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Localization of Chloride Transporters in Gill Epithelia of the Grass Pufferfish, *Takifugu niphobles*

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ABSTRACT

Grass pufferfish (*Takifugu niphobles*) is a euryhaline species of seawater origin and widely distributed along the western seashore of Taiwan. In order to reveal the basis of Cl^- transport mechanisms in gills of this euryhaline species, the immunolocalization of Cl^- transporters, i.e., $Na^+/K^+/2Cl^-$ cotransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR), anion exchanger 1 (AE1), and chloride channel 3 (CLC-3) was performed. In order to illustrate the distributions and numbers of those Cl^- transporters in gill epithelia, the grass pufferfish was acclimated to fresh water (FW) or seawater (SW, 35 ‰) for more than one month, and cryosections of gills were observed and analyzed by the confocal laser scanning microscope after double-immunofluorescent staining of the Cl^- transporters with Na^+/K^+-ATPase (NKA). The antibody staining results showed that in gills of grass pufferfish, NKA as well as NKCC, AE1, and CLC-3 were colocalized in the basolateral parts of immunoreactive epithelial cells of FW- or SW-acclimated individuals, while CFTR were localized only in the apical sides of NKA immunoreactive cells in SW fish. In FW or SW grass pufferfish, immunoreactive cells were only distributed in gill filaments. This study provided direct in vivo evidence of simultaneous immunolocalization of Cl^- transporters in gills of SW- or FW-acclimated euryhaline teleosts.

Key words: *Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, chloride channel 3, gill, salinity, grass pufferfish, immunofluorescent staining.*

INTRODUCTION

Euryhaline teleosts are able to survive in environments with a broad spectrum of salinities. Acclimation to changing environmental salinity requires pre-existing mechanisms to respond to altering conditions. Gills secrete ions in SW-acclimated fish, and in FW-acclimated fish, gills absorb ions and maintain acid-base balance (Wood and Marshall, 1994). Effective mechanisms of ionoregulation thus enable teleosts to retain an osmotic and ionic constancy in their internal milieus and survive in hypertonic or hypotonic environments.

Gill epithelium is characterized by the presence of three cell types of interests: (1) pavement cells (PVCs), (2) mitochondrion-rich cells (MRCs), and (3) mucous cells (Laurent *et al*., 1985). More than 90% of the gill surface epithelium, and usually all lamellar surface, are characterized by PVCs. Although these cells, especially on the lamellae, are assumed to be the site of transepithelial gas transfer, recent evidence suggested they also played a role in ion and acid-base regulation (Evans *et al*., 2005). MRCs (i.e., chloride cells) in the gill epithelium and opercular membrane are important osmoregulatory sites in maintaining ionic balance in fish (Marshall, 1995; McCormick, 1996). The cells are characterized by the presence of a rich population of mitochondria and an extensive

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tubular system in the cytoplasm. The tubular system is continuous with the basolateral membrane, resulting in a large surface area for the placement of ion transporting proteins such as Na^+/K^+-ATPase (NKA), a key enzyme for chloride cell activities (Hootman and Philpott, 1980). The MRCs have been implicated in ion secretion in SW and possibly in ion uptake in FW (Evans *et al*., 2005; Marshall, 2002; Hirose *et al*., 2003).

According to current models for transcellular ion transport in MRCs, various ion transporting proteins are placed in either the apical or basolateral membrane (Katoh and Kaneko, 2003). In SW teleosts, Cl^- is eliminated by secondary active transport in branchial MRCs. The key ion-transport proteins associated with this process are NKA, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Marshall, 2002; Evans *et al*., 2005). The NKCC is an integral membrane protein found in numerous epithelia among many different species of vertebrates including the rat, duck, rabbit, dog, cow, and human (Lytle *et al*., 1995) with functions of cell volume regulation and ion transport (Ecelbarger *et al*., 1996; Ginns *et al*., 1996; McCormick *et al*., 1990, 1995). CFTR is a phosphorylation-dependent epithelial Cl^- channel. NKA maintains the Na^+ gradient in the cell, which is used by NKCC to cotransport Cl^- against its electrochemical gradient. The intracellular Cl^- exits the cell via the apical CFTR Cl^- channel down its electrochemical gradient (Wilson *et al*., 2004). The driving force for Cl^- secretion is the Na^+ electrochemical gradient established by NKA in the basolateral membrane (Marshell, 2002; Katoh and Kaneko, 2003). Meanwhile, Na^+ secretion occurs down its electrochemical gradient *via* a cation-selective paracellular pathway (Katoh and Kaneko, 2003).

Current model of transepithelial ion movements in FW fish gills, although not fully understood, indicated that active Cl^- uptake is mediated by the MRCs and PVCs equipped with NKA and Cl^-/HCO_3^- anion exchanger (AE) (Flik *et al*., 1995; Perry, 1997; Marshall, 2002; Hirose *et al*., 2003; Evans *et al*., 2005). In the FW model, Cl^-/HCO_3^- exchange was thought to occur in the apical membrane of MRCs. Theoretically, local acidification in the apical crypt (or at the base of apical microvilli) of MRCs by H^+-ATPase will lower HCO_3^- activity sufficiently to drive the exchange together with Cl^- uptake (Marshall, 2002). In addition to CFTR, there are other Cl^- channels that may play roles in ionoregulation. Recently, one member of the CLC chloride ion channel was cloned from tilapia kidney (OmCLC-K). The amino acid sequence of tilapia OmCLC-K was 90.5% identical to rat CLC-3 (Miyazaki *et al*., 1999). This Cl^- channel was expressed in osmoregulatory tissues such as gills, intestines and kidneys (Miyazaki *et al*., 1999).

The grass pufferfish (*Takifugu niphobles*) is an advanced tetraodontidae teleost whose native range covers the rivers and estuaries of Northwest Pacific, from Japan and southern Korea to Viet Nam (Masuda *et al*., 1984). Recently, Kato *et al*. (2005) have demonstrated its strong adaptability in SW. In FW-acclimated *T. niphobles* serum Cl^- was decreased (Kato *et al*., 2005). The identities of mitochondrial 16S rRNA for grass pufferfish within the six *Takifugu* species were 99% whereas those between six species of *Takifugu* Spp. and *Tetraodon nigroviridi* were 87% (Kato *et al*., 2005). Thus, in order to reveal the Cl^- transport mechanisms in gills of this euryhaline species, immunolocolization of Cl^- transporters by double-immunofluorescent staining and confocal laser scanning microscopic observation were used in this study to identify differential localization of NKA, NKCC, CFTR, AE1, and CLC-3 in branchial epithelial cells of FW- or SW-acclimated grass pufferfish.

MATERIAL AND METHODS

Fish and experimental conditions

The grass pufferfish (*Takifugu niphobles*), with 7.8±1.7 cm total length and 7.4±2.2 g body weight, were captured from Kao-Mei wetland in Taichung, Taiwan. All fish used

in the present study were kept in seawater (SW, 35‰) at 28±1°C with a daily 12 h photoperiod for at least 4 weeks. SW were prepared from local tap water added with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, Ohio, USA). The SW-acclimated grass pufferfish were then transferred to either SW ($[Na^+]$ 582.86 mM; $[K^+]$ 10.74 mM; $[Ca^{2+}]$ 15.75 mM; $[Mg^{2+}]$ 32.92 mM; $[Cl^-]$ 520.84 mM), or FW ($[Na^+]$ 2.6 mM; $[K^+]$ 0.04 mM; $[Ca^{2+}]$ 0.58 mM; $[Mg^{2+}]$ 0.16 mM; $[Cl^-]$ 0.18 mM) for more than 4 weeks before experiments. The water was continuously circulated through fabric-floss filters. Fish were fed a daily diet of dry shrimp. The rate of diet mass per body mass was about 1/25.

Antibodies

For staining of Na^+/K^+-ATPase (NKA), a rabbit polyclonal antiserum (Ab-TG3) was kindly provided by Prof. Pung-Pung Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan) raised against 565 amino acid of α-subunit of NKA of tilapia (Hwang *et al*., 1998). A 1:100 dilution was used for immunofluorescent detection of NKA. On the other hand, a mouse monoclonal antibody (α5) directed against the α-subunit of the avian NKA (Takeyasu *et al*., 1988) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). A 1:200 dilution was used for immunofluorescent detection of NKA.

For staining of $Na^+/K^+/2Cl^-$ cotransporter (NKCC), a mouse monoclonal antibody (T4) directed against the 310 amino acids at the carboxyl terminus of the human colonic NKCC was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The concentration of 0.25 g ml^{-1} (1:16 in dilution) was used for detection of NKCC. This antibody has been shown to be specifically immunoreactive with NKCC (both secretory and absorptive forms) from many vertebrates, including teleost fish (Pelis *et al*., 2001; Marshall *et al*., 2002; McCormick *et al*., 2003; Wu *et al*., 2003; Wilson *et al*., 2004; Hiroi *et al*., 2005).

For staining of cystic fibrosis transmembrane conductance regulator (CFTR), a mouse monoclonal antibody (R&D Systems, Boston, MA, USA) directed against 104 amino acids at the carboxyl terminus of the human CFTR was used at the concentration of 0.4•g•ml^{-1} (1:500 in dilution) to detect CFTR. The carboxyl terminus of CFTR is highly conserved among vertebrates, and this antibody has previously been shown to be specifically immunoreactive with CFTR of teleost fish (Marshall *et al*., 2002; Katoh and Kaneko, 2003; McCormick *et al*., 2003; Wilson *et al*., 2004; Hiroi *et al*., 2005).

For staining of anion exchanger (AE1), a rabbit polyclonal antiserum raised against 300 amino acids of AE1 of tilapia was kindly provided by Prof. Pung-Pung Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan). This antiserum with a dilution of 1:100 was used for immunofluorescent detection of whose anion exchanger.

For staining of chloride channel (CLC-3), a rabbit polyclonal antibody (Anti-CLCn3; Alomone labs, Jerusalem, Isreal) directed against residues 592-661 (70 amino acids) of CLC-3 of rat which is highly conserved among vertebrates. To our knowledge, this antibody has never been used for immunofluorescent detection of CLC-3 on teleost fish. A 1:100 dilution was used in this study.

The Alexa-Fluor 488 conjugated goat anti-mouse and Alexa Fluor 546 conjugated goat anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies and diluted in 1:50 and 1:500, respectively, to detect primary antibodies from mouse or rabbit. The antibodies used in this study has been demonstrated to be specific to teleosts in the other paper (Tang and Lee, 2007) by different negative controls of the immunoblots.

Fixation and cryosection of gills

First gill arches of left and right sides were excised and fixed immediately in a mixture of methanol and DMSO (4:1 v/v) at -20°C for 3 h. Fixed samples were then

washed with phosphate buffer saline (PBS; 137.00 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH=7.4). The arch and one row of the filaments of each gill sample were removed. The remaining filaments were perfused with 30% sucrose in PBS for 1 h at room temperature. The tissue was then mounted in O.C.T. (optimal cutting temperature) compound (Tissue-Tek, Sakura, Torrance, CA, USA) for cryosection. Longitudinal and cross sections of gills were cut at 5-7 μm thick using the Cryostat Microtome (Microm HM 505E, Walldorf, Germany) at -25°C. The sections were placed on 0.01% poly-L-lysine (Sigma, St. Louis, MO., USA) coated slides, and kept in slide boxes at -20°C before staining.

Immunofluorescent double staining

Cryosections were rinsed with PBS three times for 3 min and then incubated in 5% bovine serum albumin (Sigma, St. Louis, MO., USA) and 2% Tween 20 (Merck, Hohenbrunn, Germany) in PBS for 0.5 h at room temperature. The cryosections were then washed three times with PBS, and incubated with primary polyclonal antibodies (i.e., Ab-TG3, AE1 or CLCn3) diluted in PBS for 1 h at room temperature. After incubation, the cryosections were washed several times with PBS, exposed to secondary antibody (Alexa-flour 546 goat anti-rabbit antibody) at room temperature for 2 h, and then washed several times with PBS. After the first staining, the cryosections were subsequently incubated with primary monoclonal antibodies (i.e., α5, T4 or CFTR) diluted in PBS and incubated overnight at 4 °C. After incubation, the cryosections were washed several times with PBS, exposed to secondary antibody (Alexa-Fluor 488 goat anti-mouse antibody) at room temperature for 2 h and then washed several times with PBS. Then cryosections were mounted with clearmount™ mounting solution (Zymed, South San Francisco, CA, USA), covered by cover slip, and examined with an Olympus fluorescent microscope (Olympus BX50, Tokyo, Japan). Micrographs were taken within 3 h after staining by the confocal laser scanning microscopy.

Confocal laser scanning microscopy

In order to determine and compare the localization of ion transporters among three euryhaline teleosts, immunofluorescent stained cryosections of gills were examined using a Zeiss LSM 510 inverted laser scanning microscope equipped (Hamburg, Germany) with an argon laser (488 and 543 nm) for excitation. The immunofluorescent images of NKA, NKCC, CFTR, AE1, and CLC-3 were obtained with the Alexa-Fluor 488/546 filter set (BP505-530 for 488 and LP 560 for 546) controlled by the Zeiss LSM image software. With the filter set, the emission wavelengths of Alexa-488 and Alexa-546-conjugated antibodies were separated and transmitted to different photomultipliers. The micrographs taken from each photomultiplier were subsequently merged for simultaneously visualization of the labels of different colors. At least three individuals of SW or FW groups (n = 3) were examined to confirm the results of immunolocalization for each transporters of grass pufferfish.

RESULTS

Distributions of NKA and NKCC were determined using the rabbit polyclonal antiserum (NKA Ab-TG3) to the α-subunit of the NKA and the monoclonal antiserum (T4) to NKCC, respectively. In gills of SW-grass pufferfish, the immunoreactions of NKA and NKCC were colocalized at the afferent regions (cross sections; Figs. 1e, f, g) of the filaments (longitudinal sections; Figs. 1a, b, c). Both NKA and NKCC immunoreactions were distributed basolaterally in the epithelial MRCs (Figs. 1d, h).

Distributions of NKA and CFTR were determined using the rabbit polyclonal antiserum (NKA Ab-TG3) to the α-subunit of the NKA and the monoclonal antibody of CFTR, respectively. NKA distributed basolaterally in gill epithelial cells of SW-acclimated fish (Figs. 2b, f). In SW pufferfish, CFTR were localized in the apical

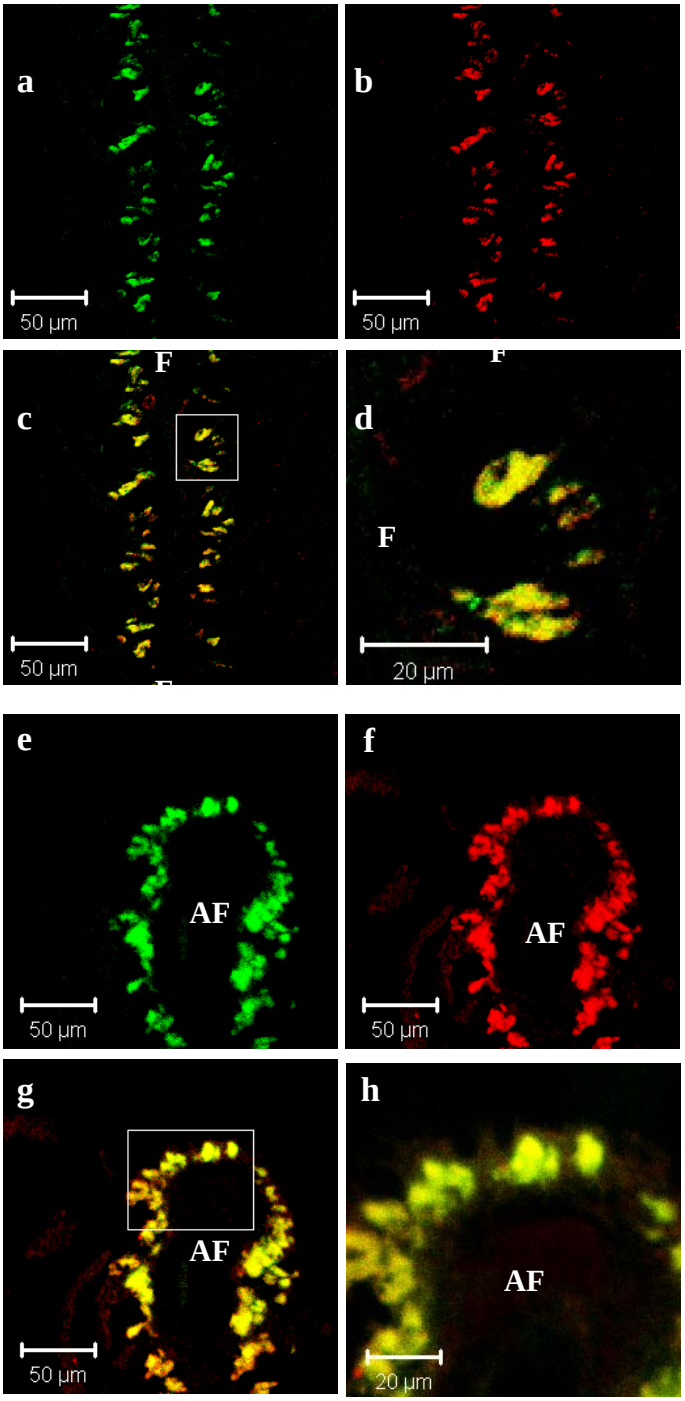


Fig. 1. The immunolocalization of Na^+/K^+-ATPase (NKA; red) and $Na^+/K^+/2Cl^-$cotransporter (NKCC; green) in frozen longitudinal (a,b,c,d) and cross sections (e,f,g,h) of gills of grass pufferfish acclimated to seawater. After immunofluorescent staining of NKA and NKCC, respectively, on the same cryosection, merged images (c and g) revealed that NKA and NKCC were colocalized on the basolateral membrane of the filamental epithelial cells. d and h were higher magnifications of the squares in c and g, respectively. AF, afferent region of the filament. F, filament.

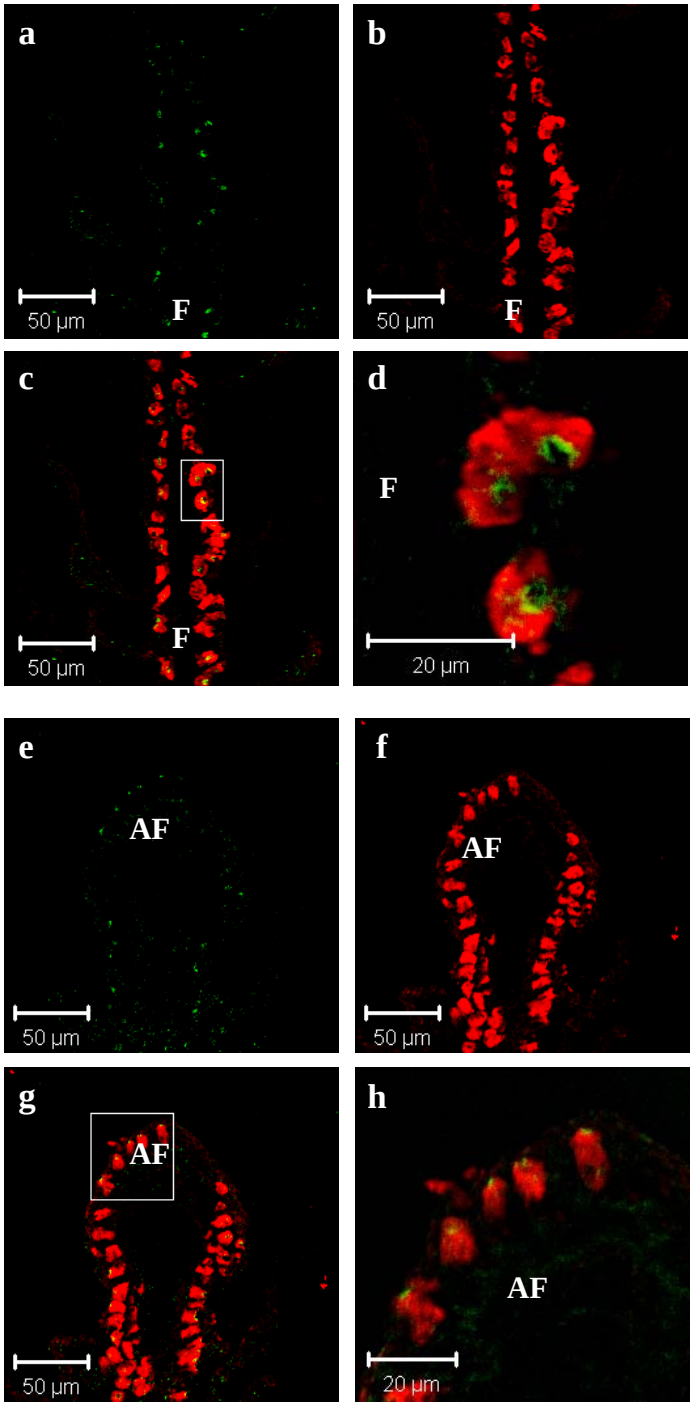


Fig. 2. The immunolocalization of Na^+/K^+-ATPase (NKA; red) and cystic fibrosis transmembrane conductance regulator (CFTR; green) in frozen longitudinal (a,b,c,d) and cross sections (e,f,g,h) of gills of grass pufferfish acclimated to seawater. After immunofluorescent staining of NKA and CFTR, respectively, on the same cryosection, merged images (c and g) revealed that NKA was exhibited on the filamental epithelial cells, and CFTR appeared on the apical sides of cells. d and h were higher magnifications of the squares in c and g, respectively. AF, afferent region of the filament. F, filament.

regions of NKA immunoreactive cells on the filaments (Figs. 2a, e). Both NKA and CFTR immunoreactions were found in the same epithelial cells of SW-acclimated fish (Figs. 2c, d, g, h).

Distributions of NKA and AE1 were determined using the monoclonal antiserum (α5) to the α-subunit of the NKA and the polyclonal antiserum (AE1) to the anion exchanger, respectively. In gills of FW-grass pufferfish, NKA and AE1 were colocalized on the afferent regions (cross sections; Fig. 3e, f, g) of the filaments (longitudinal sections; Figs. 3a, b, c). Both NKA and AE1 immunoreactions were distributed basolaterally in the epithelial cells (Figs. 3d, h).

Distributions of NKA and CLC-3 were determined using the monoclonal antiserum (α5) to the α-subunit of the NKA and polyclonal antibody (CLCn3) to CLC-3, respectively. In gills of FW-grass pufferfish, the immunoreaction of NKA and CLC-3 were colocalized on the afferent regions (cross sections; Figs. 4e, f, g) of the filaments (longitudinal sections; Figs. 4a, b, c). Both NKA and CLC-3 immunoreactions were distributed basolaterally in the epithelial cells (Figs. 4d, h).

DISCUSSION

The euryhaline teleost having the capacity to resist dramatic changes in environmental salinities is a feature found among teleost lineages and has apparently evolved many times (Evans, 1984). Mitochondrion-rich cells (MRCs) in gills of euryhaline teleosts have been considered to be responsible for two opposite vectorial ion movements: ion secretion in hyperosmotic seawater (SW) and ion absorption in hyposmotic fresh water (FW) (Chang *et al*., 2002; Marshall, 2002; Perry *et al*., 2003; Evans *et al*., 2005; Hiroi *et al*., 2005). In FW- and SW-adapted euryhaline teleosts, MRCs are generally found to be abundant in epithelia of gill filaments (Wilson and Laurent, 2002). MRCs also appeared on the lamellar epithelia in some stenohaline FW teleosts (Lee *et al*., 1996; Lin and Sung., 2003) or FW-adapted euryhaline species (Wilson and Laurent, 2002). Immunocytochemical studies on gill sections as well as biochemical studies on isolated MRCs have demonstrated that these epithelial cells contained most NKA protein in gills (Dang *et al*., 2000; Lee *et al*., 2000; Sakamoto *et al*., 2001). This study demonstrated that in both SW and FW grass pufferfish, NKA-immunoreactive (NKA-IR) cell/MRCs were distributed only in gill filaments.

Localizations of ion transporters on apical or basolateral membranes of MRCs are important for determining the functions of transport of these cells (Hiroi *et al*., 2005). Identical to NKA, NKCC immunoreactivity in gills of SW grass pufferfish occurred throughout MRCs, except for the nucleus (Fig. 1). These results were similar to mudskipper (Wilson *et al*., 2000a), salmon (Pelis *et al*., 2001), and killifish (Marshall *et al*., 2002). NKCC as well as NKA was present at low or non-detectable levels in other branchial cell types. Since NKA and NKCC were presented on the basolateral membrane of MRCs, positive NKCC-IR cells were identified as MRCs on the basis of their location in gills, size, and the same immunolocalization with NKA. Meanwhile, positive CFTR occurred at detectable levels only in MRCs with clear and consistent apical distribution in SW grass pufferfish (Fig. 2), similar to the other euryhaline teleosts, i.e., mudskipper (Wilson *et al*., 2000a), Hawaiian goby (McCormick *et al*., 2003), and killifish (Marshall *et al*., 2002). Current SW model of Cl^- transport in gill epithelia describes basolateral localization of NKA and NKCC and apical localization of CFTR in MRCs. Our results of the grass puffefish, as well as those found in SW-acclimated mudskipper (Wilson *et al*., 2000b), killifish (Marshell *et al*., 2002), and Hawaiian goby (McCormick *et al*., 2003), confirmed the model. Recently, similar distributions were found in the type IV MRCs in the embryonic yolk-sac membrane in Mozambique tilapia (Hiroi *et al*., 2005). Taken together, basolateral NKA were proposed to provide driving force in the form of transmembrane Na^+ gradient to transport Cl^- into the cell via NKCC in the basolateral

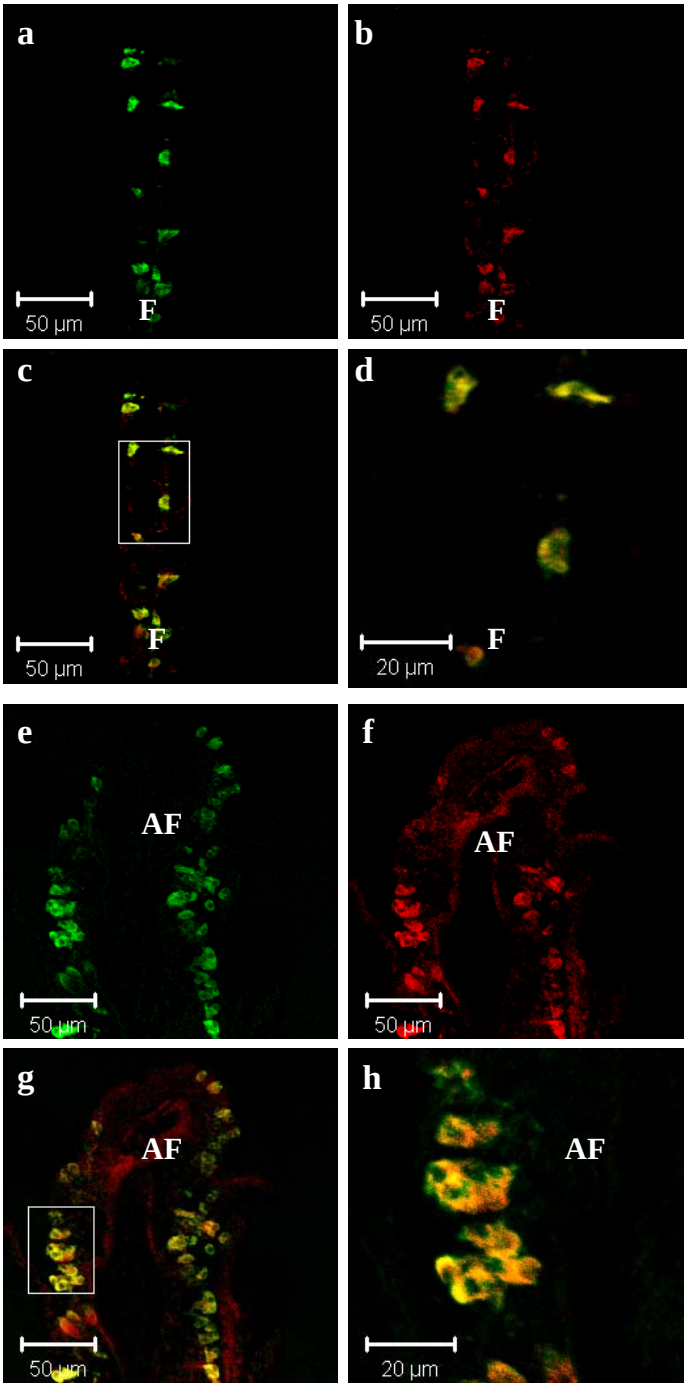


Fig. 3. The immunolocalization of Na^+/K^+-ATPase (NKA; green) and anion exchanger (AE1; red) in frozen longitudinal (a,b,c,d) and cross sections (e,f,g,h) of gills of grass pufferfish acclimated to fresh water. After immunofluorescent staining of NKA and AE1, respectively, on the same cryosection, merged images (c and g) revealed that NKA and AE1 were colocalized on the basolateral membrane of filamental epithelial cells. d and h were higher magnifications of the squares in c and g, respectively. AF, afferent region of the filament. F, filament.

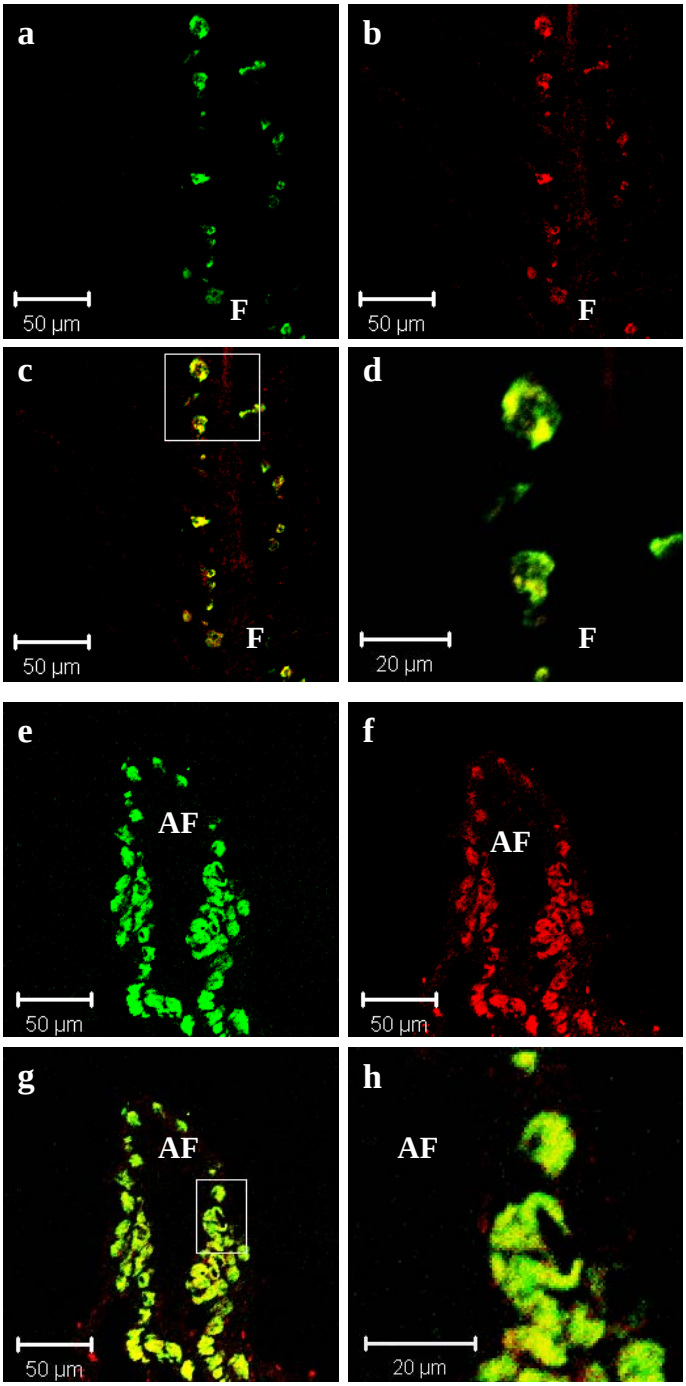
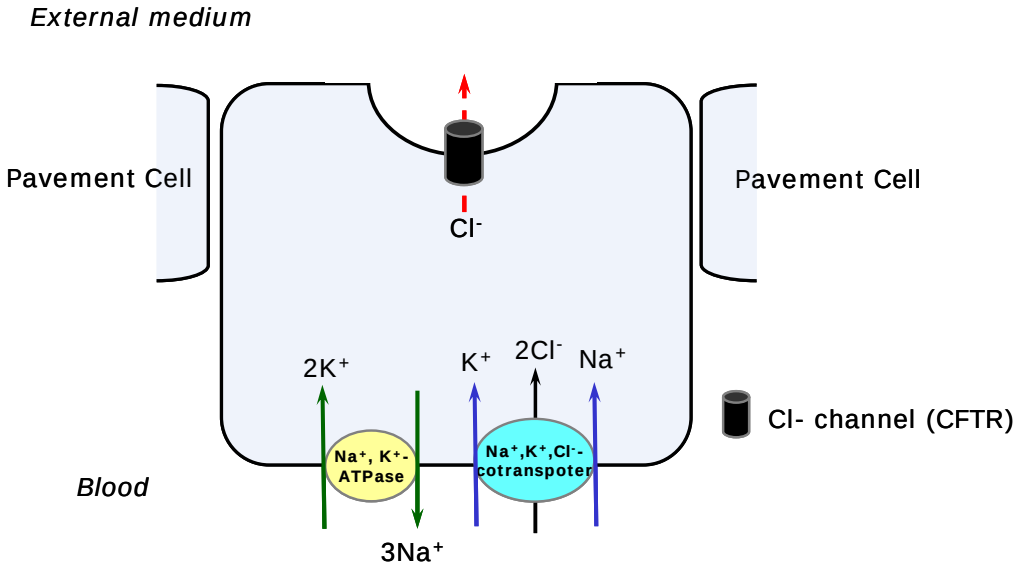


Fig. 4. The immunolocalization of Na^+/K^+-ATPase (NKA; green) and chloride channel (CLC-3; red) in frozen longitudinal (a,b,c,d) and cross sections (e,f,g,h) of gills of grass pufferfish acclimated to fresh water. After immunofluorescent staining of NKA and CLC3, respectively, on the same cryosection, merged images (c and g) revealed that NKA and CLC3 were colocalized on the basolateral membrane of the filamental epithelial cells. d and h were higher magnifications of the squares in c and g, respectively. AF, afferent region of the filament. F, filament.

a. SW model



b. FW model

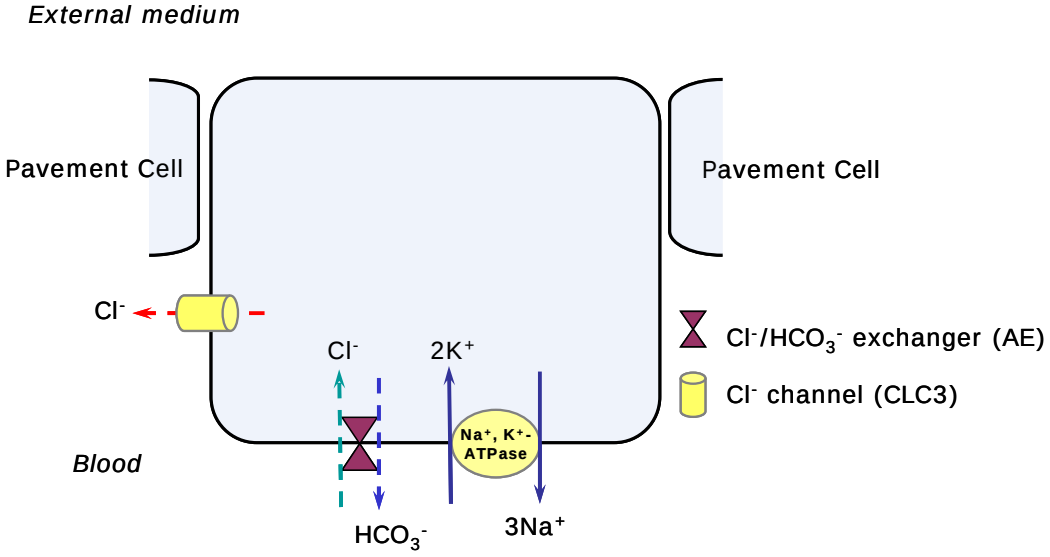


Fig. 5. The proposed model of localization of Cl^- transport in gill epithelium of seawater and freshwater grass pufferfish. Basolateral Na^+/K^+-ATPase provided driving force in the form of transmembrane Na^+ gradient to transport Cl^- into the cell via the $Na^+/K^+/2Cl^-$ cotransporter (NKCC type 1). (a) In the SW there were NKCC and anion exchanger (AE) get Cl^- into the cell in the basolateral membrane. Cl^- exited across via CFTR anion channels in the apical membrane and chloride channels (CLC-3) in the basolateral membrane. (b) In the FW grass pufferfish there were NKCC, AE on the basolateral membrane to get Cl^- into the cell, and Cl^- exited across via CLC-3 in the basolateral membrane into blood vessel. Moreover, the apical AE uptake Cl^- into the cells. Dotted lines were presented as passive transport.

membrane. Then Cl^- exits via CFTR anion channels in the apical membrane (Fig. 5).

Cl^- uptake by FW fish gill was generally considered to be via Cl^-/HCO_3^- anion exchanger (AE1) (Evans *et al*., 2005). Sullivan *et al*. (1996) localized band 3 mRNA by *in situ* hybridization using a 28-mer oligonucleotide probe to the filament interlamellar epithelium, which was typically populated by MRCs. Wilson *et al*. (2000b; 2002) used a polyclonal antibody raised against rainbow trout AE1 (anion exchanger, isoform 1) to demonstrate that AE1 were localized to the apical surface of gill NKA-IR cells in tilapia and coho salmon. Consistent with the apical distribution of Cl^-/HCO_3^- exchanger, Perry and Randall (1981) reported that apically applied SITS would inhibit Cl^- uptake and cause alkalosis in the trout. Thus, it appeared that fish made use of an apical band-3-like anion exchanger in gill epithelial cells. In the present study, however, NKA and AE1 cells were colocalized in MRCs of FW-acclimated grass pufferfish (Fig. 3). Since the antibody (tAE1) used in this study was produced based on a sequence highly homologous in AE1 and AE2 (anion exchanger, isoform 2), it might recognize both AE1 and AE2. Because AE2 expressed in basolateral membrane of renal epithelial cells in mammals and ductal segment in the zebrafish (Shmukler *et al*., 2005), the presence of AE in the NKA-IR cells could be the AE1 or AE2 and thus might reflect the demand for intercellular Cl^- absorption as well as pH regulation. Meanwhile, FW grass pufferfish, CLC-3-IR cells were identified as MRCs on the basis of their location in gills, size, and the same localization with NKA (Fig. 4). Miyazaki *et al*. (2002) have cloned kidney-specific chloride channel (omCLC-K) involved in Cl-reabsorption from tilapia which were highly expressed in the osmoregulatory organs (Miyazaki *et al*., 1999). To our knowledge, however, this is the first study demonstrating the immunolocalization of CLC-3 in FW fish gills.

Taken together, immunolocolization of transporters in this FW-acclimated euryhaline teleosts depicted the Cl^- transport model in FW gill epithelium (Fig. 5): basolateral NKA in MRCs provided driving forces in the form of transmembrane Na^+ gradient to uptake Cl^- into the cell via apically distributed AE1, or basolaterally AE2, then the Cl^- went out of MRCs to blood through basolateral CLC-3 chloride channel.

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以免疫螢光染色定位氯離子運輸蛋白在星點東方鮭(*Takifugu niphobles*)鰓表皮上之分佈

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星點東方鮭(*Takifugu niphobles*)是棲息於海洋的廣鹽性魚類，廣泛分佈於台灣西部沿岸。為了解此一廣鹽性魚種的氯離子調節機制之基礎，本實驗以免疫螢光染色法定位氯離子運輸蛋白，包括$Na^+/K^+/2Cl^-$ cotransporter (NKCC)，cystic fibrosis transmembrane conductance regulator (CFTR)，anion exchanger 1 (AE1)，及chloride channel 3 (CLC-3)在鰓表皮上的分佈。將適應在海水或淡水中一個月以上的星點東方鮭的鰓取下後作冷凍切片，之後以上述氯離子運輸蛋白的抗體配合Na^+/K^+-ATPase (NKA)做雙重免疫螢光染色，繼之以共軛焦顯微鏡觀察照相。結果顯示，適應在海水或淡水中的星點東方鮭，鰓表皮上的NKCC，AE1及CLC-3都與NKA染色呈現在相同的細胞，而CFTR只呈現在海水魚鰓表皮的NKA免疫反應細胞頂端。海水及淡水的星點東方鮭鰓表皮的免疫反應細胞都出現在鰓絲而非鰓薄板上。本研究提供直接的證據，證明廣鹽性魚類在海水與淡水中，鰓表皮同時存在的數種氯離子運輸蛋白。

關鍵詞：Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, chloride channel 3，鰓，鹽度，星點東方鮭，免疫螢光染色。

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