Editorial Manager(tm) for Fish Physiology and Biochemistry
Manuscript Draft

Manuscript Number:

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Article Type: Original research

Keywords: Na+/K+-ATPase; Na+/K+/2Cl- cotransporter; cystic fibrosis transmembrane conductance regulator; anion exchanger 1; chloride channel 3; salinity; gill; teleost.

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Submitted to the Fish Physiology and Biochemistry 2010 Sep. 22nd

Immunolocalization of Chloride Transporters to Gill Epithelia of Euryhaline Teleosts with Opposite Salinity-induced Na^+/K^+-ATPase Responses

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Running title: CHLORIDE TRANSPORTERS IN GILLS OF EURYHALINE TELEOSTS
Grant sponsor: National Science Council of Taiwan; Grant number:

NSC-93-2313-B-005-007

Abstract

Opposite patterns of branchial Na^+/K^+-ATPase (NKA) responses were found in euryhaline milkfish (*Chanos chanos*) and pufferfish (*Tetraodon nigroviridis*) upon salinity challenge. Because the electrochemical gradient established by NKA is thought to be the driving force for transcellular Cl^- transport in fish gills, the aim of this study was to explore whether the differential patterns of NKA responses found in milkfish and pufferfish would lead to distinct distribution of Cl^- transporters in their gill epithelial cells indicating different Cl^- transport mechanisms. In this study, immunolocalization of various Cl^- transport proteins, including $Na^+/K^+/2Cl^-$ cotransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR), anion exchanger 1 (AE1), and chloride channel 3 (ClC-3), were double-stained with NKA, the basolateral marker of branchial mitochondrion-rich cells (MRCs), to reveal the localization of these transporter proteins in gill MRC of FW- or SW-acclimated milkfish and pufferfish. Confocal microscopic observations showed that the localization of these transport proteins in the gill MRCs of the two studied species were similar. However, the number of gill NKA immunoreactive (IR) cells in milkfish and pufferfish exhibited to vary with environmental salinities. An increase in the number of NKA-IR cells should lead to elevation of NKA activity in FW milkfish and SW pufferfish. Taken together, the opposite branchial NKA responses observed in

milkfish and pufferfish upon salinity challenge could be attributed to alterations in the number of NKA-IR cells. Furthermore, the localization of these Cl^- transporters in gill MRCs of the two studied species was identical. It might depict these two studied species possess similar Cl^- transport mechanisms in gills.

Keywords Na^+/K^+-ATPase . $Na^+/K^+/2Cl^-$ cotransporter . cystic fibrosis transmembrane conductance regulator . anion exchanger 1 . chloride channel 3; salinity . gill . teleost

Introduction

Euryhaline teleosts are able to survive in environments with a broad range of salinities, and excellent osmoregulatory mechanisms are required. SW teleosts drink water and actively excrete excess NaCl via the gills to compensate for passively lost water and gained salt. In contrast, freshwater (FW) teleosts actively absorb NaCl through the major osmoregulatory organ, which is the gill (Evans et al. 2005).

Gill epithelium is characterized by the presence of three major cell types: (1) pavement cells (PVCs), (2) mitochondrion-rich cells (MRCs; chloride cells), and (3) mucous cells (Laurent et al. 1985). More than 90% of the gill surface epithelium and usually all lamellar surfaces are characterized by PVCs. Although these cells, especially on the lamellae, were thought to be the site of transepithelial gas transfer, recent evidence suggested they might have the roles in ion and acid-base regulation (Evans, 1987; Evans et al. 2005). Branchial MRCs are characterized by the presence of high levels of mitochondria and an extensive tubular system continuous with the basolateral membrane in the cytoplasm (Evans et al. 2005; Hwang and Lee, 2007). These cells have been reported to be the major site for conducting osmoregulation and ionoregulation (for review, see Marshall, 2002; Hirose et al. 2003; Evans et al. 2005; Hwang and Lee, 2007).

NKA is a ubiquitous, membrane-bound enzyme that actively transports Na^+ out of and K^+ into animal cells (Post and Jolly, 1957). Immunocytochemical studies demonstrated that NKA is mainly localized in the basolateral membrane of MRCs (McCormick, 1995; Dang et al. 2000; Lee et al. 2000; Wilson and Laurent, 2002). In some euryhaline teleosts, NKA-immunoreactive (IR) cells are not only presented in gill filaments but also appeared in the lamellar epithelium (reviewed by Hwang and Lee, 2007). Furthermore, the current model of osmoregulation in gill MRCs illustrates that basolateral NKA is essential to create the electrochemical gradient to trigger the secondary ion transport system during salinity challenge (Marshall, 2002; Hirose et al. 2003; Hwang and Lee, 2007). Moreover, most euryhaline teleosts exhibit acclimated changes in NKA activity following salinity changes (Marshall, 2002), and two opposite patterns of NKA responses have been reported: (1) higher NKA in hyperosmotic media, and (2) higher NKA in hyposmotic media (Hwang and Lee, 2007).

According to the current model of the Cl^- transport mechanisms in MRCs, the driving force for Cl^- secretion is the electrochemical gradient established by NKA in the basolateral membrane, and various Cl^- transporting proteins are localized in either the apical or basolateral membrane to deliver Cl^- (Hirose et al. 2003; Evans et al. 2005). In SW teleosts, the key Cl^- transport related proteins are thought to be NKA,

Na^+/ K^+ /2Cl^- cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR) (Hirose et al. 2003; Evans et al. 2005). Meanwhile, the current model of transepithelial Cl^- movements in FW fish gills is controversial. Wilson et al. (2000a) postulated that AE1 expressed in the apical surface of gill epithelial cells might play a role in Cl^- uptake in FW tilapia and coho salmon (*Oncorhynchus kisutch*; Wilson et al. 2002). In addition, the basolateral exit step for Cl^- absorption is less studied. The candidates for the basolateral Cl^- channel including the members of the chloride channel (CLC) family that are highly expressed in the osmoregulatory organs (Hirose et al. 2003). Miyazaki et al. (1999) demonstrated that transcripts of ClC-3, a member of the CLC family, are highly expressed in the osmoregulatory organs of Mozambique tilapia (*Oreochromis mossambicus*). Moreover, basolaterally located ClC-3 was demonstrated in the spotted green pufferfish (*Tetraodon nigroviridis*) (Tang et al., 2010) and Mozambique tilapia (Tang and Lee, 2010). Therefore, ClC-3 was a target candidate to study the basolateral chloride channel in the present study.

Milkfish (*Chanos chanos*) is a marine inhabitant widely distributes throughout the tropical and subtropical Indo-Pacific Ocean (Bagrinao, 1994). It occurs naturally and is commercially cultured in fresh, brackish, and oceanic waters as well as in hypersaline lagoons (Crear, 1980). Spotted green pufferfish (*T. nigroviridis*) is a

peripheral FW inhabitant whose native range covers the rivers and estuaries of the Southeast Asia (Rainboth, 1996). Both species are able to tolerate a direct transfer from FW to SW or vice versa in experimental manipulation (Lin et al. 2003; 2004; 2006). Compared with SW conditions, milkfish have higher gill NKA responses in FW (described as the "higher NKA in hyposmotic media" response) (Lin et al. 2003; 2006). However, the pattern of branchial NKA responses in pufferfish is opposite to that of milkfish (described as the "higher NKA in hyperosmotic media" response) (Lin et al. 2004). Therefore, the aim of this study was to investigate whether the different/opposite NKA responses would trigger distinct Cl^- transport mechanisms indicated by differential localization of Cl^- transporters in gill MRCs of euryhaline teleosts. Furthermore, changes in the distribution and number of the immunoreactive cells in gills were also compared to discuss the acclimation strategies of the two studied species.

Materials and methods

Fish and experimental conditions

Juvenile milkfish (*Chanos chanos*) with 15.7±5.2 cm total length and 19.1± 5.9 g body weight were obtained from a fish farm in Chia-Yi, Taiwan. Spotted green pufferfish (*Tetraodon nigroviridis*) with 6.7±1.9 cm in total length and 7.4±2.6 g body weight were purchased from a local aquarium.

All fish used in this study were kept in seawater (SW; 35‰) at 28±1°C with a daily 12 h photoperiod for at least 4 weeks. SW was prepared from local tap water by the addition of proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The SW-acclimated milkfish and 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 fresh water (FW; $[Na^+]$, 2.60 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 commercial fodder (for milkfish) or dry shrimp (for pufferfish). The rate of diet mass per body mass was about 1/25.

Antisera/antibodies

Specificity of antibodies for Na^+/K^+-ATPase (NKA) and the other chloride

transporters (i.e., $Na^+/K^+/2Cl^-$ cotransporter, NKCC; cystic fibrosis transmembrane conductance regulator, CFTR; chloride channel 3, ClC-3) to fish was previously demonstrated by immunoblotting (Marshall et al. 2002; Katoh and Kaneko, 2003; Hiroi and McCormick, 2007; Tang and Lee, 2007). The antisera/antibodies used in the present study are listed below. (1) Ab-TG3 is a rabbit polyclonal antiserum that recognizes the NKA α-subunit and was kindly provided by Prof. Pung-Pung Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan). This antiserum was raised against a 565-amino-acid portion of the tilapia NKA α-subunit (Hwang et al. 1998) and recognizes all α-isoforms. A 1:100 dilution was used for immunofluorescent detection of NKA. (2) α5 is a mouse monoclonal antibody against the avian NKA α-subunit (Takeyasu et al. 1988). This antibody was purchased from the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA, USA). A 1:200 dilution was used for immunofluorescent detection of NKA. This antibody has been widely used to detect gill NKA of many fish species including milkfish (Lin et al. 2003, 2006) and pufferfish (Lin et al. 2004). (3) T4 is a mouse monoclonal antibody against the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and is directed against the 310 amino acids at the carboxyl terminus of the human colon NKCC. This antibody was purchased from the DSHB (Iowa City, IA, USA). The concentration of 0.25 $g \cdot ml^{-1}$ (1:16 dilution) was used for detection of NKCC in gills of the two studied species.

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; Hiroi and McCormick, 2007). (4) A mouse monoclonal antibody of cystic fibrosis transmembrane conductance regulator (CFTR) (R&D Systems, Boston, MA, USA) that is directed against 104 amino acids at the carboxyl terminus of the human CFTR was used at a concentration of 0.4 $g \cdot ml^{-1}$ (1:500 in dilution). The carboxyl terminus of CFTR is highly conserved among vertebrates. 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). (5) A rabbit polyclonal antiserum of anion exchanger 1 (AE1) 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). The similarity between the antigen of AE1 antiserum and the putative AE1 protein in pufferfish (accession number: CAF92111) is 74%. This antiserum was used at a dilution of 1:100 for immunofluorescent detection of AE1. (6) Anti-ClCn3 was a rabbit polyclonal antibody against chloride channel 3 (ClC-3) (Alomone Labs, Jerusalem, Israel) directed against the highly conserved residues 592-661 (70 amino acids) of rat ClC-3. This antibody was used for immunofluorescent detection of

ClC-3-like protein in gills of pufferfish (Tang and Lee, 2007; Tang et al. 2010) and tilapia (Tang and Lee, 2010). A 1:100 dilution was used in this study. (7) 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 1:50 and 1:500, respectively, to detect primary antisera/antibodies from mouse or rabbit. Preliminary experiments of negative controls (cryosections stained with only primary or secondary antibodies) demonstrated that either non-specific staining or over-staining of the background was not found (data not shown).

Fixation and cryosections of gills

First, gill arches of the 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 (Lin et al., 2003; 2004; 2006). Fixed samples were then washed with phosphate buffered 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 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 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 (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 washed several times with PBS. After the first staining, the cryosections were subsequently incubated with primary monoclonal antibodies (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, then washed several times with PBS. Then, cryosections were mounted with Clearmount^{TM} mounting solution (Zymed, South San Francisco, CA, USA), covered by cover slips, and examined with an Olympus

fluorescent microscope (Olympus BX50, Tokyo, Japan). Micrographs were taken within 3 h after staining by confocal laser scanning microscopy or the fluorescent microscope to recognize immunolocalization for subsequent immunoreactive cell counting and morphometric analyses.

Confocal laser scanning microscopy

To determine and compare the localization of ion transporters between milkfish and pufferfish, double immunofluorescent stained cryosections of gills were examined with a Zeiss LSM 510 inverted laser scanning microscope (Hamburg, Germany) equipped 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 Flour 488- and Alexa Flour 546-conjugated antibodies were separated and transmitted to different photomultipliers. The micrographs taken from each photomultiplier were subsequently merged for simultaneous 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 different Cl^- transporters of milkfish or pufferfish.

Quantification of NKA- and NKCC-Immunoreactive (IR) cells in gills of the milkfish

Preliminary observation revealed that all NKA-IR cells were NKCC-IR in SW-acclimated milkfish. However, only a portion of NKA-IR cells expressed NKCC in FW-acclimated milkfish. Hence, NKA- and NKCC-IR cells in FW milkfish gills were quantified to clarify differential localizations of these two transporters. After double staining of longitudinal cryosections with NKA and NKCC primary antibodies, the immunoreactive cells of the cryosections of gills were observed and quantified directly using a fluorescent microscope (Olympus BX50). NKA- and NKCC-IR cells in the filaments (F) and lamellae (L) were counted separately on every 100 μm of gill filament of the cryosections. Lamellar regions of the gills were defined as the parts projecting from filaments. The interlamellar regions of filaments, including the regions between bases of lamellae, were considered as filament areas. Three regions on each filament and five filaments of each individual were randomly selected for quantification. Results were expressed as the numbers of immunoreactive cells (i) per 100 μm of filaments and (ii) per 100 μm of lamellae. Mean numbers of NKA- or NKCC-IR cells were obtained from eight individuals (n=8) of either the SW or FW group.

Quantification of NKA-IR cells in gills of the pufferfish

Cryosections (7 μm) of gills of FW- or SW-acclimated pufferfish were cut parallel to the long axis of the filament and mounted onto slides coated with poly-L-lysine. To quantify the distribution of NKA-IR cells, cryosections were immunohistochemically stained with the monoclonal antibody to the NKA α-subunit (α5) followed by a commercial kit (PicTure™, Zymed, South San Francisco, CA, USA) for visualization of the immunoreaction. The immunostained cryosections were then counterstained with hematoxylin (Merck, Hohenbrunn, Germany) and observed under a microscope (Olympus BX50).

Preliminary observation revealed that in either SW- or FW-acclimated pufferfish, NKA-IR cells were mainly distributed in gill filaments. Hence, the numbers of NKA-IR cells in the filaments were counted. The filament regions can be defined as the basal part of the lamella extending 5 μm up from the filament plus the width of the filament itself. For each individual, 15 areas on the filaments were randomly selected. Length of the filament was also measured to normalize cell counts to a fixed length (100 μm). Results are expressed as the numbers of immunoreactive cells per 100 μm of the filaments. NKA-IR cells were obtained from eight pufferfish (n=8) of either the SW or FW group.

Statistical analysis

Values are expressed as the means ± S.E.M. For milkfish, data were compared using a one-way analysis of variance (ANOVA). Post hoc comparisons were made using a Tukey's test with the differences considered to be statistically significant at $P<0.05$. For pufferfish, unpaired Student's t-test ($P<0.05$ was set as the level of significance) was used for analysis.

Results

Immunolocalization of Cl^- Transporters

Milkfish

Localization of Na^+/K^+-ATPase (NKA) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) or cystic fibrosis transmembrane conductance regulator (CFTR) on cryosections was determined by using monoclonal antibodies against NKCC (T4) or CFTR with rabbit polyclonal antiserum (Ab-TG3) to the α-subunit of NKA for double staining. Immunolocalization of NKA in the frozen longitudinal sections from the gills of seawater (SW) milkfish was mainly observed in the filaments (Figs. 1b, h). In freshwater (FW) milkfish, however, both filaments and lamellae were positively immunostained with the NKA antiserum (Figs. 1e, k). The amount of fluorescence in the negative controls was used to define the background staining (data not shown). No other type of cells in the gills could be immunostained with signals higher than background. The immunolocalization of NKCC in SW milkfish gills (Fig. 1a) was identical to that of NKA (Fig. 1b), with basolateral localization. In FW milkfish, only a portion of NKA-immunoreactive (IR) cells expressed basolateral NKCC in both gill filaments and lamellae (Fig. 1f). CFTR was detected in the apical regions of the NKA-IR cells of SW-acclimated milkfish (Figs. 1g, i), and no immunoreactivity for CFTR was observed in FW individuals (Fig. 1j, l).

Distribution of NKA and anion exchanger 1 (AE1) or chloride channel 3 (ClC-3) on longitudinal sections was examined by using a polyclonal antiserum to AE1 or a polyclonal antibody (ClCn3) to ClC-3 together with a monoclonal antibody to the α-subunit of NKA (α5) for double staining. The immunolocalization of NKA detected by the monoclonal antibody (α5) was the same as the result using polyclonal antiserum (Ab-TG3) and revealed the basolateral localization of NKA in filaments of SW fish (Figs. 2g, h) and in both filaments and lamellae of FW individuals (Figs. 2e, k). The results showed that AE1 colocalized with NKA in the basolateral membrane of SW (Fig. 2c) and FW milkfish (Fig. 2f). Furthermore, ClC-3 was also basolaterally colocalized with NKA in the gills of SW- (Figs. 2g, h) and FW-acclimated (Figs. 2j, k) milkfish (Figs. 2i, l).

Pufferfish

The immunoreactions for NKCC (Figs. 3a, d) and NKA were colocalized in the basolateral membrane of NKA-immunoreactive (NKA-IR) cells in the gill filaments (Figs. 3c, f). The Cl^- secretion channel, CFTR, (Fig. 3g) was localized to the apical membrane of NKA-IR cells in the gill filaments of SW pufferfish (Fig. 3i). Immunoreactivity of branchial CFTR, however, was undetectable in FW individuals (Fig. 3j, l).

In the gills of SW- and FW-acclimated pufferfish, AE1 (Figs. 4a, d) was

colocalized with NKA (Figs. 4b, e) in the gill filaments (longitudinal sections; Figs. 4c, f). Moreover, the immunoreactions of ClC-3 (Figs. 4g, h) and NKA (Figs. 4h, k) in SW or FW pufferfish gills were also colocalized (Figs. 4i, l).

Quantification of the Number of Immunoreactive Cells

Fig. 5 illustrates that the numbers of NKA- and NKCC-IR cells in filaments of SW milkfish were significantly higher than those in lamellae. Compared to those of SW milkfish, however, the numbers of NKA- and NKCC-IR cells in the lamellae of FW milkfish were evidently increased. The number of NKCC-IR cells but not NKA-IR cells was significantly higher in the filaments of SW milkfish than in those of FW milkfish. In addition, the number of NKCC-IR cells in both filaments and lamellae were significantly lower than NKA-IR cells in FW milkfish.

In pufferfish, NKA-IR cells were mainly presented in the gill filaments in either SW or FW individuals (Figs. 6A, B). Meanwhile, the number of NKA-IR cells in the gill filaments of SW individuals (19.133 ± 0.32) was significantly higher than that of FW fish (13.467 ± 0.59) (Fig. 6C).

Discussion

Localizations of Chloride Transporters in the Gills of SW-acclimated Teleosts

Branchial mitochondrion-rich cells (MRCs) of fish are the major site responsible for ion secretion and absorption in hyperosmotic seawater (SW) and hyposmotic fresh water (FW), respectively (Hirose et al. 2003; Evans et al. 2005; Hwang and Lee, 2007). Identifying the localization of ion transport proteins in apical or basolateral membranes of MRCs is essential for studying the potential functions in ionoregulation (Pelis et al. 2001; Wilson et al. 2000a; 2000b; 2002; McCormick et al. 2003; Hiroi et al. 2005). A working model for the mechanisms of Cl^- secretion by the gill MRCs of SW teleosts is that basolateral NKA provides the driving force in the form of an electrochemical gradient for the transport of Cl^- from plasma into the cell via $Na^+/K^+/2Cl^-$ cotransporter (NKCC) in the basolateral membrane. Then, Cl^- is secreted via apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels. Because anti-NKCC (T4) and anti-CFTR (R&D Systems, Boston, MA, USA) antibodies have been used to detect NKCC and CFTR in several teleost species and the specificity has been confirmed (Wilson et al. 2000b; Pelis et al. 2001; Marshall et al. 2002; Katoh and Kaneko, 2003; Wu et al. 2003; Hiroi et al. 2005; Hiroi and McCormick, 2007; Tang and Lee, 2007), these two antibodies were used to detect the localization of NKCC and CFTR in the present study. The results showed that the

localization of NKCC and CFTR was basolateral and apical, respectively, in branchial MRCs of SW milkfish and pufferfish. The results show that the Cl^- secretion mechanism is identical in these two studied euryhaline species despite they have opposite NKA expression patterns upon salinity challenge. In addition, NKCC includes two isoforms, NKCC1 and NKCC2. Because of the prominent expression of NKCC1 on basolateral confinement and it has been confirmed to operate in chloride-secreting function, NKCC1 is considered to be the secretory isoform. However, NKCC2 is a kidney-specific isoform and function to regulate in Cl^- absorption (Lytle et al. 1995). Therefore, the basolaterally localized gill NKCC in milkfish and pufferfish should be NKCC1.

Localizations of Chloride Transporters in Gills of FW-acclimated Teleosts

Cl^- uptake by FW fish gill is generally considered to be via Cl^-/HCO_3^- anion exchanger (AE) (Evans et al. 2005). AE1 is a member of the SLC4 gene family, and is the most abundant transmembrane protein of the human erythrocytes (Davis et al. 2002). Sullivan et al. (1996) verified that AE1 mRNA was typically observed by MRCs by *in situ* hybridization. Furthermore, Wilson et al. (2000a; 2002) used a polyclonal antibody raised against rainbow trout AE1 to verify that branchial AE1 was expressed in the apical surface of MRCs in tilapia and coho salmon. The AE1 antiserum used in the present study has been demonstrated by immunoblotting to

reveal a single immunoreactive band in the gills of pufferfish (Tang and Lee, 2007). However, the localization of branchial AE1 in FW- and SW-acclimated two studied species differs from previous findings in tilapia and coho salmon (Wilson et al. 2000a; 2002). In zebrafish (*Danio rerio*), mRNA of the AE1 paralog was localized to a specific group of skin and gill cells, whereas AE1 mRNA expression was not affected by low-Cl^- artificial FW (Hwang, 2009). Similar to zebrafish, branchial AE1 in milkfish and pufferfish might not be involved in Cl^- absorption. The non-erythrocyte AE1 protein is expressed in the basolateral membrane of alpha-intercalated cells in mammalian kidney (Alper et al. 1989) and has a role in acid secretion. Accordingly, AE1 might have a role in acid-base regulation or modulation of intracellular [Cl^-] homeostasis in fish gills. Meanwhile, the basolateral localization of gill AE1 implied that another AE protein may be presented in the apical membrane that is responsible for Cl^- uptake. Piermarini et al. (2002) illustrated that pendrin was expressed in the apical membrane of specialized ionocytes in the gill of the Atlantic stingray (*Dasyatis sabina*) and might be involved in Cl^- uptake. Recent studies further verified that SLC26 anion transporters mediated branchial chloride uptake in adult zebrafish (Perry et al. 2009). The members of the SLC26 family are, therefore, proposed to be the candidates for performing the function of Cl^- absorption in the apical membrane of branchial MRCs (Tresguerres et al. 2006).

Cl^- uptake mechanisms are accomplished through apical AE and the basolateral chloride channel of branchial MRCs. However, the basolateral chloride channel of MRCs is less studied. The CLC family has multiple roles in biological membranes, and ClC-3 is a member that is ubiquitously expressed in diverse tissues of mammals (Jentsch et al. 2002; 2005). In hyposmotic medium, ClC-3 represents a major molecular entity responsible for native volume sensing of outwardly rectifying anion channels in various cell types (Kawasaki et al. 1994; Duan et al. 1997; 2001; Hermoso et al. 2002; Wang et al. 2000; 2003; Vessey et al. 2004). In fish, ClC-3 was cloned from cDNA libraries of the euryhaline tilapia (*Oreochromis mossambicus*), and it was found that OmClC-3 mRNA was broadly expressed in different tissues in FW- and SW-acclimated tilapia (Miyazaki et al. 1999). Using the whole-mount staining method, our previous study demonstrated that ClC-3-like protein and NKA were co-localized in the basolateral membrane of gill MRCs of pufferfish (Tang et al. 2010). The present study simultaneously examined the localization of gill ClC-3-like protein in two euryhaline species by double-immunostaining of cryosections and found that ClC-3-like protein were basolaterally localized in gill NKA-IR cells in both two studied species. It was thus suggested that ClC-3-like protein might have a role in basolateral Cl^- uptake in the gill MRCs of euryhaline teleosts. To our knowledge, this study is the first to detect the basolateral chloride channel in marine euryhaline

teleosts (i.e., milkfish).

NKCC-Immunoreactive (IR) Cells in Gills of Euryhaline Teleosts

The most widely accepted model of Cl^- regulation in FW MRCs includes NKA, Na^+/Cl^- cotransporter (NCC) and AE proteins, but not NKCC (Hirose et al. 2003; Evans et al. 2005; Hwang and Lee, 2007; Evans, 2010). In this study, however, immunological detection revealed that basolateral NKCC exists in gill MRCs of FW-acclimated milkfish and pufferfish. The basolaterally localized NKCC was also reported in gills of FW-acclimated Atlantic salmon (*Salmo salar*) (Pelis et al. 2001; Hiroi and McCormick, 2007), Hawaiian goby (*Stenogobius hawaiiensis*) (McCormick et al. 2003), lake trout (*Salvelinus namaycush*) and brook trout (Salvelinus fontinalis) (Hiroi and McCormick, 2007). Pelis et al. (2001) suggested that branchial NKCC presented in FW MRCs might conduct the function of cell volume regulation. Immunoreactivity against NKCC was found in some but not all NKA-IR cells (MRCs) in the gills of Atlantic salmon parr (Pelis et al. 2001) and FW milkfish, and the number of gill NKCC-IR cells evidently decreased when compared with Atlantic salmon smolt (Pelis et al. 2001) and SW milkfish, implying that NKCC has a less critical role in ion absorption in these two species. In contrast to milkfish, immunoreactivity against NKCC was found in all MRCs of SW- and FW-acclimated pufferfish. However, our previous study has used immunoblotting with the same

antibody to detect the protein expression of NKCC in gill membrane fraction and revealed that the NKCC protein was detectable only in SW pufferfish (Tang and Lee, 2007). Therefore, we presumed that gill NKCC might exist mainly in the cytoplasm rather than the plasma membrane of the MRCs in FW pufferfish.

Na^+/K^+-ATPase (NKA) Immunoreactive Cells in Gills of Euryhaline Teleosts

NKA is highly expressed in the gill epithelia and the distribution of NKA-immunoreactive (IR) cells in the gill of euryhaline teleosts could be classified into three categories: (1) NKA-IR cells appeared only in the filamental epithelia of both FW- and SW-acclimated fish, (2) in addition to filaments, NKA-IR cells occurred abundantly in the lamellar epithelia of the FW-acclimated individuals, and (3) NKA-IR cells were found in both gill filaments and lamellae of either FW- or SW-acclimated fish (reviewed in Hwang and Lee, 2007). The distribution of NKA-IR cells in milkfish and pufferfish could be classified into categories (2) and (3), respectively. Although MRCs in lamellae were proposed to be the site of ion uptake in hyposmotic conditions while those in the filaments were proposed to be for salt secretion in hyperosmotic conditions in several studies (Uchida et al. 1996; Sasai et al. 1998; Hirai et al. 1999). Lin and Sung (2003) supported the hypothesis of Laurent and Perry (1990) and Dang et al. (2000) that the functions of filament and lamellar MRCs are identical. In this study, the identical patterns of immunolocalization of various Cl^-

transporters in NKA-IR cells in both filaments and lamellae of FW-acclimated milkfish provided more evidence that MRCs in filaments and lamellae might have similar functions.

Milkfish is a “higher NKA in hyposmotic media ” species, and the number of NKA-IR cells was significantly higher in FW milkfish due to a significant increase of NKA-IR cells in lamellae. Hence, the lamellar MRCs are likely crucial for the elevation of gill NKA responses in milkfish challenged by hypotonic conditions. Whereas pufferfish is a “higher NKA in hyperosmotic media” species, and the distribution pattern of branchial NKA-IR cell was different from that of milkfish. In contrast to milkfish, the number of NKA-IR cells in SW pufferfish was significantly higher than that of the FW individuals. This pattern is similar to the observations made in Hawaiian goby, in which higher NKA-IR cell numbers and NKA activity were reported in gills of the SW-acclimated group compared to the FW group (McCormick et al. 2003). Therefore, an increase in the number of gill NKA-IR cells in SW pufferfish might lead to higher NKA activity (Lin et al. 2004) as well as the relative protein abundance of NKA, NKCC, and CFTR (Tang and Lee, 2007) in this group of euryhaline teleosts.

Conclusion

The present study discussed whether different NKA expression patterns might lead to

distinct Cl^- transport mechanisms in the gills of euryhaline teleosts. However, our results showed that the Cl^- regulatory mechanisms were similar between the two studied species. This similarity might reflect that those Cl^- transporter proteins are important for euryhaline teleosts acclimated to various salinities, although their primary nature habitats are different. Moreover, the opposite salinity-induced NKA responses of the two studied species may result from the increase in NKA-IR cells in lamellae of FW milkfish and filaments of SW pufferfish, respectively. The results of this study depict a more detailed Cl^- transport model than was previously described in the FW gill MRCs, and the mechanisms of Na^+ absorption will be investigated in our future studies.

Acknowledgements

The rabbit polyclonal antiserum to tilapia AE1 was kindly provided by Prof. Pung-Pung Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan). The monoclonal antibodies T4 and α5 were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA. This study was supported by a grant to T.H.L. from the National Science Council of Taiwan (NSC 93-2313-B-005-007).

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Figure legends

Fig. 1. The immunolocalization of Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) in frozen longitudinal sections of seawater (SW) and freshwater (FW) milkfish gills. The cryosections were double stained with anti-NKCC (green; a and d) or anti-CFTR (green; g and j) and anti-NKA (red; b, e, h and k). The merged (c and f) images of double-stained gill sections show that NKCC is colocalized with NKA in the basolateral membrane of NKA-immunoreactive cells (mitochondrion-rich cells; MRCs) in FW and SW milkfish. Moreover, i and l show that CFTR is present in the apical membrane of MRCs in SW-acclimated milkfish rather than FW-acclimated individuals. F, filament; L, lamella.

Fig. 2. The immunolocalization of Na^+/K^+-ATPase (NKA), anion exchanger 1 (AE1), and chloride channel 3 (ClC-3) in frozen longitudinal sections of seawater (SW) and freshwater (FW) milkfish gills. The cryosections were double stained with anti-AE1 (red; a and d) or anti-ClC-3 (red; g and j) and anti-NKA (green; b, e, h and k). The merged images of double-stained gill sections show that AE1(c and f) and ClC-3 (i

and l) are colocalized with NKA in the basolateral membrane of NKA-immunoreactive cells (mitochondrion-rich cells; MRCs) in FW- and SW-acclimated milkfish. F, filament; L, lamella.

Fig. 3. The immunolocalization of Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) in frozen longitudinal sections of seawater (SW) and freshwater (FW) pufferfish gills. The cryosections were double stained with anti-NKCC (green; a and d) or anti-CFTR (green; g and j) and anti-NKA (red; b, e, h and k). The merged (c and f) images of double-stained gill sections show that NKCC is colocalized with NKA in the basolateral membrane of NKA-immunoreactive cells (mitochondrion-rich cells; MRCs) in FW and SW pufferfish. Moreover, i and l show that CFTR is present in the apical membrane of MRCs in SW-acclimated pufferfish but not FW-acclimated fish. F, filament; L, lamella.

Fig. 4. The immunolocalization of Na^+/K^+-ATPase (NKA), anion exchanger 1 (AE1), and chloride channel 3 (ClC-3) in frozen longitudinal sections of seawater (SW) and freshwater (FW) pufferfish gills. The cryosections were double stained with anti-AE1 (red; a and d) or anti-ClC-3 (red; g and j) and anti-NKA (green; b, e, h and k). The merged images of double-stained gill sections show that AE1 (c and f) and ClC-3 (i and l) are colocalized with NKA in the basolateral membrane of

NKA-immunoreactive cells (mitochondrion-rich cells; MRCs) in FW and SW pufferfish. F, filament; L, lamella.

Fig. 5. The number of Na^+/K^+-ATPase (NKA)-immunoreactive (IR) cells and $Na^+/K^+/2Cl^-$ cotransporter (NKCC)-IR cells in frozen longitudinal sections of gills of milkfish acclimated to seawater (SW) or fresh water (FW). Different letters indicate significant differences between cell numbers in filaments and lamellae or SW and FW acclimation. The asterisk indicates that the number of NKCC-IR cells in filaments or lamellae is significantly lower than that of NKA-IR cells in FW milkfish (n=8, one way ANOVA followed by Tukey's comparison, $P<0.05$). Values are represented as means ± S.E.M.

Fig. 6. Na^+/K^+-ATPase (NKA) immunostaining in longitudinal sections of gills of (A) seawater (SW)- and (B) freshwater (FW)-acclimated pufferfish. (C) Quantification of NKA-immunoreactive (IR) cells in gills of SW and FW fish (n=8). The asterisk indicates that the number of NKA-IR cells in filaments of SW pufferfish is significantly higher than that of FW individuals (Student's *t*-test; $P < 0.05$). Values were represented as means ± S.E.M. F, filaments; L, lamellae; arrows, NKA-immunoreactive cells.

Fig. 7. The proposed model of distribution of Na^+/K^+-ATPase (NKA) and chloride transporters in gill epithelial cells of milkfish and pufferfish. (A) In the

SW-acclimated fish, basolateral Na^+/K^+-ATPase (NKA) provides the driving force in the form of a transmembrane Na^+ gradient for transport of Cl^- into the cell via the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and the anion exchanger 1 (AE1). Cl^- leaves the cell to external media via cystic fibrosis transmembrane conductance regulator (CFTR) anion channels in the apical membrane, and feedback enters the blood through Cl^- channels (ClC-3) in the basolateral membrane. (B) The MRCs of FW milkfish and pufferfish utilize basolateral NKA to provide the driving force in the form of a transmembrane Na^+ gradient for the uptake of Cl^- into the cell via an unknown Cl^- transporter. Then, the Cl^- exits the cells to the blood through a basolateral ClC-3 chloride channel. Dotted lines represent passive transport.

colour figure

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Fig. 1

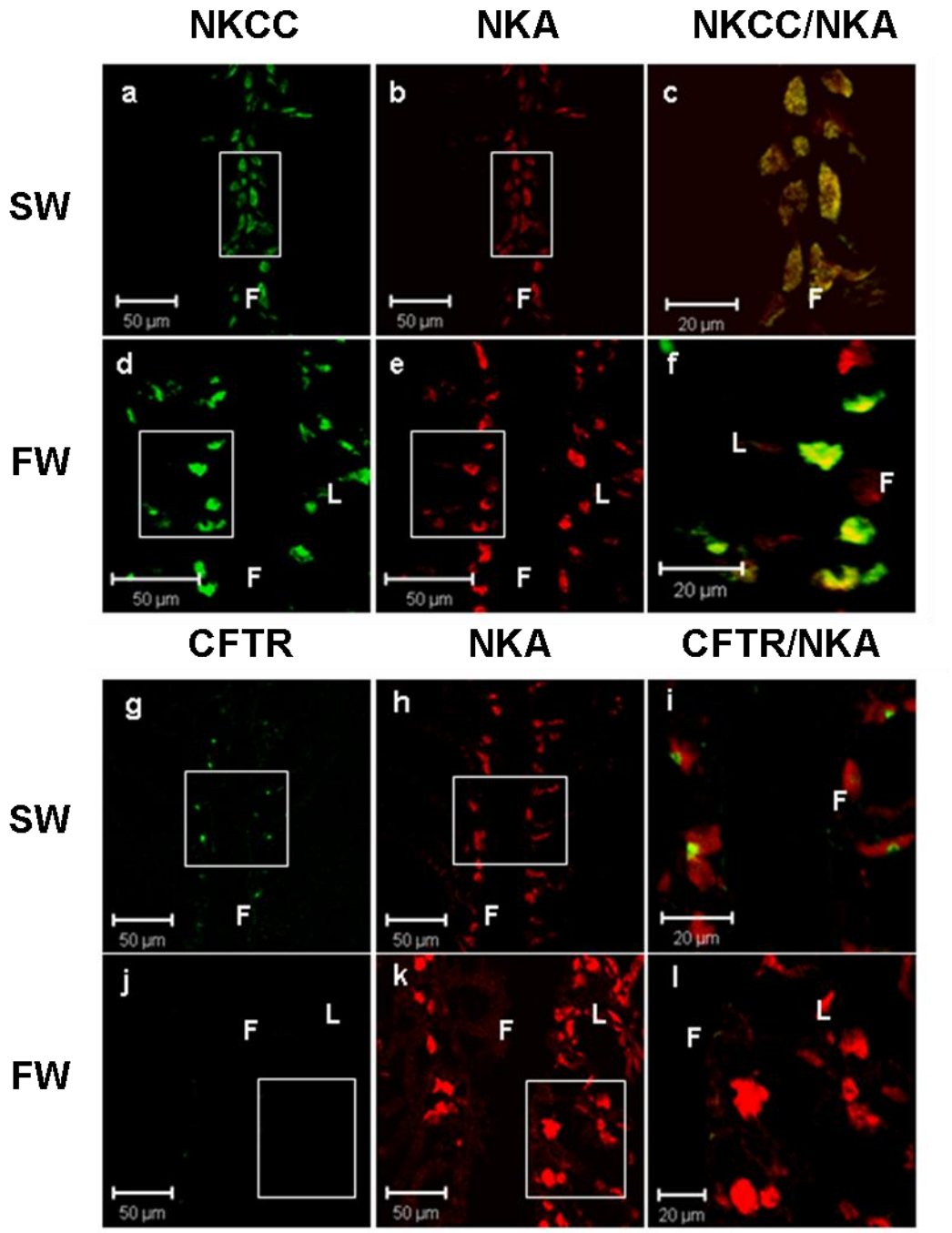


Fig. 2

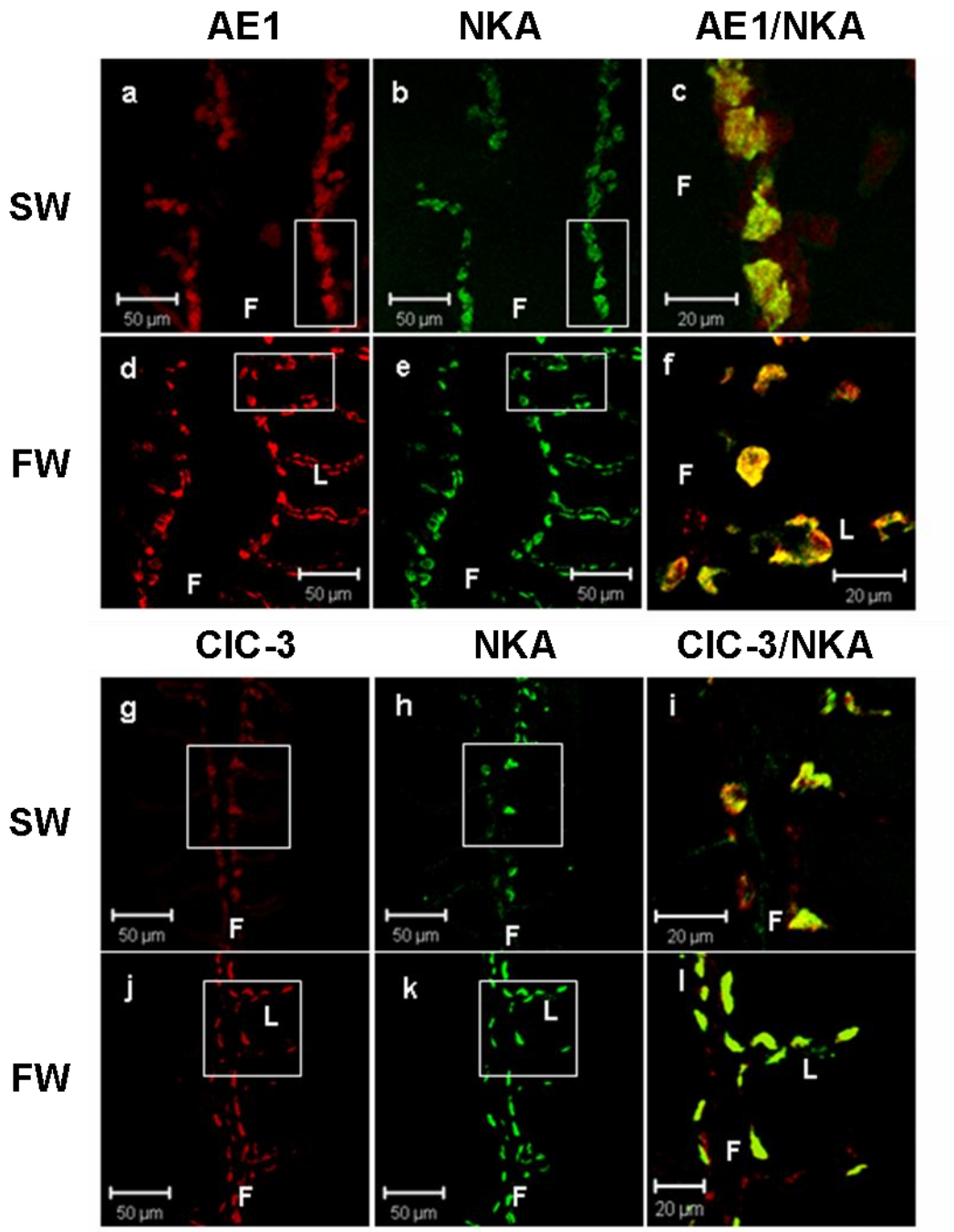

AE1
NKA
AE1/NKA
SW
FW
a
b
c
d
e
f
F
L
50 µm
20 µm
CIC-3
NKA
CIC-3/NKA
SW
FW
g
h
i
j
k
l
F
L
50 µm
20 µm

Fig. 3

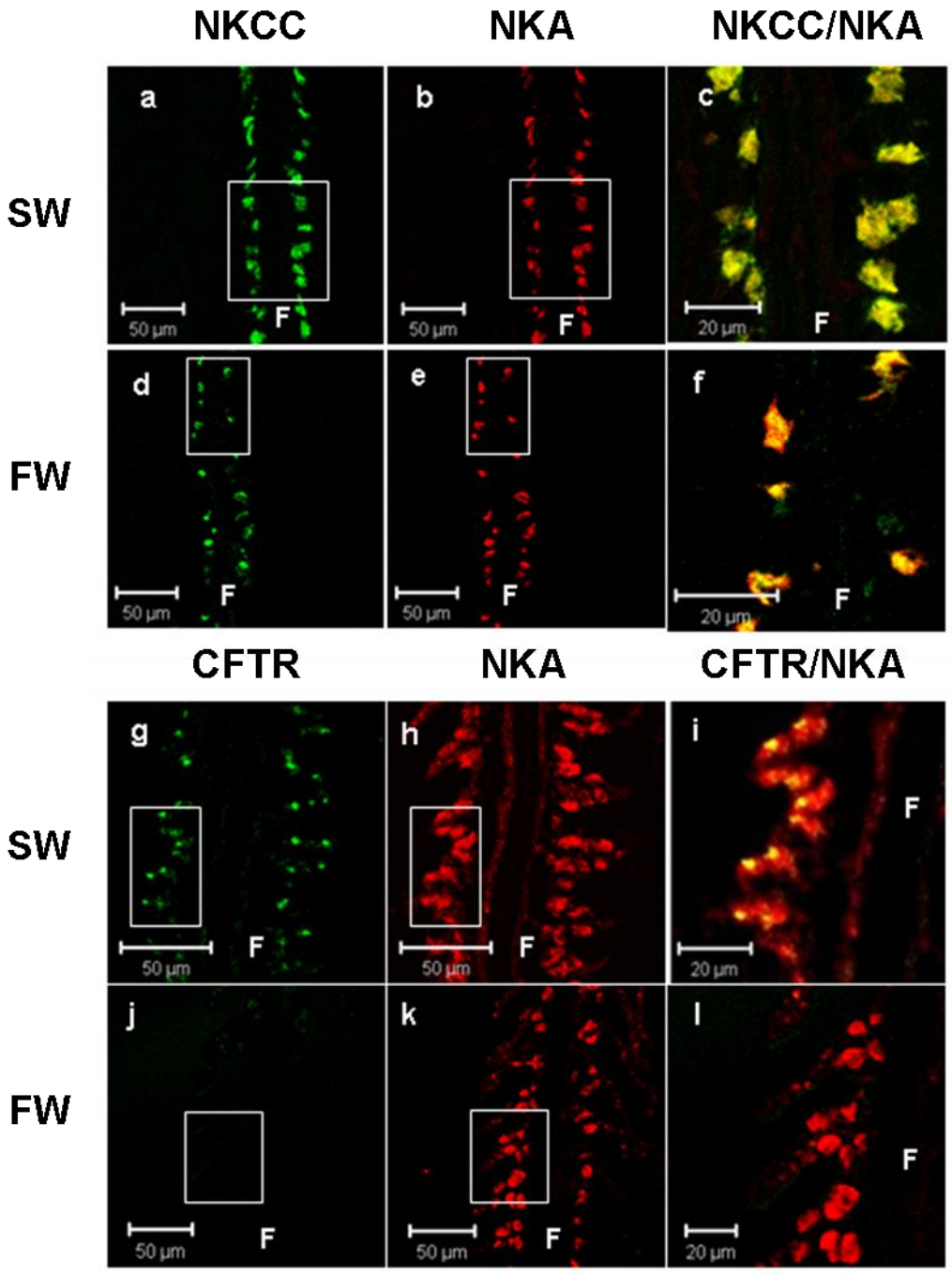

NKCC
NKA
NKCC/NKA
SW
FW
a
b
c
d
e
f
50 µm
20 µm
F
CFTR
NKA
CFTR/NKA
SW
FW
g
h
i
j
k
l
50 µm
20 µm
F

Fig. 4

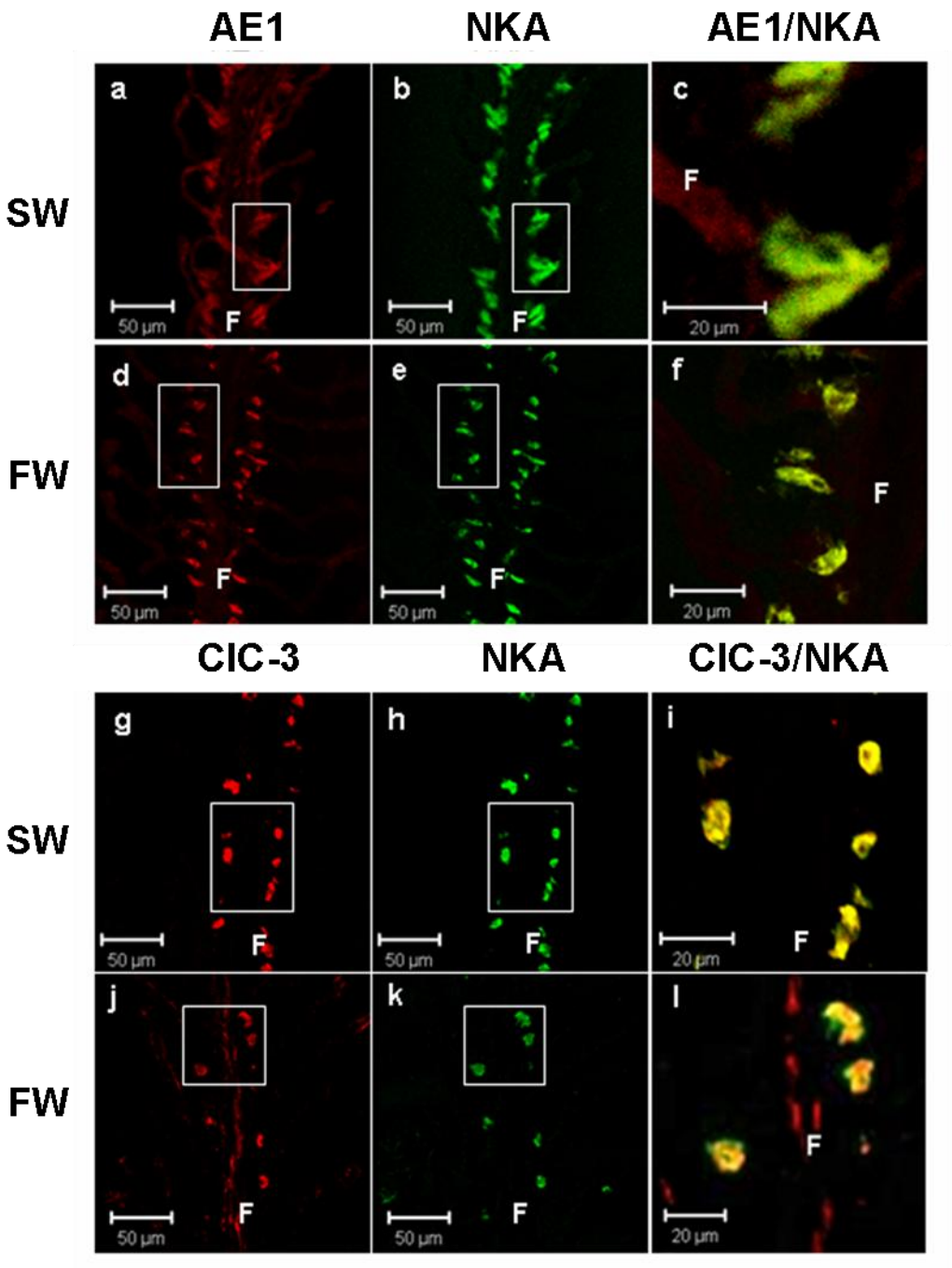

AE1
NKA
AE1/NKA
SW
FW
a
b
c
d
e
f
F
50 µm
20 µm
CIC-3
NKA
CIC-3/NKA
SW
FW
g
h
i
j
k
l
F
50 µm
20 µm

Fig. 5

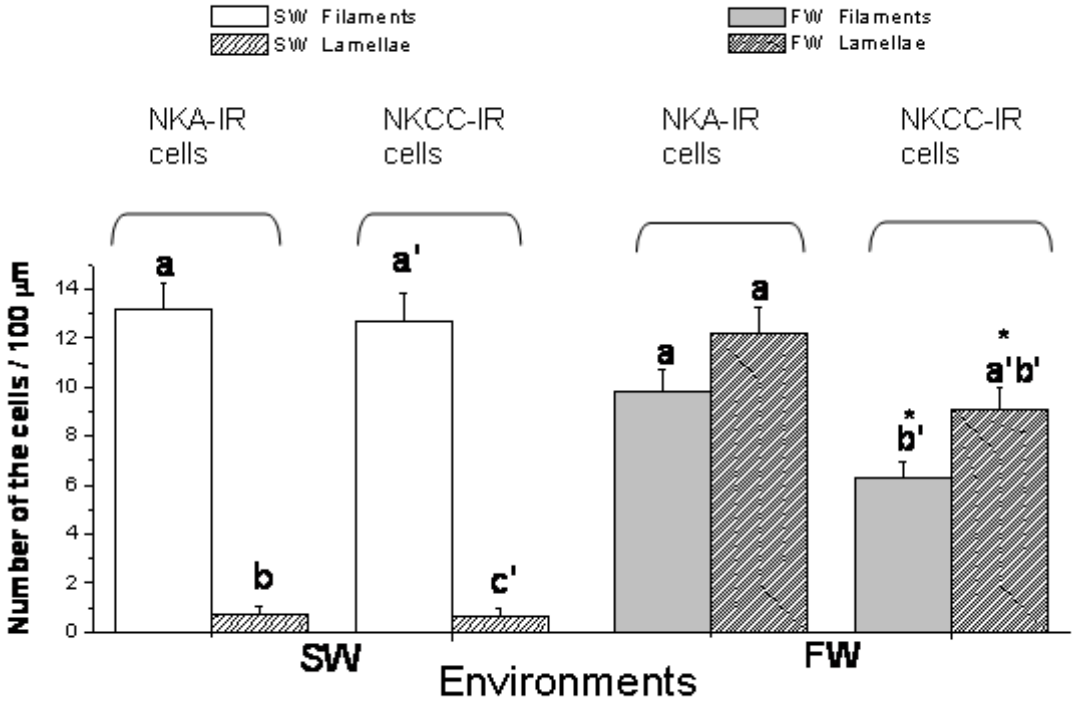

SW Filaments
SW Lamellae
FW Filaments
FW Lamellae
NKA-IR cells
NKCC-IR cells
NKA-IR cells
NKCC-IR cells
Number of the cells / 100 µm
0
2
4
6
8
10
12
14
a
b
a'
c'
a
a
*
b'
*
a'b'
SW
FW
Environments

Fig. 6

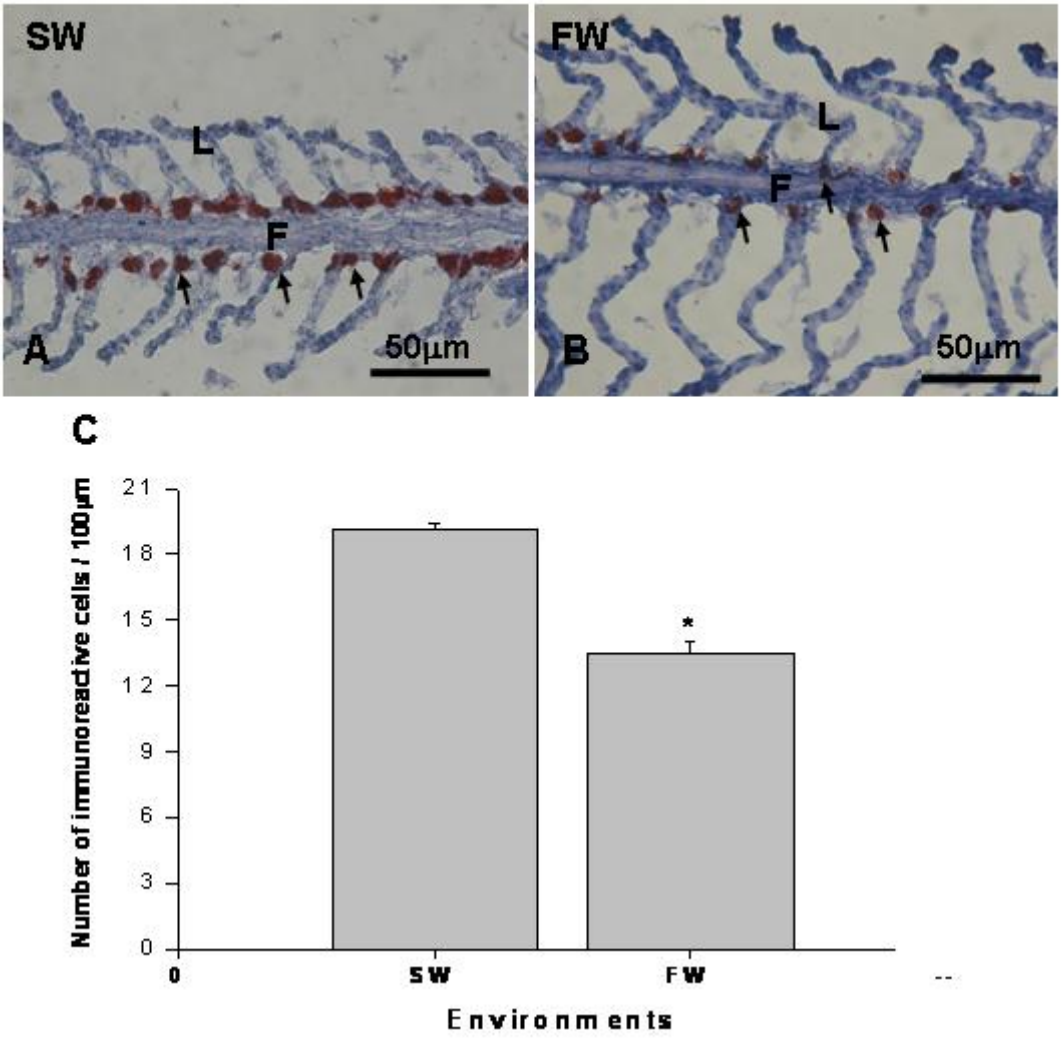
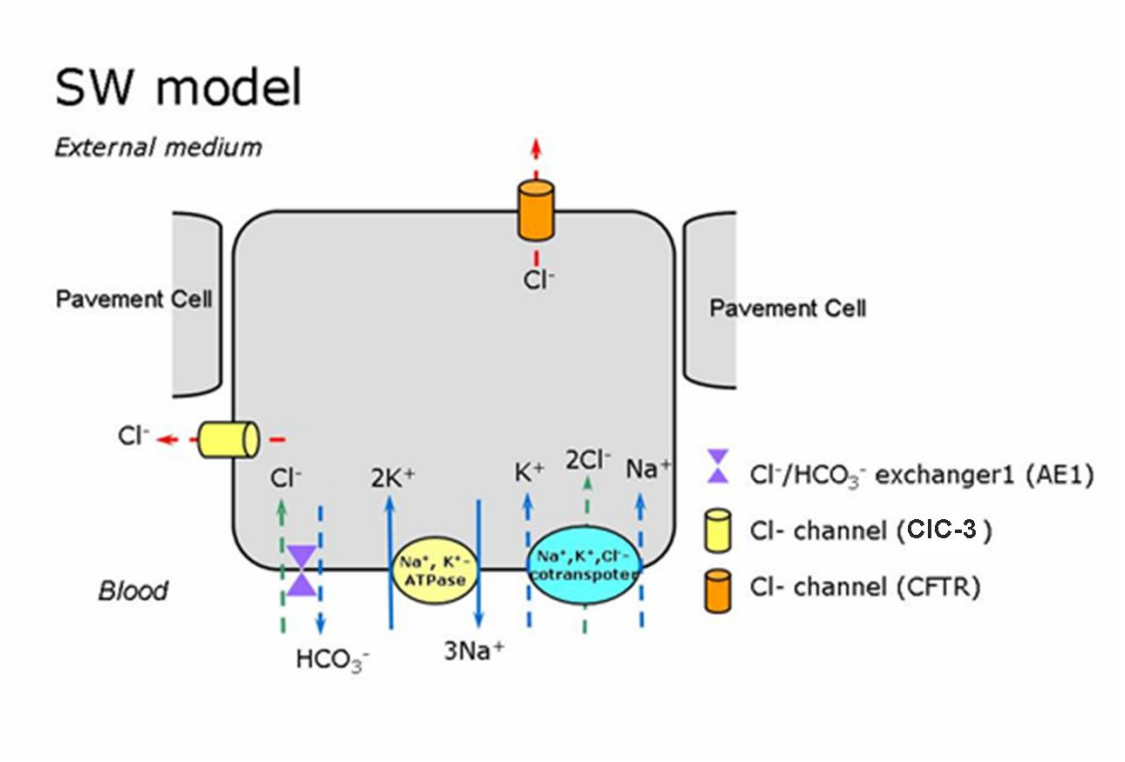
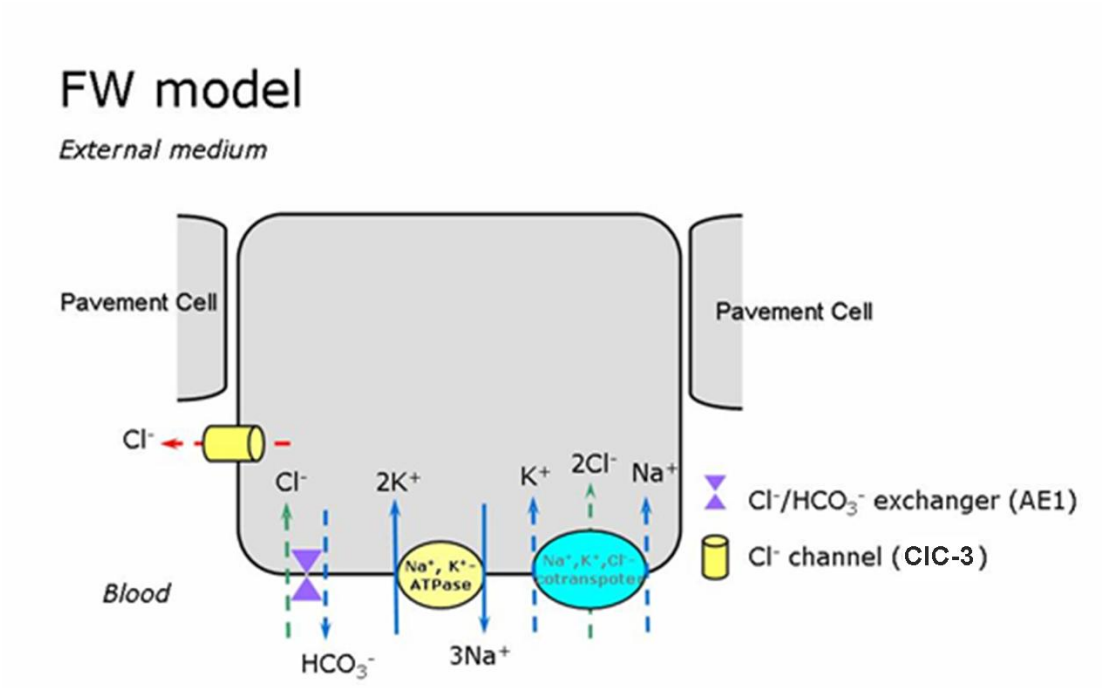

SW
L
F
A
50µm
FW
L
F
B
50µm
C
Number of immunoreactive cells / 100µm
21
18
15
12
9
6
3
0
*
0
SW
FW
Environments

Fig. 7

(A)


SW model
External medium
Pavement Cell
Pavement Cell
Cl-
Cl-
Cl-
2K+
K+
2Cl-
Na+
Na+, K+-ATPase
Na+,K+,Cl- cotranspoter
Blood
HCO3-
3Na+
Cl-/HCO3- exchanger1 (AE1)
Cl- channel (CIC-3)
Cl- channel (CFTR)

(B)


FW model
External medium
Pavement Cell
Pavement Cell
Cl-
Cl-
2K+
K+
2Cl-
Na+
Na+, K+-ATPase
Na+,K+,Cl- cotranspoter
Blood
HCO3-
3Na+
Cl-/HCO3- exchanger (AE1)
Cl- channel (CIC-3)