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$Na^+,K^+,2Cl^-$-cotransporter: A Novel Marker for Identifying Freshwater- and Seawater-type Mitochondria-rich Cells in Gills of the Euryhaline Tilapia, *Oreochromis mossambicus*

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Yu-Ching Wu, Li-Yih Lin and Tsung-Han Lee (2003) $Na^+,K^+,2Cl^-$-cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. *Zoological Studies* **42**(1): 186-192. The abundance and location of the branchial $Na^+,K^+,2Cl^-$-cotransporter (NKCC) were examined in freshwater- and seawater-adapted tilapia. Immunoblots revealed 4 bands with molecular masses centered at 282, 208, 122, and 105 kDa, respectively. Gill NKCC in tilapia is upregulated after seawater adaptation. Confocal laser scanning micrographs showed that in seawater-adapted tilapia, NKCC as well as Na,K-ATPase exhibited identical diffuse distribution confined to the basal portion of branchial mitochondria-rich cells. However, in freshwater-adapted tilapia, NKCC was displayed only in the apical region of Na,K-ATPase-immunoreactive cells. Polarized distribution of NKCC makes it a novel marker for recognizing freshwater- or seawater-type MR cells in euryhaline tilapia. http://www.sinica.edu.tw/zool/zoolstud/42.1/186.pdf

Key words: NKCC, Teleosts, Branchial epithelium, Chloride cells.

The $Na^+,K^+,2Cl^-$-cotransporter (NKCC) represents a class of integral membrane proteins that mediate the movement of Na, K, and Cl ions across the plasma membrane of animal cells in an electrically neutral manner (Payne and Forbush 1995). In euryhaline fish, NKCC is important in maintaining a plasma osmolarity of ~300 mOsmol/l in an ambient seawater (SW) salinity of ~1000 mOsmol/l (Evans 1999). Recent studies on molecular cloning and functional expression revealed at least 2 distinct isoforms: NKCC1 and NKCC2. NKCC1 is a basolateral secretory isoform with a wide epithelial distribution including in shark rectal gland, avian salt gland, and human colon (Xu et al. 1994, Lytle et al. 1995, Payne et al. 1995). NKCC2, an apical absorptive isoform, appears to be expressed in the vertebrate kidney (Gamba et al. 1994, Payne and Forbush 1995). Suvitayavat et al. (1994) first demonstrated the molecular characterization of the intestinal NKCC of the winter flounder (*Pseudopleuronectes americanus*). In SW, fish obviously drink the hypertonic saline copiously, and much of the ingested salt is absorbed in the intestine by an NKCC mechanism located on the apical surface of their intestinal epithelium. Marvao et al. (1994) provided good support for the hypothesis that NKCC occurs in the apical barrier of eel (*Anguilla anguilla*) intestine. Subcellular fractionation revealed a single prominently labeled NKCC protein of ~175 kDa in the brush-border membrane but not in basolateral membranes (Suvitayavat et al. 1994). Salt is then excreted through the gill epithelium. Flik et al. (1997) described how SW adaptation of trout (*Oncorhynchus mykiss*) leads to increased activity of NKCC in the gill epithelium. Pelis et al. (2001) also reported that gill NKCC abundance in Atlantic salmon (*Salmo salar*) increased during smolting and SW acclimation.

Gill mitochondria-rich (MR) cells were

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demonstrated to be the location of active Cl^- secretion and high ionic permeability in SW fish by using the vibrating probe technique (Foskett and Scheffey 1982). Using a Ussing chamber combined with inhibitors, i.e., furosemide and ouabain, Zadunaisky et al. (1995) reported that NKCC and Na,K-ATPase in the basolateral membrane of epithelial MR cells participated in the process of Cl^- secretion. Immunofluorescent staining of Na,K-ATPase and NKCC in gills of the Atlantic salmon (*S. salar*) and the mudskipper (*Periophthalmodon schlosseri*) showed an essentially identical diffuse cytoplasmic distribution confined to epithelial MR cells (Wilson et al. 2000, Pelis et al. 2001, Marshall et al. 2002). Hence, in the current model of the SW MR cell, basolaterally located Na,K-ATPase maintains the transmembrane electrochemical gradient for sodium ions, making NKCC feasible and energizing the transcellular, uphill transport of chloride ions and the paracellular downhill transport of sodium ions (Evans et al. 1999). Basically, gill MR cells are involved in ion extrusion in SW-acclimated fish and may also be implicated in ion uptake in freshwater (FW)-acclimated fish (Evans et al. 1999). Kirschner (1991) stated that in hypotonic milieus, Cl^- must be absorbed across the apical membrane owing to the negative potential of the cell interior, whereas the ions can pass into the blood by diffusion. The function of NKCC in gills of FW teleosts remains a matter of conjecture.

Actually, electron microscopic studies have revealed that there are 2 types of MR cells exhibited in gills of euryhaline teleosts: the FW and SW types (Hossler et al. 1985, Pisam et al. 1988, King and Hossler 1991, Kültz et al. 1995, Lee et al. 1996). Different types of MR cells obviously play different physiological roles in fish adapted to environments with varied salinities (Lee et al. 2000, Chang et al. 2001). Thus, understanding the process of changes between different types of MR cells upon salinity challenge will be the key to realizing mechanisms of salinity adaptation in euryhaline teleosts; and a marker for distinguishing FW- and SW-type MR cells will be very helpful for related studies. Since NKCC is exhibited in both secretory and absorptive epithelia of different species of vertebrates (Payne and Forbush 1995), it is possible that in the gill epithelium of euryhaline teleosts, NKCC is displayed in the basolateral membrane of SW MR cells as the "secretory" isoform and in the apical membrane of FW MR cells as the "absorptive" isoform. Polarized distribution of NKCC thus could be a useful marker for identifying SW- and FW-MR cells of euryhaline fish.

The aim of the present study was to evaluate the statement concerning the polarized distribution of NKCC and to determine the possibility of using NKCC as a marker for distinguishing SW- and FW-MR cells. For this purpose, a monoclonal antibody of NKCC recognizing both NKCC1 and NKCC2 was used for immunoblotting and immunofluorescent staining of tilapia gills and was observed using a confocal laser scanning microscope.

MATERIALS AND METHODS

Animals

Euryhaline tilapia, *Oreochromis mossambicus*, ranging in weight from 6 to 12 g were obtained from laboratory stock. Fish for the experiments were reared separately in aerated fresh water (FW) and artificial seawater (SW) at 27-29°C with a daily 12-h photoperiod for at least 2 wk. Artificial seawater was made of a mixture of synthetic sea salt called "Instant Ocean" (Aquarium Systems, Sarrebourg, France) and aerated fresh water. The water was continuously circulated through a fabric-floss filter and partially refreshed every week. Fish were fed on a daily diet of commercial pellets.

Experiments

Gills of tilapia kept in either FW or SW were dissected out for the following immunoblotting and immunofluorescent staining experiments.

Antibodies

Antibodies against NKCC and Na,K-ATPase were used as the primary antibodies in the present study. The antibody (T4) against NKCC was raised against human colonic NKCC; T4 recognizes both NKCC1 (secretory isoform) and NKCC2 (absorptive isoform) in a variety of animal tissues (Lytle et al. 1995) including teleosts (Wilson et al. 2000, Pelis et al. 2001). The antibody (NAK121) against the Na,K-ATPase α-subunit was kindly provided by Dr. Kaneko (Ocean Research Institute, University of Tokyo, Japan) (Uchida et al. 2000). The monoclonal antibody T4 was obtained from the Developmental Studies Hybridoma Bank (DSHB; John Hopkins University, Baltimore, USA). The secondary antibody for immunoblotting was alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce), while for immunofluorescent staining,

it was FITC-conjugated goat anti-rabbit IgG and Texas-red-conjugated goat anti-mouse IgG (Jackson).

Immunoblotting

The epithelial membrane fraction of tilapia gills was prepared as described previously (Weng et al. 1997). Procedures for immunoblotting were as described by Lee et al. (2000) with little modification. Briefly, aliquots of 160μg of membrane protein and pre-stained molecular weight standards (BioRad) were heated at 37°C for 15 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from unstained gels to polyvinylidene difluoride membranes (PVDF-Plus, MSI) using a tank transfer system (Electrotransfer, TE22, Hoefer). Blots were preincubated for 2 h in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% (v/v) Tween 20, pH 7.4) containing 1%-2% (wt/v) nonfat dried milk to minimize non-specific binding, then incubated overnight with primary antibody (T4) diluted in PBST (1: 5000). The blot was washed in PBST, followed by a 1-h incubation with secondary antibody diluted 2500x in PBST. Blots were visualized after incubation with 0.015% nitroblue tetrazolium and 0.07% bromochloroindolyl phosphate in a reaction buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Immunoblots were photographed using a digital vedio camera (Sony TRV900), imported as JPG files into a commercial software package (Kodak Digital Science 1D, 1995), and the results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands. To obtain the staining intensity, an entire lane on a Western blot was chosen, and the software scanned the selected part of the image from top to bottom, averaging the 8-bit gray scale values on each horizontal line. The average 8-bit gray scale values on each horizontal line were then summed to gain cumulative 8-bit gray scale values for each particular band. NKCC abundance, as measured by staining intensity, was recorded as a cumulative 8-bit gray scale. Western blot analysis for FW and SW groups were repeated 4 times using different sample fish. Values of abundance were compared using one-way analyses of variance (ANOVA) (Tukey's pair-wise method). Values are expressed as the means ± SEM (the standard error of the mean).

Cryosectioning and immunofluorescent staining

Gills were excised and immediately fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 10 min at 4°C; samples were then immersed in acetone followed by ethanol at -20°C for 10 min each. After being perfused by 30% sucrose in PBS for 1 h at room temperature, the tissue was mounted in OCT, and 10-μ-thick frozen cross sections of the gill filaments were made. The subsequent procedures for immunostaining were similar to those described before (Lee et al. 1998). The monoclonal antibody T4 and the polyclonal antibody NAK121 were used in immunostaining for colocalization of NKCC and Na,K-ATPase. NKCC and Na,K-ATPase were independently visualized owing to the different colors (red and green) of the secondary antibody. Control sections were processed in parallel without a primary antibody or substitution of the primary antibody with nonimmune normal mouse serum.

Confocal laser scanning microscopy

Stained gill filaments were observed using a Leica TCS NT confocal laser scanning microscope equipped with an argon laser (488 and 514 nm) for excitation, and a Leica DMRE microscope was attached. The stained images of Na,K-ATPase and NKCC were obtained with the use of a FITC/Texas-red filter set (BF 525/50 for FITC and BP 600/30 for Texas red) controlled by Leica TSC NT software. With the filter set, the emission wavelengths of FITC- and Texas-red-conjugated antibodies were separated and transmitted to different photomultipliers. The pictures from each photo multiplier were subsequently merged to simultaneously visualize the labels.

RESULTS

To identify the presence of NKCC in the gill of *Oreochromis mossambicus*, gill homogenate was subjected to immunoblotting. To localize NKCC and Na,K-ATPase, both the T4 and NAK121 antibodies were applied to the gill filaments for immunofluorescent staining and confocal microscopic observation.

Expression of NKCC protein in SW-adapted tilapia was greater than that in FW-adapted fish. The immunoblot of NKCC revealed 4 bands with molecular masses centered at 282, 208, 122, and 105 kDa, respectively (Fig. 1). The immunoreac-

tive bands appeared to be more intense in SW-adapted tilapia than in FW-adapted fish (Fig. 1). Image analysis of the immunoblots indicated that the average amount of NKCC expressed in the SW group was almost 2-fold higher than that in the FW group according to Western blotting (Fig. 2).

Figure 3 shows the confocal images of frozen cross-sections of gill filaments of FW- and SW-adapted tilapia stained with antibodies specific for either NKCC or the Na,K-ATPase α-subunit. Lower magnification of the sections revealed that the immunoreactive cells were concentrated on the afferent regions of the filaments, but no positive reaction was found on the efferent filamental epithelia or lamellae (Fig. 3a). Merged images of NKCC (red) and Na,K-ATPase (green) of SW tilapia revealed identical localization (red fluorescence with green counterstain yielding yellow) in gill epithelium (Fig. 3a) which indicated that NKCC was also exhibited in Na,K-ATPase-immunoreactive (NKIR) cells. On the other hand, in FW fish, double labeling of NKCC and Na,K-ATPase showed that the main reaction of NKCC occurred in the apical regions (yellow) of the epithelial cells rich in Na,K-ATPase (green) (Fig. 3b). Negative control experiments, in which normal goat serum was used instead of the primary antibodies, were conducted (data not shown) to clarify the above positive results.

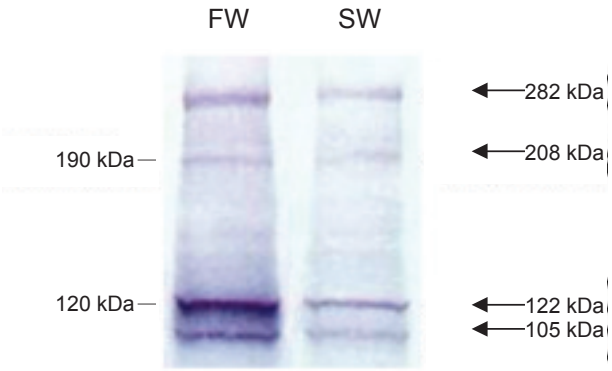

Fig. 1. Immunoblots of *Oreochromis mossambicus* gill epithelia probed with a monoclonal antibody (T4) to NKCC. The immunoreactive bands had molecular masses centered at 282, 208, 122, and 105 kDa, respectively, in SW- and FW-adapted tilapia gill. The immunoreactive bands of SW fish were more intense than those of FW fish. Markers are 190 and 120 kDa.

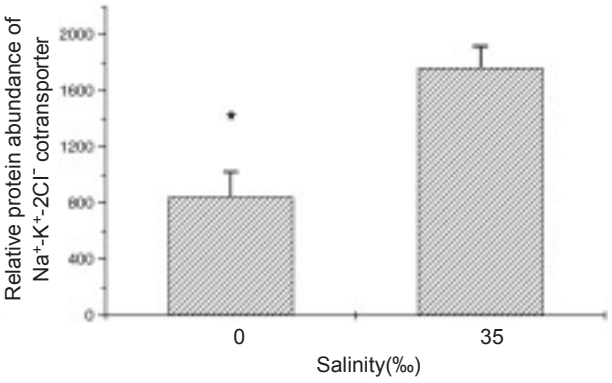

Fig. 2. Relative abundance of NKCC protein (bars; n = 5, mean ± SEM) expressed in gills of tilapia adapted to SW and FW. * Significantly different, $p < 0.05$, by Student's t-test.

DISCUSSION

The T4 antibody has been shown to recognize both the secretory (NKCC1) and absorptive (NKCC2) isoforms of NKCC from various cell types among a wide variety of species, ranging in mass from 145 to 285 kDa, including the rectal gland of shark (*Squalus acanthias*), the intestine of the flounder (*Pseudopleuronectes americanus*), and gills of the Atlantic salmon (*Salmo salar*) and killifish (*Fundulus heteroclitus*) (Suvitayavat et al. 1994, Lytle et al. 1995, Pelis et al. 2001, Marshall et al. 2002). With the use of the T4 antibody, immunoblots in the present study revealed that tilapia gill contained 4 bands with molecular masses from 105 to 282 kDa (Fig. 1). Using the same antibody (T4), Marshall et al. (2002) reported that immunoreactive bands appeared in killifish gills at 150.0 ± 2.0, 92.7 ± 0.3, and 87.8 ± 0.3 kDa,

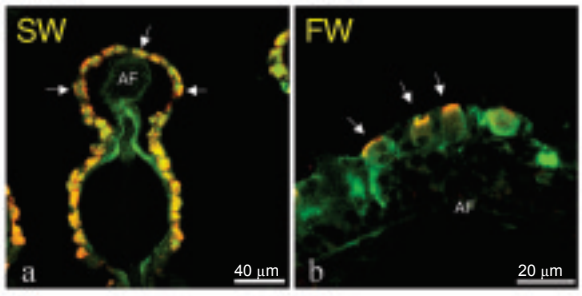

Fig. 3. Confocal laser scanning micrographs of the colocalization of NKCC and the Na,K-ATPase α-subunit in frozen cross-sections of gill filaments from SW- and FW-adapted tilapia. Merged images of NKCC and Na,K-ATPase labeling show polarized exhibition of NKCC: basal distribution throughout the cells excluding the nuclei identical to that of Na,K-ATPase in SW fish (a) and apical expression in FW fish (b). The red fluorescence of NKCC with green counterstaining (Na,K-ATPase) yields yellow. Arrows indicate immunoreactive epithelial cells. AF, afferent region of the filament.

respectively. In addition, Pelis et al. (2001) stated that the Western blot of salmon gills revealed several bands with sizes from 120 to 285 kDa. According to their description, the lower-molecular-mass bands (122 and 105 kDa) in our Western blot likely represent degradation products, the band at 208 kDa represents the core mass of the cotransporter, while the band at 282 kDa likely represents the glycosylated form. Similar to results in the salmon (Pelis et al. 2001), gill NKCC abundance in tilpia increased significantly after seawater adaptation (Fig. 2). Upregulation of NKCC during seawater adaptation provides further evidence for the role of this protein in secretion by the gill. Although there is strong evidence for the role of the NKCC in ion secretion, the presence of this protein in gills of FW-adapted fish is less easily explained since the current model of ion uptake by gill epithelium in FW lacks NKCC (Evans et al. 1999). However, euryhaline tilapia can tolerate gradual increases in environmental salinity (Hwang et al. 1989). Expression of the NKCC protein may serve a physiological function in FW and indicate a moderate level of readiness for SW challenge.

Gill NKIR cells were demonstrated to be mitochondria-rich (MR) cells (Dang et al. 2000, Lee et al. 2000). In SW-adapted tilapia, positive NKCC-immunostaining cells were identified as MR cells on the basis of their location, size, morphology, and the same localization with Na,K-ATPase. NKCC as well as Na,K-ATPase was present at low or non-detectable levels in other branchial cell types. Except for the nucleus, NKCC immunoreactivity in gills of SW-adapted tilapia, as in the mudskipper (*Periophthalmodon schlosseri*), salmon, and killifish (Wilson et al. 2000, Pelis et al. 2001, Marshall et al. 2002), occurred throughout MR cells, and the immunoreactivity was similar to that exhibited by Na,K-ATPase (Fig. 3). Immunocytochemical labeling and electron microscopic observation of tilapia demonstrated that Na,K-ATPase is present in the basolateral membrane-formed tubular system extending to the cytoplasm of MR cells (Lee et al. 1995, Dang et al. 2000). Hence, similar immunofluorescent staining results of NKCC and Na,K-ATPase in this study suggest that in SW, the transmembrane cotransporter is also present on the basolateral surface of MR cells and supports the current model of SW MR cells (Evans et al. 1999). Conversely, in FW-adapted tilapia, NKCC was ascribed specifically to the apical region of NKIR cells, which differs from the even distribution of Na,K-ATPase (Fig. 3). Although MR cells in FW were implicated in ion uptake and acid-base regulation, the most widely accepted model for these functions include Na,K-ATPase but not NKCC (Evans et al. 1999). Since the antibody recognized both NKCC1 and NKCC2, the presence of NKCC in the apical region of MR cells could be the absorptive isoform (NKCC2) and may reflect the demand for ion uptake in FW tilapia. Unlike that which was found in tilapia, in FW-acclimated parr of Atlantic salmon, NKCC immunoreactivity also occurred throughout MR cells and was found only in some but not all MR cells exhibiting Na,K-ATPase immunoreactivity (Pelis et al. 2001). In FW-adapted killifish, eccentric localization of NKCC in MR cells was found, and a relatively slow redistribution of NKCC in SW-adapted fish (with an even including distribution in the basal portion of MR cells) was suggested (Marshall et al. 2002). Different NKCC distributions in branchial epithelial cells of FW salmon, FW killifish, and FW tilapia reveal the possibility that dissimilar pathways of ion uptake might exist in different species of euryhaline teleosts.

Previous ultrastructural studies show that in euryhaline teleosts, i.e., Atlantic salmon, killifish, striped bass (*Morone saxatilis*), and tilapia (*Oreochromis mossambicus*), at least 2 types of MR cells exist: the FW and the SW types (Hossler et al. 1985, Pisam et al. 1988, King and Hossler 1991, Kültz et al. 1995, Lee et al. 1996). Changes of branchial MR cell types with altered salinity as well as alterations of other physiological indicators such as ion fluxes and sodium pump activities imply that FW- and SW-type MR cells are 2 functionally distinct groups of MR cells (Hossler et al. 1985, King and Hossler 1991, Kültz et al. 1995, Lee et al. 2000). The polarized distribution of NKCC found in this study in FW- and SW-adapted tilapia provides additional evidence for the existence of absorptive and secretory types of MR cells. Polarized display features of NKCC also make this cotransporter a novel marker for identifying functionally distinct FW- and SW-type MR cells in gills of euryhaline tilapia. Thus, NKCC, a novel marker of FW- and SW-type MR cells will be applied in future studies on MR cell turnover in euryhaline tilapia adapted to different salinities.

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