected (Fig. 1C), but plasma osmolality was significantly lower at the 12 h groups (Fig. 1D).

3.1.2 Plasma cortisol and gill mRNA levels of GR1, GR2 and MR

Plasma cortisol levels showed a significant effect of time, with elevated levels at 24 h, and a return to initial levels at 72 h (Fig. 2A). In the gills, an effect of time was detected in the mRNA expression levels of GR1 (Fig. 2B) and MR (Fig. 2D), both being low at 72 h after the onset of the experiment. The patterns of GR2 mRNA levels were similar to those of GR1 and MR, but no significant effects were detected (Fig. 2C)

3.1.3 Pituitary mRNA levels of GH, PRL, SL, and POMC

There were no significant effects of treatment among any pituitary transcripts (GH, PRL, SL, and POMC) following H-K treatment (Fig. 3), while a significant effect of time was detected for PRL (Fig. 3B) and SL (Fig. 3C). SL mRNA levels were temporally reduced at 12 h fish independent of H-K treatment (Fig. 3C). On the contrary, PRL mRNA increased at the same time point (Fig. 3B). GH and POMC mRNA expression levels were stable (Fig. 3A, D).

3.1.4 Gill mRNA levels of ROMKa, NKCC1a, CFTR, and NKA α 1b

A significant interaction of time and H-K treatment was detected for ROMKa (Fig. 4A) and NKA α 1b (Fig. 4D), a significant main effect of time was detected for NKCC1a (Fig. 4B) and CFTR (Fig. 4C), and a main effect of H-K treatment was detected in NKCC1a (Fig. 4B). ROMKa mRNA levels in H-K treated fish increased by 6 h, reached peak levels at 24 h, and remained higher than control levels at 72 h (Fig. 4A). NKCC1a mRNA was significantly greater in H-K groups than in control groups, and was significantly low at 6 h group compared with 0 h (Fig. 4B). CFTR mRNA

was low at 6 and 72 h samples compared with that at 24 h (Fig. 4C). NKA α 1b mRNA was decreased in the control SW group compared with 0 h at 72 h after the onset of the experiment (Fig. 4D).

3.2 Experiment 2. *In vitro* effects of K^+ concentration on ROMKa expression in incubated gill filaments

To examine the direct responses of gill filaments to high K^+ stress, isolated gill filaments were incubated for 6 h in media with control or high K^+ concentration (Figs. 5 and 6). In the triple-color immunofluorescence staining, ROMKa (Figs. 5A, E, I) and CFTR (Figs. 5C, G, K) immunosignals were detected in apical membrane, and NKA (Figs. 5B, F, J) was in basolateral membrane of the ionocytes, consistent with previous observations (Furukawa et al., 2014). Notably, ionocytes of the gills incubated in H-K medium tended to show stronger signals of ROMKa than those in the control medium (Fig. 5E, I).

To assess the responses of branchial ion transporter mRNAs to *in vitro* H-K stress, mRNA levels of ROMKa, NKCC1a, CFTR, and NKA α 1b were measured after 6 h of incubation (Fig. 6). Among the mRNAs assessed, ROMKa and NKCC1a increased in response to H-K condition (Fig. 6A, B). Also, NKCC1a mRNA was increased after incubation in the control medium (Fig. 6A). Meanwhile, CFTR, and NKA α 1b mRNA levels did not change after incubation in both control and H-K media (Fig. 6C, D).

4. Discussion

In teleosts, it has been unclear whether the endocrine systems direct the expression of branchial ROMKa in response to high plasma K^+ stress. In the present study, we first examined changes in organismal parameters of SW-acclimated tilapia following exposure to H-K SW. Fish exposed to H-K SW showed a marked increase in plasma K^+ levels, reflecting the impact of a 10 mM increase in external K^+ . On the other hand, a slight decrease was detected in Na^+ level at 6 h after exposure to H-K. Since many of the transporters for these ions, such as NKCC and NKA, are expressed in a variety of cells for cell volume regulation (Hoffmann et al. 2009), these transporters may contribute to mitigating the K^+ -related perturbation of plasma Na^+ levels, although the detailed mechanism underlying this phenomenon is unknown. There were significant decreases in plasma Na^+ and osmolality in both groups at 12 h after the onset of the H-K exposure experiment. Although there is no clear explanation for these patterns, it is possible that tilapia exhibited circadian rhythms in plasma ion levels and

osmolality, as reported in several euryhaline species (Peterson and Gilmore, Jr., 1988).

Next, we quantified plasma cortisol levels and its receptor mRNAs in the gill following K^+ stress. Gill mRNA levels of GR1, GR2, and MR showed no distinct changes due to H-K treatment. On the other hand, an increase in plasma cortisol was observed at 24 h after the onset of H-K treatment. This response seems somewhat slow compared with the rapid rise often seen in fish following exposure to stressors (Wendelaar Bonga, 1997). Since ROMKa mRNA in the gills increased by 6 h and remained high without a concomitant increase in plasma cortisol, a link between increased cortisol and ROMKa mRNA seems unlikely. In zebrafish (*Danio rerio*), cortisol promotes differentiation of epithelial ionocytes via Foxi3 transcription factors (Cruz et al., 2013). Also, glucocorticoids are known to protect keratinocytes from apoptosis in human epithelia (Stojadinovic et al., 2007). Since plasma cortisol tended to be higher in the H-K group at 24 h, the contribution of cortisol to K^+ homeostasis cannot be ruled out from this study. Further study will be necessary for precise understanding of this issue, addressing cell differentiation or maintenance of the ionocytes.

We also quantified mRNA levels of pituitary hormones (GH, PRL, SL and POMC), which were considered as candidate hormones that could regulate body K^+ via branchial ROMKa. In the present H-K exposure experiment, there were no significant differences in mRNA levels between control and H-K groups, but PRL mRNA was increased at 12 h, and SL mRNA showed an inverse pattern to that of PRL. It is likely that the levels of PRL mRNA are related to the decreased plasma osmolality levels seen in these groups, although the increases in PRL mRNA levels were far more modest than those reported in freshwater-acclimating fish (Breves et al., 2011; Seale et al. 2012). At present, we have no clear explanation for changes in SL mRNA levels. Since time-course changes were detected irrespective of H-K treatment in plasma Na^+ , osmolality, cortisol, and mRNA expression of PRL and SL, it is also possible that these factors were affected by systemic biological rhythms which may have developed in outdoor tanks where the fish were kept before the experiment, with slight fluctuations in temperature (25-28°C) and light conditions. Branchial ROMKa, NKCC1a and CFTR mRNAs were also quantified in this experiment to characterize branchial transcriptional responses to altered environmental K^+ levels. As expected, ROMKa mRNA was markedly up-regulated in response to H-K SW exposure. Importantly, the onset of this increase was not accompanied by changes in the endocrine parameters assessed in the present study. We therefore conclude that the pituitary hormones assessed in the present study do not appear to be involved in regulating ROMKa gene expression.

NKCC1a mRNA was highly expressed in the gills of H-K fish compared with controls. A similar tendency was observed in artificial H-K SW-acclimated fish (Furukawa et al., 2012), but not in H-K freshwater fish (Furukawa et al., 2014). These patterns suggest that branchial NKCC1a may support K^+ excretion in hypertonic environments. The stable CFTR mRNA levels, along with the plasma Cl^- concentrations, indicate that a 10 mM increase in environmental Cl^- was physiologically negligible for SW-acclimated fish. NKA α 1b mRNA levels were not significantly different between H-K and control fish. Although we did not measure enzymatic activity of NKA, our results are consistent with ROMKa being the major regulator of plasma K^+ in tilapia gills.

Since H-K SW exposure did not elicit clear changes in endocrine-related parameters *in vivo*, we subsequently tested the direct effect of K^+ concentration on ROMKa expression in ionocytes through an *in vitro* incubation experiment. In morphological observations, SW-type ionocytes, which express CFTR (Type-IV ionocytes, Hiroi et al., 2008), were maintained in gill filaments incubated in both the control and H-K media for 6 h. Interestingly, stronger signals of ROMKa were detected in filaments maintained in the H-K medium compared with filaments in the control medium, suggesting that high K^+ can stimulate expression of ROMKa in ionocytes.

Gene expression patterns *in vitro* revealed that ROMKa and NKCC1a mRNAs, but not the other transporter mRNAs quantified, were stimulated by incubation in the H-K medium. This is the first study to report that a change in K^+ concentration can alter mRNA levels of specific transporters in teleost ionocytes. In opercular membrane of killifish (*Fundulus heteroclitus*), it has been reported that elevated basolateral osmolality evokes apical Cl^- secretion rate, suggesting that ionocytes are osmosensitive (Marshall et al., 2000; Hoffmann et al., 2002). However, there have been no studies reporting the responses of ionocytes to specific ions. In the present study, we cannot conclude whether the expression of ROMKa within ionocytes was induced by K^+ concentrations at the apical and/or basolateral surface, because the ionic conditions at both surfaces are identical in our incubation system. *In vivo* ionocytes (in SW-acclimated animals) are exposed to ~10 mM and ~4 mM K^+ at the apical and basolateral membrane, respectively. Therefore, in theory, the control medium (5.4 mM K^+) used in this study was hypokalemic, while the H-K medium (10.4 mM K^+) was nearly isokalemic, to the ionocytes via apical membranes. On the other hand, control K^+ condition was nearly isokalemic, and H-K condition was hyperkalemic relative to the plasma in SW fish. The expression level of ROMKa in the incubated gill filaments

with the H-K medium significantly increased compared with that in preincubated filaments, whereas ROMKa expression in the control incubated filaments remained stable. While 6-h incubation of the gills in control media did not affect ROMKa mRNA levels, H-K media significantly increased ROMKa mRNA levels. Based on these results, it is most likely that SW-type ionocytes can sense K^+ concentration in the plasma at the basolateral membrane but not in the environmental water at the apical one. Although the mechanism underlying the response of the ionocytes to K^+ is unknown, some voltage-gated channels (Kültz, 2012) might be involved in this response, since increases in extracellular K^+ concentration can activate voltage-gated channels via membrane depolarization. In rats with impaired aldosterone secretion following adrenalectomy, ROMK mRNA in the renal medulla was regulated by serum K^+ levels, instead of aldosterone-based ROMK control (Wald et al., 1998). Since the maintenance of plasma K^+ concentration is critical to vertebrates, particular ionocytes may have evolved a cell-autonomous K^+ regulatory mechanism to promptly regulate internal K^+ levels.

In summary, we sought to identify endocrine factors that control plasma K^+ balance via branchial ROMKa expression in SW-acclimated tilapia. There were no marked changes in any of the candidate hormone or hormone receptor mRNA levels following H-K treatment. On the other hand, branchial ROMKa mRNA increased within hours after H-K treatment, implying the existence of other more proximate means to regulate the expression of this K^+ channel. Utilizing isolated gill filaments *in vitro*, we found that a high K^+ condition can stimulate ROMKa expression in ionocytes. We therefore propose that ionocytes may directly sense extracellular K^+ conditions. Alternatively, ROMK expression may be controlled by paracrine factors released from other cells in the gill filament. In future studies, it will be interesting to look at intracellular factors that mediate K^+ -induced ROMKa expression in teleost ionocytes.

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Figure Legends

Fig. 1.

Changes in plasma concentrations of K^+ (A), Na^+ (B), and Cl^- (C), and plasma osmolality (D) in tilapia maintained in seawater (SW, open circles) or in tilapia exposed to high- K^+ (H-K) SW (closed circles). Data are expressed as means $\pm$ S.E.M. (n=5-8). *int indicates significant interaction of the two factors (time and treatment). *time indicates a significant main effect of time. Different letters indicate significant differences ($P < 0.05$) between time points within each treatment group (a, b, c, ... for SW, and x, y, z, ... for H-K SW; in D, the two treatment groups are combined), and asterisks indicate the significant differences between treatments at the same time points.

Fig. 2.

Changes in plasma cortisol levels (A) and relative mRNA expression levels (mRNA/18S rRNA) of GR1 (B), GR2 (C) and MR (D) in the gills of tilapia maintained in seawater (SW, open circles) or in tilapia exposed to high- K^+ (H-K) SW (closed circles). mRNA expression levels at 0 h are designated as an arbitrary value of 1. Data are expressed as means $\pm$ S.E.M. (n=5-8). *time indicates a significant main effect of time. Different letters indicate significant differences at $P < 0.05$ between

time points (SW and H-K SW are combined). NS, not significant.

Fig. 3.

Changes in relative mRNA expression levels (mRNA/18S rRNA) of GH (A), SL (B), PRL (C), and POMC (D) in the pituitary of tilapia kept in seawater (SW, open circles) or in tilapia exposed to high- K^+ (H-K) SW (closed circles). mRNA expression levels of samples at 0 h are designated as an arbitrary value of 1. Data are expressed as means $\pm$ S.E.M. (n=5-6). *time indicates significant main effect of time. Different letters indicate significant differences at $P < 0.05$ between time points (SW and H-K SW are combined). NS, not significant.

Fig. 4.

Changes in relative mRNA expression levels (mRNA/18S rRNA) of ROMKa (A), NKCC1a (B), CFTR (C), and NKA α 1b (D) in the gills of tilapia maintained in seawater (SW, open circles) or in tilapia exposed to high- K^+ (H-K) SW (closed circles). mRNA expression levels at 0 h are designated as an arbitrary value of 1. Data are expressed as means $\pm$ S.E.M. (n=5-6). *int indicates significant interaction of the two factors (time and treatment). *time and *treatment indicate significant main effects of time and treatment, respectively. Different letters indicate significant differences ($P < 0.05$) between time points within each treatment group (a, b, c, ... for SW, and x, y, z, ... for H-K SW; in B and C, the two treatment groups are combined), and asterisks indicate the significant differences between treatments at the same time points.

Fig. 5.

Immunofluorescence micrographs of the pre-incubation gill filaments of seawater-acclimated tilapia (A-D) and the filaments incubated for 6 h in the control (E-H) and high- K^+ (I-L) media, stained with anti-ROMKa (A, E, I; green), anti-NKA (B, F, J; red), and anti-CFTR (C, G, K; blue). The RGB images for ROMKa/NKA/CFTR were merged in (D, H, L). Bar, 20 μ m.

Fig. 6.

Relative mRNA expression levels (mRNA/18S rRNA) of ROMKa, NKCC1a, CFTR, and NKA α 1b in pre-incubated gill filaments (Pre) of seawater-acclimated tilapia and the filaments incubated for 6 h in control (Ctrl) and high- K^+ (H-K) media. Data are expressed as means $\pm$ S.E.M. (n=6). The expression levels of pre-incubation samples

612 were designated as an arbitrary value of 1. Different letters indicate significant
613 differences between experimental groups ($P<0.05$).

Table 1. Furukawa et al.

Table 1. *Primers used for cloning and quantitative PCR (qPCR)*

Primers	Sequence (5'-3')	Notes
GRs-dF	CARYTNGTNCCNACNATG	degenerate PCR; aa, "QLVPTM"
GRs-dR	RAANCKYTGCCARTTYTG	degenerate PCR; aa, "QNWQRF"
GR1-GSP1R	GGTAAGACCTCCAGCCCAAAC	5' RACE
GR1-GSP2R	AAGCCTCGTCAGAGCACAC	qPCR
GR1-GSP3F	TCCTCCCAGTAACGACCATC	qPCR
GR2-GSP1R	CCCACTTGACTGCAGAGACAAC	5' RACE
GR2-GSP2R	CCGTTAATGGCATCCAGAG	
GR2-GSP3F	AATGGGCATACAAGACCAGAAG	qPCR
GR2-GSP4R	GGACGCGACAGGAGTGAG	qPCR
MR-dF	ATGGGNYTNTAYATGAAYG	degenerate PCR; aa, "MGLYMNA"
MR-dR	CATYTCNGGRAAYTCNAC	degenerate PCR; aa, "VEFPEN"
MR-GSP1F	ACAGCCTGACACCACTGAC	qPCR, 3' RACE
MR-GSP2R	GGCAAAAGGAGGACAGACAC	qPCR, 5' RACE
GH-F	CCGATCGACAAACACGAGAC	qPCR
GH-R	GACAGAGAGCGACTGGGAAAC	qPCR
PRL-F	ACCAAGCACTTCAAGTGTCAGAGT	qPCR, Watanabe and Kaneko (2010)
PRL-R	TGAAGTGATGGCCTGAGCA	qPCR, Watanabe and Kaneko (2010)
SL-F	TCCCTTTGCGACTTCAGAG	qPCR
SL-R	CAGAGTGAGCAACCATTTATC	qPCR
POMC-F	CTCTGTCACTCTGACCTCAC	qPCR
POMC-R	GTGAGGAGGCGTCTGATG	qPCR
ROMKa-F	GATTAGCCCCAGAAACAACATATC	qPCR, Furukawa et al. (2012)
ROMKa-R	TGTCTCCCCATCTTGTGTG	qPCR, Furukawa et al. (2012)
NKCC1a-F	GGAGGCAAGATCAACAGGATTG	qPCR, Hiroi et al. (2008)
NKCC1a-R	AATGTCCGAAAAGTCTATCCTGAACT	qPCR, Hiroi et al. (2008)
CFTR-F	TCACCAGCATCGCTGTAGATG	qPCR
CFTR-R	GGTTGTGCGATGACGATATCAGG	qPCR
NKA α 1b-F	GGAGCGTGTGCTTCATCACT	qPCR, Tipsmark et al. (2011)
NKA α 1b-R	ATCCATGCTTTGTGGGGTTA	qPCR, Tipsmark et al. (2011)
18S rRNA-F	CGATGCTCTTAGCTGAGTGT	qPCR, Watanabe et al. (2008)
18S rRNA-R	ACGACGGTATCTGATCGTCT	qPCR, Watanabe et al. (2008)

Mixed bases: N, A/T/C/G; H, A/C/T; Y, C/T; R, A/G; K, G/T. Corresponding amino acid (aa) residues of degenerate primers are written in the notes.

Figure 1. Furukawa et al.

Figure 2. Furukawa et al.

Figure 3. Furukawa et al.

Figure 4. Furukawa et al.

Figure 5. Furukawa et al.

Figure 6. Furukawa et al.