Physiological studies on potassium and cesium excretion and its regulatory mechanisms in gill ionocytes of Mozambique tilapia

ティラピアの鰓塩類細胞におけるカリウム及びセシウムの排出と
その調節機構に関する生理学的研究

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General Introduction

Body fluid homeostasis is a critical regulatory mechanism for all animals to maintain proper function of their body organs and thus to survive. Among various parameters of body fluid, osmolality, defined as the magnitude of osmotic pressure produced by electrolytes, or osmolytes, in a solution, is an important factor. Osmolality of vertebrate plasma is predominantly made up of two inorganic ions, Na^+ (~145 mM) and Cl^- (~116 mM), and its regulation, referred to as osmoregulation, mostly represents the regulation of Na^+ and Cl^- levels in plasma. Vertebrate animals maintain their plasma osmolality within physiological ranges, through the regulation of Na^+ and Cl^-.

Osmoregulation in fish

Teleost fishes are a large group of vertebrate animals, inhabiting a variety of aquatic environments, such as rivers, lakes and the ocean. Since the fish live in the aquatic environments with varying levels of osmolality, they are always challenged by diffusional movement of electrolytes and water across the skin: seawater (SW) fish suffer from ion gain and water loss, and freshwater (FW) fish face ion loss and water gain. To compensate for the osmotic stresses, SW fish drink the ambient water and excrete Na^+ and Cl^- from the gills, while divalent ions are eliminated in the kidney. On the other hand, FW fish absorb Na^+ and Cl^- from the gills, and produce dilute urine to discharge excess water. Among 32,500 fish species reported (FishBase, 2013), the majority of fish can live only in either SW or FW, whereas an estimated 3 to 5% of all fish species can freely move between SW and FW, being called euryhaline fish (Schultz and McCormick, 2013). Osmoregulatory mechanisms in fish have been well studied for decades, often using euryhaline fish as model species. Recently, precise molecular mechanisms of Na^+ and Cl^- handling in the major osmoregulatory organs, the gill, kidney and intestine are increasingly clear (Marshall and Grosell, 2006).

Plasma potassium balance in vertebrates

In addition to osmolality, plasma levels of various ions have significant impacts on the body functions of the animals. Body fluid of vertebrate animals is divided into two major compartments, extracellular fluid (ECF) and intracellular fluid (ICF). As is the case with

plasma, the vast majority of electrolyte in ECF is the two inorganic ions, Na^+ and Cl^-. On the other hand, K^+ is the overwhelmingly major ion (~140 mM) in ICF (Their, 1986), due to the action of sodium pump, Na^+/K^+-ATPase (NKA), which actively transports extracellular K^+ into the cell in exchange for intracellular Na^+. The chemical gradient of K^+ and Na^+ across the cell membrane creates membrane potential, which is vital to all types of cells. For this reason, most vertebrate animals finely regulate plasma K^+ levels nearly 4 mM to maintain normal functions of the cells (Their, 1986). K^+ regulatory mechanisms have been studied extensively in mammals, where the kidney plays a central role by modulating renal K^+ secretion (Giebisch et al., 2007). In fish, whereas regulatory mechanisms of plasma Na^+ and Cl^- have been precisely understood, those of K^+ remain unclear. For example, in SW fish, although K^+ tends to be excessive due to diffusional gain and drinking the external water, the small amount of urine does not appear to match the demand of eliminating excess K^+ (Hickman, 1968; Shehadeh and Gordon, 1969; Smith, 1930). In the present study, I aimed to clarify K^+ regulatory mechanisms in fish, mostly focusing on the gills as a K^+ regulatory organ.

Gill ionocytes

As described above, the gill is one of the important sites for ion transport (Evans et al., 2005). In the gills, there are ionocytes, which are responsible for active excretion and absorption of the ions (Kaneko et al., 2008). Also, embryonic skin has functional ionocytes before development of the gills (Kaneko et al., 2008). Since the ionocytes are epithelial cells, their cell membrane is divided into apical and basolateral membranes, facing environmental water and ECF, respectively, in the gills. Within the ionocytes, there are numerous mitochondria and an extensive tubular system derived from the basolateral membrane. The basolateral membrane of ionocytes is enlarged by the presence of the tubular system, and is rich in NKA, a key enzyme of ion transport in ionocytes. NKA establishes robust electrochemical gradients across the cell membranes, and provides driving force for ion transport through a variety of ion channels or transporters in apical or basolateral membrane of ionocytes. By expressing various transporters with different substrates, ionocytes play crucial roles not only in osmoregulation, but also in acid-base regulation, excretion of

nitrogenous waste, and Ca^{2+} absorption (Hwang et al., 2011). Although ionocytes are often referred to as "mitochondrion-rich cells" or "chloride cells" elsewhere in other articles, I will use the term "ionocytes" throughout this thesis.

Mozambique tilapia

In the present study, I adopted a euryhaline tropical fish, Mozambique tilapia *Oreochromis mossambicus*, as an experimental model fish. In common, "tilapia" refers to the three cichlid genera, *Oreochromis*, *Sarotherodon* and *Tilapia*, which are indigenous to Africa. Tilapias are important fish for aquaculture, since they ingest a wide variety of food organisms and are tolerant of poor water quality. Especially, their tolerance to high water salinity, high water temperature, low dissolved oxygen, and high ammonia concentrations is outstanding among most commonly farmed FW fish (Popma and Masser, 1999). Among tilapia species, Mozambique tilapia is a suitable model for studies on ion regulatory mechanisms, because this tilapia is adaptable to a wide range of salinity from deionized FW to concentrated SW (Uchida et al., 2000).

Tilapia ionocytes

In tilapia, ionocytes are divided into two FW types, one SW type, and one type without any known functions, using embryonic ionocytes in the skin for the first time (Figure 0-1; Hiroi et al., 2008; Inokuchi et al., 2008; Inokuchi et al., 2009). Ion-absorbing FW type ionocytes include: Type II cells, expressing Na^+/Cl^- cotransporter (NCC) at apical membrane for Na^+ and Cl^- uptake; Type III cells, expressing Na^+/H^+ exchanger 3 (NHE3) at apical membrane for Na^+ uptake and H^+ excretion. Type I cells do not possess any known characteristic transporters or channels other than basolaterally-expressed NKA. Type IV SW-type cells express cystic fibrosis transmembrane conductance regulator (CFTR) and NHE3 apically, and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) 1a basolaterally. NKCC1a and CFTR play central roles in excretion of Cl^- to external water. Type IV cells often form complexes with accessory cells or other type IV cells, to build "leaky" junctions between those cells and allow Na^+ secretion through the narrow space between the cells (paracellular pathway).

Potassium excretion through ROMKa potassium channel expressed in gill ionocytes of SW-acclimated tilapia

As described above, SW fish may well have extrarenal mechanisms for K^+ excretion. In Chapter 1, I tested if SW-acclimated tilapia excrete excess K^+ from the gills, the major ion regulatory organ in fish. Through the invention of a new technique, I clearly showed that SW-acclimated tilapia excrete K^+ from gill ionocytes. Among molecular candidates that may contribute to K^+ excretion from the ionocytes, a K^+ channel homologous to renal outer medullary K^+ channel (ROMK or Kir1.1), whose ortholog was originally found from the rat (*Rattus norvegicus*) kidney, was highly expressed in tilapia gills in response to high K^+ environment. A putative paralog of this gene was further found in Chapter 3 of this thesis. Thus, the ROMK found in Chapter 1 and 3 were tentatively designated as ROMKa and ROMKb, respectively, although I did not discuss about ROMKb in Chapter 1, 2, and 4. My subsequent studies in Chapter 1 clearly demonstrated that tilapia ROMKa was strongly expressed in apical openings of SW-type ionocytes, being the primary pathway that allows K^+ extrusion from the ionocytes.

Concerns for radioactive cesium after the fallout of Fukushima Daiichi nuclear power plant

On March 11, 2011, a magnitude 9.0 earthquake occurred off the coast of Japan and triggered powerful tsunami, causing destruction in northeastern Japan. This disaster severely damaged the Fukushima Daiichi Nuclear Power Plant, leading to emissions of radioactive materials. Among these materials, cesium-137 (^{137}Cs), a 30.1-year half-life radioactive material, caused large concern due to its harmful effects on fishery, agriculture, stock farming, and ultimately on human life for decades (Yasunari et al., 2011). Since Japan is one of the most fish-consuming countries in the world, there is a large concern on the contamination of the fish captured in northeastern Japan with ^{137}Cs (Japanese Fisheries Agency, 2012). Now, it is an urgent issue not only to assess the deposition of ^{137}Cs in the captured fish, but also to understand its behavior within fish bodies.

Excretion of cesium and rubidium via the branchial potassium-transporting pathway in

Mozambique tilapia

It is empirically established that K^+, Cs^+ and Rb^+ behave similarly in physiological systems of animal bodies, thus they are considered as biochemical analogs. In the animal bodies, Cs^+ is highly deposited in muscle (Peters et al., 2006), and high-K^+ diet helped eliminating Cs^+ from rats (Sato et al., 1997). In fish bodies, "biological" half-life of ^{137}Cs is much shorter than true half-life of ^{137}Cs, suggesting the presence of active excretory mechanisms for Cs^+ in the fish (Kasamatsu, 1999). Importantly, SW fish are prone to have shorter biological half-life of ^{137}Cs than FW fish. Also, in a study using rainbow trout, high-K^+ environments facilitated the elimination rate of ^{137}Cs from the fish (Kasamatsu, 1999). These facts led me to the hypothesis that Cs^+ is actually excreted via branchial ROMKa, the novel K^+ excretory pathway that was activated by high environmental K^+. In chapter 2, I used stable isotope of Cs and Rb to see if Cs^+ and Rb^+ are excreted from ionocytes, applying the K^+ insolubilization technique that I invented in Chapter 1. My findings showed that ionocytes in SW-acclimated tilapia actually excreted both Cs^+ and Rb^+ along with K^+, suggesting that ROMKa contributes as a molecular pathway involved in this mechanism.

Expression of ROMKa in gill ionocytes of freshwater-acclimated tilapia

Inhabiting dilute water environments, FW fish should usually reduce diffusional loss of Na^+ and Cl^- to the environment and maintain plasma osmolality. On the other hand, it seems still important to excrete K^+ occasionally, since K^+ is abundant in their food (natural plants and animals) as a primary intracellular ion. Also, there are temporal risks of K^+ overload, such as acidosis and tissue breakdown (Weiner and Wingo, 1998; Clausen, 2010). In Chapter 3, I assessed the responses of FW fish to high-K^+ FW (H-K FW, 10 mM KCl) environment, to understand the mechanisms of K^+ excretion in FW-adapted fish. Since FW fish excrete a considerable amount of urine for osmoregulation, the kidney was focused on as a possible K^+ regulatory organ, as well as the gills. After 1-week exposure to normal or H-K FW, mRNA levels of the molecular candidates of K^+ regulation in the gill and kidney were assessed. The results showed that H-K FW tilapia strongly expressed ROMKa in the gills, whereas kidney mRNAs did not change. In addition, triple-color immunofluorescence staining revealed that ROMKa were expressed in apical openings of Type-III ionocytes. The

results suggest that FW fish can excrete K^+ from the gills, and the branchial K^+ excretion is accomplished by Type-III ionocytes.

Differential regulation of ROMKa expression in ionocytes of freshwater- and seawater-acclimated tilapia: hormonal vs. cell-autonomous regulation

Throughout vertebrates, the endocrine system mediates proper physiological responses of osmoregulatory organs to homeostatic demands of salt and water transport (McCormick and Bradshaw, 2006). Among various endocrine factors, a primary hormone responsible for mammalian renal K^+ regulation is aldosterone, the main mineralocorticoid that is produced in the adrenal gland. In rats, aldosterone up-regulates mRNA expression of ROMK, the major pathway for K^+ excretion in the kidney, and reduces plasma K^+ (Beesley et al., 1998, Wald et al., 1998). Teleost fish lack aldosterone (Arterbery et al., 2011), and the main corticosteroid is cortisol, a glucocorticoid produced in the interrenal gland. In teleosts, glucocorticoid receptor (GR) 1, GR2, and mineralocorticoid receptor (MR) act as receptors for cortisol and several exogenous corticosteroids with different affinities and transactivation efficiencies of the target genes (Bury and Sturm, 2007; Arterbery et al., 2011; Greenwood et al., 2003). In Chapter 4, I first aimed to assess the effect of cortisol on the gill expression of ROMKa mRNA, using a separated gill filament incubation system (Ito, 2011). Surprisingly, only FW-acclimated tilapia gills showed marked response to cortisol in incubation media, expressing ROMKa mRNA more than twice as high as control tissues without cortisol treatment. On the other hand, in SW-acclimated gills, cortisol appeared merely to maintain ionocytes, or basal expression levels of ROMKa mRNA. Finally, I found that increased K^+ concentration in the incubation media triggered twice more expression of ROMKa mRNA than moderate K^+ control. My findings suggest that up-regulation of ROMKa mRNA levels for K^+ excretion in gill ionocytes was directly induced by increased plasma K^+ concentration in SW fish, but was mediated by cortisol as an endocrine factor in FW fish.

In the present studies on molecular mechanisms of K^+ regulation in fish gills, I have successfully broken new ground in the field of fish fluid homeostasis. I do believe that this

rapidly-growing research field is further fertilized by the knowledge obtained in the present study.

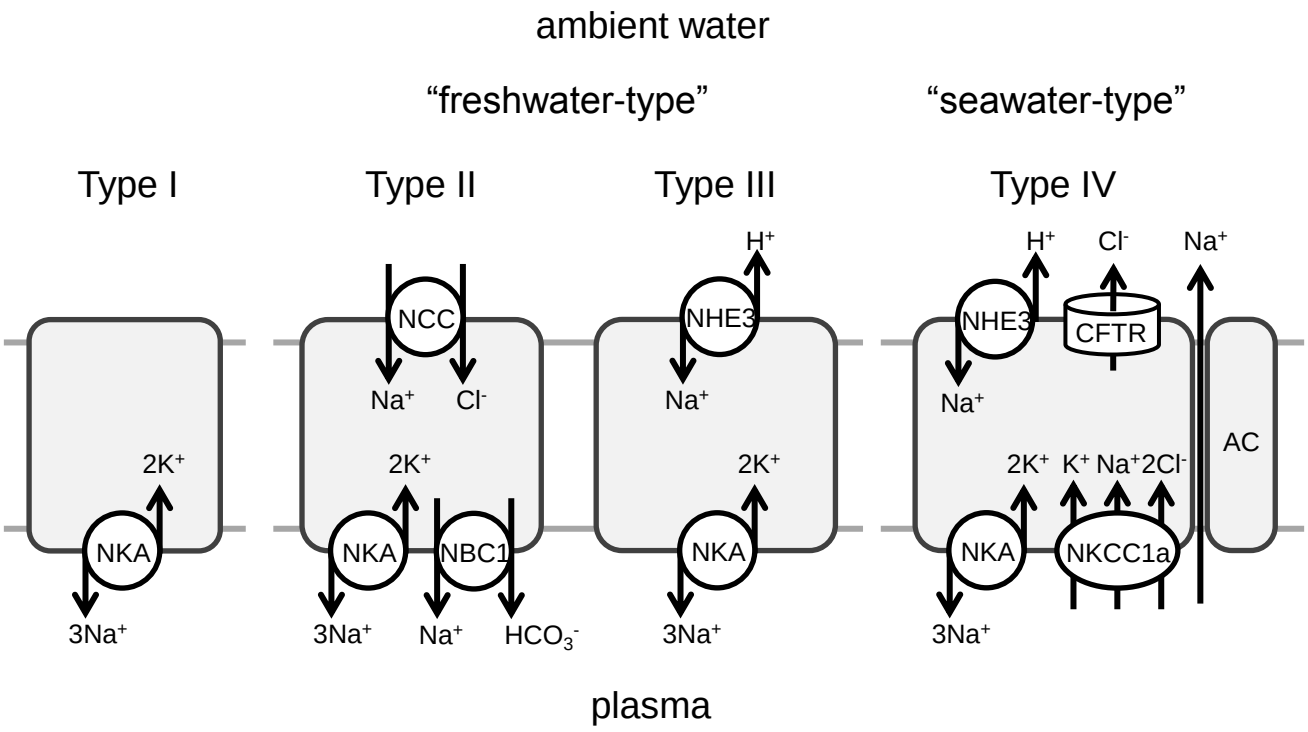


Fig. 0-1. Models of ion transport in gill ionocytes of Mozambique tilapia. Type-I, ionocytes without known functions. Type-II and Type-III, freshwater-type ionocytes. Type-IV, seawater-type ionocytes. AC, accessory cell. NKA, Na^+/K^+-ATPase; NCC, Na^+/Cl^- cotransporter; NBC1, Na^+/HCO_3^- cotransporter-1; NHE3, Na^+/H^+ exchanger-3; CFTR, cystic fibrosis transmembrane conductance regulator; NKCC1a, $Na^+/K^+/2Cl^-$ cotransporter-1a. The model was constructed in reference to Hiroi et al. (2008), Inokuchi et al. (2008), and Furukawa et al. (2011).

1. Potassium excretion through ROMKa potassium channel expressed in gill mitochondrion-rich cells of Mozambique tilapia

1-1. Introduction

Among various physiological phenomena occurring in animals, body fluid homeostasis is one of the most important mechanisms. In most teleost species, the gills have important roles in body fluid regulation (Marshall and Grosell, 2006). In gill epithelia, ionocytes are mainly involved in ion and acid-base regulation (Evans et al., 2005). The basolateral membrane of ionocytes is equipped with NKA, a key enzyme which provides an electrochemical gradient for the ion-transporting functions of ionocytes (Kaneko et al., 2008). Recently, molecular mechanisms of Na^+, Cl^- and acid-base regulation and those of nitrogen metabolite excretion in fish ionocytes have become increasingly clear (Hwang et al., 2011). However, the regulatory mechanism of K^+ in fish is yet to be identified.

In general, K^+ is a major monovalent cation in the bodies of vertebrate animals (Their, 1986). Due to the action of NKA on the cell membrane, a large proportion of K^+ is confined within the cells, while plasma K^+ concentration is maintained at a relatively low level. Terrestrial animals regulate their plasma K^+ levels within physiological ranges by modulating renal K^+ excretion into the urine. In the mammalian kidney, renal outer medullary potassium channel (ROMK, or Kir 1.1), located in the apical membrane of tubular epithelial cells from the thick ascending limb (TAL) through the outer medullary collecting duct (OMCD), is the main route for K^+ secretion to the lumen (Hebert et al., 2005; Giebisch et al., 2007; Wang and Giebisch, 2007). Other proteins involved in K^+ handling are also expressed in the mammalian kidney, such as potassium large conductance Ca^{2+}-activated channel, subfamily M (Maxi-K or BK), and K^+/Cl^- cotransporters (KCC1, KCC3 and KCC4) (Pácha et al., 1991; Gillen et al., 1996; Hiki e al., 1999; Velázquez and Silva, 2003; Najjar et al., 2005; Wang and Giebisch, 2007).

SW contains K^+ more than twice as much as typical plasma in vertebrate animals. However, SW-inhabiting fish produce a small amount of K^+-poor isosmotic urine, which is insufficient to maintain an appropriate internal K^+ level (Hickman, 1968; Shehadeh and Gordon, 1969; Smith, 1930). For these reasons, SW fish are considered to have an

extrarenal mechanism to eliminate excess K^+ in the body fluid. Since the gill is one of the primary osmoregulatory organs in fish (Evans et al., 2005), it is most likely that SW fish excrete excess K^+ through the gills. However, there has been no direct evidence to confirm K^+ excretion from the gills so far.

In Chapter 1, I aimed to demonstrate the excretion of K^+ from the gills and reveal its molecular mechanism. I first confirmed that K^+ was excreted from gill ionocytes of SW-acclimated tilapia, using a newly-developed technique that insolubilized and visualized excreted K^+. After determining sequences of cDNAs encoding ROMKa, Maxi-K α subunit, KCC1, KCC2 and KCC4, I assessed mRNA expression levels of above K^+ channels and transporters, along with a basolateral K^+ transporter, NKCC1a, in the gills of tilapia acclimated to environments with different K^+ concentrations. As ROMKa appeared to be the primary K^+ channel involved in plasma K^+ regulation, further analyses were conducted to define ROMKa as a major pathway for K^+ excretion expressed in the gill ionocytes of tilapia.

1-2. Materials and Methods

Experimental animals

Mozambique tilapia (*Oreochromis mossambicus*) were maintained in tanks supplied with recirculating FW or SW at 25°C. The fish were fed commercial pellets (Tilapia 41M; JA Nishi-Nihon Kumiai Shiryo, Hyogo, Japan) once a day. The fish were anesthetized with 0.1% 2-phenoxyethanol before blood sampling or 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.

Visualization of potassium excreted from gill ionocytes

To determine the site of K^+ excretion, K^+ was reacted with sodium tetraphenylborate (TPB) to form precipitation of K-TPB (Engelbrecht and McCoy, 1956). The gills of SW-acclimated tilapia were dissected out, and washed in 0.025% $KMnO_4$ in PBS for 1 min to remove mucus from the filament surface. After washing with mannitol solution (320 mM mannitol, 1.8 mM $CaCl_2$), the gill filaments were placed in reaction solution containing (in mM) 30 Na-TPB, 265 mannitol, 1.8 $CaCl_2$, and 50 HEPES (TPB solution). After 30 sec,

TPB solution was removed and filaments were washed with mannitol solution. In order to label ionocytes, the gill filaments were then immersed in a solution containing (in mM) 0.05 rhodamine123, 2 HEPES, 2.7 KCl, 0.4 $MgCl_2$, 158 NaCl and 1.8 $CaCl_2$ for 15 min. The samples were observed with a confocal laser scanning microscope (C1; Nikon, Tokyo, Japan). The wavelengths of excitation and recorded emission for rhodamine 123 were 543 nm and 572.5-637.5 nm, respectively. The differential interference contrast (DIC) images of the precipitates formed on the gill surface and the corresponding panfocal fluorescent images of ionocytes were taken to localize the K^+-excreting site on the gill surface.

The TPB-treated gill filaments were also analyzed by energy dispersive X-ray spectrometry (EDS) to confirm K existence in the precipitates observed at the apical openings of ionocytes. After fixation in 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4) for 24 h, the gill filaments were washed with distilled water and snap-frozen in liquid nitrogen. The frozen filaments were dried in a freeze-dryer (Neocool; Yamato Scientific, Tokyo, Japan). The dried filaments were mounted on specimen stubs, coated with gold in an ion coater (IB-3; Eiko Engineering, Ibaraki, Japan), and then observed with an EDS system attached to a scanning electron microscope (SEM) (S-4800; Hitachi, Tokyo, Japan), at 20 kV accelerating voltage. The TPB contains atomic elements of C and B, and reacts with K^+ and NH_4^+ to form insoluble precipitates. Therefore, the X-ray spectrometry was applied to detect K, B, C and N to clarify an element involved in the precipitate formation.

Molecular cloning

Gill filaments were removed from SW-acclimated 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), first-strand cDNA was synthesized from the total RNA with SuperScript III (Life Technologies). The cDNA fragments of ROMKa, Maxi-K, KCC1 and KCC4 were amplified by degenerate PCR with a cDNA template obtained from tilapia gills. The degenerate primers corresponding to the conserved amino acid residues of known above channels/transporters were designed (Table 1-1). Since a specific PCR product of KCC2 did not appear using the gill total cDNA as a template, total cDNA from the brain was used to amplify a cDNA fragment of KCC2. Although we

initially intended to clone KCC3 from tilapia, its cloning was not performed in practice, since there was no DNA sequence homologous to the mammalian KCC3 clade in all fish genome databases available. The PCR products were ligated into pGEM T-Easy vector (Promega, Madison, WI) and sequenced using an ABI Prism 310 sequencer (Life Technologies). For ROMKa, complete cDNA sequence was determined using CapFishing^{TM} kit (Seegene, Seoul, Korea), and deduced amino acid sequence was analyzed with TMHMM (Center for Biological Sequence, Copenhagen, Denmark; http://www.cbs.dtu.dk/services/TMHMM/) for putative transmembrane regions.

Tissue distribution analyses

The tissue distribution patterns of mRNA expression of ROMKa, Maxi-K, KCC1, KCC2 and KCC4 in tilapia was analyzed by RT-PCR, using the first-strand cDNA obtained from the brain, gill, heart, stomach, liver, kidney, anterior and posterior intestines, rectum, skin and muscle. Tissues from both SW- and FW-acclimated tilapia were analyzed. The cDNA fragments were amplified with primers listed in Table 1-1. The PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide.

Acclimation to environments with different K^+ concentrations

Tilapia (69-190 g) were transferred to a 100-l tank, containing FW, SW or artificial SW containing moderate (M-K) or high (H-K) concentration of K^+, and acclimated for 1 week. Each group consisted of 6 individuals. At the start of the acclimation, FW-reared tilapia were transferred to the experimental tank with FW, while SW-reared tilapia were used for SW, M-K, and H-K groups. From 2 days prior to the experiment, the fish were not fed to avoid any effect of electrolytes in the feed. After 1-week acclimation, concentrations of Na^+, Cl^-, K^+, and Ca^{2+} in experimental media were determined with an ion analyzer (IA-200; TOA-DKK, Tokyo, Japan) and a digital chloridometer (Labconco, Kansas, MO). Table 1-2 shows ion concentrations and water pH on the last day of the experiments.

Plasma ion concentrations and osmolality

Plasma osmolality and concentrations of Na^+, K^+, and Cl^- were measured in tilapia

acclimated to experimental conditions. After anesthetizing the fish, blood was collected from the caudal vessels with a heparinized syringe and needle. Blood plasma was separated by centrifugation at 5,000 g for 10 min and stored at -20°C. Plasma concentrations of Na^+ and K^+ were measured with the IA-200 ion analyzer, Cl^- concentration with the digital chloridometer, and plasma osmolality with a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT).

Quantitative real-time PCR

The mRNA levels of ROMKa, Maxi-K α subunit, KCC1, KCC4, and NKCC1a (GenBank accession no. AY513737) were assessed by quantitative real-time PCR (qPCR) with a LightCycler® 480 system II (Roche diagnostics, Basel, Switzerland) using LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche diagnostics). I did not quantify the KCC2 mRNA since its expression was not detected in the gills (Fig. 1-3). From the gills of experimental fish, the total RNA was extracted with Isogen (Nippon Gene), and reverse-transcribed with SuperScript III (Life Technologies). The copy numbers of the transcripts were calculated with reference to parallel amplifications of known concentrations of the respective cloned PCR fragments, and the specificity of each PCR was checked by melting curve analysis. The data were normalized to the corresponding 18S rRNA levels. The primers used are shown in Table 1-1.

Antibody

A rabbit polyclonal antiserum was raised against a synthetic peptide corresponding to a part near the cytosolic C-terminal of tilapia ROMKa (amino acid residues 350-364, Fig. 1-2). The antigen conjugated with keyhole limpet hemocyanin (KLH) was emulsified with Freund's complete adjuvant, and immunization was performed in a New Zealand white rabbit. The antiserum was obtained after several booster injections with Freund's incomplete adjuvant, and the specific antibody was affinity-purified from the antiserum with the antigen peptide (Protein Purify, Gunma, Japan). The specificity of anti-ROMKa was confirmed by western blot analysis according to Watanabe and Kaneko (2008). The protein samples prepared from the gills of FW- or SW-acclimated tilapia were heat-denatured at 45°C for 30

min, and separated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the protein was transferred to a PVDF membrane, which was stained with anti-tilapia ROMKa diluted 1:2000 with blocking buffer overnight at 4°C.

Immunohistochemistry

The second gill arches were dissected out from FW tilapia, fixed in 4% PFA in 0.1 M phosphate buffer (PB, pH 7.4) for 24 h at 4°C, and stored in 70% ethanol. After a brief wash in 0.01 M phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBST), the filaments were incubated 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. The filaments were then incubated in NB-PBS containing anti-ROMKa diluted 1:1000 overnight at 4°C. After a brief wash in PBS, the gill filaments were incubated overnight at 4°C with goat anti-rabbit IgG labeled with Alexa Fluor 555 (Life Technologies) diluted 1:1000 in PBS. The filaments were washed in PBST and post-fixed in 4% PFA for 5 min. After a brief wash in PBST, the samples were incubated overnight at 4°C with fluorescein isothiocyanate (FITC)-labeled anti-NKA diluted 1:500 in NB-PBS (Choi et al., 2010). The samples were observed with the C1 confocal laser scanning microscope. The wavelengths of excitation and recorded emission for each dye were as follows: Alexa Fluor 555, 543 nm and 572.5-637.5 nm; FITC, 488 nm and 500-530 nm.

Confirmation of potassium excretion through ROMKa

The K^+ secretory function of ROMKa was examined by exposing the gills to TPB solution containing Ba^{2+}, a nonselective blocker for ROMK and Maxi-K, or iberiotoxin (IBTX), a specific blocker for Maxi-K (Segal and Reuss, 1990; Meera et al., 1997; Sohma et al., 1998; Bailey et al., 2006). The gills were removed from SW-acclimated tilapia, and washed in 0.025% $KMnO_4$ in PBS for 1 min. The gill filaments were separated into 3 groups, each being washed with mannitol solution, that with 5 mM $BaCl_2$, or that with 200 nM IBTX. After washing, the gill filaments were exposed to control TPB solution, TPB solution with 5 mM $BaCl_2$ or TPB solution with 200 nM IBTX for 1 min, followed by washing with mannitol solution. The gill filaments were observed with an inverted

microscope (Ti-S; Nikon).

Statistics

The data from the acclimation experiment were expressed as means ± S.E.M. The significance of differences at $P<0.05$ was examined by one-way analysis of variance (ANOVA) followed by Tukey's HSD test.

1-3. Results

Visualization of potassium excretion from gill ionocytes

When the TPB-treated gills were observed by DIC microscopy, precipitates of possible K-TPB were detected as particles on the gills (Fig. 1-1A). The particles were localized to the apical openings of ionocytes, as confirmed by merging the DIC image and fluorescent image showing ionocytes in the same area (Fig. 1-1B). In the SEM observation, the precipitates appeared as amorphous structures attaching to the surface of the gill filaments (Figs. 1-1C). The EDS analysis showed that the signals of K were closely associated with the precipitates (Figs. 1-1, D and H). The signals of C and B were also mapped at the K-existing sites (Figs. 1-1, E and F), whereas no clear signal of N was observed near the precipitates (Fig. 1-1G).

Molecular cloning of tilapia ROMKa

The cDNA of tilapia ROMKa (AB669173) consisted of 1557 bp, encoding a protein with 374 aa (Fig. 1-2). The deduced amino acid sequence of ROMKa shared identity with ROMK from human (56%), rat (55%) and zebrafish (64%), and contained two putative transmembrane regions (Fig. 1-2). In the tilapia ROMKa sequence, several consensus residues or domains of ROMK were found: the K^+ channel signature sequence "TXXTXGYG" in the pore region, the asparagine (N) residues corresponding to N_{171} of human ROMK, and arginine (R) residues corresponding to R_{188}, R_{203} and R_{217} of human ROMK. The amino acid sequences deduced from the partial cDNA fragments of Maxi-K (AB669174), KCC1 (AB669175), KCC2 (AB669176) and KCC4 (AB669177) shared identity with those from human: Maxi-K, 89%; KCC1, 82%; KCC2, 76%; KCC4, 51% (sequence data not shown).

Tissue distribution patterns of ROMKa, Maxi-K, KCC1, KCC2 and KCC4 mRNAs

The mRNA expression of ROMKa was found in many tissues of both SW- and FW-acclimated fish (Fig. 1-3). Among them, gills from SW fish showed distinct mRNA expression of ROMKa. The Maxi-K mRNA was detected in all tissues examined (Fig. 1-3). Broad expression patterns of KCC1 and KCC4 mRNA were observed, although the levels seemed to be low in the muscle (Fig. 1-3). The KCC2 mRNA was expressed exclusively in the brain (Fig. 1-3).

Plasma ion concentrations and osmolality

Plasma K^+ concentration was higher in H-K fish than in SW or M-K fish (Table 1-3). FW fish showed high K^+ levels compared to SW-acclimated fish (Table 1-3). In the fish exposed to M-K and H-K, plasma Na^+ concentration was significantly lower than that in SW fish (Table 1-3). Among the four groups, there was no significant difference in plasma Cl^- concentration nor osmolality (Table 1-3).

Quantitative PCR

The relative mRNA expression levels of ROMKa were significantly higher in H-K fish, marking over 5-fold increase compared to SW or M-K fish (Fig. 1-4A). Although there was no significant difference, mRNA levels of ROMKa in FW fish tended to be low, being approximately one-third of SW fish (Fig. 1-4A). There was no difference among the groups in the mRNA expression levels of Maxi-K α subunit and KCC1 (Figs. 1-4, B and C). In FW fish, KCC4 mRNA was higher than in SW and H-K, although there was no difference when compared to that in M-K (Fig. 1-4D). NKCC1a mRNA expression was 10-fold higher in SW than in FW, and was even higher in H-K fish than in SW fish (Fig. 1-4E).

Western-blotting

Western blot analysis detected three bands immunoreactive to the antibody against tilapia ROMKa in the sample only from SW-acclimated tilapia. Two bands were located at ~45 kDa, and one band was approximately at 90 kDa (Fig. 1-5).

Immunohistochemistry

In all fish examined, ionocytes were detected with FITC-labeled anti-NKA (Figs. 1-6, A-D and I-L). Whereas the signal of ROMKa was faint in FW fish (Figs. 1-6, E and I), ROMKa was detected at apical openings of ionocytes (Fig. 1-6M) in SW, M-K and H-K fish (Figs. 1-6, F-H and J-L). The signal in H-K fish was strongest in the four groups (Figs. 1-6, H and L).

Confirmation of potassium excretion through ROMKa

In the control, the precipitates of K-TPB appeared on the surface of the gills (Fig. 1-7A). The treatment with 5 mM Ba^{2+} extinguished the K-TPB precipitates (Fig. 1-7B), whereas 200 nM IBTX did not inhibit the formation of precipitates (Fig. 1-7C).

1-4. Discussion

In the present study, I could successfully visualize the excretion of K^+ from gill ionocytes, applying the chemical reaction that K^+ reacts with TPB to form insoluble precipitates of K-TPB. At the sites where precipitates were formed, intense signals of K, C and B were detected, suggesting that K-TPB was produced in these areas. On the other hand, although teleost fish excrete ammonia through the gills (Evans et al., 2005), there was no clear signal of N in the present study. This result possibly indicates that K^+ is excreted at a much higher rate than NH_4^+ under SW condition. However, the absence of N may not necessarily negate NH_4^+ secretion, since the rates of precipitate formation for K-TPB and NH_4-TPB may differ due to the characteristics of coupling ions.

Ho et al. (1993) were the first to examine the molecular features of ROMK, using an expression cloning technique and functional analyses. Among all K^+ channels expressed in the mammalian kidney, ROMK is best studied for its structure, localization and functions (Hebert et al., 2005). All ROMKs examined so far are a pore-forming homotetrameric channel, with weak inward rectification, ATP dependence, and Ba^{2+} sensitivity (Hebert et al., 2005; Welling and Ho, 2009). The deduced amino acid sequence of cDNA obtained in the present study shared several consensus amino acid residues with the known ROMK sequences.

Those were (in human): N_{171}, responsible for ROMK-type modest inward rectification (Wible et al., 1994); "TXXTXGYG" in the pore-forming region, the K^+ channel signature sequence (Heginbotham et al., 1994); and R_{188}, R_{203} and R_{217}, critical residues in ATP-binding segment (183-221 aa) (Dong et al., 2002). Although I did not assess the functions of these residues, the coherence among definitive residues, together with the sequence homology shared by ROMK from other species, indicates that the cDNA cloned in the present study encodes tilapia ROMKa.

Previous studies showed that several mRNA isoforms of ROMK were expressed predominantly in the kidney of both rat and human (Shuck et al., 1994; Yano et al., 1994; Zhou et al., 1994). In the present study using a euryhaline teleost species of Mozambique tilapia, mRNA expression of ROMKa was robust in the gills of SW fish, implying functional significance of the gills for K^+ excretion in SW fish. At the same time, other tissues also expressed ROMKa mRNA. Although I did not analyze the full-length sequences of ROMKa mRNAs expressed in the tissues other than the gills, it is possible that another ubiquitous ROMKa splicing variant, sharing most nucleotide sequences, is expressed in those tissues, as reported previously (Kondo et al., 1996). The broad mRNA expression patterns of Maxi-K α subunit, KCC1and KCC4, and brain specific expression of KCC2 are consistent with the previous findings (Hebert et al., 2005; Hoffmann et al., 2009; Berkefeld et al., 2010), suggesting conserved roles of these channels and transporters in a wide range of vertebrate species.

In this study exposing euryhaline tilapia to environments with various K^+ concentrations, tilapia could regulate plasma K^+ within a narrow range, although a slight effect of external K^+ was observed in H-K fish. Interestingly, a higher plasma K^+ level was detected in FW fish than in SW fish, implying existence of mechanisms for K^+ retention in FW fish. Also, reciprocal relationship between plasma K^+ and Na^+ in SW and H-K fish implies connections between the regulatory mechanisms of K^+ and Na^+. Further studies for K^+ retention mechanisms and for K^+/Na^+ relationship are required for a comprehensive understanding of K^+ metabolism in fish. Similar to the present study, rainbow trout (*Oncorhynchus mykiss*) maintained their plasma K^+ in a narrow range of approximately 4 mM, regardless of rearing condition of SW or FW (Shehadeh and Gordon, 1969). These facts

indicate the importance of strict regulation of K^+ in the body fluid.

Among the mRNA expression levels examined, ROMKa mRNA was highly responsive to the external K^+ level, suggesting its significance in K^+ regulation by the gills. Also, basolateral NKCC1a tended to be highly expressed in high K^+ environments. NKCC1a was expressed in the basolateral membrane of SW-type ion-secretory ionocytes (Hiroi et al., 2008; Inokuchi et al., 2008). Although K^+ enters into ionocytes primarily through basolateral NKA, the up-regulation of NKCC1a in tilapia acclimated to high K^+ conditions may contribute to additional K^+ entry, supporting K^+ excretion via apical K^+ channels or transporters. The mRNA expression levels of Maxi-K α-subunit and KCC1 were not influenced by external K^+ concentrations. In general, Maxi-K is widely expressed and involved in various body functions, such as controls of vascular tone and cell volume regulation (Berkefeld et al., 2010). KCCs also take several roles in cell physiology, represented by cell volume regulation (Hebert et al., 2005, Hoffmann et al., 2009). The present mRNA expression study suggests that branchial Maxi-K and KCC1 are not likely responsible for plasma K^+ regulation, but for some other functions described above. Several significant differences were detected in KCC4 mRNA levels among the four groups examined. However, mRNA levels showed no clear tendency linked to the environmental K^+ levels. In the human kidney, KCC4 is expressed in the basolateral membrane of α-intercalated cells and extrudes K^+ and Cl^-, which serves as a Cl^- recycling pathway for a proper function of basolateral anion exchanger 1 (Boettger et al., 2002). For a comprehensive understanding of the role of KCC4 in the gills, it will be necessary to study other branchial ion transport mechanisms yet to be identified.

The antibody raised against tilapia ROMKa detected two bands around ~45 kDa and one distinct band at the position of approximately 90 kDa in the SW gill sample, although the predicted molecular mass of tilapia ROMKa was 42.5 kDa. ROMK is known to be glycosylated and form homotetrameric channel (Shuck et al., 1994; Hebert et al., 2005). Similar to the previous ROMK western blot studies (Xu et al., 1997; Kohda et al., 1998; Spector et al., 2008), the two bands around 45 kDa may represent unglycosylated and glycosylated forms of ROMKa, whereas the band at 90 kDa might be dimeric ROMKa. All these bands were not detected in FW-acclimated tilapia gills, suggesting a limited amount of

ROMKa protein in gills of FW-acclimated tilapia.

In the gills of SW, M-K and H-K fish, the clear immunosignals for tilapia ROMKa were detected in the apical openings of ionocytes. Whereas ordinary cells possess ~140 mM K^+ in the cytoplasm (Their, 1986), ionocytes are likely to be more rich in K^+ due to active basolateral uptake of K^+ through NKA. Thus, the concentration gradient across the apical membrane of ionocytes is most likely to be in favor of K^+ excretion through ROMKa to the external environment. The strong ROMKa immunoreactivity of ionocytes in H-K fish indicates higher density of ROMKa in the apical openings of ionocytes, suggestive of elimination of excessive K^+ at a higher rate. To confirm the involvement of branchial ROMKa in K^+ excretion, I further examined the effects of putative ROMKa vs. Maxi-K channel blockers. My finding that the specific Maxi-K channel blocker (IBTX) did not reduce K-TPB deposition, but the specific ROMK channel blocker (Ba^{2+}) did, supports the conclusion that K^+ excretion occurs mainly through ROMKa.

Although molecular mechanisms for regulation of Na^+, Cl^- and other electrolytes have become increasingly clear in fish, less attention has been paid to K^+ handling. The present study is the first to demonstrate that fish excrete K^+ from gill MR cells through apically-expressed ROMKa, and that the expression levels of ROMKa respond to external K^+ concentration. In addition to diffusional gain of K^+, seawater fish actively absorb K^+ in the intestine (Hickman, 1968; Shehadeh and Gordon, 1969). Further studies addressing the influence of dietary K^+ intake will be necessary for a better understanding of fish ion homeostasis.

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

Primers	Sequence (5'-3')	Notes
ROMKa-dF	ACNACNATHGGNTAYGG	degenerate PCR; aa, "TTIGYG"
ROMKa-dR	RTCYTTNCCNGCRTCNAC	degenerate PCR; aa, "VDAGKD"
ROMKa-F	GATTAGCCCCAGAAACAACTATC	RT-PCR, qPCR, 3'-RACE
ROMKa-R	TGTCTCCCCATCTTGTGTG	RT-PCR, qPCR, 5'-RACE
Maxi-K-dF1	ACNCARCCNTTYGCNTGYG	degenerate PCR; aa, "TQPFACG"
Maxi-K-dR	CNCCRTARCANCCNCCRTC	degenerate PCR; aa, "DGGCYGD"
Maxi-K-dF2	SNATGGTNACNTTYTTYG	degenerate PCR; aa, "SMVTFFG"
Maxi-K-F	CACTCCTCATCCTGGGAAAC	RT-PCR, qPCR
Maxi-K-R	GGGCACCAGTGAAACATC	RT-PCR, qPCR
KCC1-dF1	ATGGGNTGGCCNTAYGG	degenerate PCR; aa, "MGWPYG"
KCC1-dR	TCRTCCATYTGNGCNAC	degenerate PCR; aa, "VAQMDD"
KCC1-dF2	TNGCNYTNTTYGARGARG	degenerate PCR; aa, "LALFEEE"
KCC1-F	CAGCCAGCTGTGGGATAAG	RT-PCR, qPCR
KCC1-R	TCTAATAATTCCGCTGCCAAG	RT-PCR, qPCR
KCC2-dF1	ATGTGYTAYATGTTYGTNG	degenerate PCR; aa, "MCYMFVN"
KCC2-dR	RAANCCYTTNACYTTYTC	degenerate PCR; aa, "EKVKGF"
KCC2-dF2	GARTTYGGNGGNGCNGTNG	degenerate PCR; aa, "EFGGAVG"
KCC2-F	ACACTGCTGAGGACACCAAAC	RT-PCR, qPCR
KCC2-R	AGACAGACCCCGTATTCCATC	RT-PCR, qPCR
KCC4-dF	TTYGARCCNCCNGAYTTYC	degenerate PCR; aa, "FEPPDFP"
KCC4-dR	TTNACNACRTANGGCAT	degenerate PCR; aa, "MPYVVN"
KCC4-F	CAATGCCACATGCAACGAG	RT-PCR, qPCR
KCC4-R	TTTCCACCAACATACCGAGAG	RT-PCR, qPCR
NKCC1a-F	GGAGGCAAGATCAACAGGATTG	qPCR, Hiroi et al. (2008)
NKCC1a-R	AATGTCCGAAAAGTCTATCCTGAACT	qPCR, Hiroi et al. (2008)
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; S, C/G. Corresponding amino acid (aa) residues of degenerate primers are written in the notes.

Table 1-2. *Ion concentrations (mM) and pH of 4 experimental media*

	$[Na^+]$	$[K^+]$	$[Cl^-]$	$[Ca^{2+}]$	$[Mg^{2+}]$	pH
FW	0.748	0.0747	0.523	0.608	0.211	7.61
SW	470	7.95	541	9.90	52.3	7.70
M-K	457	7.83	532	9.83	51.5	7.65
H-K	439	17.1	521	9.55	50.6	7.67

Table 1-3. *Plasma ion concentrations (mM) and osmolality (mOsm/kg H_2O) in tilapia acclimated to 4 experimental media*

	$[K^+]$	$[Na^+]$	$[Cl^-]$	Osmolality
FW	3.83 ± 0.2^{ab}	164 ± 2^{ab}	144 ± 1	313 ± 4
SW	2.77 ± 0.08^{c}	167 ± 3^{a}	145 ± 3	323 ± 3
M-K	2.86 ± 0.13^{ac}	156 ± 3^{b}	141 ± 2	320 ± 3
H-K	4.42 ± 0.53^{b}	157 ± 3^{b}	141 ± 1	325 ± 4

Different letters indicate significant difference at $P<0.05$.

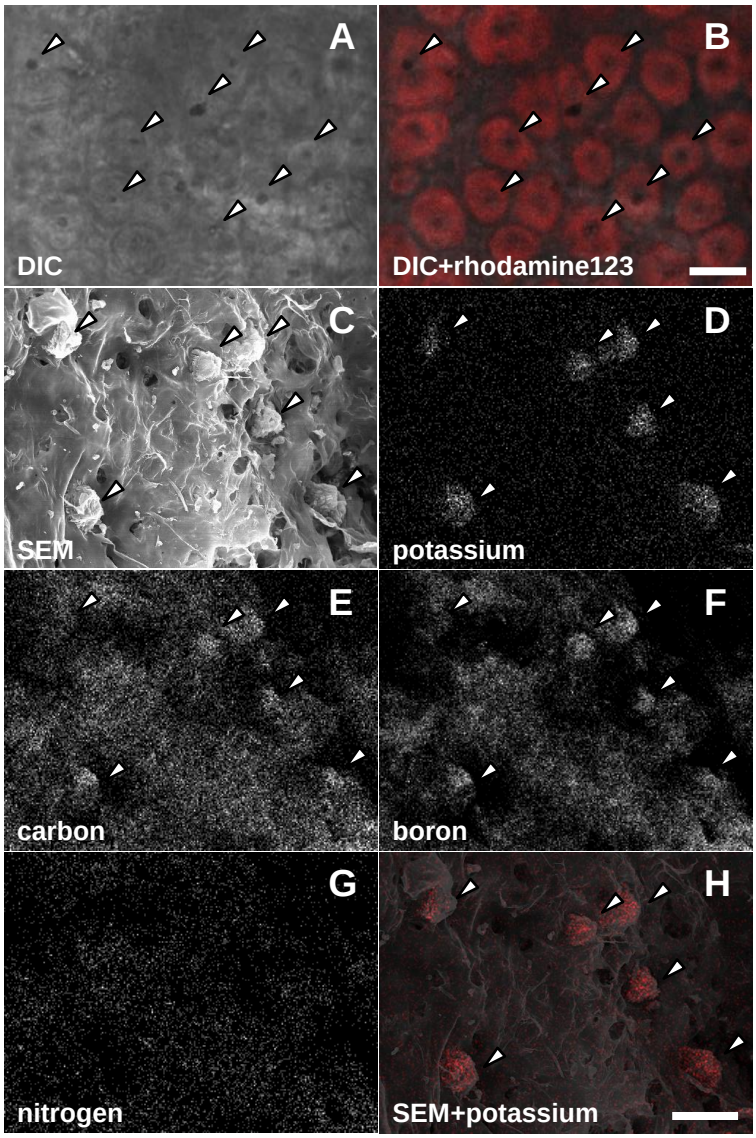


Fig. 1-1. A transmitted differential interference contrast (DIC) image of the precipitate above the gills (**A**) and the DIC image merged with a panfocal fluorescent micrograph showing ionocytes (red) in the same visual field (**B**). A scanning electron micrograph (SEM, **C**) and mapping images of potassium (**D**), carbon (**E**), boron (**F**) and nitrogen (**G**) obtained by energy dispersive X-ray spectrometry (EDS) analysis. In **H**, SEM image and EDS signals of K (red) are merged. Arrowheads indicate the positions where intense EDS signals of K were observed on the precipitates. Bars, 20 μm.

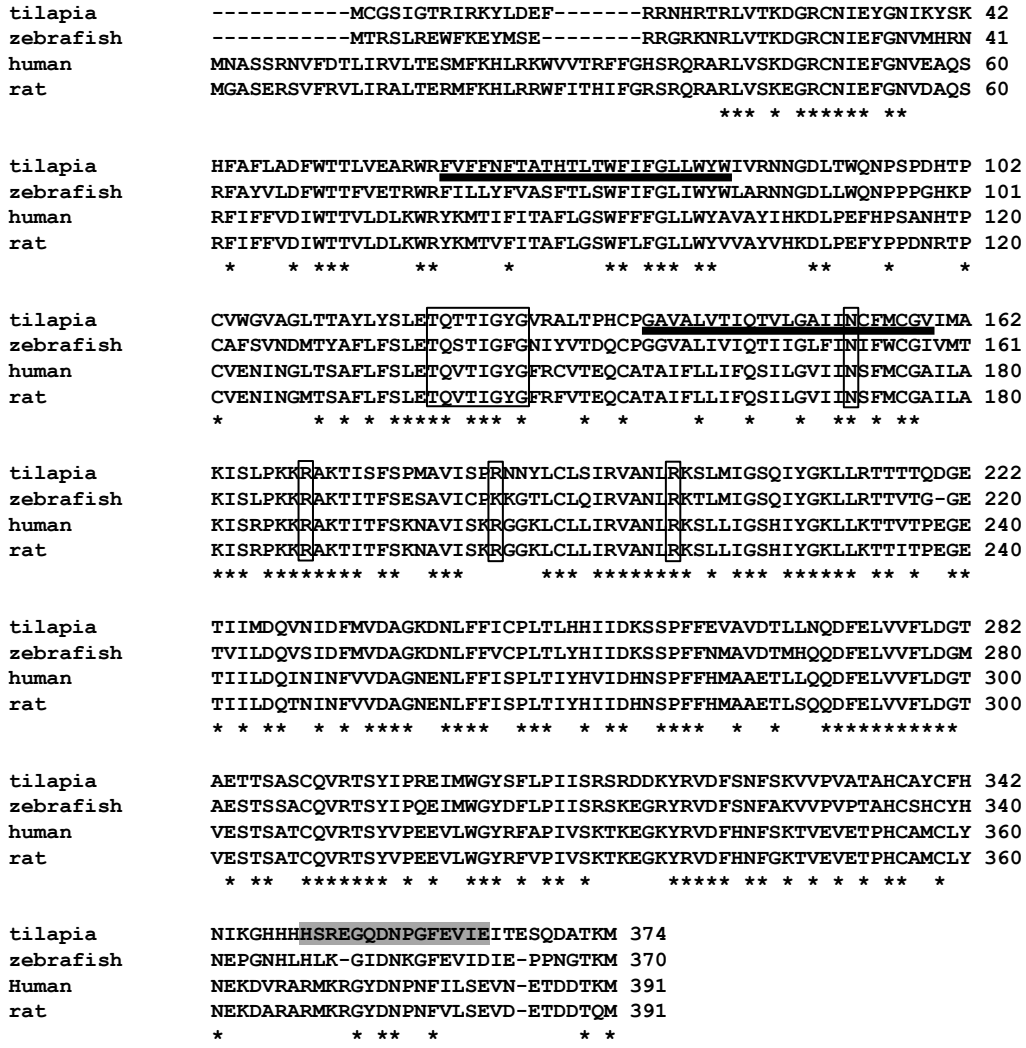


Fig. 1-2. Deduced tilapia ROMKa sequence (**AB669173**) was aligned with ROMKs of zebrafish (**AAH055133.1**), human (**AAA61712.1**) and rat (**CAA51068.1**). Bars indicate 2 putative transmembrane regions of tilapia ROMKa, and the shaded sequence indicates the peptide used to raise the antiserum against tilapia ROMKa. The boxed sequence and residues indicate the K^+ channel signature sequence "TXXTXGYG" and the consensus residues, N_{153}, R_{170}, R_{185} and R_{199}, respectively.

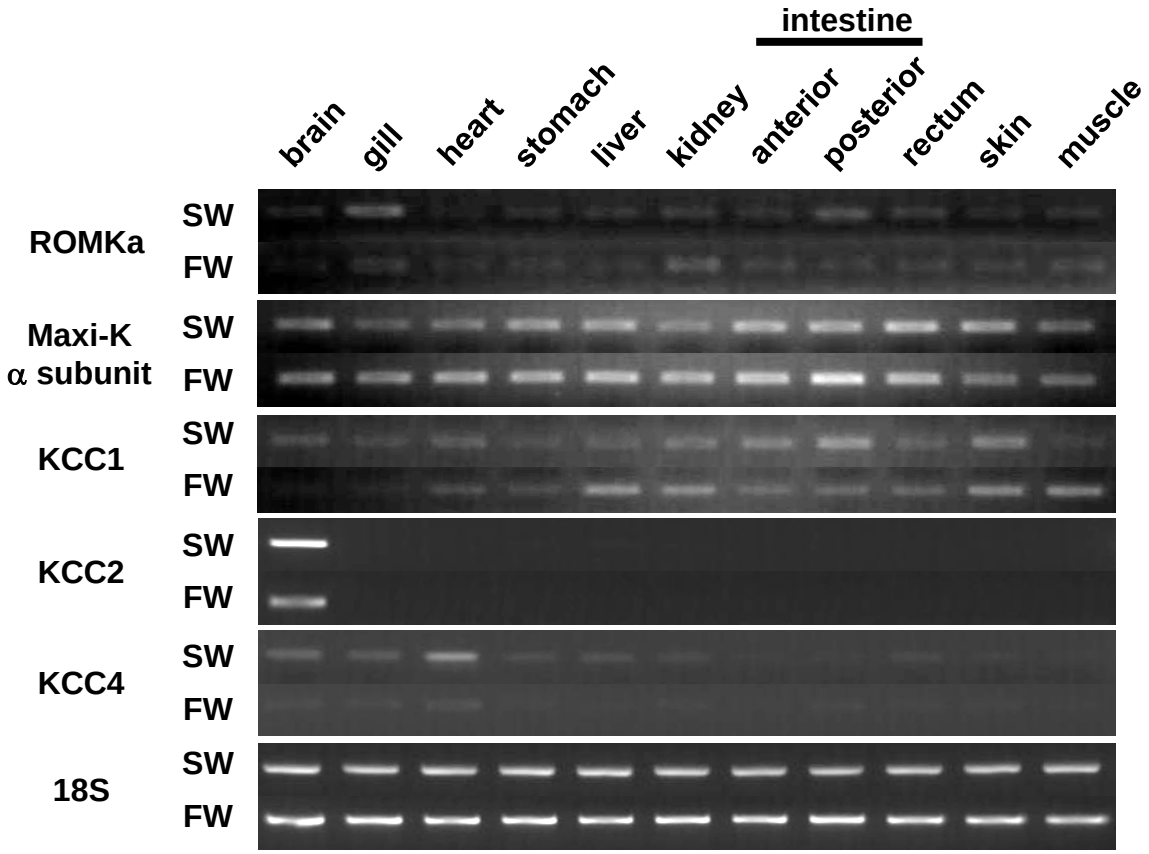


Fig. 1-3. Tissue distribution analysis of tilapia ROMKa, Maxi-K α subunit, KCC1, KCC2 and KCC4 in tilapia acclimated to seawater (SW) or freshwater (FW). 18S, internal control.

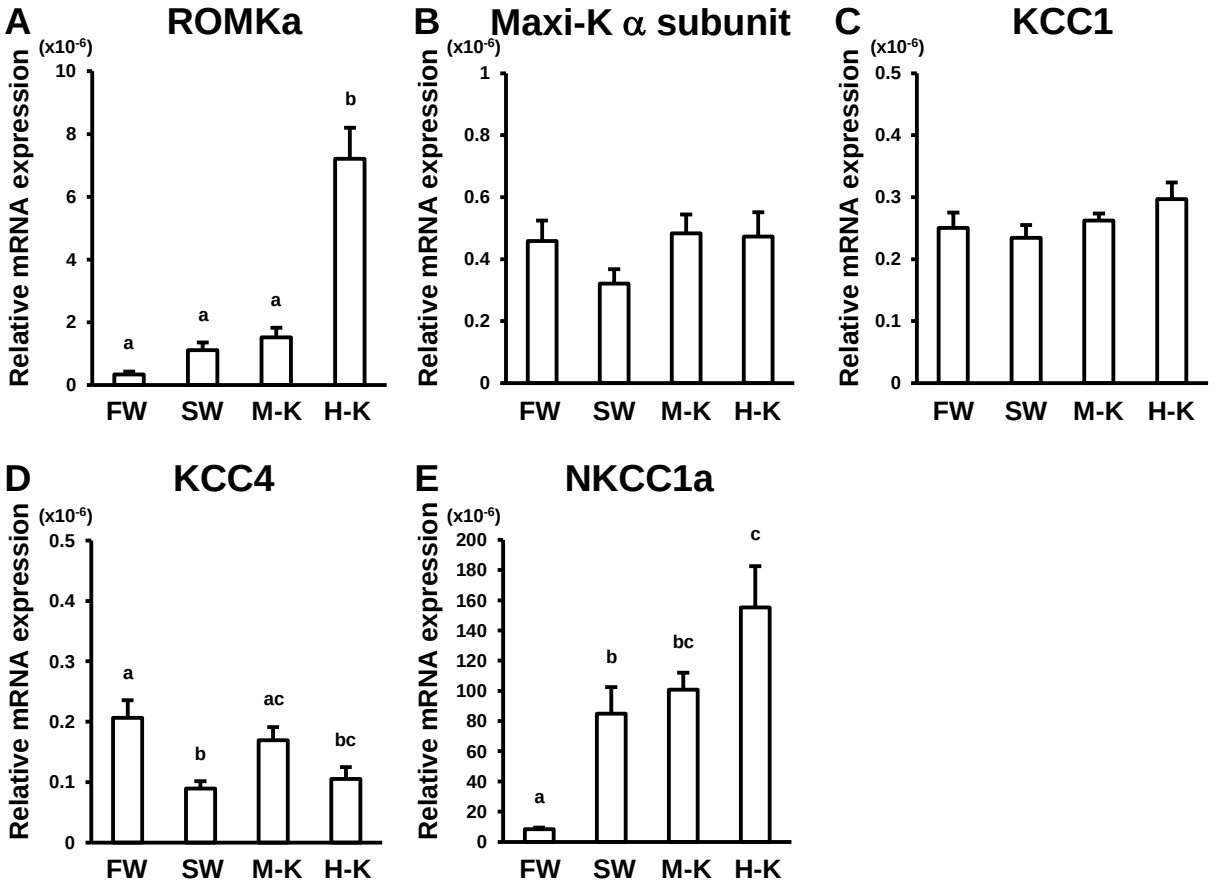


Fig. 1-4. Relative mRNA expression levels (mRNA/18S rRNA) of ROMKa, Maxi-K α subunit, KCC1, KCC4 and NKCC1a in the gills of tilapia acclimated to freshwater (FW), seawater (SW), artificial seawater with moderate (M-K) and high (H-K) K^+ concentrations. Data are expressed as means ± S.E.M. (n=6). Different letters indicate significance of differences at $P<0.05$.

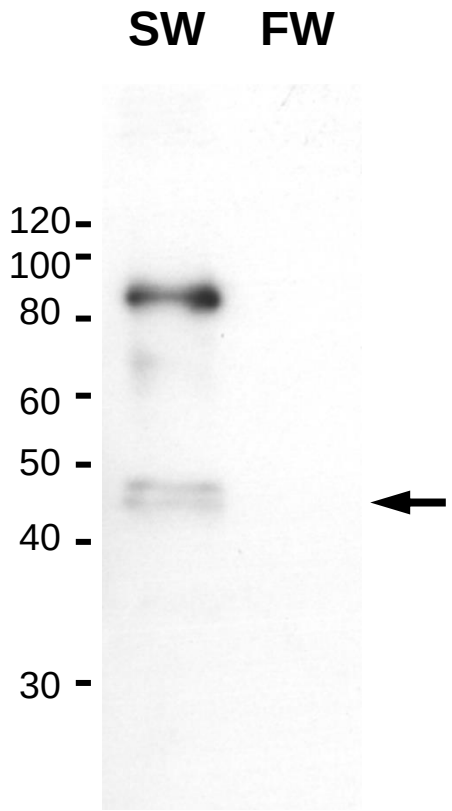


Fig. 1-5. The immunoreactive bands obtained in western blot analysis for ROMKa expressed in the gills. The two bands around ~45 kDa may represent unglycosylated and glycosylated forms of ROMKa, while 90 kDa band may represent a homodimer of ROMKa protein. The arrow indicates the expected molecular mass of tilapia ROMKa.

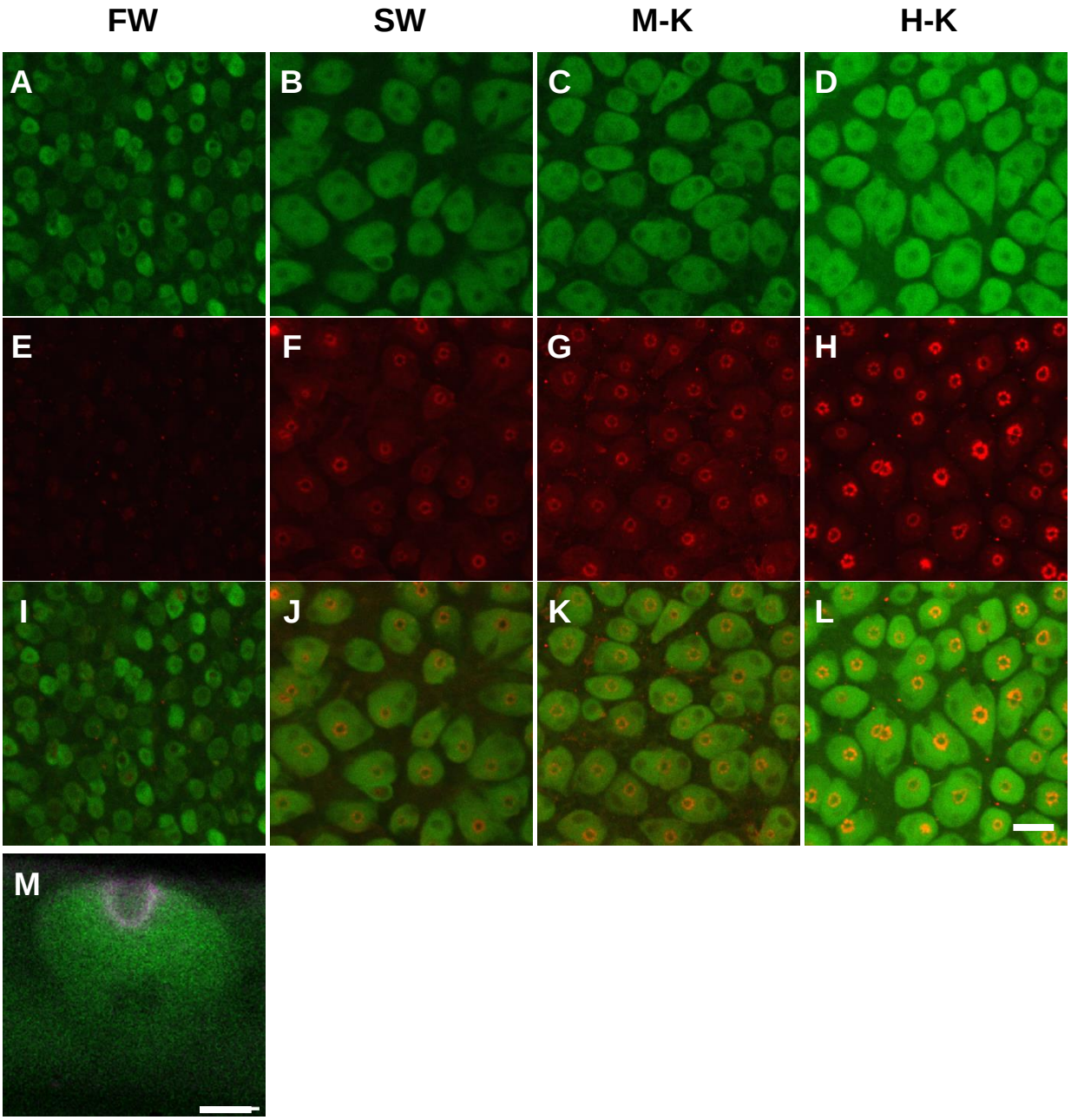


Fig. 1-6. Immunofluorescence micrographs of the gills in tilapia, stained with anti-Na^+/K^+-ATPase (NKA, green) and anti-ROMKa (red). Overhead views of the gills in tilapia acclimated to freshwater (**A**, **E**, **I**), seawater (**B**, **F**, **J**), artificial seawater with moderate K^+ concentration (**C**, **G**, **K**) and high K^+ concentration (**D**, **H**, **L**). Immunoreactions for NKA (**A**-**D**), ROMK (**E**-**H**), and merged images (**I**-**L**). Bar, 20 μm. Magnified lateral view of a representative ionocyte expressing ROMK (magenta, **M**). Bar, 5 μm.

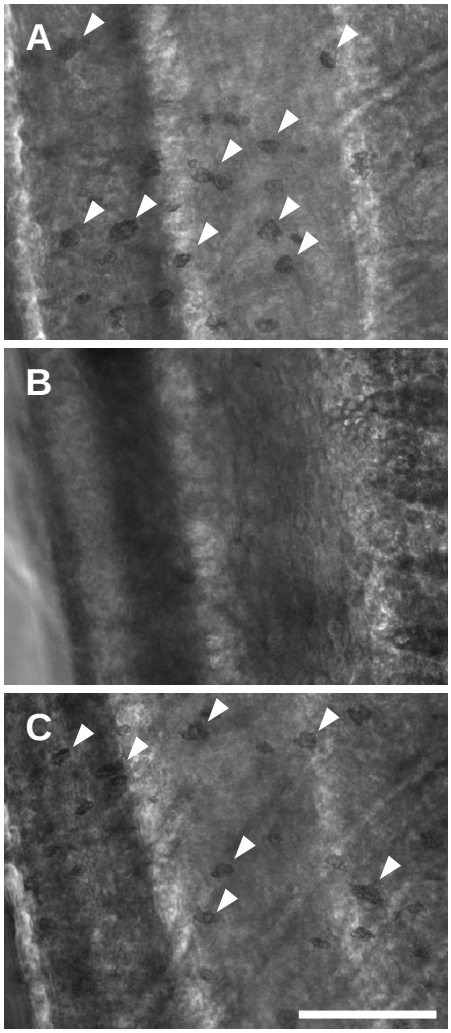


Fig. 1-7. The surface of the tilapia gills after exposure to control tetraphenylborate (TPB) solution (**A**), TPB with Ba^{2+} (**B**), and TPB with iberiotoxin (**C**) for 1 min. Arrowheads indicate the precipitations emerging on the gill surface. Afferent vesicular sides of the gill filaments to the left. Bar, 100 μm.

2. Excretion of cesium and rubidium via the branchial potassium-transporting pathway in Mozambique tilapia

2-1. Introduction

In the intestine of marine teleosts, Na^+, Cl^- and K^+ are actively absorbed from the ingested SW as a part of osmoregulatory water absorption (Marshall and Grosell, 2006). Therefore, fish adapted to SW should cope not only with excess Na^+ and Cl^- but also with chronic K^+ overload. In mammalian species, excess K^+ is excreted by producing urine containing high concentration of K^+ (Wang and Giebisch, 2009). In marine teleosts, on the other hand, urinary K^+ excretion is less likely because they produce a small amount of urine containing low concentration of K^+ (Marshall and Grosell, 2006). In Chapter 1, I found that K^+ is excreted via gill ionocytes in Mozambique tilapia, and that ROMKa located in the apical membrane of ionocytes is the key molecule for the K^+-excreting mechanism.

Small amounts of rubidium (Rb^+) and cesium (Cs^+) ions, being generally considered as biochemical analogs of K^+ (Relman, 1956), are detectable in both fishes and normal SW, although their concentrations are much lower than that of K^+ (Bolter et al., 1964; Ribeiro Guevara et al., 2006). The distributions of Rb^+ and Cs^+ have also been studied in fish; those ions are mainly located in the muscle (Peters et al., 2006). Whereas these trace elements seem to have little impact on physiological events of marine teleosts, it has been reported that sub-millimolar concentration of Rb^+ could suppress spermatogenesis of Japanese eel *Anguilla japonica in vitro* (Yamaguchi et al., 2007). Although the elimination route of Rb^+ and Cs^+ remains unclear, the K^+-excreting pathway in the branchial epithelium is a strong candidate for the elimination mechanism of those ions.

In my previous study, excreted K^+ was insolubilized and visualized on the surface of the gills by a chemical reaction with Na-TPB, and the site of K^+ excretion was determined in isolated gills. In the present study, using this insolubilization technique in combination with element analysis, we investigated the possibility of excretion of Rb^+ and Cs^+ from gill ionocytes in Mozambique tilapia acclimated to SW.

2-2. Materials and methods

Experimental animals

Mozambique tilapia were maintained for more than 6 months in tanks supplied with recirculating SW at 25°C. The fish were fed on commercial pellets (Tilapia 41M; JA Nishi-Nihon Kumiai Shiryo) once a day. The fish (100-150 g) were anesthetized with 0.1% 2-phenoxyethanol before removal of the gills.

Detection of Rb^+ and Cs^+ excreted from the gills

To detect Rb^+ and Cs^+ excreted from the gills, we applied the K^+ insolubilization technique (Chapter 1 of this thesis) with some modifications, as Na-TPB reacts not only with K^+ but also with Rb^+ and Cs^+ to form insoluble precipitates (Pflaum and Howick, 1956). A gill arch of SW-acclimated tilapia was cut at the ventral end, and infused intra-arterially for over 1 min with 1 ml of balanced salt solution (BSS, in mM; 140 Na^+, 146 Cl^-, 2.95 K^+, 1.5 Ca^{2+}, 1.29 Mg^{2+}, 5 HEPES; pH 7.5), whose K^+ was replaced with the same amount of Rb^+ (Rb-BSS) or Cs^+ (Cs-BSS). After replacement of blood with Rb-BSS or Cs-BSS, the gills were dissected out and incubated in BSS for 10 min to allow infused Rb^+ or Cs^+ to spread. Without the incubation after infusion of Rb-BSS or Cs-BSS, neither Rb^+ nor Cs^+ was detected in the following analyses (data not shown). After washing procedures to remove mucus and K^+ remaining on the branchial surface, the gills were incubated in TPB solution (Chapter 1) for 30 sec, and then fixed in 4% PFA in 0.1 M PB (pH 7.4) for 3 h. After washing in distilled water, the gills were snap-frozen in liquid nitrogen, and lyophilized in a freeze-drying device (Neocool; Yamato Scientific). The dried samples were mounted on specimen stubs and coated with gold in an ion coater (IB-3; Eiko Engineering).

The TPB-treated gill filaments were analyzed with a SEM (S-4800; Hitachi) that was equipped with an EDS system (Emax Energy; Horiba). The existence of K, Rb and Cs in the precipitates was assessed by spectrum analysis and line-scanning analysis of the EDS. In the spectrum analysis, characteristic X-ray energy patterns were obtained from designated sample areas. In the line-scanning analysis, the distribution pattern of the X-ray characteristic of each element (K, 3.31 keV; Rb, 1.69 keV; Cs, 4.29 keV) was assessed along the line across a lump of the precipitates.

To simultaneously detect ion excretion and ionocytes, Rb-BSS or Cs-BSS containing 50 μM rhodamine 123 (a fluorescent vital staining dye for mitochondria) was infused into some gills. After the gills were incubated in TPB solution and fixed in 4% PFA as described above, the samples were examined under a confocal laser scanning microscope (C1; Nikon). The wavelengths of excitation and recorded emission for rhodamine 123 were 543 nm and 572.5-637.5 nm, respectively.

2-3. Results

After treatment with TPB solution, the precipitates were observed as black particles on the openings of ionocytes (Fig 2-1), in a similar fashion to my previous observations (Chapter 1). Precipitate areas (Figs. 2-1, A and E) and those of the precipitate-free areas (Figs. 2-2, C and G) on the gill surface were subjected to EDS analysis to obtain the X-ray energy spectra. At the precipitate area of Rb-BSS-infused gills, a peak of X-ray energy characteristic of Rb was detected (Figs. 2-2, A and B). Similarly, the X-ray energy peaks of Cs were detected at the precipitate area of Cs-BSS-infused gills (Figs. 2-2, E and F). Those precipitates also showed a sharp X-ray energy peak characteristic of K (Figs. 2-2, B and F). On the other hand, there were no clear X-ray energy peaks of K, Rb and Cs at the areas that were devoid of the precipitates, irrespective of samples infused with Rb-BSS (Figs. 2-2, C and D) or with Cs-BSS (Figs. 2-2, G and H).

The distribution patterns of K, Rb and Cs on the lines across the precipitates were examined by the line-scanning analysis of the EDS (Fig. 2-3). In Rb-BSS-infused gills, there was a clear correspondence of distribution patterns between K and Rb, and both elements were specifically detected on the precipitate (Figs. 2-3, A, C, and E). Similarly, the characteristic X-rays of K and Cs were specifically detected on the precipitate in Cs-BSS-infused gills (Figs. 2-3, B, D, and F), although the background signals of Cs appeared stronger than those of K and Rb.

2-4. Discussion

In this chapter, I verified the hypothesis that Cs^+ and Rb^+, the biochemical analogs of K^+, were eliminated via the branchial K^+-excreting pathway in SW-acclimated tilapia. In the

previous studies with radioactive ^{137}Cs, the excretion of Cs from rainbow trout was increased by loading of K^+ to environmental FW, and the biological half-life of radioactive Cs in euryhaline Japanese seabass (*Lateolabrax japonicus*) was lengthened as environmental salinity decreased (Kasamatsu, 1999). These findings strongly suggest that the metabolism of Cs^+ is closely associated with that of K^+ in teleosts. In the present study, I confirmed the formation of the precipitates on the surface of the gill filaments in SW tilapia infused intra-arterially with Cs^+ and Rb^+. The EDS analysis clearly showed that K, Cs and Rb were distributed specifically at the area of the formed precipitates. These results suggest that Cs^+ and Rb^+ were excreted via the K^+-transporting pathway in the gill ionocytes, and that non-specific leakage, which serves as an indicator of epithelial cell collapse, did not occur in the Cs^+ and Rb^+-treated gills in agreement with the previous chapter. It is known that Cs^+ and Rb^+ have little impact on biological activities (Relman, 1956). Actually, the branchial K^+-excreting mechanism was not suppressed by these elements, as K was also contained in the precipitates formed on the surface of the Cs^+- and Rb^+-infused gills.

In the present study, excretion of Cs^+ and Rb^+ through the transcellular pathway of ionocytes was detected 10 min after infusion, whereas these elements were not detected in the precipitates formed just after infusion. To excrete intra-arterially infused Cs^+ and Rb^+ via the K^+-transporting pathway in the gills, these ions should first be moved into branchial ionocytes across the basolateral cell membrane, and then excreted to the external environment via apically located ROMKa channel. At the time just after infusion, it is most likely that Cs^+ and Rb^+ has not yet transported into ionocytes, and that only preexisting intracellular K^+ may be excreted through the apical membrane of ionocytes. According to the report by Burrows and Lamb (1962), entire intracellular K^+ in the cardiac muscle cells of chick embryos was exchanged in 75 min, implying that majority of administrated Cs^+ and Rb^+ remained in the extracellular fluid of the isolated gills even 10 min after infusion, although the K^+ turnover rate in branchial ionocytes is unknown. This may explain the less intense characteristic X-rays for Cs and Rb compared with those of K.

My findings clearly showed the branchial Cs^+- and Rb^+-eliminating pathway via the ROMKa-based K^+-excreting mechanism of ionocytes in SW-acclimated tilapia. The fallout of radioactive Cs caused by the recent nuclear disaster at the Fukushima Daiichi Nuclear

Power Plant in Japan is one of the most serious environmental issues to be solved promptly. The present study provides significant information about the possible eliminating route of Cs in marine teleosts, which is definitely important in a better understanding of the metabolism of radioactive Cs in teleosts. The excretion of the biochemical analogs of K^+ reported in this study is most likely to account for the shorter biological half-life of ^{137}Cs in marine teleosts, as compared with FW ones (Kasamatsu, 1999). In euryhaline tilapia, ROMKa is expressed in the gills not only in SW but also in FW, although its expression levels are lower in FW than in SW (Chapter 1), suggesting the possibility that FW teleosts also possess the ability to excrete Cs^+ through ionocytes. The activation of ROMKa in ionocytes in combination with supplementation of K^+ could more rapidly eliminate unnecessary biochemical analogs of K^+, serving as an effective countermeasure against radioactive Cs in aquaculture.

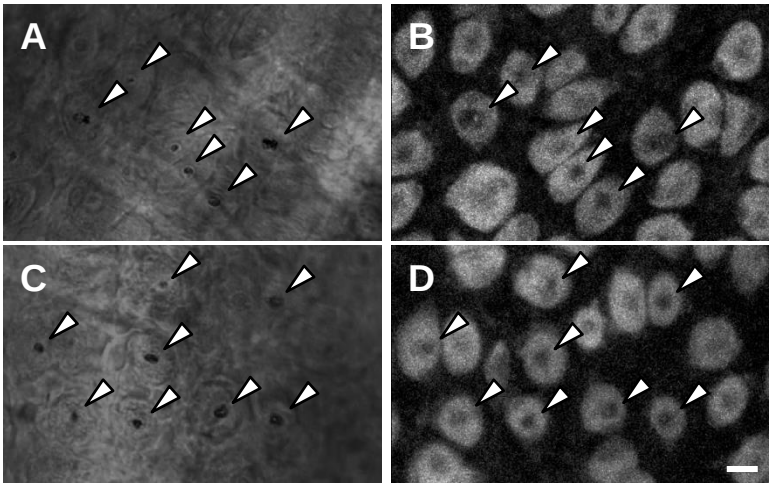


Fig. 2-1. Light microscopic images (**A, C**) and the corresponding fluorescent images (**B**, **D**) of the gills infused with Rb^+ (**A**, **B**) and Cs^+ (**C**, **D**), observed by confocal laser scanning microscopy. The precipitates (arrowheads) are seen on mitochondrion-rich cells stained with rhodamine123 (**B**, **D**) in the gill epithelia. Bar, 10 μm.

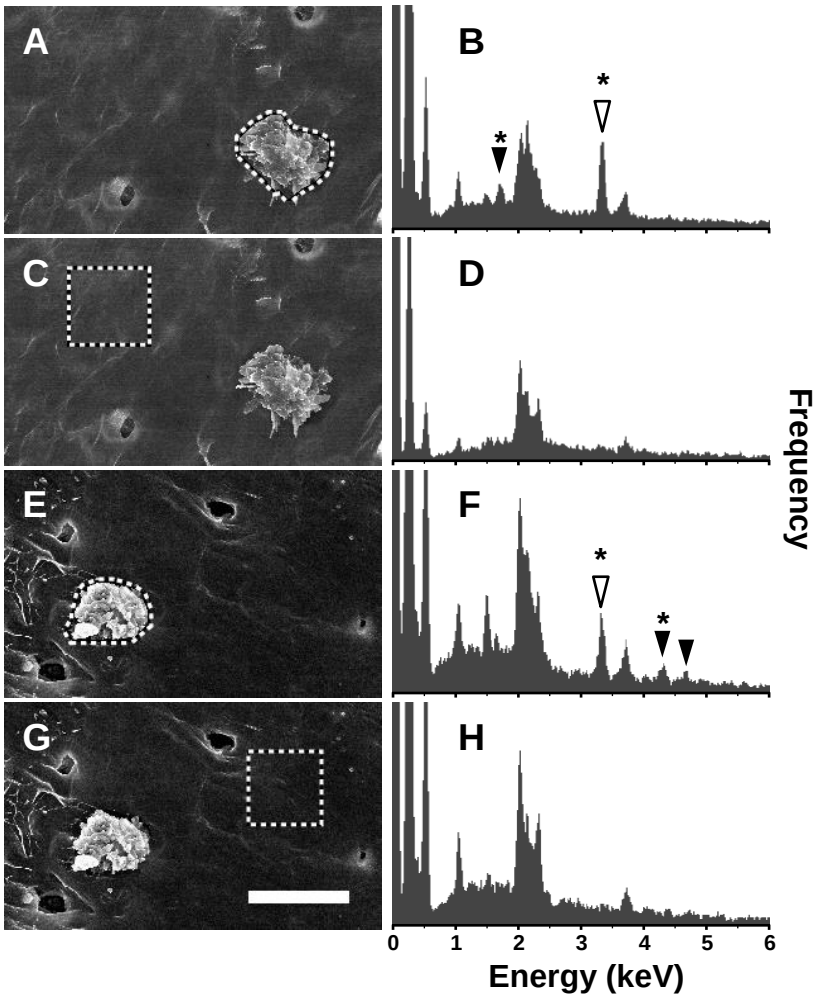


Fig. 2-2. Scanning electron micrographs (**A**, **C**, **E**, **G**), and energy-dispersive X-ray spectrometry (EDS) profiles (**B**, **D**, **F**, **H**) of gills infused with Rb^+ (**A**-**D**) and Cs^+ (**E**-**H**). The amorphous and square lines (**A**, **C**, **E**, **G**) indicate the areas subjected to EDS analysis (**B**, **D**, **F**, **H**). White arrowheads indicate the representative X-ray energy peak of K (a combined peak of $K\alpha_1$ and $K\alpha_2$, 3.31 keV). A black arrowhead in the panel **B** indicates an energy peak of Rb ($L\alpha_1$, 1.69 keV), and black arrowheads in the panel **F** indicate those of Cs ($L\alpha_1$, 4.29 keV; $L\beta_1$, 4.62 keV). Asterisks indicate the energy levels counted in the line-scanning analysis in Fig. 2-3. Bar, 10 μm.

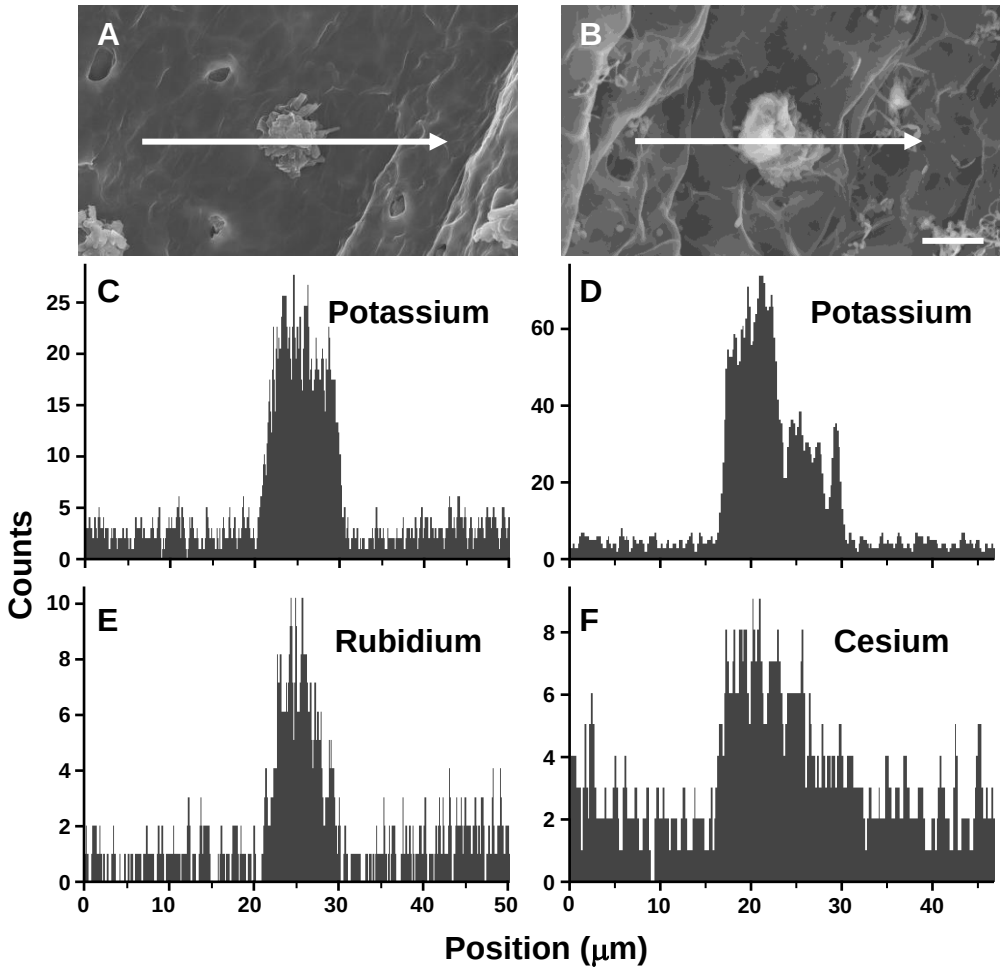


Fig. 3. Line-scanning element analyses of gills infused with Rb^+ (**A**, **C**, **E**) and Cs^+ (**B**, **D**, **F**). Line-scanning analyses were performed along *arrows* crossing precipitates in panels **A** and **B** at 3.31 keV for K (**C**, **D**), 1.69 keV for Rb (**E**), and 4.29 keV for Cs (**F**). Bar,10 μm

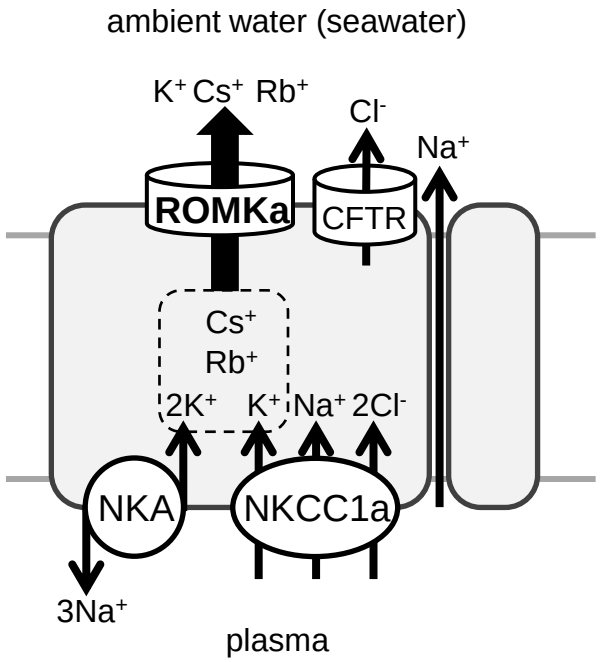


Fig. 2-4. A schematic model showing a possible Rb^+- and Cs^+-excretory mechanism in ionocytes of SW- adapted Mozambique tilapia. Rb^+ and Cs^+ are possibly first absorbed by basolateral NKA and NKCC1a, and then excreted via apical ROMKa. The model was constructed in reference to Hiroi et al. (2008) and my previous observation (Chapter 1).

3. Expression of ROMKa in gill ionocytes of freshwater-acclimated tilapia

3-1. Introduction

Inhabiting dilute aquatic environments, FW fish face diffusional ion loss and water load. To overcome these osmotic problems, FW fish usually absorb ions in the gills, while discharging excess water by producing dilute urine in the kidney. The mechanisms of active ion absorption in the gill ionocytes have become increasingly clear for the two major ions in plasma, Na^+ and Cl^- (Hwang et al., 2011). On the other hand, for K^+, the most abundant cation in the body, it is unknown how FW fish maintain plasma levels of this ion, except that FW fish reduce diffusional loss of K^+ through the integument (Eddy, 1985). It appears still important for FW fish to have K^+ excretory mechanisms, in preparation for risks of temporal plasma K^+ overload, such as eating K^+-rich food, metabolic acidosis, and tissue breakdown (Weiner and Wingo, 1998; Clausen, 2010). Whereas it became clear that SW-acclimated tilapia excrete K^+ from gill ionocytes (Chapter 1), the mechanisms of K^+ regulation in FW fish need further investigation, since the gill ionocytes in FW fish are different from those in SW fish. To cope with this question, I assessed the physiological responses of FW fish to high-K^+ (H-K) FW environment. Since FW fish excrete considerable amount of urine for osmoregulation, I focused on the kidney as well as the gills. As possible candidate of K^+ handling in the kidney, a putative paralogous product of ROMKa was cloned, and tentatively named “ROMKb”. After 1-week exposure to normal or (H-K) FW, mRNA levels of the molecular candidates of K^+ handling were examined in the gill and kidney. The results showed that FW tilapia strongly expressed ROMKa in the gills when they were exposed to high K^+ concentration. Also, immunofluorescence staining showed that branchial ROMKa was expressed at the apical opening of type-III ionocytes. These results suggested that freshwater fish could excrete K^+ from the gill ionocytes in response to H-K conditions.

3-2. Materials and Methods

Experimental animals

Mozambique tilapia were maintained in a stock tank supplied with recirculating FW at 25°C. Fish were fed on commercial tilapia pellets “Tilapia 41M” (Nishi-Nihon Kumiai

Shiryo) once a day. The fish were anesthetized with 0.1% 2-phenoxyethanol before sampling of tissues.

Molecular cloning of ROMKb

A cDNA fragment encoding a putative paralogous gene product of ROMKa, ROMKb, was cloned from the tilapia kidney. The kidney was removed from FW-acclimated tilapia, and total RNA was extracted from the kidney with Isogen (Nippon Gene). First-strand cDNA was synthesized from the total RNA with SuperScript III (Life Technologies), and a cDNA fragment of ROMKb was amplified by degenerate PCR with the primers used to clone ROMKa from tilapia gills (Chapter 1). The PCR products were ligated into pGEM T-Easy vector (Promega) and sequenced using an ABI Prism 310 sequencer (Life Technologies). A phylogenetic tree was constructed for amino acid sequences of tilapia ROMKa and ROMKb, along with those of human inwardly rectifying K^+ (Kir) channel subfamilies, which include human ROMK (Kir1.1), zebrafish homolog of Kir1.1 (Kcnj1; Abbas et al., 2011), and eel Kir (eKir, Suzuki et al., 1999), using CLUSTAL W program and neighbor-joining method, in reference to Hibino et al. (2010). The GenBank accession numbers are as follows: ROMKa, AB669173; ROMKb, AB771746; Kcnj1, AAH055133.1; eKir, BAA76936.1; Kir1.1, NP_000211; Kir2.1, NP_000882; Kir2.2, NP_066292; Kir2.3, CAG30393; Kir2.4, AAF97619; Kir3.1, NP_002230; hKir3.2, NP_00223; Kir3.3, AAF89098; Kir3.4, AAH69571; Kir4.1, AAI31628; Kir4.2, NP_733933; Kir5.1, NP_733937; Kir6.1, NP_004973; Kir6.2, NP_000516; Kir7.1, NP_002233. For more detailed insight into phylogenetic relationship among ROMKs of various species, another phylogenetic tree was constructed with amino acid sequences of sea lamprey (*Petromyzon marinus*), zebrafish, Atlantic cod (*Gadus morhua*), Japanese medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), Nile tilapia (*Oreochromis niloticus*), pufferfish (*Takifugu rubripes*), xenopus (*Xenopus tropicalis*), rat (GenBank accession no. CAA51068.1), and human, with human Kir2.1 and Kir2.4 assigned to the outgroup. The amino acid sequences of putative ROMK homologs are obtained from Ensembl database (http://www.ensembl.org/index.html).

Tissue distribution analysis of ROMKb mRNA

The tissue distribution pattern of ROMKb mRNA expression in tilapia was analyzed by RT-PCR, using the first-strand cDNA obtained from the brain, gill, heart, stomach, liver, kidney, anterior and posterior intestines, rectum, skin and muscle. Tissues from both FW- and SW-acclimated tilapia were analyzed. The cDNA fragments were amplified with primers listed in Table 3-1. The PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide.

Acclimation to H-K FW

Freshwater-acclimated tilapia (93-180 g) were transferred to a 100-l tank, containing either control FW (in mM; 0.84 Na^+, 0.10 K^+, 0.75 Ca^{2+}, 0.25 Mg^{2+}, 0.62 Cl^-; pH=7.6) or H-K FW (in mM; 0.79 Na^+, 10 K^+, 0.77 Ca^{2+}, 0.24 Mg^{2+}, 11 Cl^-; pH=7.3), and acclimated for 1 week. Both groups consisted of 6 individuals. From 2 days prior to the experiment, the fish were not fed to avoid any effect of electrolytes in the feed. Concentrations of Na^+, Cl^-, K^+, and Ca^{2+} in experimental media were determined with an ion analyzer (IA-200; TOA-DKK) and a digital chloridometer (Labconco) after 1-week acclimation.

Plasma and urine ion concentrations and plasma osmolality

Plasma and urine concentrations of Na^+, K^+ and Cl^-, and plasma osmolality were measured in tilapia acclimated to experimental conditions. After anesthetizing the fish, blood was collected from the caudal vessels with a heparinized syringe and needle, and the urine was taken from the urinary bladder with a syringe and needle. Blood plasma was separated by centrifugation at 5,000 g for 10 min and stored at -20°C. Concentrations of Na^+ and K^+ in plasma and urine, and Cl^- in the urine were measured with the IA-200 ion analyzer, plasma Cl^- concentration with the digital chloridometer, and plasma osmolality with a vapor pressure osmometer (Vapro 5520; Wescor).

qPCR

The mRNA levels of several ion transporters or channels were assessed: ROMKa, ROMKb (AB771746), Maxi-K α subunit, KCC1, KCC4, NHE3 (AB326212), NKAα1a (GR645170 and GR644771), and NKAα1b (U82549) in the gills and kidney; NCC

(EU518934) and NKCC1a in the gills; and NKCC2 (AY513739) in the kidney. The expression levels of those ion transporters or channels were determined by qPCR with a LightCycler® 480 system II (Roche diagnostics) using LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche diagnostics). The total RNA was extracted from the gills and kidney of experimental fish with Isogen (Nippon Gene), and reverse-transcribed with SuperScript III (Life Technologies). The copy numbers of the transcripts were calculated with reference to parallel amplifications of known concentrations of the respective cloned PCR fragments. The data were normalized to the corresponding 18S rRNA levels. For ROMKa, Maxi-K α subunit, KCC1, KCC4, and NKCC1a, the primers used are the same as Chapter 1. The other primers used are listed in Table 3-1.

Triple-color immunofluorescence staining

The second gill arches were dissected out from FW tilapia, fixed in 4% PFA in 0.1 M PB (pH 7.4) for 24 h at 4°C, and stored in 70% ethanol. After a brief wash in 0.01 M PBST, the gill filaments were incubated in NB-PBS for 30 min to block nonspecific binding sites. First, the filaments were stained for ROMKa, NCC, and NKA. The filaments were incubated in NB-PBS containing T4 (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-NKA (NAK121) both diluted 1:500 overnight at 4°C. After a brief wash in PBST, the gills were incubated overnight at 4°C with goat anti-mouse IgG labeled with Alexa Flour 405 (Life Technologies) and goat anti-rabbit igG labeled with Alexa Flour 555 (Life Technologies) diluted 1:1000. The samples were washed in PBS, post-fixed in 4% PFA for 15 min, and then incubated in anti-ROMKa (raised in Chapter 1) labeled with Zenon Alexa Fluor 488 Rabbit IgG Labeling Kit (Life Technologies) diluted 1:1000. Triple-color immunofluorescence staining for ROMKa, NHE3, and NKA was also conducted. In brief, the samples after blocking were incubated with anti-NHE3 (Watanabe et al., 2008) diluted 1:500 overnight at 4°C. After a brief wash in PBS, the gill filaments were incubated overnight at 4°C with goat anti-rabbit IgG labeled with Alexa Fluor 405 (Life Technologies) diluted 1:1000 in PBS. The samples were washed in PBS, post-fixed in 4% PFA, and then incubated in anti-ROMKa labeled with Zenon Alexa Fluor 488, and with Cy3-labeled anti-NKA diluted 1:500 in NB-PBS overnight at 4°C. The samples were observed with the

C1 confocal laser scanning microscope (Nikon). The wavelengths of excitation and recorded emission for each dye were as follows: Alexa Fluor 555, 543 nm and 572.5-637.5 nm; Alexa Fluor 488, 488 nm and 500-530 nm; Alexa Flour 405, 405 nm and 432.5-467.5.

Statistics

The data from the acclimation experiment were expressed as means ± S.E.M. The significance of differences between treatment groups at $P<0.05$ was examined by Student's *t*-test.

3-3. Results

Phylogenetic analysis and tissue distribution patterns of tilapia ROMKb

The amino acid sequence deduced from the cDNA fragment cloned by degenerate PCR for ROMKb, a putative paralogous product of ROMKa, showed 74% identity with tilapia ROMKa (sequence data not shown). Also, the above sequence was assigned to the Kir1.1 clade in the phylogenetic tree, together with a zebrafish homolog of ROMK, Kcnj1 (Fig. 3-1A). In the detailed tree of putative ROMK homologs (Fig. 3-2), fish ROMKs were separated into two major clades. Surprisingly, in addition to well-characterized zebrafish homolog of Kcnj1 (Abbas et al., 2011), zebrafish have 6 putative ROMK homologs, 5 of which formed a clade with Kcnj1. The mRNA expression of ROMKb was found in all tissues of both SW- and FW- acclimated fish (Fig. 3-1B).

Ion concentrations and osmolality of plasma and urine

After 1-week acclimation of tilapia to H-K FW, plasma levels of all ions measured and osmolality were not significantly different from those of control FW (Table 3-2). In the urine, K^+ concentration was significantly elevated in H-K FW fish (Table 3-2). Urine Na^+ and Cl^- concentrations were not significantly different between the two groups.

mRNA levels of ion transporters and channels in the gill and kidney

The mRNA levels of the following transporters and channels were assessed in tilapia acclimated to control FW and H-K FW: ROMKa, ROMKb, Maxi-K α subunit, KCC1, KCC4,

NHE3, NKAα1a, and NKAα1b in the gill and kidney; NCC in the gill; and NKCC2 in the kidney (Fig. 3-3). Gill ROMKa mRNA levels changed most markedly in response to H-K condition, being approximately 5 times that of control FW fish (Fig. 3-3A). For the other channels and transporters, there was no significant difference between control FW and H-K FW groups (Figs. 3-3, B-I), although gill NKAα1a mRNA tended to be lower in H-K FW fish (Fig. 3-3G).

Triple-color Immunofluorescent microscopy

The gill filaments of control FW and H-K FW fish were subjected to two combinations of triple-color immunofluorescence staining: ROMKa, NCC and NKA; and ROMKa, NHE3 and NKA (Fig. 3-4). In both fish groups, NKA immunosignals reveled the distribution of ionocytes (Figs. 3-4, A, E, I, and M). In control FW fish, immunosignals of ROMKa were detected in several ionocytes (Figs. 3-4, B and J), and those in H-K FW fish were clear (Figs. 3-4, F and N) and localized to apical openings of ionocytes (Figs. 3-4, Q and R). The immunosignals of NCC (Figs. 3-4, C and G) and NHE3 (Figs. 3-4, K and O) were detected in the both groups. In the merged image, the signals of ROMKa were not colocalized with those of NCC (Figs. 3-4, D and H), but with those of NHE3 (Figs. 3-4, L and P) at the apical opening of the ionocytes.

3-4. Discussion

In Chapter 1, I found that the gills are the primary site of K^+ excretion in SW-acclimated fish, and that ROMKa expressed in the gill ionocytes was responsible for K^+ excretion. I did not focus on the kidney in Chapter 1, because of a modest amount of urine from SW fish reported previously (Smith, 1930; Hickman, 1968; Shehadeh and Gordon, 1969). In the present chapter, I used tilapia kept in FW, where the fish produce a large amount of urine to keep plasma ion levels higher than those of external water.

To understand the K^+ regulatory mechanisms of FW-adapted fish, the responses of the fish exposed to high external K^+ concentration were investigated. Although H-K FW environment contained 10 mM KCl, those fish showed physiological levels of plasma ions

and osmolality. This fact indicates that the fish could successfully acclimated to the H-K FW environment within 1 week, and that not only SW fish, but also FW fish have mechanisms to eliminate excess K^+. In H-K fish, urine K^+ levels were significantly higher than that of control FW, indicating the roles of the kidney in K^+ regulation. It is possible, however, that K^+ is merely "less reabsorbed" by the renal tubule cells, due to slightly higher plasma K^+ in H-K fish (P=0.13). Further studies with radioactive Rb^+ or Cs^+, the K^+ analogs, will be necessary to elucidate actual K^+ excretion/absorption rate and contribution of kidney in K^+ regulation. Tentatively, the possible roles of kidney in K^+ regulation are discussed below, with reference to the ion transporters expressed in the kidney.

To assess the possible role of ROMKs in the kidney of tilapia, I cloned the putative paralogous product of tilapia ROMKa, and tentatively named the newer clone "ROMKb". In a phylogenetic tree of putative ROMK homologs of various vertebrate species, fish ROMKs were divided into two clades, indicating the result of "fish-specific genome duplication" that occurred ~350 million years ago (Meyer and Van der Peer, 2005). Surprisingly, zebrafish have 7 putative ROMK homologs, and 6 of which, containing Kcnj1, formed a clade. The 6 genes coding for these putative ROMK homologs in this clade are on the same chromosome (chromosome 18), and probably derived by the tandem gene duplication (Lu et al., 2012).

To elucidate molecular pathways that underlie K^+ elimination in FW-acclimated fish, mRNA levels of ROMKa, ROMKb, Maxi-K α subunit, KCC1, KCC4, NHE3, NKAα1a, NKAα1b, NCC, NKCC1a, and NKCC2 in the gills and/or kidney were quantified in control FW and H-K FW fish. Similar to the fish acclimated to high-K^+ artificial SW (Chapter 1), the fish exposed to H-K FW expressed ~5 times higher levels of gill ROMKa mRNA than control fish, suggesting that gill ROMKa has still an important role in fish in hypotonic environments. However, the mRNA expression of NKCC1a, the possible basolateral K^+ transporting pathway into the ionocytes (Chapter 1), was not stimulated by H-K treatment. It is of interest for a future study, to assess the differences in NKCC1a mRNA regulation between FW and SW fish. In the mammalian kidney tubule, predominant K^+ conductance is represented by ROMK (Welling and Ho, 2009). In tilapia, on the other hand, the expression level of ROMKa mRNA in the kidney was approximately 1/500 that of the gills (control FW

fish, gill : kidney = 1 : 0.0022). Also, ROMKb mRNA was expressed marginally in both gills and kidney, and H-K condition did not alter its mRNA levels. These results indicate that tilapia ROMKs have only modest roles on kidney K^+ handling. Maxi-K, KCC1, and KCC4 did not show any difference between the experimental groups, being consistent with the results of SW -acclimated tilapia gills (Chapter 1). Interestingly, KCC1 mRNA was highly expressed in the kidney compared to the gills (control FW fish, gill : kidney = 1 : 29), suggesting its significant role in renal K^+/Cl^- handling. A recent study (Ubels et al., 2006) showed that KCC1 was expressed at the apical membrane of the rat lacrimal gland. Also, an apical K^+/Cl^- cotransport activity was detected in distal nephron of rat kidney (Amorim et al., 2002). Considering that plasma Cl^- tended to be low (P=0.056) in H-K fish compared to control fish, there may be a link(s) between K^+ and Cl^- handling in the kidney. In this regard, further investigation on KCC1 in the kidney might be an interesting subject, for a comprehensive understanding of K^+ regulation in FW-acclimated teleosts. I also quantified mRNA expression levels of other transporters in the gills and kidney, as important transporters involved in plasma ion homeostasis (Hiroi et al., 2008, Tipsmark et al., 2011). NKAα1a and NKAα1b are suggested to be important for FW- and SW-adaptation, respectively (Tipsmark et al., 2011). The mRNA levels of these transporters were not affected by the H-K treatment, suggesting that these transporters do not have important roles in K^+ regulation in FW-adapted fish.

The gill filaments of the experimental fish were also subjected to triple-color immunofluorescence staining for NKA/ROMKa/NCC and NKA/ROMKa/NHE3. Among the ionocytes of tilapia, Type-II and Type-III cells are considered as ion-absorbing ionocytes that are responsible for FW adaptation (Hiroi et al., 2008; Inokuchi et al., 2008, 2009; Choi et al., 2010). The immunofluorescence staining study clearly demonstrated that FW tilapia express ROMKa in Type-III cells, as evidenced by the colocalization of ROMKa with NHE3, but not with NCC, at the apical openings of the ionocytes (Fig. 3-5). Also, consistent with the results of the mRNA quantification study, convincingly intense immunosignals of ROMKa was detected at the apical openings of the ionocytes in H-K FW fish. It is reasonable that ROMKa is expressed in Type-III cells, since these cells are considered to transform to Type-IV “ion-excretory” ionocytes upon acclimation to SW (Hiroi et al., 1999;

Choi et al., 2010; Inokuchi et al., 2012; Hiroi and McCormick, 2012). Indeed, other ion transporters such as apical NHE3 and basolateral NKCC1a are expressed in common in these two types of cells, although the expression levels are different: NHE3 is highly expressed in Type-III cells of FW fish, while NKCC1a is high in Type-IV cells of SW fish (Hiroi et al., 2008; Inokuchi et al., 2008). The difference in expression levels of ROMKa between SW and FW fish may reflect the magnitude of K^+ excretion in usual conditions. The results of the present study indicate that FW-acclimated fish have an ability to actively excrete K^+ by expressing branchial ROMKa, when body K^+ becomes excessive.

In summary, I found that FW-acclimated tilapia express ROMKa in the apical openings of Type-III ionocytes in a modest level, and can up-regulate its expression levels in response to H-K stress. Also, the fact that urine K^+ concentration was significantly elevated in H-K fish suggests some roles of the kidney in K^+ excretion, although the molecular pathway and contributing rate of kidney are unknown. Since mRNA expression levels of ROMKa, as well as ROMKb, were low in the kidney, tilapia ROMKa has probably evolved to function mainly in the gills rather than in the kidney, showing a sharp contrast to ROMK in mammals. In a previous study using zebrafish, mRNA of Kcnj1, an ROMK homolog, was localized to the pronephros and epithelial ionocytes in the larvae (Abbas et al., 2011). For further discussion on the evolution of K^+ regulatory organs and the underlying molecular mechanisms in the vertebrate animals, it will be informative in the future to study ROMK isoforms expressed in evolutionarily ancient species, such as lamprey and hagfish, together with cartilaginous fish.

4. Differential regulation of ROMKa expression in ionocytes of freshwater- and seawater-acclimated tilapia: hormonal vs. cell-autonomous regulation

4-1. Introduction

Regulation of plasma K^+ level is crucial to all vertebrate animals. Plasma K^+ regulatory mechanisms are extensively understood in mammals, where the kidney plays a key role in modulating K^+ excretion rate in urine (Wang and Giebisch, 2009). In the kidney, ROMK is expressed at the apical membrane of tubule cells from the TAL through the OMCD, and its expression mainly affects K^+ excretion (Hebert et al., 2005). In fish, although the K^+ regulatory mechanisms have been largely unknown, the mechanisms were studied in Chapter 1 and 3 of this thesis, and it is now evident that ROMKa expressed in gill ionocytes of tilapia is the primary pathway for K^+ regulation. In both mammals and fish, the levels of ROMK expression are regulated to meet the demands of plasma K^+ homeostasis (Wang and Giebisch, 2009; Chapter 1 and 3). Now, another question arises: how expression levels of ROMK are regulated in fish? In mammals, aldosterone, the main mineralocorticoid hormone synthesized in the adrenal cortex, is known as the major regulator of ROMK, accelerating mRNA expression and surface expression of mature protein (Beesley et al., 1998; Yoo et al., 2003). Teleost fish lack aldosterone, and the main corticosteroid is cortisol, a glucocorticoid produced in the interrenal gland located in the head kidney (Bury and Sturm, 2007; Arterbery et al., 2011). 11-deoxycorticosterone (DOC), a new member of mineralocorticoid, has been found in some salmonid fish; its physiological significance is controversial due to its extremely low plasma concentration (McCormick et al., 2008). Although fish do not have a distinct mineralocorticoid, they have two types of glucocorticoid receptors (GRs), GR1 and GR2, and one type of mineralocorticoid receptor (MR). All of them can bind to cortisol, and mediate the downstream actions (Bury and Sturm, 2007; Arterbery et al., 2011). Multiple roles have been reported for cortisol in teleosts, including ion regulation, energy metabolism, reproduction, and growth (Mommsen et al., 1999). In this chapter, I first aimed to clarify the effects of cortisol on ROMKa expression in the gills. Eventually, cortisol seemed to be the primary regulator of ROMKa mRNA only in FW-acclimated tilapia, as no marked response to cortisol was seen in SW-acclimated tilapia gills. For SW tilapia, I then focused on other

Table 3-1. *Primers used for quantitative PCR (qPCR)*

Primers	Sequence (5'-3')	Notes
ROMKb-F	GGCTCTAATTGTCATCCAGTCTC	
ROMKb-R	TCGTTGTCCTCAGCAACTTC	
NCC-F	CCGAAAGGCACCCTAATGG	Hiroi et al. (2008)
NCC-R	CTACACTTGCACCAGAAGTGACAA	Hiroi et al. (2008)
NHE3-F	ATGGCGTGTGGAGGCTTG	Inokuchi et al. (2008)
NHE3-R	CCTGTCCCAGTTTCTGTTTGTG	Inokuchi et al. (2008)
NKCC2-F	CCAGAATTTAGGGACGGTGAG	
NKCC2-R	GAGCCATTGCCAGATAGGTG	
NKAα1a-F	AACTGATTTGGTCCCTGCAA	qPCR, Tipsmark et al. (2011)
NKAα1a-R	ATGCATTTCTGGGCTGTCTC	qPCR, Tipsmark et al. (2011)
NKAα1b-F	GGAGCGTGTGCTTCATCACT	qPCR, Tipsmark et al. (2011)
NKAα1b-R	ATCCATGCTTTGTGGGGTTA	qPCR, Tipsmark et al. (2011)

Table 3-2. *Plasma and urine concentrations of ions (mM) and plasma osmolality (mOsm/kg H_2O) in tilapia acclimated to control FW and H-K FW*

	$[K^+]$	$[Na^+]$	$[Cl^-]$	Osmolality
control FW plasma	3.54±0.14	163±1	143±2	310±5
H-K FW plasma	3.83±0.13	163±1	138±1	306±3
control FW urine	1.36±0.13	8.16±1.36	10.3±1.15	-
H-K FW urine	1.94±0.24*****	7.65±1.51	15.9±5.26	-

An asterisk indicates significant difference from control value (P<0.05).

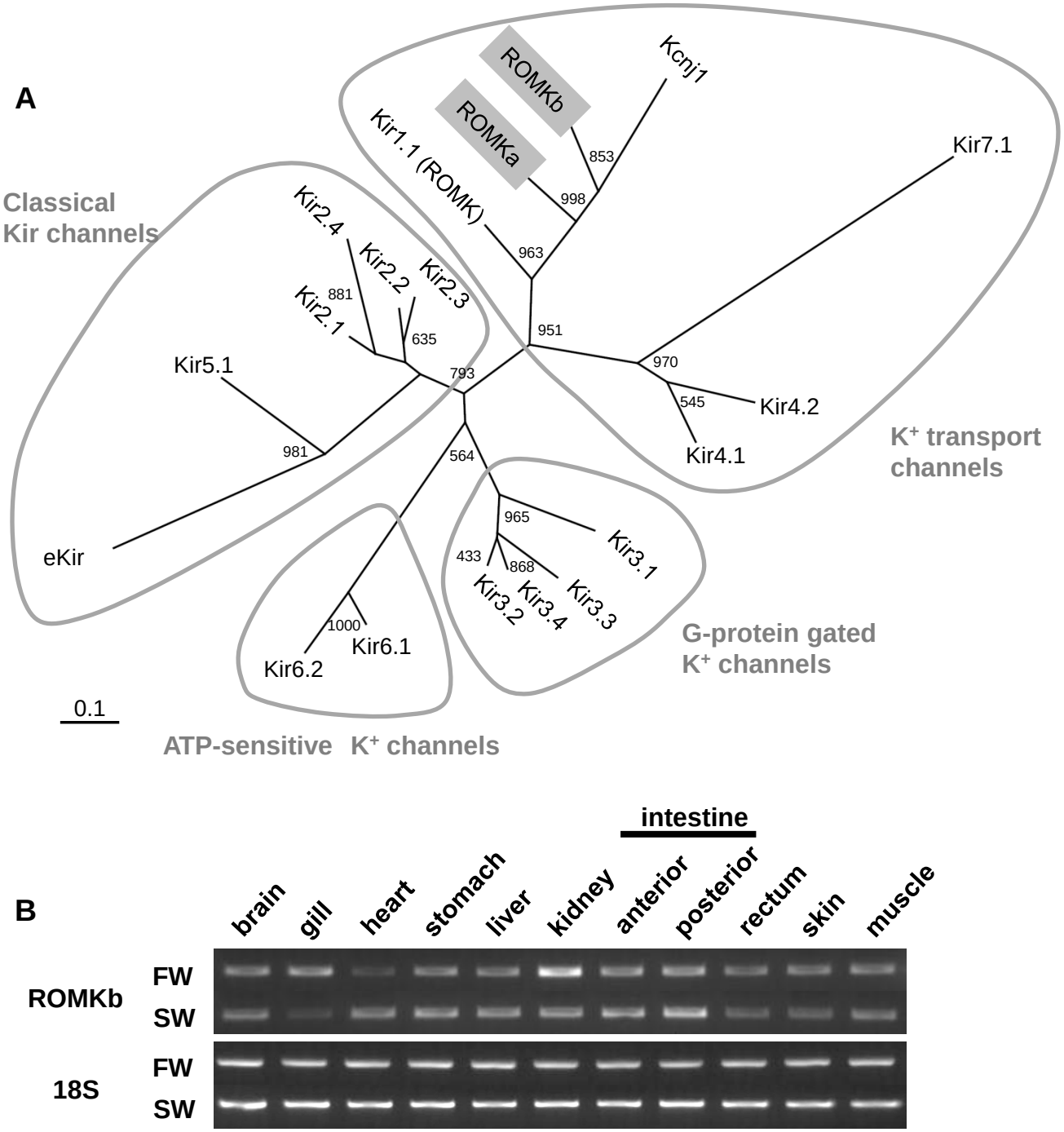


Fig. 3-1. (**A**) A phylogenetic tree of amino acid sequences of human inwardly rectifying K^+ (Kir) channels, tilapia ROMKs, zebrafish Kcnj1, and eel Kir (eKir) channel, constructed by the neighbor-joining method. Tilapia ROMKa and ROMKb are shaded. The sequences are categorized into four functional groups: classical Kir channels, G-protein gated K^+ channels, ATP-sensitive K^+ channels, and K^+ transport channels. Bar, evolutionary distance of 0.1 amino acid substitution per site. The tree was constructed in reference to Hibino et al. (2010). (**B**) Tissue distribution analysis of ROMKb in tilapia acclimated to seawater (SW) or freshwater (FW). 18S, internal control.

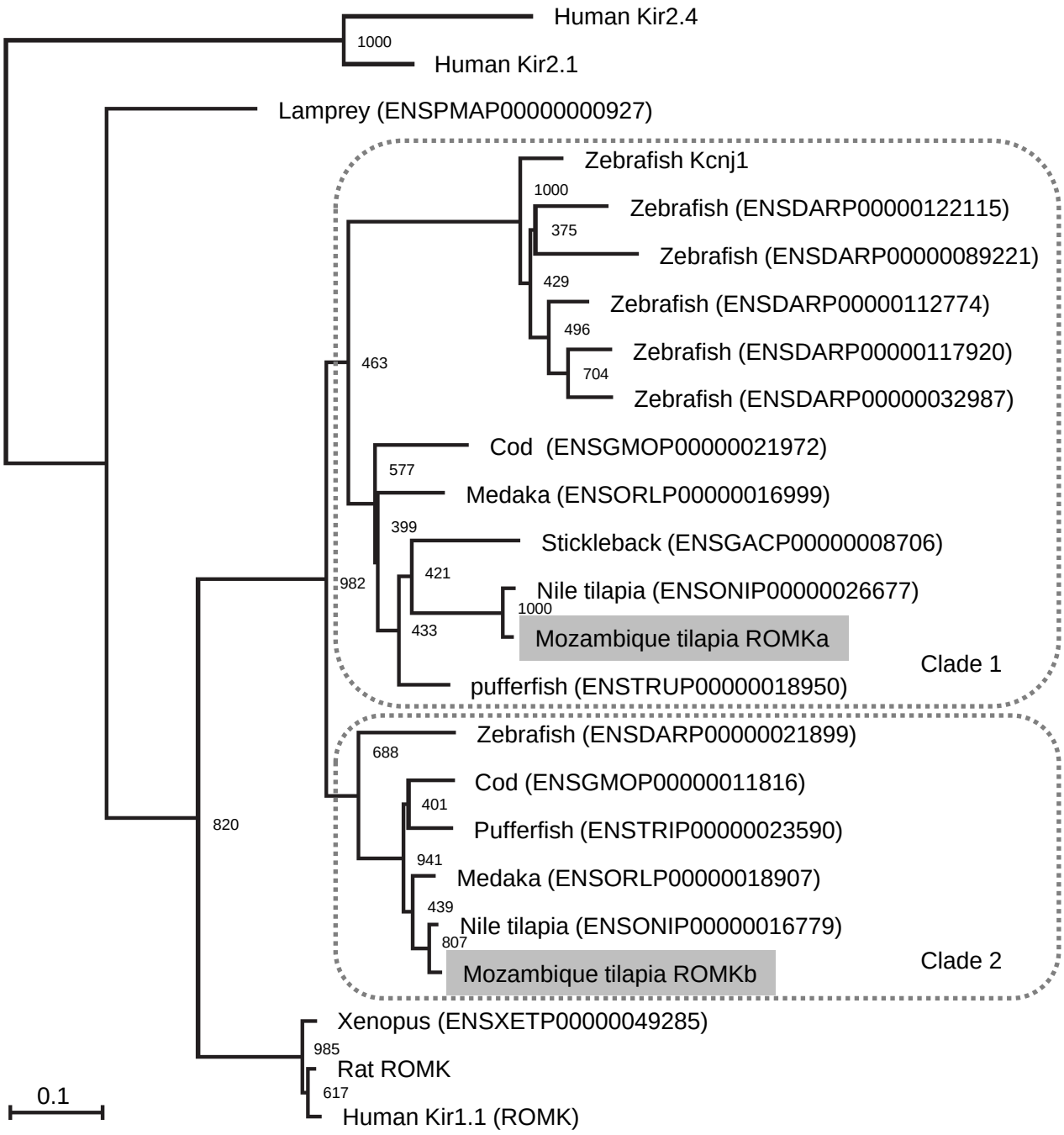


Fig. 3-2. A phylogenetic tree of amino acid sequences of ROMKs: human Kir1.1 tilapia ROMKa and ROMKb, and zebrafish Kcnj1 along with presumable ROMK homologs of Sea lamprey, zebrafish, Atlantic cod, Japanese medaka, stickleback, Nile tilapia, pufferfish, and xenopus, constructed by the neighbor-joining method, with human Kir2.1 and Kir2.4 assigned to the outgroup. The teleost ROMKs were divided into two clades (Clade 1 and 2). The sequences of putative ROMKs were obtained from Ensembl database, and the protein IDs are shown in the parentheses. Mozambique tilapia ROMKa and ROMKb are shaded. Bar, evolutionary distance of 0.1 amino acid substitution per site.

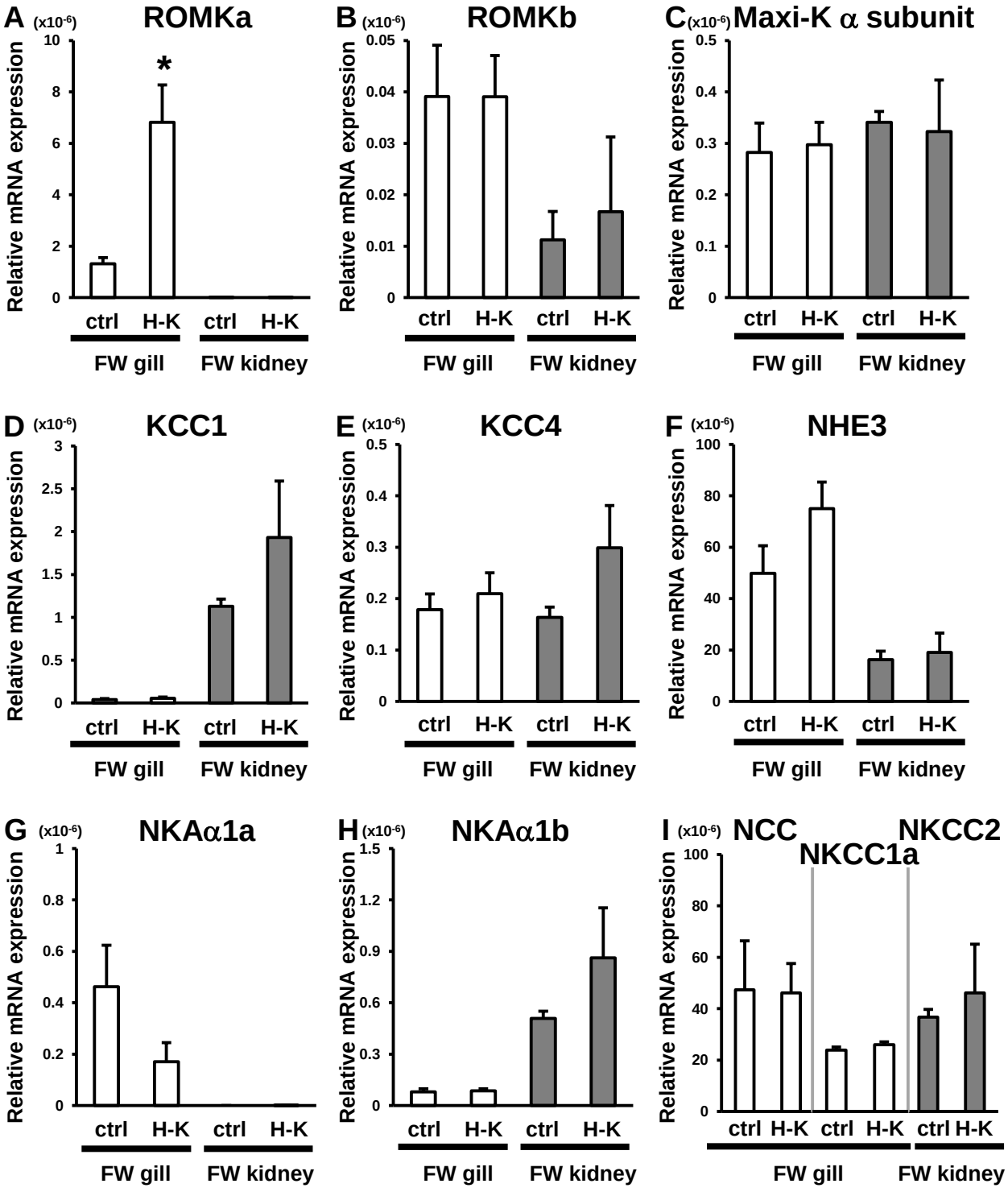
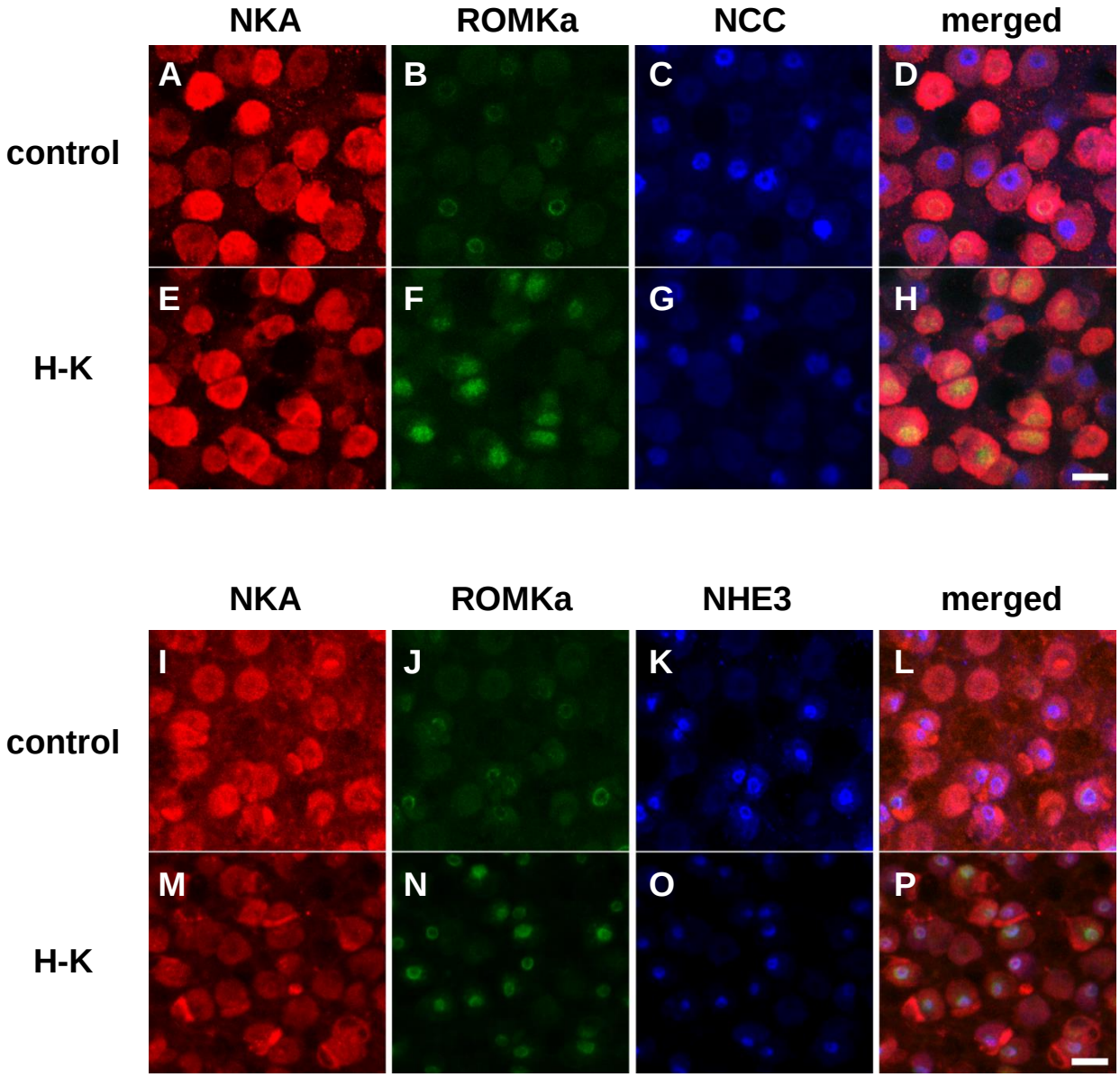


Fig. 3-3. Relative mRNA expression levels (mRNA/18S rRNA) of ion transporters in the gills and kidney of tilapia exposed to control (ctrl) freshwater (FW) or high-K^+ (H-K) FW: ROMKa, ROMKb, Maxi-K α subunit, KCC1, KCC4, NHE3, NKAα1a, and NKAα1b in the gill and kidney; NCC and NKCC1a in the gills; NKCC2 in the kidney. Data are expressed as means ± S.E.M. (n=5-6). An asterisk indicates significance of differences compared to control fish at $P<0.05$.



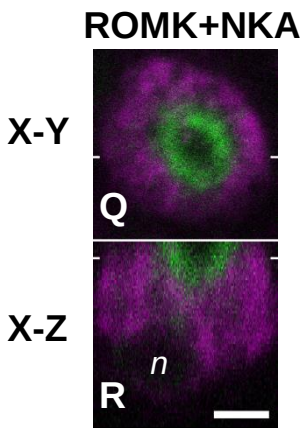


Fig. 3-4. Immunofluorescence micrographs of the gills in tilapia acclimated to control freshwater (FW; **A**-**D**, **I**-**L**) and high-K^+ (H-K) FW (**E**-**H**, **M**-**P**), stained with anti-NKA (**A**, **E**, **I**, **M**; red), anti-ROMKa (**B**, **F**, **J**, **N**; green), anti-NCC (**C**, **G**; blue), and anti-NHE3 (**K**, **O**; blue). The RGB images for NKA/ROMKa/NCC and NKA/ROMKa/NHE3 were merged in (**D**, **H)** and (**L**, **P**), respectively. Bar, 10 μm. A representative ROMK-expressing ionocyte (**Q**, **R**) stained with anti-ROMK (green) and anti-NKA (magenta). Lines in the images at optical sections of X-Y (**Q**) and X-Z (**R**) indicate the position of the optical section of each other. *n*, nucleus. Bar, 3 μm.

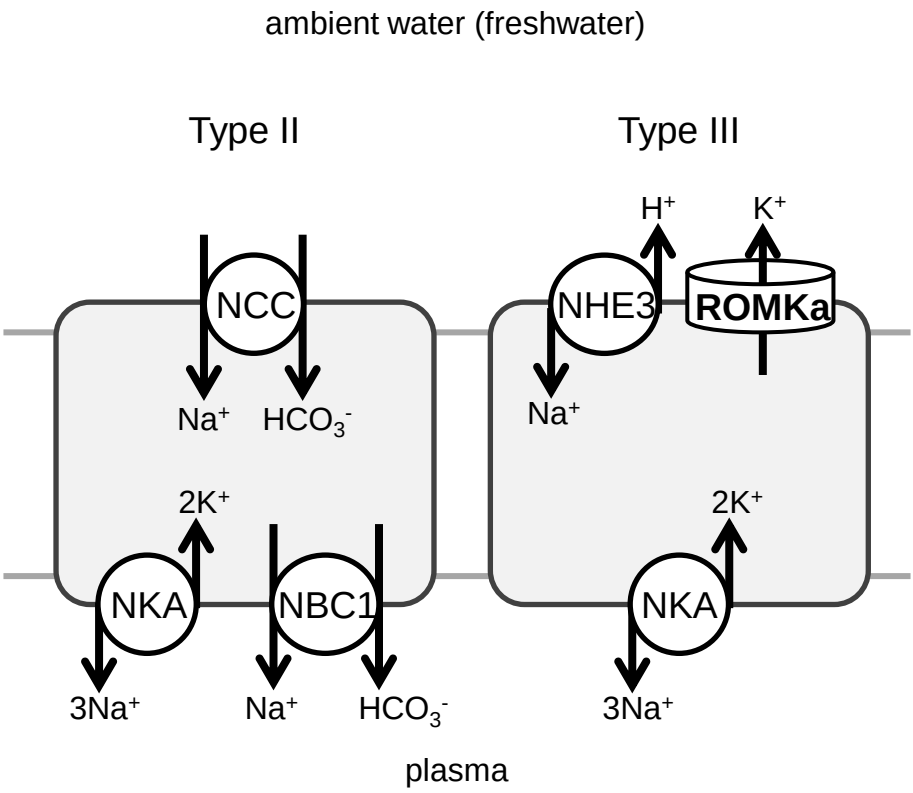


Fig. 3-5. A schematic model showing a possible K^+-excretory mechanism in ionocytes of FW-adapted Mozambique tilapia. In Type-III cells, K^+ is first absorbed via basolateral NKA, and then excreted via apical ROMKa. The model was constructed in reference to Hiroi et al. (2008), Inokuchi et al. (2008), and Furukawa et al. (2011).

hormones as possible factors that mediate K^+ regulation in SW tilapia: growth hormone (GH), prolactin (PRL), somatolactin (SL), and proopiomelanocortin (POMC), all of which are expressed in the pituitary gland. I transferred SW-acclimated tilapia to high-K^+ (H-K) SW, and examined their physiological responses. None of the candidate hormones markedly responded to H-K exposure, although plasma K^+ level and gill ROMKa mRNA expression were elevated within 6 h after transfer to H-K media. Finally, I found that an increased level of ambient K^+ *per se* triggered high mRNA expression of ROMKa in incubated gills. As a consequence of several experiments in this chapter, I hypothesized that ROMKa in gill ionocytes was regulated by an endocrine factor and in a cell-autonomous fashion in FW- and SW-acclimated fish, respectively.

4-2. Materials and Methods

Experimental animals

Mozambique tilapia were maintained in tanks supplied with recirculating FW or SW at 25°C. The fish were fed commercial pellets (Tilapia 41M; JA Nishi-Nihon Kumiai Shiryo) once a day. The fish (100-250 g) were anesthetized with 0.1% 2-phenoxyethanol before blood sampling or removal of tissues.

I conducted an H-K SW transfer experiment in Hawaii Institute of Marine Biology (HIMB), University of Hawaii, where a large number of the fish is available. In HIMB, Mozambique tilapia were reared in 700-l outdoor flow-through tanks at 25-28°C. Fish were fed daily with Silver Cup Trout Chow (Nelson and Sons, Murray, UT).

cDNA cloning of GR1, GR2 and MR

Gill filaments were removed from FW-acclimated tilapia, and total RNA was extracted from the filaments with Isogen (Nippon Gene). After digestion of genomic DNA with DNase I (Life Technologies), 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. The degenerate primers corresponding to the conserved amino acid residues of known GRs and MR were designed (Table 4-1). The PCR products were ligated into pGEM T-Easy vector

(Promega) and sequenced using an ABI Prism 310 sequencer (Life Technologies). 5'- and 3'-RACE was conducted using CapFishing^{TM} kit (Seegene, Seoul, Korea).

Incubation of separated gill filaments

To investigate the effects of corticosteroids on gill mRNA levels of ROMKa and other ion transporters, I incubated separated gill filaments in media containing corticosteroids (cortisol or aldosterone) with or without possible specific corticosteroid antagonists, RU486 or spironolactone (Mahajan, 1997; Delyani, 2000), according to the method established in our laboratory. Briefly, the gill was removed from FW-acclimated tilapia and washed in 0.025% $KMnO_4$ in BSS for 1 min. After washing in BSS, gill filaments were separated from the gill arch, and cut with a fine knife into afferent and efferent halves, to allow the incubation media to spread easily throughout the filament. Two filaments were incubated in 1 ml L-15 (Life Technologies) with 1 × antibiotic-antimycotic solution (Life Technologies), containing five different combinations of corticosteroid and antagonist: control, vehicle; cortisol, 5 μg cortisol; cortisol + RU486, 5 μg cortisol and 10 μg RU486; cortisol + spironolactone, 5 μg cortisol and 10 μg spironolactone; aldosterone, 5 μg aldosterone. Ligands (cortisol and aldosterone) and antagonists (RU486 and spironolactone) were first dissolved in ethanol and dimethylsulfoxide (DMSO), respectively. The final concentrations of the vehicles in media were 0.1 % and 0.2% (v/v), respectively. After 6 h of incubation, total RNA was extracted from the filaments with Isogen (Nippon Gene), and cDNA was synthesized with ReverTra Ace -α- (Toyobo, Osaka, Japan).

The gill filaments of SW-acclimated fish were also subjected to *in vitro* incubation to evaluate the effects of K^+ levels on mRNA expression levels of ROMKa. For SW-acclimated tilapia gills, I used 1 ml modified L-15, containing 10% charcoal/dextran-treated fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA), and 5 μg cortisol. The filaments were incubated in control medium (5.4 mM K^+) and H-K medium (10.4 mM K^+). To maintain activities of SW-type ionocytes (Marshall et al., 2000; Hoffmann et al., 2002), osmolality of both media was adjusted to 430 mOsm/kg H_2O by addition of appropriate amounts of NaCl and KCl.

Transfer of SW tilapia to H-K SW

SW-acclimated tilapia (10-40 g) were transferred to 5-l glass tanks filled with SW, each tank containing 8 fish. The fish were fasted 2 days prior to the experiment, and then KCl was added to the H-K treatment tanks. The ion concentration and pH of the two experimental water were: control SW (in mM), 483 Na^+, 7.47 K^+, 545 Cl^-, 10.7 Ca^{2+}, 52.7 Mg^{2+}, pH=7.8; H-K SW (in mM), 483 Na^+, 17 K^+, 551 Cl^-, 10.8 Ca^{2+}, 52.7 Mg^{2+}, pH=7.7. The sampling was conducted before (0 h) and 6 h, 12 h, 24 h, 72 h after the addition of KCl. 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), and Cl^- with chloridometer (Labconco). The gills were removed and total RNA was extracted with Tri Reagent® RT (Molecular Research Center, Inc., Cincinnati, OH). Pituitaries were removed and stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany), 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- (Toyobo).

Radioimmunoassay (RIA)

Plasma cortisol levels were measured by RIA according to Witt et al. (2009) in H-K SW transfer experiment. The plasma sample (5 μl) was diluted with 95 μl assay buffer (0.01 M PBS, 1% BSA, pH 7.3), mixed with 150 μl ^{3}H-labelled cortisol (9,000 cpm) and anti-cortisol antibody (Fitzgerald Industries, North Acton, MA) in 1:5000 dilution. The sample mixtures were incubated at room temperature for 2 h. To remove unbound cortisol, 400 μl dextran-coated charcoals (Sigma) was added to the samples, mixed and incubated on ice for 15 min. Finally, the sample tubes were centrifuged at 3000 rpm for 20 min, and 450 μl supernatant was transferred to 4 ml scintillation fluid (Scinti Safe Econo 1, Fisher Scientific, Pittsburgh, PA, USA) and counted in a gamma counter (Cobra II Auto-Gamma; Packard, Prospect, CT). Plasma concentration of cortisol was calculated with reference to a standard curve drawn by parallel assays of known concentrations of cortisol standards.

qPCR

In gill incubation experiment, mRNA expression levels were quantified for ROMKa, NKCC1a, NHE3, NCC, NKAα1a, and NKAα1b. GR1 (AB771724), GR2 (AB771725), and MR (AB771726) mRNA levels were also assessed in incubated FW gills. In the H-K SW transfer experiment, mRNAs of GR1, GR2, MR, ROMKa, NKCC1a, and CFTR (AB601825) in the gills, and GH (AF033806), PRL (X923280), SL (AB442015), and POMC (AF116240) in the pituitaries were quantified. In the gill incubation experiment of SW acclimated fish, mRNA levels of ROMKa, NKCC1a, NHE3, CFTR, NKAα1a, and NKAα1b were assessed. For GR1, GR2, MR, and CFTR, the primers used are listed in Table 4-1, and the primers used to assess the other transporter mRNAs were same as Chapter 3. The copy numbers of the 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 the corresponding 18S rRNA levels.

Statistics

The data were expressed as means ± S.E.M. The significance of differences at $P<0.05$ was examined by one-way analysis of variance (ANOVA) in gill incubation experiments, or by two-way ANOVA in the H-K SW transfer experiment, followed by Tukey's HSD test. Significant differences between each sample were shown when the interaction of the two factors (time x treatment) was detected (Figs. 4-3, A and B; Fig. 4-5A)

4-3. Results

Experiment 1. Effects of corticosteroids on mRNA expression levels of FW-acclimated tilapia gills

The responses of mRNA levels of several ion transporters were quantified in the gill incubation experiment of FW-acclimated fish. Compared to pre-incubation samples, incubation of the gill filaments in control L-15 media increased NKCC1a mRNA, and decreased NHE3, NCC and NKAα1a mRNAs (Figs. 4-1, B-E). ROMKa mRNA levels clearly increased in the samples treated with cortisol, and the increase was completely canceled by RU486 (Fig. 4-1A). NKCC1a and NHE3 mRNAs were also increased by cortisol treatment, but these effects were inhibited by both RU486 and spironolactone (Figs.

4-1, B and C). NCC also showed a similar tendency, but there was no significant effect of cortisol (Fig.4-1D). NKAα1a and NKAα1b were both increased by cortisol treatment, and the effect was blocked by RU486 for NKAα1a mRNA, whereas both RU486 and spironolactone blocked the effect of cortisol on NKAα1b (Figs. 4-1, E and F). There was no effect of aldosterone on transporter mRNA expression examined (Fig. 4-1). At the same time, the mRNA expression levels of the corticosteroid receptors (GR1, GR2 and MR) were assessed (Fig. 4-2). GR1 and GR2 mRNA were increased after 6-h incubation (Fig. 4-2). GR1 mRNA was decreased by RU 486, but increased by aldosterone (Fig. 4-2A). GR2 and MR mRNAs were down-regulated in response to any of corticosteroids/antagonists administration (Figs. 4-2, C and D).

Experiment 2. Transfer of SW tilapia to H-K SW

Plasma ion concentration and osmolality

After transfer to H-K SW, plasma K^+ levels of H-K fish increased within 6 h, and were always higher than those of control SW fish (Fig. 4-3A). Plasma Na^+ levels of H-K fish slightly decreased by 6 h after transfer, but were resumed within 24 h (Fig. 4-3B). Plasma Cl^- levels were not affected (Fig. 4-3C), but plasma osmolality was low at 12 h (Figs. 4-3D).

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

Plasma cortisol levels were high at 24 h, and the levels returned to initial level in at 72 h (Fig. 4-4A). Also, plasma cortisol tended to be high in H-K treated fish (P=0.075; Fig. 4-4A). In the gills, the mRNA expression levels of GR1 became transiently low at 72 h after transfer (Fig. 4-4B). The patterns of GR2 mRNA levels were similar to that of GR1, but no significant difference was detected (Fig. 4-4C). MR mRNA also showed a pattern similar to the other receptor mRNAs assessed, and was significantly low at 72 h after acclimation to the media (Fig. 4-4D).

Gill mRNA levels of ROMKa, NKCC1a, and CFTR

The changes in branchial expression levels of ROMKa, NKCC1a and CFTR mRNAs were examined (Fig. 4-5). ROMKa mRNA of H-K treated fish increased at 6 h, being

reached the peak level at 24 h after transfer, and still higher than normal level at 72 h (Fig. 4-5A). NKCC1a mRNA was significantly greater in fish transferred to H-K SW than in control fish, and significantly low at 6 h group (Fig. 4-5B). CFTR mRNA was low at 6 h and 72 h samples (Fig. 4-5C).

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

In the pituitary, mRNA expression levels of GH, PRL, SL, and POMC were assessed (Fig. 4-6). There was no statistically significant difference detected among treatment groups and after transfer to H-K SW. Among those hormones, however, GH and SL mRNAs tended to be reduced until 12 h after transfer of the fish (Figs. 4-6, A and C). On the contrary, PRL mRNA increased after transfer, and the level was significantly higher in control group than initial level at 12 h (Fig. 4-6B). POMC mRNA expression was stable (Fig. 4-6D).

Experiment 3. Effects of ambient K^+ concentration on gill mRNA expression

The responses of the gill mRNA expression to high K^+ concentration were assessed in gill filament incubation study (Fig. 4-7). Among mRNAs assessed, only NKCC1a was increased after 6-h incubation in control media (Fig. 4-7A). The mRNA up-regulation in response to H-K media was seen in ROMKa and NKCC1a (Figs. 4-7, A and B). NHE3, CFTR, NKAa1a, and NKAa1b showed responses neither to incubation itself nor to H-K media (Figs. 4-7, C-F).

4-4. Discussion

The effects of cortisol on mRNA expression levels of branchial ion transporters and cortisol receptors

In the present chapter, I investigated the factors that possibly regulate expression levels of ROMKa, the primary K^+ channel that is responsible for K^+ excretion from the gills. First, I focused on cortisol, as an analogous substance of aldosterone, a mineralocorticoid known as a the principal regulator of ROMK in the mammalian kidney (Wang and Giebisch, 2009). In the gill incubation experiment, the effects of corticosteroids and antagonists on branchial ion transporter mRNA levels were assessed. As SW-acclimated tilapia gills showed responses

neither to cortisol nor to aldosterone in the preliminary incubation experiment (data not shown), the corticosteroids/antagonists treatment study was conducted using only FW-acclimated tilapia gills.

The present study clearly demonstrated that mRNA expression levels of ROMKa, NKCC1a, NHE3, NKAα1a, and NKAα1b in the gills of FW-acclimated tilapia were up-regulated by *in vitro* cortisol treatment. Cortisol has long been considered as a SW-adapting hormone in fish (Mommsen et al., 1999), but FW-adapting roles are also suggested in a variety of fish (McCormick et al., 2001; Kiirelich et al., 2007; McCormick et al., 2008). Also in the present study, cortisol displayed the "dual osmoregulatory roles", by up-regulating mRNA levels of ion transporters responsible for ion secretion (ROMKa, NKCC1a, and NKAα1b) and absorption (NHE3 and NKAα1a). The fact that the effects of cortisol on ROMKa and NKAα1a mRNA levels were abolished by RU486 treatment, but not by spironolactone, indicates that mRNA up-regulation of these genes is preferentially mediated by GRs. Both RU486 and spironolactone blocked the effect of cortisol on NKCC1a, NHE3, and NKAα1b, implying that the transporters mentioned above are targets of GRs- and MR-mediated transactivation. In the samples incubated in control L-15, mRNA up-regulation of NKCC1a and down-regulation of NHE3, NCC, and NKAα1a were detected after 6-h incubation. In the previous study, Hoffmann et al. (2002) reported that NKCC1a mRNA expression was induced by hyperosmotic stress to the incubated gills. Since the epithelial cells of FW fish gills should usually be exposed to hyposmotic stress via the apical membrane, incubation with L-15 (320 mOsm/kg H_2O) may have caused hyperosmotic responses and up-regulation of NKCC1a in FW gills. Although the reasons for NHE3 mRNA decrease are unknown, decrease in NCC and NKAα1a mRNA levels is probably due to the lack of prolactin, a FW-adapting hormone that maintains expression of these transporters (Breves et al., 2010; Tipsmark et al., 2011), in the incubating medium. It would be interesting to reveal plasma factors that regulate mRNA expression levels of NHE3 in future studies.

In addition to ion transporters, the mRNA levels of GR1, GR2 and MR were assessed to examine the possible feedback system on these receptor mRNAs in the gills. Cortisol repressed mRNA expression of GR2 and MR, and aldosterone repressed MR mRNA. Also,

RU486 and spironolactone further down-regulated GR2 and MR mRNA. Corticosteroid -mediated gene repression occurs via GR-corepressor complexes, and GR antagonists are known to enhance such transrepression activities by inhibiting GR-coactivator interaction, but promoting GR-corepressor interaction (Bosscher et al., 2002; Presman et al., 2010). The result of this experiment implies the presence of above gene repression system in the gills of tilapia. In the gills, such negative feedback mechanism via repression of corticosteroid receptor mRNA levels probably regulates cellular responsiveness to cortisol. Interestingly, aldosterone up-regulated GR1, but down-regulated GR2 and MR, being partly consistent with the study in Atlantic salmon (Kiirelich et al., 2007), where MR controlled GR1. These facts suggest that MR in the gills regulates GR, although its physiological significance needs further research to be understood.

The physiological responses after transfer from SW to H-K SW

Since there was no clear up-regulation of ROMKa in response to *in vitro* cortisol treatment of SW-acclimated tilapia gills, another approach was conducted for SW fish. I transferred SW fish to H-K SW, and examined the temporal changes in ion and cortisol levels in blood, and mRNA levels of gill ion transporters, gill cortisol receptors, and pituitary hormones. In plasma, there was a marked increase in K^+ levels of H-K fish, reflecting the impact of 10 mM increase in external K^+. The other ion levels and osmolality remained constant, although there was slight decrease in Na^+ levels, being consistent with the plasma data in Chapter 1. A slight decrease in plasma osmolality at 12 h indicates possible stresses due to transfer of fish.

Although cortisol did not affect gill ROMKa mRNA in the incubation study, the *in vivo* contribution of this hormone to K^+ regulation still cannot be ruled out. For this reason, I also quantified plasma cortisol levels and gill receptor mRNA levels in H-K SW transfer study. An increase in plasma cortisol was found at 24 h after transfer. This response seems somewhat slow compared to classic rapid rise (Wendelaar Bonga, 1997). In this regard, the possible roles of cortisol on K^+ homeostasis in SW are discussed below. In the gills, mRNA levels of GR1 and MR were transiently lower than those of control fish at 12 h after transfer. This phenomenon might be a response of the gills to transfer stresses, but the direct causes are

still unknown.

ROMKa, NKCC1a and CFTR mRNAs were also quantified in this experiment. As expected, ROMKa mRNA was markedly up-regulated in response to H-K SW transfer. Interestingly, the onset of this increase was well before that of cortisol, indicating that ROMKa mRNA up-regulation in SW fish can take place without a surge of cortisol. NKCC1a mRNA was highly expressed in H-K SW fish compared to control. A similar tendency was observed in artificial SW-acclimated fish (Chapter 1), and these facts together suggest that NKCC1a also contribute to K^+ excretion to external water. The stable CFTR mRNA levels, along with the plasma Cl^- concentrations, indicate that 10 mM increase of environmental Cl^- is a physiologically negligible increase for SW-acclimated fish.

Pituitary hormone (GH, PRL, SL, and POMC) mRNA levels were quantified as the candidate hormones that could mediate up-regulation of ROMKa. The roles of the candidate hormones have been discussed in the context of body fluid homeostasis so far: GH and PRL are considered as important hormones for SW- and FW-acclimation, respectively (Sakamoto and McCormick, 2006; Breves et al., 2010); a physiological role of SL is still not clear (Kaneko, 1996; Fukamachi et al., 2005; Fukamachi and Meyer, 2007), but several studies have implied its relation to acid-base regulation (Kakizawa et al., 1996; Kakizawa et al., 1997; Furukawa et al., 2010); POMC is a precursor of adrenocorticotropic hormone (ACTH), which is an upstream hormone of cortisol in the hypothalamus-pituitary-interrenal gland axis (Mommsen et al., 1999). In the present transfer experiment, there were no significant differences among control and H-K groups, but PRL mRNA was highly expressed at 12 h, and SL and GH mRNAs showed inverse patterns to those of PRL mRNA levels. Considering that SW and H-K SW fish showed similar mRNA patterns, it is possible that the changes observed are due to circadian rhythms reported previously (Spieler et al., 1978; Squier, 2007). All in all, I conclude that pituitary hormones are not particularly involved in plasma K^+ regulation.

High external K^+ concentration augments branchial expression of ROMKa mRNA in vitro

Although the H-K SW transfer study showed an increase in plasma cortisol levels 24 h, the rapid fraction (within 6 h) of ROMKa mRNA up-regulation in response to H-K exposure

was quite unlikely to be under the control of the hormones assessed. Based on these results, I subsequently tested the direct effect of K^+ concentration on ROMKa mRNA expression in ionocytes by the *in vitro* incubation experiment. Interestingly, ROMK and NKCC1a mRNAs were stimulated by H-K treatment, without effects on the other transporter mRNAs quantified. This is the first knowledge that changes in K^+ concentration alter mRNA levels of specific transporters in the ionocytes. Although the mechanism underlying this response is unknown, some voltage-gated channels or osmosensors (Kültz, 2012) might be involved in this response, since increases in extracellular K^+ concentration can activate above molecules via membrane depolarization and K^+ intrusion, respectively.

Differential regulation of plasma K^+ with respect to the role of cortisol

Throughout the series of experiments, I demonstrated that gill ROMKa mRNA expression in FW-acclimated tilapia was markedly up-regulated by *in vitro* cortisol treatment, and that GRs are the probable mediator of this effect. In contrast, ROMKa mRNA levels of SW-acclimated fish were not activated by cortisol, but by increased extracellular concentration of K^+. The difference may reflect the different situations of K^+ handling between FW and SW. FW fish need to excrete K^+ only when plasma K^+ exceeds its physiological ranges (for example, feeding K^+ diet, acidosis, and tissue breakdown). In mammals, plasma K^+ overload, acidosis, and stress are accompanied by an elevation of plasma cortisol levels (Ueda et al., 1982; Charmandari et al., 2005; Koeppen, 2009). In tilapia, plasma cortisol levels increase in the early stages of salinity acclimation (Kammerer et al., 2010). Thus, the responsiveness of ROMKa mRNA to cortisol in FW fish may be a preparatory mechanism for above physiological events. In contrast to FW fish, SW fish constantly excrete ions, including K^+, Na^+, and Cl^- from the gills (Hwang et al., 2011; Chapter 1 of this thesis), and this is accomplished by Type-IV cells in tilapia. The role of cortisol, along with GH and insulin-like growth factor I (IGF-I), on SW adaptation has long been discussed (Mommsen et al., 1999; McCormick, 2001). The present study is the first to successfully maintain initial gill transporter mRNA levels of SW fish *in vitro*, but only when incubated in the presence of FBS and cortisol. Taken together, the primary role of cortisol in SW fish may be maintenance of "ion-secretory" cells, or Type-IV cells of tilapia. Recently,

Cruz et al. (2012) found that cortisol induced cell differentiation into ionocytes in zebrafish epithelia. Considering above effects of cortisol, the tendency of plasma cortisol levels to be high in H-K fish of the present transfer experiment may reflect the active engagement of the fish in maintenance of or differentiation into the ionocytes. In conclusion, I suggest differential regulatory mechanisms of ROMKa mRNA expressions between FW and SW: FW, under the control of cortisol and GRs; SW, a cell-autonomous response to K^+. Further investigation will be necessary to confirm this hypothesis; for example, by *in vivo* cortisol injection, and by observation of cell proliferation and differentiation.

Table 4-1. *Primers used for cloning, quantitative PCR (qPCR) and RT-PCR*

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, "VEFPEM"
MR-GSP1F	ACAGCCTGACACCACTGAC	qPCR, 3'RACE
MR-GSP2R	GGCAAAAGGAGGACAGACAC	qPCR, 5'RACE
CFTR-qF	TCACCAGCATCGCTGTAGATG	qPCR
CFTR-qR	GGTTGTCGATGACGATATCAGG	qPCR
POMC-F	CTCTGTCACTCTGACCTCAC	qPCR
POMC-R	GTGAGGAGGCGTCTGATG	qPCR

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.

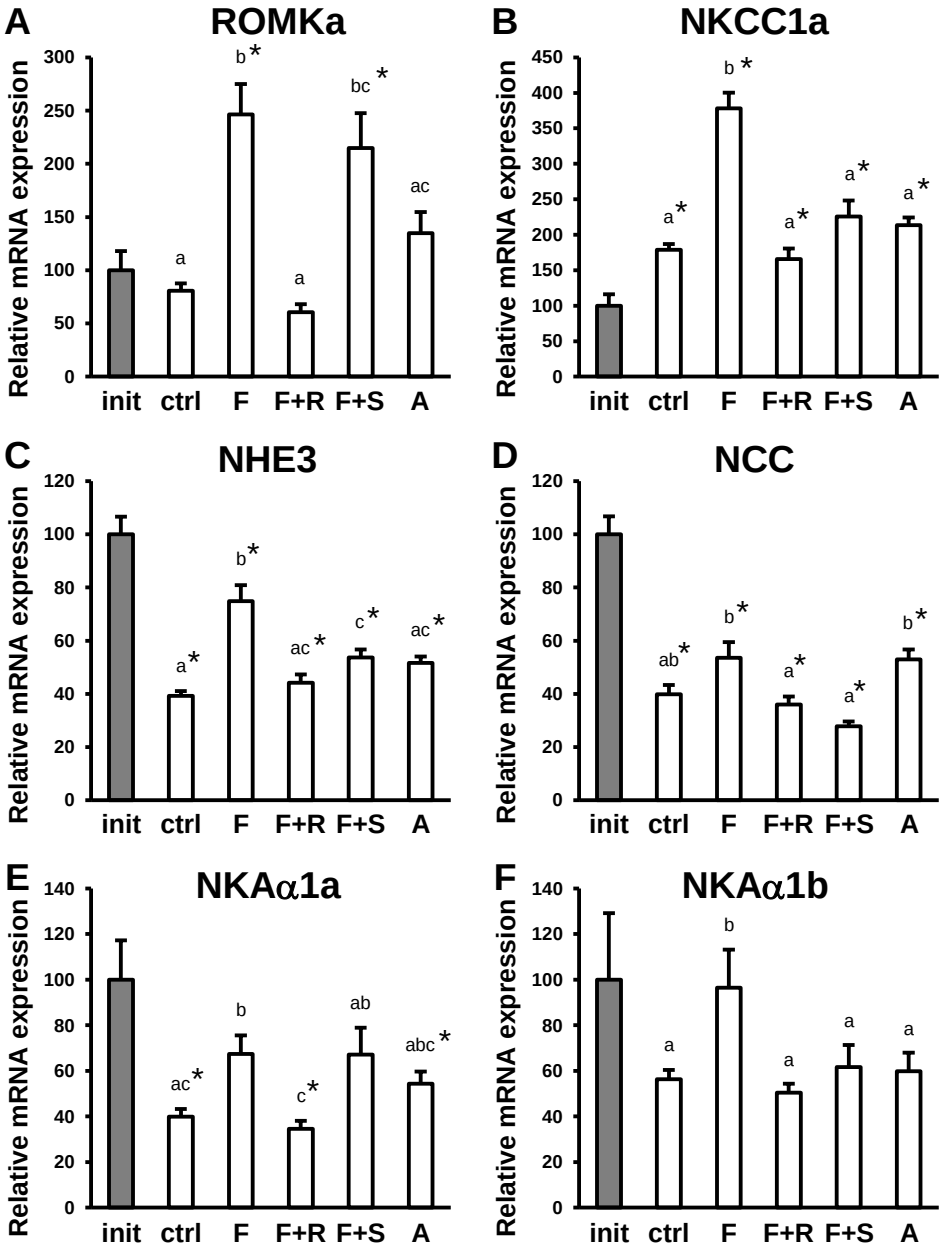


Fig. 4-1. Relative mRNA expression levels (mRNA/18S rRNA) of ROMKa, NKCC1a, NHE3, NCC, NKAα1a, and NKAα1b in gill filaments of freshwater-acclimated tilapia at the initial (init) time point and after 6-h incubation in 5 different media: control (ctrl), cortisol (F), cortisol+RU486 (F+R), cortisol+spironolactone (F+S), aldosterone (A). Data are expressed as means ± S.E.M. (n=6). The expression levels of initial samples were designated as an arbitrary value of 100. Different letters indicate significant differences among incubated samples, and asterisks indicate significant differences from initial value (P<0.05).

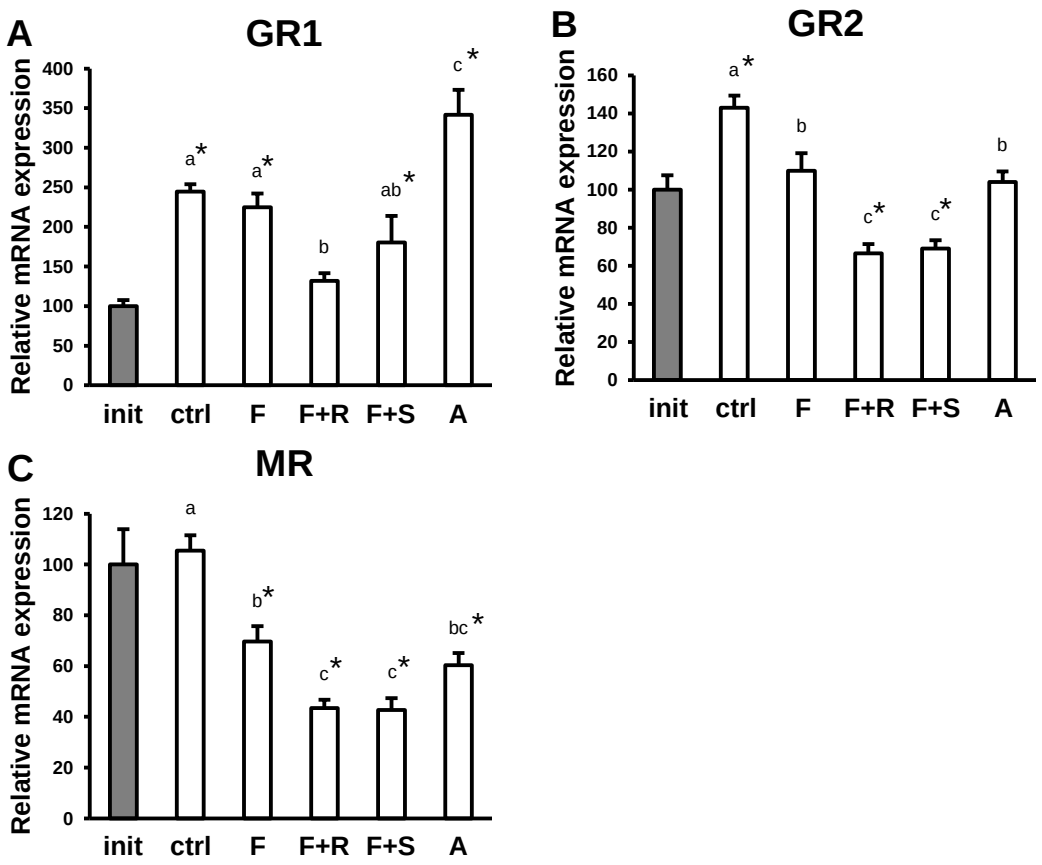


Fig. 4-2. Relative mRNA expression levels (mRNA/18S rRNA) of GR1, GR2, and MR in gill filaments of freshwater-acclimated tilapia at the initial (init) time point and after 6-h incubation in 5 different media: control (ctrl), cortisol (F), cortisol+RU486 (F+R), cortisol+spironolactone (F+S), aldosterone (A). Data are expressed as means ± S.E.M. (n=6). The expression levels of initial samples were designated as an arbitrary value of 100. Different letters indicate significant differences among incubated samples, and asterisks indicate significant differences from initial value (P<0.05).

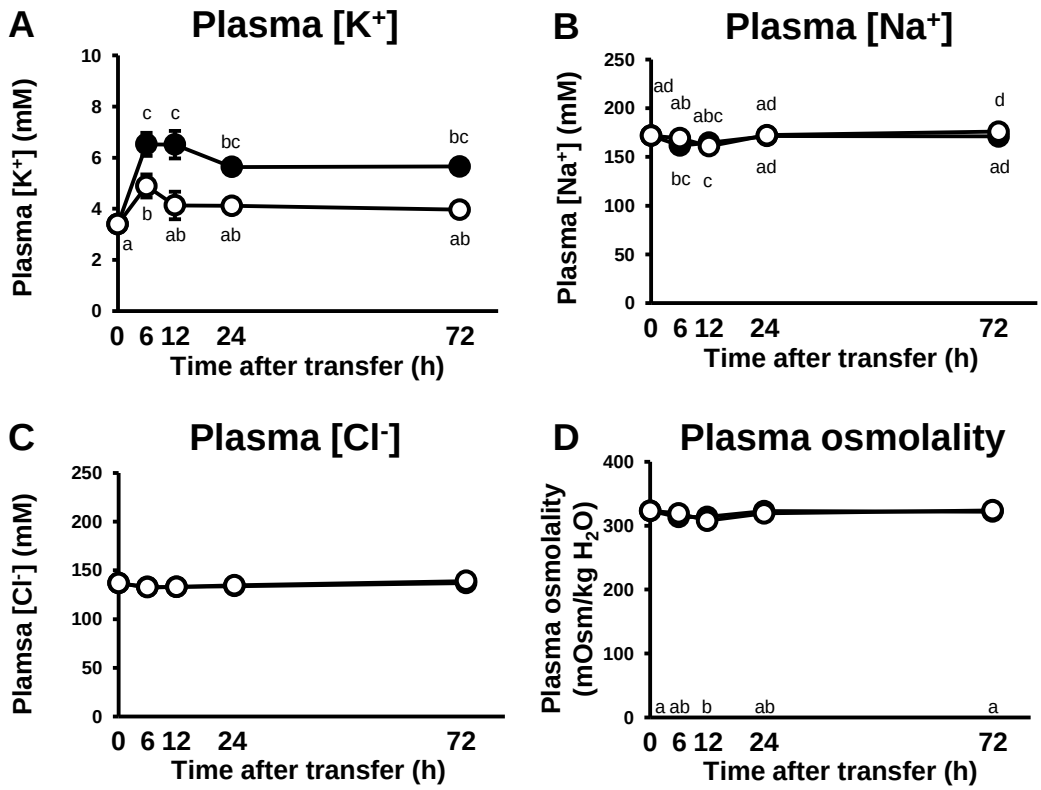


Fig. 4-3. Changes in plasma concentrations of K^+, Na^+, and Cl^-, and plasma osmolality in tilapia kept in seawater (SW, open circles) or in those transferred to high-K^+ (H-K) SW (closed circles). Data are expressed as means ± S.E.M. (n=5-8). Different letters on time axes indicate significant differences in the factor of time, and those on each sample indicate significant differences between each sample (P<0.05).

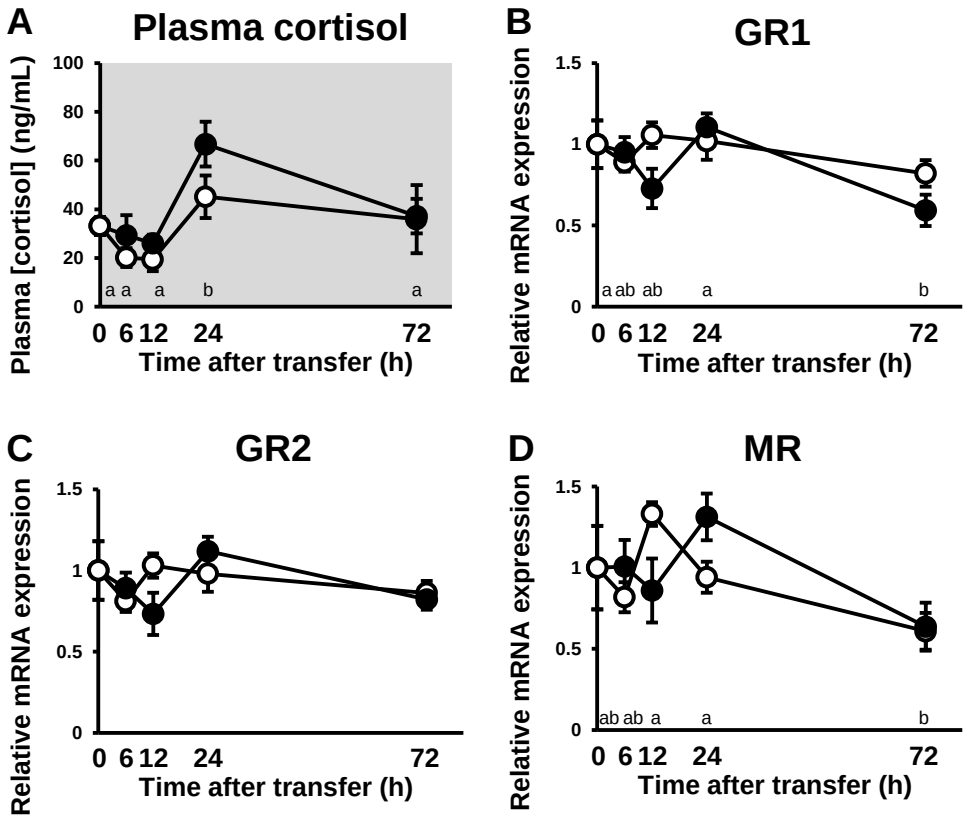


Fig. 4-4. Changes in plasma cortisol levels and relative mRNA expression levels (mRNA/18S rRNA) of GR1, GR2, and MR in gills of tilapia kept in seawater (SW, open circles) or in those transferred to high-K^+ (H-K) SW (closed circles). mRNA expression levels at 0 h are designated as an arbitrary value of 1. Data are expressed as means ± S.E.M. (n=5-8). Different letters on time axes indicate significant differences in the factor of time (P<0.05).

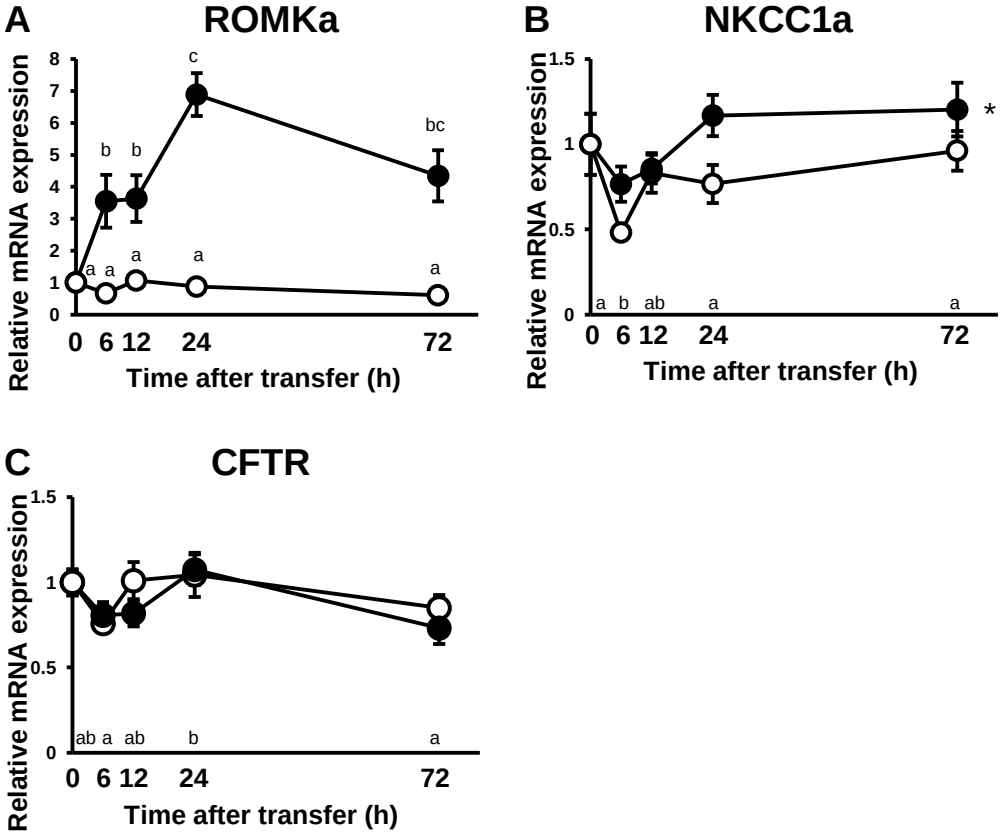


Fig. 4-5. Changes in relative mRNA expression levels (mRNA/18S rRNA) of ROMKa, NKCC1a, and CFTR in gills of tilapia kept in seawater (SW, open circles) or those transferred to high-K^+ (H-K) SW (closed circles). mRNA expression levels at 0 h are designated as an arbitrary value of 1. Data are expressed as means ± S.E.M. (n=5-6). Different letters on time axes indicate significant differences in the factor of time, and those on samples between each sample (P<0.05). An asterisk indicates significant difference between control and H-K fish (P<0.05).

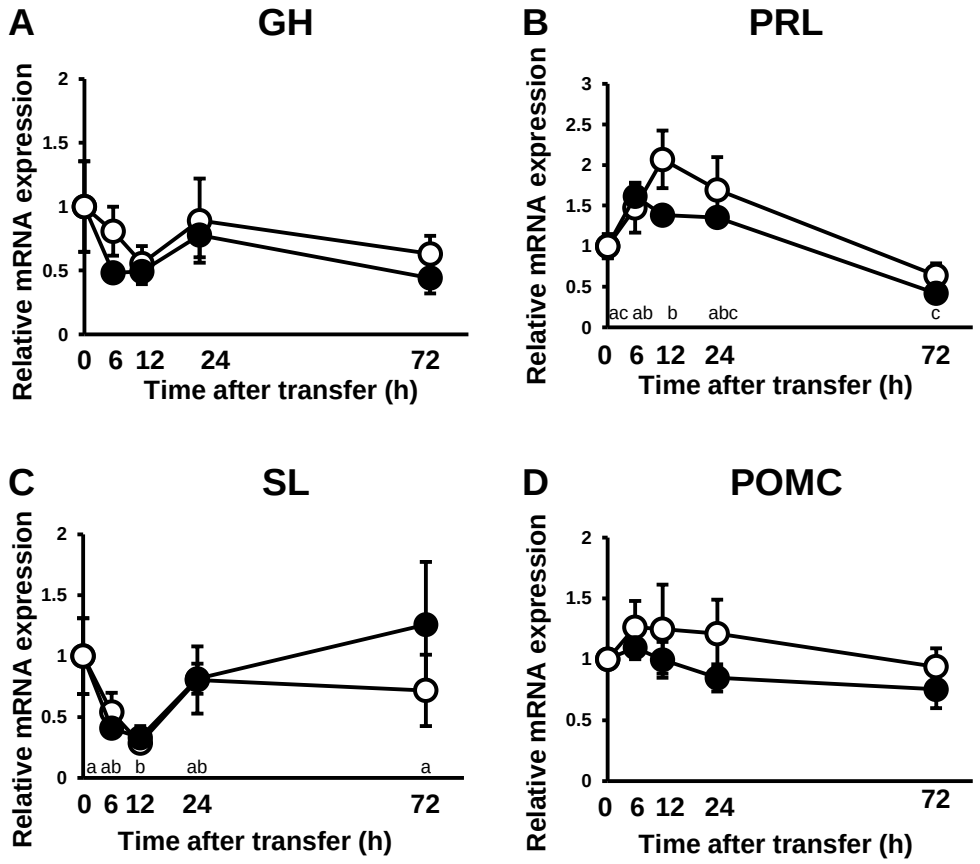


Fig. 4-6. Changes in relative mRNA expression levels (mRNA/18S rRNA) of GH, SL, PRL, and POMC in tilapia kept in seawater (SW, open circles) or those transferred to high-K^+ (H-K) SW (closed circles). mRNA expression levels of samples at 0 h are designated as an arbitrary value of 1. Data are expressed as means ± S.E.M. (n=5-6). Different letters on time axes indicate significant differences in the factor of time (P<0.05).

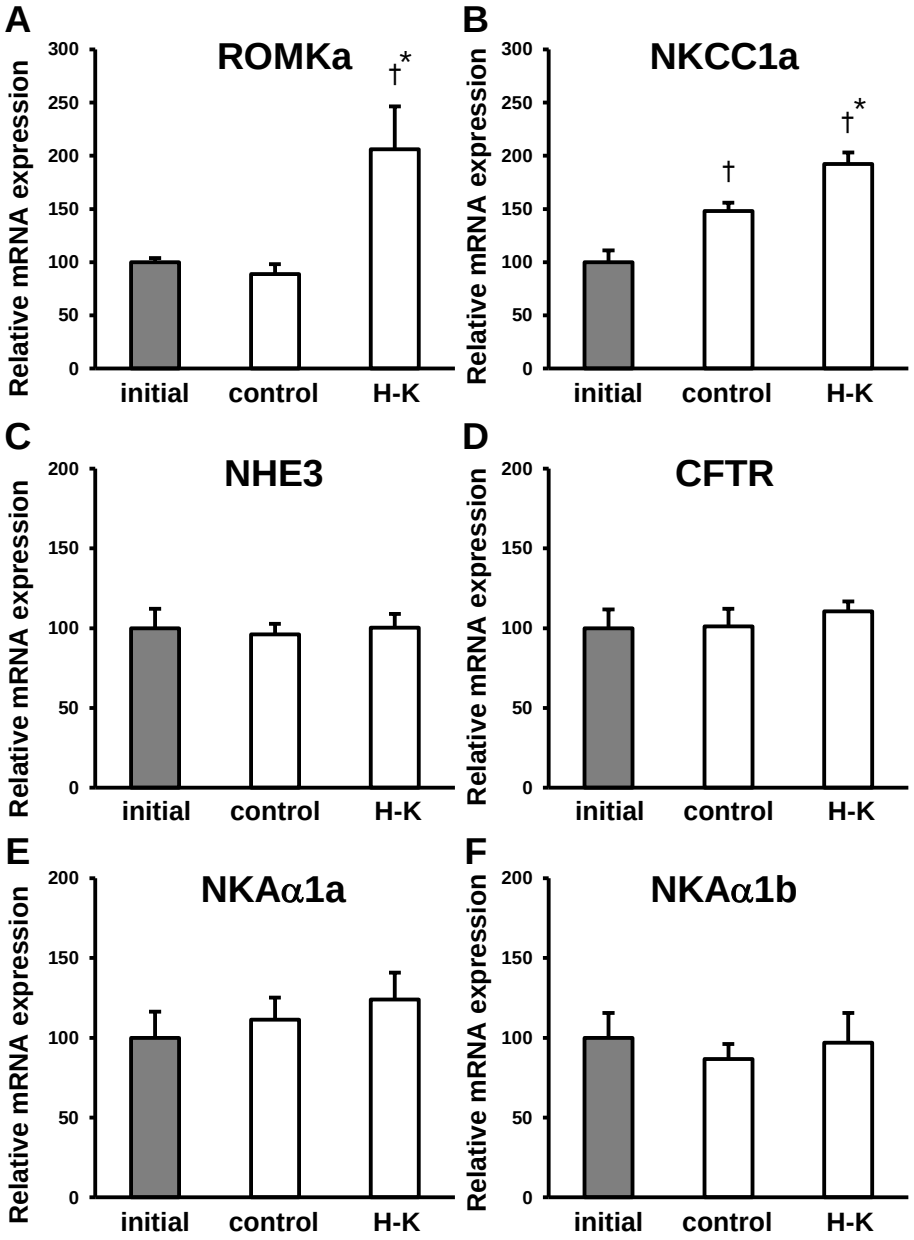


Fig. 4-7. Relative mRNA expression levels (mRNA/18S rRNA) of ROMKa, NKCC1a, NHE3, CFTR, NKAα1a, and NKAα1b in gill filaments of seawater-acclimated tilapia at the initial time point and after 6-h incubation in control and high-K^+ (H-K) media. Data are expressed as means ± S.E.M. (n=6). The expression levels of initial samples were designated as an arbitrary value of 100. Asterisks indicate significant differences between incubated samples, and daggers indicate significant differences from initial value (P<0.05).

General Discussion

Since the first discovery of ionocytes in fish (Keys and Wilmer, 1932), these cells have long been studied from the aspects of morphology, distribution, function, and evolution (Evans et al., 2005; Kaneko et al., 2008; Hiroi and McCormick, 2012). Generally, fish utilize specific types of ionocytes equipped with a different set of transporters for hypo- and hyperosmoregulation in SW or FW, respectively. With the recent progresses in fish osmoregulation research, the molecular mechanisms of ion transport within gill ionocytes have become increasingly clear (Hwang et al., 2011). In such a situation, we would have to say that K^+ is an overlooked ion species as a research subject in fish. Actually, K^+ handling mechanisms have been fairly investigated in mammals, due to its physiological importance (Giebisch et al., 2007). The present study is the first to assess fish K^+ regulatory mechanisms in detail, mainly focusing on gill ionocytes.

Detection of K^+ secreted from ionocytes

In Chapter 1, the onset of the present study on fish K^+ regulation was to detect K^+ excretion, from SW-acclimated tilapia gills. Similar to Na^+ and Cl^-, excess K^+ was most likely to be eliminated by extrarenal mechanisms of SW fish, since SW fish produce a little amount of urine in the kidney. To detect K^+ excretion, I used Na-TPB, which is known to react with K^+ to form water-insoluble K-TPB. Exposing freshly-dissected gill filaments to Na-TPB solution allowed formation of precipitates on the gill surface. Actually, this method is an application of silver staining, which has been used to detect Cl^- secretion from the gills of SW-acclimated fish (Kaneko and Shiraishi, 2001). By precipitating Cl^- immediately after secretion from the gills, this method enables visualization and localization of the Cl^- excretion. In the present study, K-TPB treatment, followed by rhodamine 123 staining for ionocytes, localized the presence of precipitates to the apical openings of the ionocytes. After lyophilizing the samples, EDS analysis was applied to confirm the presence of K in the precipitates. Thus, K^+ excretion from the gill ionocytes was demonstrated for the first time.

ROMKa expressed in tilapia Type-IV ionocytes

After localizing K^+ excretion sites to ionocytes, the molecular pathway responsible for the K^+ excretion in tilapia was determined. Among the molecular candidates cloned, ROMKa mRNA showed marked response to H-K treatment, and thus further investigations were conducted focusing on this channel. Among renal K^+ channels in mammals, ROMK is the best studied for its structure, distribution, and function (Hebert et al., 2005). Mammalian renal ROMK is localized to apical membrane of tubule cells from the TAL through the OMCD, and plays central roles in plasma K^+ regulation. Now that a variety of ion transporters homologous to those expressed in the mammalian kidney are found in fish gill ionocytes (Hwang et al., 2011), tilapia ROMKa expressed in the gills was not an unexpected result. Subsequently, immunofluorescence staining revealed that tilapia ROMKa was localized to the apical membrane of the ionocytes in SW-acclimated tilapia. Interestingly, the distribution patterns of ROMKa immunosignals were reminiscent of those of CFTR in Type-IV ionocytes (Hiroi et al., 2008). One would speculate that apical movement of Cl^- through CFTR also plays a role in establishing voltage gradients across apical membrane that favor K^+ extrusion via nearby ROMKa. Actually, the interaction between ROMK and CFTR channels has been stated previously (Hebert et al., 2005). For future studies, it may be interesting to examine the relationship between regulation of plasma K^+ and Cl^- by ROMKa and CFTR, respectively, expressed in Type-IV ionocytes.

Radioactive Cs in the living organisms

After the recent earthquake in northeastern Japan and the following nuclear disaster in Fukushima Daiichi nuclear power plant, leakage of radioactive Cs has become great concern on fishery, agriculture, stock farming, and ultimately human lives. It has been known that Rb^+ and Cs^+ are biochemical analogs of K^+, behaving in a similar manner within organisms. Empirically, radioactive Rb^+ has been used as a tracer for K^+ *in vivo*, with some coefficients to obtain more likely estimate (Sanders and Kirschner, 1983a, 1983b; Eddy, 1985; Gardaire et al., 1991; Gardaire and Isaia, 1992). Just like K^+ contained in the intracellular compartment of the body, Cs^+ does not appear much in the blood, but the most part does in the muscle (Sato et al., 1997; Peters et al., 2006). While investigations on distribution patterns of ^{137}Cs-contaminated fish in the ocean and those of ^{137}Cs *within* the fish bodies are well

conducted (Japanese Fisheries Agency, 2012), the physiological studies on molecular pathways that transport ^{137}Cs in fish bodies are lacking.

Excretion of Cs^+ and Rb^+ via Type-IV ionocytes

In a review describing ^{137}Cs accumulation in SW fish, Kasamatsu (1999) pointed out that high environmental K^+ accelerated elimination of ^{137}Cs from rainbow trout. Since this phenomenon appeared to be in good agreement with up-regulation of gill ROMKa mRNA in tilapia subjected to H-K treatment (Chapter 1), the possible contribution of this novel K^+-transporting pathway in the gills to excretion of the two biochemical analogs, Cs^+ and Rb^+, was examined in Chapter 2. I applied the K-TPB precipitation method for the purpose, since TPB has been known to react with both Cs^+ and Rb^+ in a similar manner to K-TPB formation. After intra-arterial administration of Cs^+ and Rb^+ as substitutes for K^+, the ionocytes readily took up these elements from ECF, and started excreting Cs^+ and Rb^+ within 10 min. The existence of Cs and Rb were finally confirmed by EDS analysis, and it became evident that Type-IV ionocytes in tilapia excrete Cs^+ and Rb^+, and ROMKa in the apical membrane is most likely to be the molecular pathway responsible for the excretion. In combination with the further analysis determining ROMKa as an actual $^{137}Cs^+$ transporter, this finding will have beneficial influence for future fishery surrounding the northeastern areas of Japan.

Differences in renal K^+ transporter mRNAs expressed between fish and mammals

Inhabiting ion-poor environments, FW fish take fairly different osmoregulatory strategies compared to SW fish, to counteract ion loss and water gain. Not only they absorb ions through branchial “ion-absorbing” FW-type ionocytes, they discharge excess water by producing a large amount of urine in the kidney (Marshall and Grosell, 2006). Thus, unlike the situations in SW-acclimated fish, the contribution of the kidney cannot be ignored for plasma K^+ regulation in FW fish. In Chapter 3, to understand the K^+ regulatory mechanisms in FW-acclimated fish, I exposed FW tilapia to H-K FW, and observed physiological responses of the fish, including plasma and urine K^+ concentrations and mRNA expression profiles in the gills and kidney. As a result, a significant elevation of urine K^+ concentration was detected, suggesting a possible role of the kidney in plasma K^+ regulation in

FW-acclimated tilapia.

In the mammalian kidney, the K^+ regulatory mechanisms have been extensively studied so far. The kidney executes a relatively complicated mechanism for K^+ regulation that include K^+ absorption and excretion within a nephron: (1) the blood is filtered in a glomerulus; (2) bulk of filtered K^+ is first reabsorbed as it travels through the proximal convoluted tubule; (3) K^+ is further absorbed by NKCC2 expressed in the apical membrane of the TAL; and (4) finally, K^+ is excreted into urine through ROMK expressed in the apical membrane of the distal part of the tubule. During above courses of K^+ handling, ROMK, the main regulator of plasma K^+, takes two different significant roles: apical K^+-recycling pathway for $Na^+/K^+/2Cl^-$ reabsorption through NKCC2 in the TAL, and definitive K^+ excretion in the cortical collecting duct, the section just before the OMCD. In mammals, thus, renal ROMK is important not only to maintain plasma K^+ levels, but also to regulate plasma levels of Na^+ and Cl^-. In the kidney of FW-acclimated tilapia, on the other hand, mRNA levels of ROMKa, along with that of ROMKb, a putative paralogous gene product of ROMKa, was only marginally expressed, and their expression levels did not change after H-K treatment. Instead, substantial expression of KCC1 mRNA was detected in the kidney, marking 29 times higher than those in the gills. Assuming that KCC1 is localized to the apical membrane of renal tubule, as in mammals, a possible physiological role of KCC1 would be apical recycling of K^+ for NKCC2-mediated reabsorption of Na^+ and Cl^-. Another possible role of KCC1 could be excretion of K^+ into the urine, functioning as a fish counterpart of mammalian renal ROMK either way. Considering that zebrafish larvae strongly express ROMK in the pronephros (Abbas et al., 2011), it will be interesting to assess the renal K^+ transporters in evolutionarily-ancient fish species, such as lamprey and hagfish, along with cartilaginous fish, to understand the evolution of K^+ handling in the kidney of vertebrate species.

ROMKa expressed in apical openings of Type-IV and Type-III ionocytes

In Chapter 1 and 3, ROMKa immunosignals were clearly detected at the apical openings of Type-IV and Type-III ionocytes of tilapia. Type-III ionocytes in FW-acclimated tilapia are considered to transform into Type-IV cells upon SW-acclimation and *vice versa*

(Hiroi et al., 1999; Inokuchi and Kaneko, 2012; Hiroi and McCormick, 2012). In the present study, the presence of ROMKa, together with NHE3 and NKCC1a, in both of these cells further supports this Type-III and Type-IV "mutual transformation" hypothesis.
Co-expression of ROMKa with NHE3, but not with NCC, in the ionocytes, is also reasonable in evolutionary aspects: while NHE3-expressing ionocytes have been reported in many teleost species, NCC-expressing ones are considered to be lost in a wide range of fish species (Hiroi and McCormick, 2012). Although further studies are needed to confirm ROMK in the gills of other FW-inhabiting fish species, the presence of ROMKa in both SW and FW-type ionocytes in tilapia indicates that branchial K^+ excretion, separated from hyposmoregulation or "salt secretion", is an important physiological mechanism in both SW- and FW-inhabiting fish. Since fish gills are directly exposed to external media, ion-secretory transporters expressed in apical openings of ionocytes do not seem to function for any of "recycling" mechanisms: the excreted ions should immediately disperse into the environmental water. In this regard, ROMKa expressed in the gills, but little in the kidney, may meet the specific role of K^+ excretion, with less interactive effect on the other ions regulated in the kidney.

A possible approach to reducing ^{137}Cs from FW fish

Whereas SW fish can eliminate radioactive Cs faster than true half-life of it (Kasamatsu, 1999), accumulation of Cs in FW fish has been pointed out, as an urgent issue to be solved. In Chapter 2, I showed that gill ionocytes of SW-acclimated tilapia excrete Cs, presumably through ROMKa expressed at the apical opening of the cells. The results of Chapter 3, showing ROMKa expression in FW-type ionocytes, imply a possible countermeasure for the radioactive Cs accumulated in FW fish. For example, we may allow FW fish to excrete radioactive Cs^+ together with K^+, by manual administration of exogenous K^+, with the food or the environmental water added with K^+.

GR-mediated transactivation of ROMKa mRNA in the gills of FW-acclimated tilapia

In Chapter 4, the effects of cortisol on ROMKa mRNA were investigated using *in vitro* gill incubation system. In FW-acclimated tilapia gills, ROMKa mRNA was markedly up-regulated in response to cortisol treatment, and this effect was abolished in the presence of

RU486, a GR antagonist. This is in sharp contrast with ROMK in mammals, where aldosterone, a mineralocorticoid, regulates mRNA and surface protein expression of ROMK (Schrier et al., 2010). In the distal nephron of the mammalian kidney, aldosterone induces expression of corticosteroid hormone induced factor (CHIF) and serum- and glucocorticoid-regulated kinase-1 (SGK-1), thereby activating epithelial Na^+ channels (ENaCs), NKA, and ROMKa in concert, to accelerate Na^+ retention and K^+ excretion in this segment (Fuller and Young, 2005). Such cooperative mechanisms are still not known in the gills or kidney of fish, but the difference in the corticosteroid receptor responsible for ROMKa expression in the gills implies the presence of distinct transporter regulatory mechanisms from those in the kidney. Further studies on physiological roles of GRs and MR in the gills and kidney will be helpful to understand functional differentiation of these receptors between gills and kidneys, or between kidneys of fish and mammals.

Cortisol function on Type-IV ionocytes: maintenance or differentiation?

Cortisol has long been considered as SW-adapting hormone. During acclimation to SW, cortisol highly circulates in the plasma, stimulates gill NKA activities, and increases the number of epithelial ionocytes, often in cooperation with GH and insulin-like growth factor-I (IGF-I) (Mommsen et al., 1999; McCormick, 2001). Importantly, for SW fish, there is still no direct evidence that cortisol increases ion-transporting activities of each “SW-type” ionocyte. Shaw et al. (2007) and Kiirelich et al. (2007) suggested that cortisol increases transcription of several ion transporter mRNAs in SW-type ionocytes. In these studies, however, the effect of cortisol on cell recruitment (Cruz et al., 2012), or maintenance during incubation cannot be negated. Actually, in my preliminary experiment, 6-h incubation of SW gills dramatically reduced any of the ion transporter mRNA levels, implying a major destruction of SW-type ionocytes, similar to the previous observation (Kiirelich et al., 2007). At the end of Chapter 4, I found that incubated SW gills can maintain the initial levels of several ion transporter mRNAs in the presence of FBS and cortisol. Since 6-h incubation does not seem long enough to raise newly-differentiated ionocytes highly expressing ion transporters (Chang and Hwang, 2011; Cruz et al., 2012), I would attribute the mRNA levels in FBS+cortisol incubated gills to the maintenance of the number and function of the

preexisting ionocytes. In human cultured keratinocytes, glucocorticoid-mediated inhibition of apoptosis has been reported (Stojadinovic et al., 2007). It would be interesting for future studies to assess the possible effects of cortisol on the maintenance of SW-type ionocytes, and to determine the factors in FBS that are involved in such phenomenon.

Differential regulation of ROMKa mRNA expression

In the gill incubation experiment of Chapter 4, H-K media increased mRNA expression of ROMKa in the SW-acclimated tilapia gills. Although the present study is the first to show the effects of K^+ on ionocyte mRNA expression profiles, the effects of salinity or osmolality on ionocytes has been shown previously. Shiraishi et al. (2001) and Hiroi et al. (2005) demonstrated the effect of salinity on the emergence of SW-type ionocytes using a "yolk-ball" incubation system, whereby the effects of endocrine factors emanating from the embryonic bodies are abrogated. In another study, the effect of basolateral osmolality on branchial Cl^- secretion has been shown using an Ussing chamber (Marshall et al., 2000). Although the contribution of paracrine factors from the nearby cells cannot be ruled out, these *in vitro* studies imply the presence of cell-autonomous responses of the ionocytes to the salinity or osmolality via osmosensory signaling pathways (Kültz, 2012).

Meanwhile, there has been no distinct K^+ sensing mechanism reported, probably due to the profound impacts of K^+ levels on the activities of a variety of the enzymes and channels, any of which may lead to downstream actions to restore K^+ levels. However, there is an interesting example implying K^+ sensing mechanisms in the rat kidney. In the kidney of an adrenalectomized rat, cortical ROMK mRNA is regulated by aldosterone and serum K^+, whereas medullary ROMK mRNA is regulated by serum K^+ levels irrespective of aldosterone (Wald et al., 1998). Such differentially-regulated fractions of ROMK mRNA, responding to corticosteroid or K^+ levels, share similarity with those found in FW- and SW-acclimated tilapia in the present study: "temporal K^+ secretion" and "constant K^+ secretion" fractions. In the rat kidney, cortical ROMK operates final K^+ excretion that strongly affects plasma K^+ levels, whereas constant K^+ secretion in the medulla guarantees a K^+ recycling pathway contributing to apical absorption of Na^+ and Cl^- via NKCC. In tilapia, FW gills secrete K^+ only when plasma K^+ exceeds its physiological range, whereas SW ones should constantly

eliminate excess plasma K^+. At present, it is unknown if such similarity in differential ROMK mRNA regulation is a result of a random chance or conserved phenomena across many species. Thus, studies on K^+ regulatory mechanisms, focusing on ROMK, corticosteroid, and K^+-sensing, in a variety of species would be of great interest for future studies.

Throughout this study, I clarified the K^+ excretory mechanisms in tilapia gills, where ROMKa expressed in apical openings of ionocytes play a central role. Also, the differential regulation of ROMKa expression in FW- and SW- tilapia indicates the different K^+ regulatory strategies adopted by FW- and SW-inhabiting fish, probably reflecting the different demands of K^+ excretion in their habitats. Since K^+ excretory mechanisms most likely eliminate Cs^+ accumulated in the bodies, the findings in the present study can contribute not only to the basic understanding of K^+ handling in fish, but also to the possible countermeasure against the contamination of fish by radioactive Cs^+ in northeastern Japan. The present study revealed the core mechanism of K^+ excretion in fish, but at the same time it highlighted other questions yet to be resolved, such as renal K^+ handling mechanism, functional differentiation of corticosteroid receptors, and branchial K^+-sensing mechanism. Thus, future studies should be oriented to basic studies on K^+, in combination with applied researches with $^{137}Cs^+$, both of which will be the most powerful support for the reconstruction and further advances of Japanese fishery.

Summary in Japanese

（論文の内容の要旨）

ティラピアの鰓塩類細胞におけるカリウム及びセシウムの排出と その調節機構に関する生理学的研究

魚類を含む全ての脊椎動物において、体液の恒常性を保つことは個体の生存に必須である。中でも血漿浸透圧の調節、すなわち、血漿中の主な浸透圧構成物質である Na^+や Cl^-、および溶媒である水の調節は必要不可欠である。一方で、Na^+、Cl^-以外のイオンの調節も生物の正常な活動にとって非常に重要である。体内に最も豊富に存在する一価の陽イオンである K^+は、その大部分が細胞内(約 140 mM)に局在し、細胞外の濃度は約 4 mM と、Na^+や Cl^-に比べて低い値で一定に保たれている。この細胞膜を隔てた濃度差は静止膜電位を生じる一因であり、細胞や個体の正常な生命活動を保証している。哺乳類は、腎臓で尿中への K^+排出量を調節し、血漿 K^+濃度を正常な範囲内に保つことが知られており、これまでにその詳細なメカニズムが明らかとなっている。一方、魚類においては、血漿 K^+の調節機構に着目した研究は極めて乏しい。

2011 年 3 月 11 日に発生した東日本大震災と、それに付随する福島第一原子力発電所の事故の影響により、日本国土や近海において放射性 Cs による汚染が発生した。現在も汚染による国民の健康や農林水産業への影響が懸念されており、早急に生物圏への影響を調べる必要がある。とりわけ魚類は日本において食糧としても馴染み深いものであり、魚類の汚染状況をモニタリングするのみならず、放射線物質の魚体内における動態を理解することは、日本の水産業の未来にとって重要な知見となる。生物の体内において、Cs^+は K^+と非常に類似した挙動を示すことが知られており、魚類での K^+調節機構を解明することで、Cs^+汚染へ対処する上での大変有用な情報をもたらすと考えられる。

真骨魚類の浸透圧調節では鰓に存在する塩類細胞が非常に重要な役割を担っているが、K^+調節への関与については不明である。塩類細胞は、体内側の膜(側底膜)に Na^+/K^+-ATPase (NKA)が豊富に存在し、また体外側の膜(頂端膜)と側底膜に多種多様なイオン輸送体が存在することで、Na^+や Cl^-の輸送以外にも酸塩基調節、アンモニア輸送などに関わることが知られている。このことから、本研究では真骨魚類の鰓塩類細胞に着目し、魚類では未だ知られていない K^+調節機構を明らかにすることを目的とした。

第 1 章　海水馴致ティラピアの鰓塩類細胞における K^+排出機構の解明

海水環境中では比較的高濃度に K^+が存在し、体内に K^+が過剰になる傾向にあるため、海水魚は積極的に K^+を排出する必要がある。しかし、海水に馴致した真骨魚は水分を保持するためほとんど尿を出さず、またその K^+濃度は低いことが知られている。このことから、まず本研究では主要な浸透圧調節器官である鰓に着目し、海水馴致ティラピアを用いて、鰓における K^+排出の有無を検討した。K^+と反応して不溶性の沈殿を形成するテトラフェニルほう酸を、生体から切り出した直後の鰓に反応させたところ、塩類細胞の外界への開口部に顆粒状の沈殿を得た。この沈殿をエネルギー分散型 X 線分析に供した結果、K を多量に含んでいることが判明し、魚類の鰓塩類細胞が K^+を排出することが初めて明らかとなった。さらに、塩類細胞に存在する K^+排出の分子機構を解明するため、陸上生物の腎臓等で K^+の輸送を行うことが知られる K^+輸送体、renal outer medullary K^+ channel (ROMK)、large conductance Ca^{2+} activated K^+ channel (Maxi-K)、K^+/Cl^- cotransporter (KCC)1、KCC2、KCC4 をティラピアで同定した。その後、淡水、海水、高 K^+人工海水に馴致したティラピアの鰓で、上記遺伝子の発現量を定量した。その結果、ROMK のみが環境 K^+濃度依存的に有意な発現上昇を示し、鰓での K^+排出に重要な役割を持つことが示唆された。そこでティラピア ROMK に特異的な抗体を作成し、免疫染色を行った結果、ROMK は海水馴致ティラピアの鰓にある Type-IV 塩類細胞の頂端膜に局在し、さらに高 K^+環境に馴致したティラピアでは高密度に存在することが判明した。ROMK による K^+の排出を確認するため、阻害剤を組み合わせ、再度テトラフェニルほう酸を用いた実験を行った。その結果、ROMK の阻害剤(Ba^{2+})存在下で沈殿の形成が阻害され、ティラピアの鰓塩類細胞に発現する ROMK が、主要な K^+排出経路であることが証明された。これら一連の実験により、魚類の鰓における塩類細胞を用いた K^+排出機構の存在と、その分子メカニズムが初めて明らかとなった。

第 2 章　鰓における K^+排出経路を介した Cs および Rb の排出

昨今の日本における放射性物質汚染、およびそれに付随する水産業への影響を緩和する指針を考える上で、Cs の魚体内における挙動を解明することは喫緊の課題である。この現実を踏まえ、第 2 章では、生物の体内で同族の K^+と類似した挙動を示すことが知られる Cs^+に着目し、第 1 章で示した魚類における K^+排出経路を介して Cs^+が排出される可能性を検討した。海水馴致ティラピアの鰓を切り出し、入鰓動脈に Cs^+または同族の Rb^+を含んだ平衡塩類溶液を注入し、第 1 章で用いた K^+の検出方法と同様の手法に供した。その結果、鰓の塩類細胞

の開口部に同様に沈殿が形成され、沈殿部における特性 X 線分析により、Cs^+および Rb^+の存在が明らかとなった。この結果は、K^+の排出と同様の経路で、魚体内より放射性 Cs が積極的に排出される可能性を示している。

第 3 章　淡水馴致ティラピアの鰓塩類細胞における ROMK の発現

イオン濃度の乏しい淡水中に生息する魚類は、常に体外へとイオンが流出する危険にさらされている。K^+に関しても、通常は体内に保持する必要がある。しかし、高 K^+食やアシドーシス、また細胞や体組織の崩壊などにより、一時的に血漿 K^+が過剰に上昇する危険性がある。そのため、淡水適応時の魚類でも K^+に関してはその排出機構の存在が強く考えられる。第 1 章では海水馴致ティラピアが塩類細胞から K^+を排出することを明らかにした。一方、淡水中では過剰となる水を排出するために、腎臓において多量の尿を産生することから、K^+排出における腎臓の貢献も考慮する必要がある。また、淡水に馴致したティラピアの鰓では、Type-IV 塩類細胞のみが機能的であると考えられている海水中と異なり、Type-II および Type-III という異なる 2 種類の塩類細胞を有することが知られている。

第 3 章では、淡水馴致ティラピアにおいて鰓による K^+排出の可能性と腎臓の寄与を検討するため、淡水馴致ティラピアを通常淡水と高 K^+淡水に 1 週間馴致し、血漿と尿中のイオン濃度、および鰓と腎臓における ROMKa をはじめとした各種イオン輸送体の mRNA 発現量を測定した。その結果、高 K^+馴致群で尿中の K^+濃度が有意に増加した。一方、鰓での ROMKa 発現量が約 5 倍に増加しており、鰓の ROMKa が淡水中でも K^+の排出に重要であると考えられた。また、鰓の免疫染色の結果、通常淡水群では ROMKa のシグナルが僅かであったが、高 K^+馴致群で ROMKa の免疫反応が顕著に現れ、さらに ROMKa が Type-III 塩類細胞の頂端膜に局在することが明らかとなった。

第 4 章　淡水および海水馴致ティラピアの鰓塩類細胞における ROMK 発現調節機構

第 1 章、第 3 章の結果から、海水および淡水の両方の環境中で高 K^+ストレスに応答し、鰓の ROMKa 発現量が増加することで、K^+の排出が促進されると考えられる。哺乳類の腎臓においては、副腎から分泌されるアルドステロンが ROMK の発現調節に重要であることが知られている。しかし真骨魚類の体内にはアルドステロンが存在せず、別の何らかの因子によって ROMKa を調節していることが推測される。

第 4 章では、未だ不明である魚類の ROMK 発現調節機構を明らかにすることを目的とし

た。魚類における重要なステロイドホルモンであるコルチゾルが ROMK mRNA の発現調節に関与するのかを、鰓の単離培養実験により検討した。その結果、淡水馴致ティラピアの鰓のみがコルチゾルに応答して ROMKa mRNA 発現量を上昇させ、さらにそれは糖質コルチコイド受容体(GR)のアンタゴニストである RU486 存在下でのみ阻害された。従って、淡水馴致ティラピアでは GR を介したコルチゾルによる ROMKa mRNA の発現調節が行われていると考えられる。一方、海水馴致ティラピアではコルチゾル単体の効果が見られなかったため、海水から高 K^+への移行実験を行い、ROMKa mRNA 調節因子の探索を行った。この実験では、血漿中の各種イオン及びコルチゾル濃度とともに、各種下垂体ホルモン、鰓のコルチゾル受容体、およびイオン輸送体の mRNA を定量した。その結果、高 K^+への移行 24 時間後に血中コルチゾル濃度が増加したものの、鰓 ROMKa mRNA、および血漿 K^+の上昇は移行 6 時間後から起こっており、さらに下垂体ホルモン群の mRNA 発現量に顕著な変化は見られなかった。このことから、ホルモン以外の要素が ROMKa mRNA の発現に関与している可能性が考えられたため、血漿 K^+濃度の上昇そのものが鰓 ROMKa の発現に及ぼす影響を調べた。コルチゾルと FBS を添加した L-15 培養液による海水馴致ティラピア鰓の単離培養実験系を用いて、鰓の ROMKa 発現に及ぼす培養液 K^+濃度の影響を調べた。その結果、高 K^+培養液で培養した鰓で ROMKa mRNA の有意な上昇が見られた。これらの結果から、淡水中ではコルチゾルが、一方で海水中では血漿 K^+濃度が、ティラピアの鰓 ROMKa の発現を制御していることが示唆された。

以上の結果より、海水に馴致したティラピアでは Type-IV 塩類細胞が、淡水に馴致したティラピアは Type-III 塩類細胞が、それぞれ頂端膜に ROMKa を発現することで、体内で余剰となった K^+を排出することが示された。また、海水中では血漿 K^+濃度が、淡水中ではコルチゾル濃度が、それぞれ Type-IV および Type-III 塩類細胞における ROMKa の発現量の増加を促すことが示唆された。さらに、海水馴致ティラピアの鰓塩類細胞が Cs^+を排出することから、ROMKa を介した Cs^+の排出が示唆された。本研究では、真骨魚類の鰓塩類細胞における K^+排出機構を解明したばかりでなく、その機能性を調節するメカニズムに関する新たな知見についても見出すことができた。このことは、真骨魚類のイオン浸透圧調節機構の包括的な理解に大きく貢献するものである。また、東日本大震災から復興に向かう今、本研究により魚体内における放射性 Cs の挙動が明らかとなったが、この成果は被災地の水産業の復興へ少なからず寄与するものと期待している。

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