Zoological Studies **47**(6): 733-740 (2008)

Zoological Studies

Expression of the Na-K-2Cl Cotransporter in Branchial Mitochondrion-Rich Cells of Mozambique Tilapia (*Oreochromis mossambicus*) Subjected to Varying Chloride Conditions

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(Accepted April 8, 2008)

Jiun-Lin Horng and Li-Yih Lin (2008) Expression of the Na-K-2Cl cotransporter in branchial mitochondrion-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) subjected to varying chloride conditions. *Zoological Studies* **47**(6): 733-740. The Na/K/2Cl cotransporter (NKCC) plays a critical role of active Cl^- transport through the basolateral membranes of mitochondrion-rich (MR) cells in seawater teleosts. Recently, the NKCC was also found in apical membranes of MR cells of freshwater (FW)-acclimated tilapia (*Oreochromis mossambicus*). In this study, immunocytochemistry and immunoblotting were used to analyze the expression levels of the NKCC in FW-acclimated tilapia in response to different ambient chloride levels. The amount of branchial NKCC from low-Cl^-- (0.002-0.006 mM)-acclimated tilapia was about 3- and 5.5-fold higher than that from normal- (0.49-0.51 mM) and high-Cl^- (9.8-10.2 mM)-acclimated tilapia. With confocal microscopy, the NKCC was localized mainly in the apical membranes of MR cells with Cl^--dependent abundances. The staining intensity of the NKCC in individual MR cells was significantly higher in the low-Cl^- group than in the normal and high-Cl^- groups. Results suggest that the apically localized NKCC is induced by a Cl^- deficiency and is probably also involved in the Cl^- uptake mechanism of MR cells. http://zoolstud.sinica.edu.tw/Journals/47.6/733.pdf

Key words: Mitochondrion-rich cells, Tilapia (*Oreochromis mossambicus*), Osmoregulation.

The Na-K-2Cl cotransporter (NKCC) mediates the coupled movements of Na^+, K^+, and Cl^- across plasma membranes of animal cells. The NKCC plays an important role in ion movements across polarized epithelia and is also known to be involved in regulating cell volume and intracellular Cl^- levels (Lytle and Forbush 1996, Haas and Forbush 1998). The NKCC is a member of the Na-coupled group of cation-chloride cotransporters (CCCs), a family which includes K-Cl cotransporters (KCCs). Three Na-coupled CCCs have been described to date (Mark and David 1996, Haas and Forbush 2000). The "secretory" Na-K-2Cl cotransporter, NKCC1, is widely distributed in mammalian tissues, and is especially prominent in basolateral membranes of secretory epithelial cells. Within the kidney, NKCC1 is found in epithelial cells of the collecting duct and glomeruli. The "apical" Na-K-2Cl cotransporter, NKCC2, is only found in apical membranes of epithelial cells in the thick ascending limb of the loop of Henle (Lytle et al. 1995).

In fish, the NKCC was found in the intestinal epithelium of several species including winter flounder *Pseudopleuronectes americanus* (Suvitayavat et al. 1994), European eel *Anguilla anguilla* (Culter and Cramb 2002), and killifish *Fundulus heteroclitus* (Marshall et al. 2002), and in the rectal gland of the spiny dogfish *Squalus acanthias* (Lytle et al. 1992). Pharmacological studies using loop diuretics (furosemide, bumetanide, and benzmetanide) indicated that

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the NKCC is involved in branchial salt secretion by marine teleosts (Karnaky et al. 1977, Eriksson et al. 1985). Using immunocytochemistry, the NKCC was found to be distributed in branchial mitochondrion-rich (MR) cells of several species including tilapia *Oreochromis mossambicus* (Wu et al. 2003, Hiroi et al. 2005), killifish *F. heteroclitus* (Scott et al. 2004), striped bass *Morone saxitilis* (Tipsmark et al. 2002), and 3 salmonids *Salvelinus namaycush*, *Salv. fontinalis*, and *Salmo salar* (Hiroi and McCormick 2007).

In the current model of seawater-type MR cells, basolaterally located Na/K-ATPase maintains the transmembrane electrochemical gradient for Na^+, making the NKCC feasible and energizing the active transcellular transport of Cl^- (reviewed by Evans et al. 1999, Marshall 2003, Hwang and Lee 2007). However, recent studies also found NKCC immunoreactivity in apical membranes of MR cells in gills and larval skin of FW-acclimated tilapia (*O. mossambicus*) (Wu et al. 2003, Hiroi et al. 2005), and in gills of FW-acclimated sea bass (*Dicentrarchus labrax*) (Lorin-Nebel et al. 2006), implying that the NKCC might also be involved in ion uptake of FW-type MR cells. However, no further investigations of possible functions of the apical NKCC in MR cells have been carried out. In mammals, NKCC2 is found in apical membranes of epithelial cells in the thick ascending limb (TAL) of the loop of Henle and functions in salt reabsorption (Lytle et al. 1995, Kaplan et al. 1996, Nelsen et al. 1998). By investigating the expression levels of branchial NKCCs in tilapia under varying chloride levels, we attempted to provide further evidence for ion uptake by apical NKCCs in tilapia MR cells. The abundance of the apically expressed NKCC was determined by immunocytochemistry and immunoblotting to test if the expression levels of the apical NKCC were associated with ambient Cl^- levels. In our previous studies, tilapia MR cells were found to be activated and enlarged in apical membranes when acclimating to Cl^--deficient fresh water, suggesting an increase in the putative Cl^- transporter in apical membranes of MR cells (Lin and Hwang 2001 2004). If the apical NKCC is involved in Cl^- uptake of MR cells, one would expect upregulation of the apical NKCC to occur to compensate for the Cl^- lost in tilapia acclimated to Cl^--deficient water.

MATERIALS AND METHODS

Acclimation of tilapia

Mozambique tilapia (*O. mossambicus*) of 5-10 g in body weight were obtained from laboratory stocks. All fish were maintained in a tank with circulating freshwater at 25-28°C and a photoperiod of 12 h light/12 h dark. Three artificial freshwater media were prepared by the addition of appropriate amounts of NaCl, Na_2SO_4, $MgSO_4$, K_2HPO_4, KH_2PO_4, and $CaSO_4$ to deionized water: (1) normal, which consisted of local tap water; (2) low-Cl^- content; and (3) high-Cl^- content. The final concentrations of cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were measured by atomic absorption spectrophotometry (Z-8000, Hitachi, Tokyo, Japan) and that of the Cl^- anion was measured by the ferricyanide method (Table 1). Tilapia were acclimated for 7 d to the 3 media which was maintained at 26-28°C. During acclimation, the fish were not fed, and the media were changed daily to guarantee optimal water quality.

Gill homogenates

Tilapia were anesthetized with ice and immediately biopsied. Gills were removed and weighed, followed by homogenization in 100 mM imidazole-HCl buffer (pH 7.0), containing 5 mM Na_2EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate with a motorized Teflon pestle at 600 rpm for 20 strokes on ice. After centrifugation (at 12,000 rpm and 4°C for 30 min), the supernatant was kept at -70°C until the assay was performed. The total protein content was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and calculated from a standard curve

Table 1. Ionic compositions (mM) in artificial freshwater media

Medium	normal	low Cl^-	high Cl^-
[Na^+]	0.42 - 0.51	0.48 - 0.52	0.51 - 0.52
[Cl^-]	0.49 - 0.51	0.002 - 0.006	9.8 - 10.2
[K^+]	0.14 - 0.17	0.15 - 0.17	0.15 - 0.19
[$Mg2^+$]	0.19 - 0.20	0.17 - 0.19	5.08 - 5.19
[$Ca2^+$]	0.18 - 0.19	0.18 - 0.19	0.18 - 0.21
pH	6.8 - 7.1	6.8 - 7.0	6.7 - 7.2

established from known concentrations of bovine serum albumin (BSA; Sigma, St. Louis, MO, USA).

Antibodies

The monoclonal antibody (mAb) against the NKCC (T4) was used as the primary antibody in the present study. The T4 antibody against the NKCC was raised against the human colonic NKCC and has been shown to recognize both NKCC1 (the secretory isoform) and NKCC2 (the absorptive isoform) in a variety of animal tissues (Lytle et al. 1995) including teleosts (Wilson et al. 2000, Pelis and McCormick 2001). The T4 mAb was obtained from the Developmental Studies Hybridoma Bank (DSHB, John Hopkins Univ., Baltimore, MD, USA). The secondary antibody for immunoblotting was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; Jackson Immunoresearch Laboratories, West Grove, PA, USA), while for immunofluorescent staining, a FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) was used.

Immunoblotting

The epithelial membrane fraction of tilapia gills was prepared as described previously (Lin et al. 2003). Aliquots of 200 μg of membrane protein and prestained molecular weight standards (BioRad) were heated to 95°C for 15 min and fractionated by electrophoresis on sodium dodecylsulfate (SDS)-containing 7.5% polyacrylamide gels. Separated proteins were transferred from unstained gels to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a tank transfer system (BioRad). Blots were preincubated for 2 h in phosphate buffer with salts and Tween 20 (PBST, 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.2% (vol/vol) Tween 20; pH 7.4) containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding, then incubated overnight with the primary antibody (T4) diluted in PBST (1: 5000). The blot was washed in PBST, followed by a 1 h incubation with the secondary antibody which was 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, and 5 mM $MgCl_2$; pH 9.5).

Fluorescence staining of frozen sections

Excised gills from anesthetized tilapia were immediately fixed with 4% paraformaldehyde for 1 h at 4°C and permeabilized with 90% alcohol for 10 min at -20°C. After washing with PBS, the gills were immersed in 30% sucrose for 2 h and then embedded in a Shandon Cryomatrix embedding medium (Thermo Fisher Scientific, Waltham, MA, USA) at -20°C. Frozen cross-sections at 15 μm were then cut with a cryostat (CM1900, Leica, Heidelberg, Germany) and attached to slides coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). The fixed gill sections were rinsed with PBS, blocked with 10% normal goat serum (NGS), and then incubated at 4°C overnight with the 100x-diluted T4 mAb. After washing with PBS, sections were further incubated with a goat anti-mouse IgG conjugated with 200x-diluted FITC (Jackson Immunoresearch Laboratories) at room temperature for 2 h. After washing, specimens were observed with a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Bronchial sections from normal, and high-Cl^-- and low-Cl^--acclimated tilapia were attached side by side on the same slide to compare the staining intensities. Therefore, sections from various groups could be stained under the same conditions to reduce bias from the staining process.

Acquiring and analyzing images

The period over which the image was captured, the photomultiplier tube (PMT) gain, and the scanning rules of the confocal microscope were optimized before each experiment and maintained throughout each experiment to standardize the intensity of the fluorescence among experiments. Images were quantified with MetaMorph software (Universal Imaging, Philadelphia, PA, USA). The relative fluorescent intensity of the NKCC in individual MR cells was measured and compared among treatments.

Statistical analysis

Values are presented as the mean ± S.D. One-way analysis of variance (ANOVA) was used to compare the staining intensities and areas of the 3 groups of sections.

RESULTS

Immunoblotting of the NKCC revealed 3 major bands with molecular weights of 282, 122, and 105 kDa (Fig. 1A) in tilapia gill extracts. The molecular weights of the immunoreactive bands appeared to be identical among the 3 (normal, low-Cl^-, and high-Cl^-) groups, but the intensities of the blots varied among the groups. Image analysis of the immunoblots indicated that the average amount of the NKCC expressed in the low-Cl^- group was about 3- and 5.5-fold higher than those in the normal and high-Cl^- groups, respectively (Fig. 1B). The high-Cl^- group showed the lowest intensity among the 3 groups.

Figure 2 shows confocal images of T4-immunoreactive signals in frozen sections of gill filaments from tilapia acclimated to the 3 different media. Lower magnification of the sections revealed that the immunoreactive cells were distributed in the afferent region of the gill filaments, but not in the efferent region or lamellae (Fig. 2). These T4-immunoreactive cells were previously demonstrated to be MR cells with high Na/K-ATPase expression (Wu et al. 2003).

The NKCC signals were strongly concentrated in apical membranes of MR cells in all 3 groups of tilapia (Figs. 2, 3). However, in the normal and low-Cl^- groups, NKCC signals were also dispersed throughout the entire cell except the oval nucleus (Fig. 3B, C). In the low-Cl^- group, the outline of strong NKCC signals formed a convex shape of the apical membrane of MR cells which were recognized as the wavy-convex-type of MR cells (Fig. 3C). In contrast, the most signals in the high-Cl^- group were confined to small apical membranes of MR cells, which were recognized to be deep-hole-type MR cells (Figs. 2A, 3A). The staining intensities of individual MR cells were compared among fish in the 3 groups (Fig. 4). The NKCC intensity in MR cells of the low-Cl^- group was 1.6- and 4.4-fold higher than those of the normal and high-Cl^- groups, respectively.

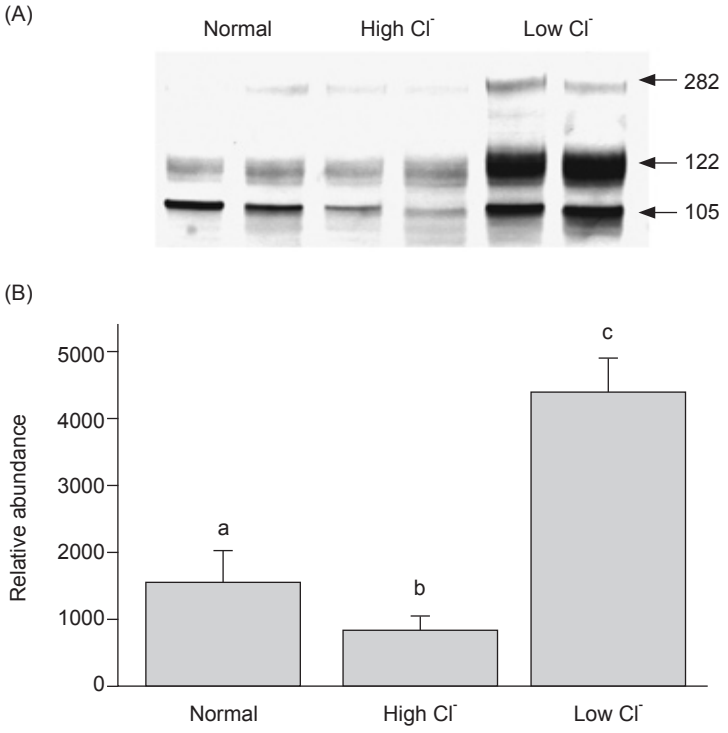

Fig. 1. Western blots of the gill Na/K/2Cl cotransporter (NKCC) (A) and relative abundances (B) in tilapia acclimated to high-Cl^-, normal, and low-Cl^- media. The Western blot was reacted with the T4 antibody, and 3 immunoreactive bands centered at 282, 122, and 105 kDa were obtained. The intensities of the immunoreactive bands were quantified and are shown in B. Values are the mean ± S.D. (n = 6), and different letters indicate significance at the $p < 0.05$ level (one-way ANOVA followed by Tukey's pair-wise comparison).

DISCUSSION

The NKCC's distribution in SW-type MR cells was found to be basolateral, as measured in vesicle experiments and detected by immunocytochemistry. Basolateral membrane vesicles from rainbow trout gills demonstrated the presence of bumetanide- and furosemide-sensitive NKCCs (Flik et al. 1997). The NKCC has been identified immnocytochemically in basolateral membranes of SW-type MR cells in the mudskipper *Periophthalmodon schlosseri* (Wilson et al. 2000), juvenile Atlantic salmon *S. salar* (Pelis and McCormick 2001), killifish *F. heteroclitus* (Marshall et al. 2002), and tilapia *O. mossambicus* (Wu et al. 2003, Hiroi et al. 2005). However, the most notable finding is the apical distribution of the NKCC in FW-type MR cells of tilapia (*O. mossambicus*, Wu et al. 2003, Hiroi et al. 2005) and sea bass (*D. labrax*, Lorin-Neel et al. 2006). Those reports showed the apical distribution of the NKCC in FW-type MR cells but

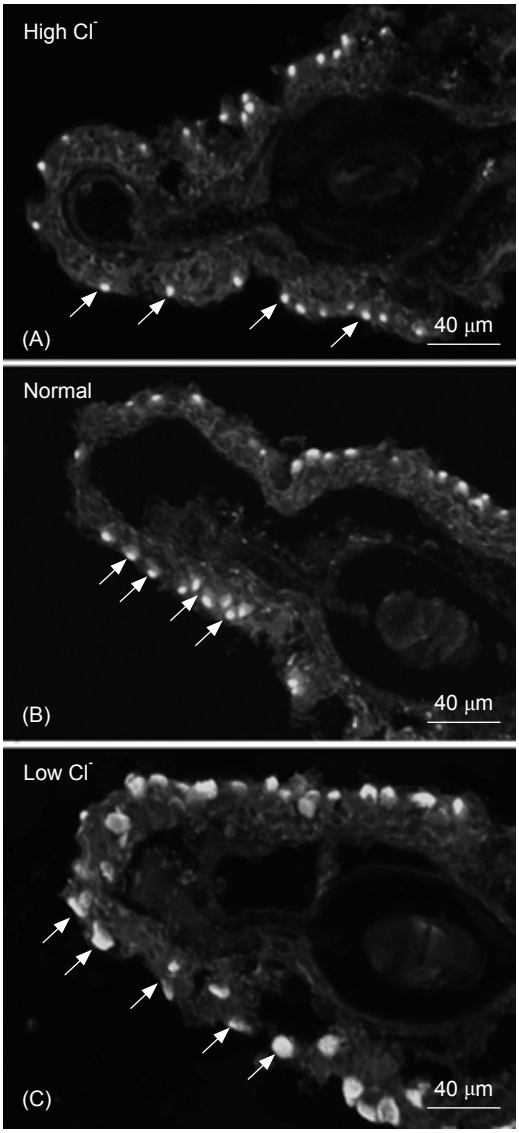

Fig. 2. Confocal laser scanning images of Na/K/2Cl cotransporter (NKCC) signals in frozen cross-sections of gills from tilapia acclimated to high-Cl^- (A), normal (B), and low-Cl^- media (C). Arrows indicate strong signals of the NKCC in the apical region of mitochondrion-rich (MR) cells.

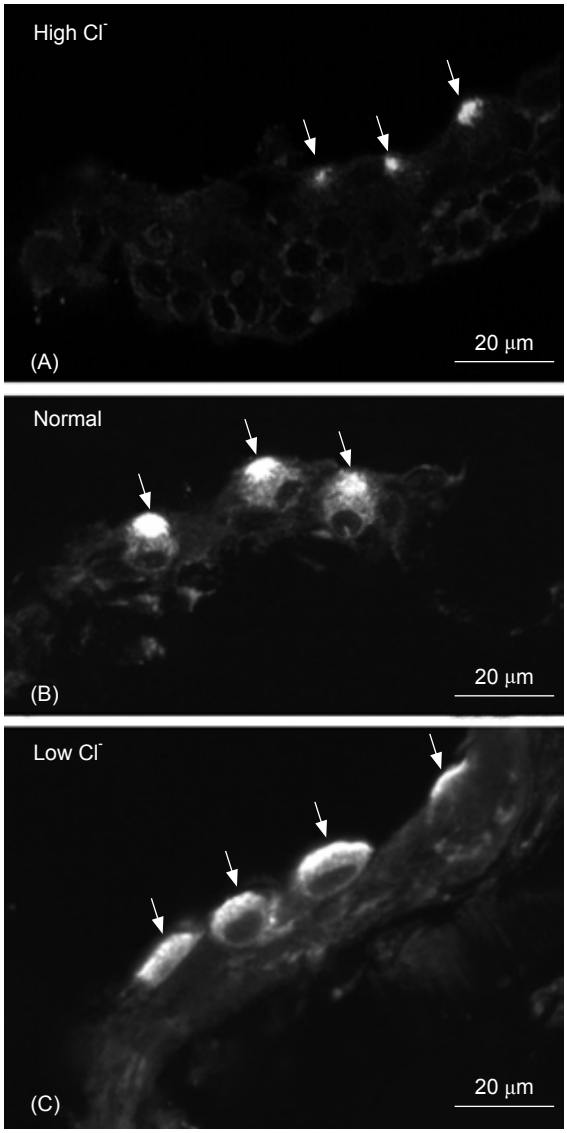

Fig. 3. Confocal laser scanning images of Na/K/2Cl cotransporter (NKCC) signals in frozen cross-sections of gills from tilapia acclimated to high-Cl^- (A), normal (B), and low-Cl^- media (C). Arrows indicate strong signals of the NKCC in the apical region of mitochondrion-rich (MR) cells.

provided no further evidence for the function of the apical NKCC. In this study, we showed that the regulatory expression of the apical NKCC in MR cells responds to varying Cl^- levels, and suggest that the apical NKCC might be involved in Cl^- uptake by MR cells.

Three types or subtypes of MR cells with distinct apical structures (wavy-convex, shallow-basin, and deep-hole) have been reported in FW-acclimated tilapia (Lee et al. 1996). Changes among these cell types due to external ionic concentrations were found in gills or larval skin of tilapia (Chang et al. 2001 2003, Lin and Hwang 2001). Low-Cl^- medium was suggested to induce activation of FW-type MR cells from small deep-hole- or basin-type to large wavy-convex-type cells of the apical membrane in tilapia larvae (Lin and Hwang 2001 2004). The present data further consolidate this suggestion. A higher expression level of the NKCC on the convex surface was coincident with a larger apical surface for Cl^- uptake in wavy-convex-type MR cells; in contrast, small deep-hole-type MR cells expressed a low level of the NKCC. In FW-acclimated tilapia, the apical structure of the deep-hole type of MR cells was similar to that of SW-type MR cells which form an apical crypt when in contact with the environment. However, the distributions of NKCCs in the apical and basolateral membranes were functionally distinct between these 2 types of MR cells. In addition to the strong signals of the NKCC in apical membranes of MR cells, we also observed relatively weak signals in the cytoplasm, particularly in the "subapical" region, which refers to the cytoplasm beneath the apical membranes of MR cells. In this region, punctate signals were observed, which implies that these NKCC proteins might be stored in secreting vesicles beneath the apical membranes.

The immunoblots in the present study revealed that tilapia gills contained 3 major bands with molecular weights of 105-282 kDa (Fig. 1). Similar results using the same antibody (T4) have been reported elsewhere. T4 immunoreacted with 3 major bands of molecular sizes ranging 120-285 kDa in Atlantic salmon gills (Pelis and McCormick 2001). Immunoreactive bands of 88, 93, and 150 kDa appeared in killifish gills (Marshall et al. 2002), while Tipsmark et al. (2002) also identified 3 T4-immunoreactive proteins with apparent MWs of 223/224, 209/214, and 156/160 kDa in both brown trout and Atlantic salmon, respectively. Recently, Lorin-Nebel et al. (2006) also reported 3 major bands in sea bass gills centered at 110, 120, and 225 kDa. This variability in T4 immunoreactive bands is due to different degrees of glycosylation (Lytle et al. 1995). In this study, all 3 bands simultaneously changed in response to treatment; thus the abundances of the 3 bands were summed to represent the total abundance of the NKCC.

The T4 antibody used in the present study was found to be capable of recognizing both the secretory (NKCC1) and absorptive (NKCC2) isoforms of the NKCC in mammals (Lytle et al. 1995). Therefore the apical NKCC in tilapia might be NKCC2 and thus plays a role in ion uptake from ambient water. Recently, Na^+/Cl^- cotransporter (NCC)-homologous genes from gills of tilapia were isolated and their expressions in apical membranes of FW-type MR cells were further

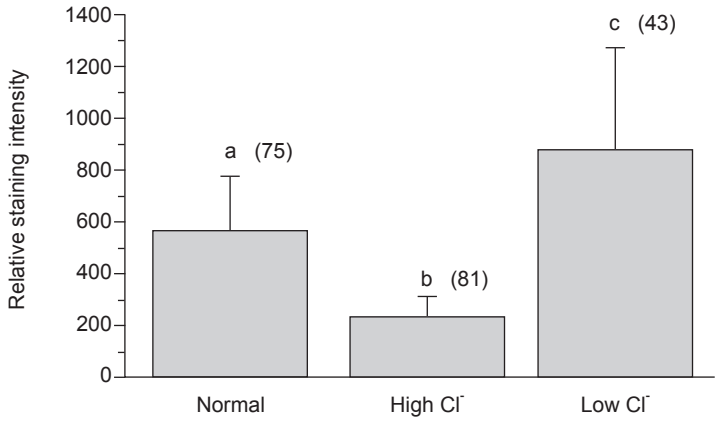

Fig. 4. Relative staining intensities of the Na/K/2Cl cotransporter (NKCC) in individual mitochondrion-rich (MR) cells from tilapia acclimated to high-Cl^-, normal, and low-Cl^- media. Values are the mean ± S.D., n = 81, 75, and 43, respectively. Different letters indicate a significant difference at the $p < 0.05$ level (one-way ANOVA followed by Tukey's pair-wise comparison).

demonstrated (Hiroi et al. 2008). Hence, it is also possible that the T4-labeled protein is the NCC instead of the NKCC. However, either the NKCC or NCC functions as a Cl^- transporter and supports the conclusion of Cl^- dose-dependent expression found in this study. If the apical NKCC or NCC is coupled to Na^+ and Cl^- transport, upregulation of NKCC expression would be expected in fish acclimated to Na^+-deficient medium. However, the NKCC was not induced by Na^+-deficient medium according to our observations (data not shown). We have an ongoing study to investigate the properties of the NKCC in tilapia using electrophysiological approaches.

Acknowledgments: We thank Dr. P.P. Hwang for assistance during the experiments and the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan for assistance with confocal microscopy. This study was financially supported by the National Science Council of Taiwan (NSC96-2313-B-003-001-MY3).

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