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Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*)

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Summary

Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR) are the three major transport proteins thought to be involved in chloride secretion in teleost fish. If this is the case, the levels of these transporters should be high in chloride cells of seawater-acclimated fish. We therefore examined the influence of salinity on immunolocalization of Na^+/K^+-ATPase, NKCC and CFTR in the gills of the Hawaiian goby (*Stenogobius hawaiiensis*). Fish were acclimated to freshwater and 20‰ and 30‰ seawater for 10 days. Na^+/K^+-ATPase and NKCC were localized specifically to chloride cells and stained throughout most of the cell except for the nucleus and the most apical region, indicating a basolateral/tubular distribution. All Na^+/K^+-ATPase-positive chloride cells were also positive for NKCC in all salinities. Salinity caused a slight increase in chloride cell number and size and a slight decrease in staining intensity for Na^+/K^+-ATPase and NKCC, but the basic pattern of localization was not altered. Gill Na^+/K^+-ATPase activity was also not affected by salinity. CFTR was localized to the apical surface of chloride cells, and only cells staining positive for Na^+/K^+-ATPase were CFTR-positive. CFTR-positive cells greatly increased in number (5-fold), area stained (53%) and intensity (29%) after seawater acclimation. In freshwater, CFTR immunoreactivity was light and occurred over a broad apical surface on chloride cells, whereas in seawater there was intense immunoreactivity around the apical pit (which was often punctate in appearance) and a light subapical staining. The results indicate that Na^+/K^+-ATPase, NKCC and CFTR are all present in chloride cells and support current models that all three are responsible for chloride secretion by chloride cells of teleost fish.

Key words: Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, NKCC, CFTR, teleost fish, chloride secretion, salinity, *Stenogobius hawaiiensis*, goby.

Introduction

Teleost fish have evolved an osmoregulatory strategy in which they maintain nearly constant internal osmotic concentration (approximately one-third seawater) irrespective of the external salinity. When in seawater, this necessitates secretion of excess sodium and chloride, which occurs through the action of specialized, mitochondrion-rich cells in the gills known as chloride cells (Keys and Willmer, 1932; Foskett and Scheffey, 1982). Silva et al. (1977) proposed a model for chloride cell function in which a basolaterally located Na^+/K^+-ATPase provides ionic and electrical gradients for a basolateral Na^+:Cl^- exchanger that brings chloride into the cell, which then leaves at the apical surface on its 'downhill' electrical gradient. Evidence gathered in the intervening years has largely supported this model, with some minor modification. Pharmacological evidence suggests that basolateral Na^+:Cl^- cotransport occurs through a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (NKCC; Degnan, 1984). Rather than being diffusive, chloride secretion at the apical surface may occur through an ion channel with characteristics similar to that of the cystic fibrosis transmembrane conductance regulator (CFTR; Marshall et al., 1995). Sodium is secreted by a paracellular pathway, probably involving Na^+/K^+-ATPase and junctions between chloride cells and adjacent accessory cells. Na^+/K^+-ATPase is found in high concentrations in chloride cells of many teleost fish and is regulated by environmental salinity

(McCormick, 1995). By contrast, characterization and immunolocalization of NKCC and CFTR have been examined in only a few teleost species. In Atlantic salmon (*Salmo salar*), NKCC immunoreactivity was found specifically in gill chloride cells and was strongly upregulated following seawater acclimation (Pelis et al., 2001). Two major isoforms of NKCC have been found in vertebrates: a secretory and an absorptive form (NKCC1 and NKCC2, respectively). Two isoforms of NKCC1 have been found in the European eel (*Anguilla anguilla*), and the transcript of one of these (NKCC1a) is found in large quantities in the gill and is upregulated after seawater exposure (Cutler and Cramb, 2002). Wilson et al. (2000) found NKCC in the basolateral membrane and tubular system of chloride cells, and CFTR in the apical region of chloride cells, of the seawater-acclimated mudskipper (*Periopthalmodon schlosseri*). CFTR is present in the apical region of chloride cells in seawater-acclimated killifish (*Fundulus heteroclitus*) but also in pavement cells of freshwater killifish (Marshall et al., 2002). CFTR has been sequenced in two teleosts to date (killifish and Atlantic salmon), and gill mRNA levels of CFTR are upregulated in both of these species following seawater exposure.

To date, no single study has examined the influence of salinity on all three of the major transporters thought to be involved in chloride secretion. Based on the current model of chloride secretion in fish, we hypothesize that chloride cells should contain high levels of Na^+/K^+-ATPase, NKCC and CFTR and that all three are potentially upregulated and their localization altered by exposure to increased salinity. In the present study, we have examined the influence of salinity on chloride cells and the immunolocalization of the Na^+/K^+-ATPase, NKCC and CFTR in the gills of the euryhaline Hawaiian goby (*Stenogobius hawaiiensis*).

Materials and methods

Sampling and rearing of fish

The Hawaiian goby (*Stenogobius hawaiiensis* Cuvier and Valenciennes) has an amphidromous life history in which spawning occurs in freshwater, and eggs or newly hatched larvae are swept out to sea. After undergoing larval and early juvenile development in the ocean, the fish return to freshwater. Juvenile gobies adapt readily to laboratory conditions and abrupt salinity changes and were therefore considered a good subject for this study. There is also little information on this order of teleost, which has a large number of euryhaline species. Fish were collected with a dip net from a small side channel of the Haleiwa River (Oahu, Hawaii) approximately 1.5 km from the river's mouth. The pool and river in this immediate area are wholly freshwater. Fish were transported to the Hawaii Institute of Marine Biology on Coconut Island and randomly placed in three 80 liter aquaria with freshwater (see Morgan et al., 1997 for details of the ionic composition). Each aquarium had charcoal filtration, aeration and several plastic tubes to provide cover. The room and aquaria were maintained at a constant temperature of 20±0.2°C under natural photoperiod conditions. Fish were fed a commercial diet (Biokyowa 1000; Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) twice per day. After two days of acclimation, the salinity in two of the tanks was raised in a stepwise, daily fashion as follows. For one tank, the daily increases were to 5‰, 10‰, 15‰ and 20‰, and the fish were thus termed the '20‰ group'. For the other tank, the daily increases were to 14.5‰, 19.5‰, 24.5‰ and 30‰, and the fish were thus termed the '30‰ group'. Salinities were achieved by removing water from the tank and adding an appropriate amount of filtered seawater (35‰, 20°C). Water in the freshwater tank was replaced at the same time as for the salinity groups. Fish were fed soon after the water changes and there was no obvious difference in feeding after salinity changes. Once the desired salinity was achieved, half the water in each tank was changed, using the appropriate salinity, every other day.

After 10 days at the acclimation salinity, fish were anesthetized with 200 mg l^{-1} MS-222, length and weight were recorded and the gill tissue was removed. Fish weighed between 0.4 g and 2.5 g, and mass did not differ significantly among groups. The branchial bone was trimmed away from two gill arches, and the primary filaments were placed in SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen on dry ice for analysis of Na^+/K^+-ATPase activity. Two gill arches were placed in 4% buffered formalin for two days and then this solution was replaced with 70% ethanol.

Antibodies

Rabbit polyclonal antisera directed against 17 amino acids from a highly conserved region of the α-subunit of salmon Na^+/K^+-ATPase (Ura et al., 1996) and diluted 1:500 was used for immunocytochemical detection of Na^+/K^+-ATPase. A mouse monoclonal antibody (T4) directed against the 310 amino acids at the carboxyl terminus of the human colonic $Na^+/K^+/2Cl^-$ cotransporter (NKCC1; obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA) was used at a concentration of 0.25 μg ml^{-1}. This antibody has been shown to be specifically immunoreactive with NKCC (both secretory and absorptive forms) from many vertebrates, including teleost fish (Lytle et al., 1992; Pelis et al., 2001). A mouse monoclonal antibody (24:1; R&D Systems, Boston, MA, USA) against 104 amino acids at the carboxyl terminus of the human CFTR was used at 0.4 μg ml^{-1}. The carboxyl terminus of CFTR is highly conserved among vertebrates, and this antibody has previously been shown to be specifically immunoreactive with CFTR from several vertebrates, including teleost fish. Alexa-Fluor 488 goat anti-mouse and Alexa Fluor 546 goat anti-rabbit (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Antibody control experiments (primary antibodies without secondary

antibody, and secondary antibody without primary antibody) showed no specific staining and low background. Double staining of the same sections was performed for Na^+/K^+-ATPase and NKCC, and Na^+/K^+-ATPase and CFTR.

Immunocytochemistry

Fixed gill tissue was rinsed in 10 mmol l^{-1} phosphate-buffered saline (PBS), placed in PBS with 30% (w/v) sucrose for one hour and then frozen in embedding medium. 7 μm sections were cut in a cryostat at –24°C, parallel to the long axis of primary filaments and perpendicular to the attachment of secondary lamellae. The tissue was placed on poly-L-lysine-coated slides, dried, rinsed with PBS and then incubated in 2% normal goat serum in PBS for 0.5 h at room temperature. Slides were exposed to primary antibody in antibody dilution buffer (0.01% NaN_3, 0.1% bovine serum albumin, 2% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS) and incubated overnight at 4°C. After incubation, the slides were rinsed several times with PBS, exposed to secondary antibody at room temperature for 2 h and then rinsed several times with PBS. The tissue was covered by a cover slip and examined with a Nikon inverted fluorescent microscope with a mercury lamp. Images were taken within 4 h of completion of staining for subsequent counting and morphometric analysis.

From each fish, immunoreactive chloride cells on the primary filament and secondary lamellae (tallied separately) were counted from sagittal sections of gill filament (300 μm of primary filament/sagittal section) and expressed per millimeter of primary filament. As in most teleost fish, there was an increasing number of chloride cells from leading to trailing edge, so only sections in the middle of the filament were used to quantify cell number. Mean numbers of chloride cells for each group were obtained using the means calculated from each fish. Cell or staining area (μm^2 $cell^{-1}$), staining intensity (mean gray scale/pixel) and shape factor were also obtained from immunoreactive chloride cells using MetaMorph 4.1.2 (Universal Imaging Corporation, West Chester, PA, USA). A single threshold level for each image and antibody was used to quantify immunoreactive regions. Background staining intensity was obtained by averaging intensity in at least two noncellular regions of each image and subtracting that from each staining intensity value obtained from that image. In order to determine whether the shape of chloride cells and their immunoreactive regions was affected by salinity, shape factor was measured. Shape factor is defined as $4\times A/p^2$ (where A and p are the area and perimeter of the immunopositive regions, respectively), with values close to 1 indicating a circular shape and 0 an elongate shape. Since the NKCC and Na/K-ATPase were measuring the same region of the cell, only the shape factor for Na/K-ATPase is presented. At least 50 immunoreactive chloride cells from several different tissue sections were analyzed from at least five fish from each salinity; more than 1000 chloride cells were examined overall.

Measurement of gill Na^+/K^+-ATPase

Within one month of sampling, gill Na^+/K^+-ATPase activity was measured according to the microassay protocol of McCormick (1993). Gill filaments were homogenized in SEI buffer containing 0.1% sodium deoxycholate. Following centrifugation (3000 ***g*** for 0.5 min) to remove large debris, Na^+/K^+-ATPase activity was determined by linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), measured at 340 nm for 10 min at 25°C in the presence or absence of 0.5 mmol l^{-1} ouabain. Protein content in the gill homogenate was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Specific activities were expressed as μmol ADP mg^{-1} protein h^{-1}.

Statistics

One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post-hoc* test was used to test for the effect of salinity on cell number and gill Na^+/K^+-ATPase activity. As multiple chloride cells were measured from each individual, measures of cell area, brightness and shape factor were analyzed with ANOVA with 'individual' nested

Table 1. *Shape factor of Na^+/K^+-ATPase and CFTR-immunoreactive areas of chloride cells, staining intensity of NKCC in chloride cells, and whole gill Na^+/K^+-ATPase activity in Hawaiian goby acclimated to freshwater, 20‰ and 30‰ seawater*

		Freshwater	20‰ Salinity	30‰ Salinity
Na^+/K^+-ATPase shape factor	Primary	0.68±0.01	0.68±0.01	0.67±0.01
	Secondary	0.61±0.01[a]	0.52±0.01[b]	0.55±0.02[b]
CFTR shape factor	Primary	0.95±0.02	0.97±0.01	0.97±0.01
	Secondary	0.94±0.02	0.96±0.01	0.95±0.01
NKCC staining intensity	Primary	98.4±1.6	94.4±1.3	98.2±1.3
	Secondary	85.9±1.7[a]	91.1±1.7[b]	95.2±1.7[c]
Gill Na^+/K^+-ATPase activity		7.6±0.5	8.9±0.5	9.4±0.6

At least 100 cells per individual and five individuals in each treatment group were measured. Gill Na^+/K^+-ATPase activity was measured in six individuals from each group. Values are expressed as means ± S.E.M.

NKCC, $Na^+/K^+/2Cl^-$ cotransporter; CFTR, cystic fibrosis transmembrane conductance regulator.

Different superscript letters indicate that the means are significantly different from one another (P<0.05, Student–Newman–Keuls test).

(contained) within 'salinity'. $P<0.05$ was used to reject the null hypothesis.

Results

There were no mortalities during the study. Fish in all groups ate at each feeding, and there were no observable differences in feeding or other behaviors as a function of salinity exposure. There were no significant differences in length or mass among the treatment groups. Gill Na^+/K^+-ATPase activity rose slightly with increasing salinity, but the means were not significantly different (Table 1).

Large columnar cells on the primary filament and somewhat smaller cells on the secondary lamellae were positively stained for Na^+/K^+-ATPase (Fig. 1). Their size, shape and location indicated that these were mitochondrion-rich chloride cells. No other cell types in the gill were stained above background levels, and antibody controls (lacking primary antibody) showed only background staining. Na^+/K^+-ATPase immunoreactivity was detectable throughout the chloride cell except for the nucleus and the most apical region of the cell (Figs 1, 2), consistent with a basolateral/tubular distribution. This pattern of staining was not altered by salinity. Fish in all salinities had Na^+/K^+-ATPase-positive chloride cells on both the primary filament and secondary lamellae, with most of the cells located on the primary filament (Figs 1, 3). The number of Na^+/K^+-ATPase immunoreactive chloride cells on the primary filament increased with increasing salinity and was 42% greater in 30‰ seawater than in freshwater. Chloride cells on the primary filament were larger than those on the secondary lamellae, and their size increased slightly but significantly in response to increased salinity (13–27%; Fig. 3). Chloride cells on the secondary lamellae were more flattened (less round with a lower shape factor; Table 1) than those on the primary filament. Shape of chloride cells on the primary filament was not altered by salinity, whereas chloride cells on the secondary lamellae were less round (lower shape factor) in 20‰ and 30‰ than in freshwater. The brightness of Na^+/K^+-ATPase immunoreactivity was similar in chloride cells in the primary filament and secondary lamellae and decreased slightly (7.0–9.4%) in response to salinity.

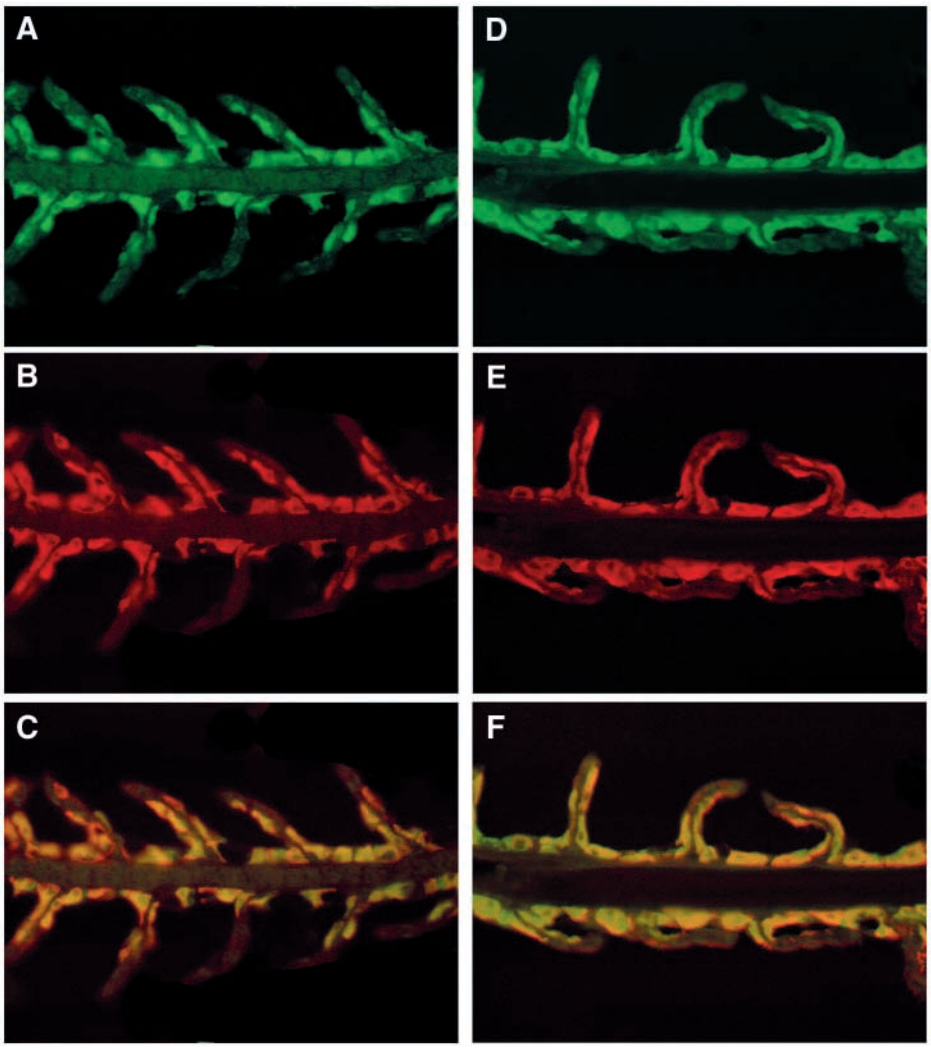

Fig. 1. Na^+/K^+-ATPase (green) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC; red) immunoreactivity in gills of Hawaiian goby acclimated to freshwater (A–C) and 30‰ seawater (D–F). Double-staining was performed on the same sections, and the images combined in C and F. Images C and F were merged (from A,B and D,E, respectively) to highlight the colocalization of Na^+/K^+-ATPase and NKCC1 (red + green is yellow-orange). The width of each image is 207 μm.

The pattern of immunoreactivity for NKCC was nearly identical to that of Na^+/K^+-ATPase (Fig. 1). All cells that stained for Na^+/K^+-ATPase also stained for NKCC, and the distribution of NKCC staining within the chloride cells (staining throughout the cell except for the nucleus and most apical region) was the same as for Na^+/K^+-ATPase immunoreactivity. NKCC staining intensity was slightly higher in chloride cells on the primary filament compared with the secondary lamellae (Table 1). NKCC staining was not significantly affected by salinity in chloride cells on the primary filament but increased with increasing salinity in chloride cells on the secondary lamellae.

The pattern of CFTR staining differed substantially from that of Na^+/K^+-ATPase and NKCC. CFTR staining was only present in cells that were Na^+/K^+-ATPase positive (i.e. chloride cells; Figs 2, 4). The distribution of staining was apical and slightly subapical with a relatively

small area of overlap with Na^+/K^+-ATPase immunoreactivity. In freshwater, CFTR immunoreactivity was not very bright and spread over a broad apical surface. In 20‰ and 30‰ salinity, CFTR was more often in a narrower apical region of the cell and extended deeper into the cell. In seawater, the brightest staining was usually in the most apical region of the chloride cell with a less bright subapical region (Fig. 2). In many chloride cells of seawater-exposed fish, there was a clear discontinuous, punctate distribution of CFTR at the apical surface (Fig. 2), although this distribution was rarely seen in cells of freshwater fish. In some chloride cells of seawater-exposed fish, there was a central area of the apical region that was not stained, indicating that the apical crypt had been cross-sectioned in these cells and that staining within the non-cellular portion of the apical crypt was absent.

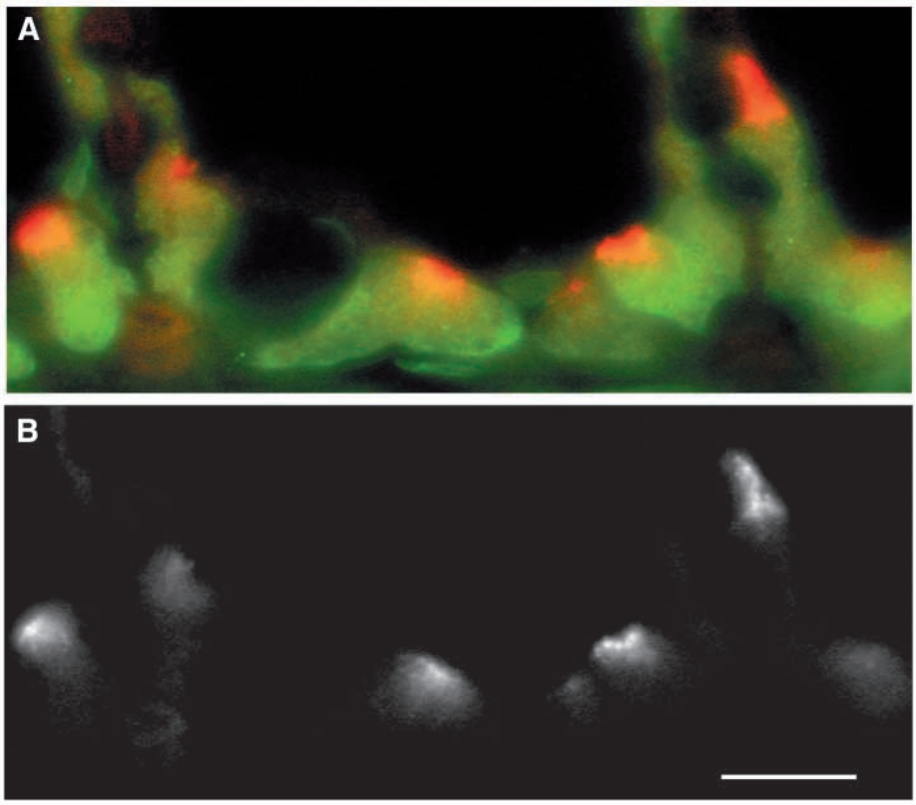

Fig. 2. Na^+/K^+-ATPase (green) and cystic fibrosis transmembrane conductance regulator (CFTR; red) immunoreactivity in gills of Hawaiian goby acclimated to seawater (A). Image B contains only CFTR staining (white). Scale bar, 12 μm.

The number of CFTR-positive cells was much greater in the 20‰ and the 30‰ groups than in the freshwater group (4- and 5-fold more, respectively; Fig. 3). The relative proportions of chloride cells with CFTR-positive staining were 19%, 62% and 67% in freshwater, 20‰ and 30‰, respectively. The area of the chloride cells staining positively for CFTR was about one-eighth of that stained by Na^+/K^+-ATPase (6–10 μm^2 *versus* 50–80 μm^2). In chloride cells on the primary filament, the CFTR positive area was 62% and 53% greater in fish in 20‰ and 30‰ seawater, respectively, relative to gobies in freshwater. The mean CFTR staining intensity also increased significantly after seawater exposure in chloride cells on both the primary filament and the secondary lamellae.

Discussion

In the gill of the Hawaiian goby, only chloride cells stained positively for Na^+/K^+-ATPase, NKCC and CFTR, whereas other cell types in the gill had only background staining. Na^+/K^+-ATPase and NKCC had a basolateral/tubular distribution, whereas CFTR immunoreactivity was present only in the apical region. In addition, increased salinity resulted in an increase in the number of chloride cells staining positively for all three transporters, but this effect was by far the most pronounced for CFTR immunoreactivity. These major findings support the current model of chloride secretion by chloride cells of teleost fish, which suggests the presence of a basolateral Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter coupled with an apical chloride channel. Our results strongly suggest that the apical chloride channel is a CFTR-like protein and that this protein is upregulated with increasing salinity.

The intracellular distribution of both Na^+/K^+-ATPase and NKCC immunoreactivity in Hawaiian goby gills was identical, staining throughout the cell except for the nucleus and the most apical region of the chloride cell. Previous studies using electron microscopy have demonstrated that Na^+/K^+-ATPase is present on both the basolateral membrane and the extensive tubular system that extends through most of the cell but that is not present in the most apical region of the cell (Karnaky et al., 1976; Wilson et al., 2000). Thus, the presence of high levels of both Na^+/K^+-ATPase and NKCC found within chloride cells in the present study is likely to represent a basolateral/tubular distribution. Marshall et al. (2002) recently found that NKCC immunoreactivity occurs throughout the chloride cell in seawater-acclimated killifish but has a more restricted and asymmetric basolateral distribution in freshwater-acclimated killifish. As in the present study, Pelis et al. (2001) found that NKCC immunoreactivity occurred throughout the chloride cell in both freshwater- and seawater-acclimated Atlantic salmon and that the number of cells staining and the amount of NKCC present in the gill was lower in freshwater. For the few species examined to date, seawater teleosts seem to have a consistent basolateral/tubular distribution and high levels of NKCC and Na^+/K^+-ATPase present in chloride cells.

CFTR was present at detectable levels only in chloride cells

and had a clear and consistent apical distribution. In freshwater, this distribution was spread over a relatively broad area of the apical surface. Previous studies have shown that chloride cells in most freshwater teleosts have a broad apical surface (Perry, 1997), and the observed CFTR immunoreactivity in freshwater may reflect the presence of CFTR over just such a broad apical membrane. In seawater, the orientation of CFTR immunoreactivity was more often perpendicular to the main axis of the cell, indicative of staining along a deep apical pit that is characteristic of seawater chloride cells. There was also a more intense, often punctate staining in the most apical region, with a less intense subapical region. The punctate distribution of CFTR may be a function of the structure of the apical pit. The apical pit is formed primarily by the chloride cell but is interdigitated by the neighboring accessory cell (Shiraishi et al., 1997). This results in a discontinuity of the chloride cell at the apical surface, and the presence of CFTR only in chloride cells would explain the punctate distribution that we observed.

Not all Na^+/K^+-ATPase-positive cells had positive CFTR staining, and this may have been due, in part, to the greater area of the Na^+/K^+-ATPase-rich cell body relative to the more narrow apical region, making it inherently less likely that the apical region would be sectioned. However, the large number

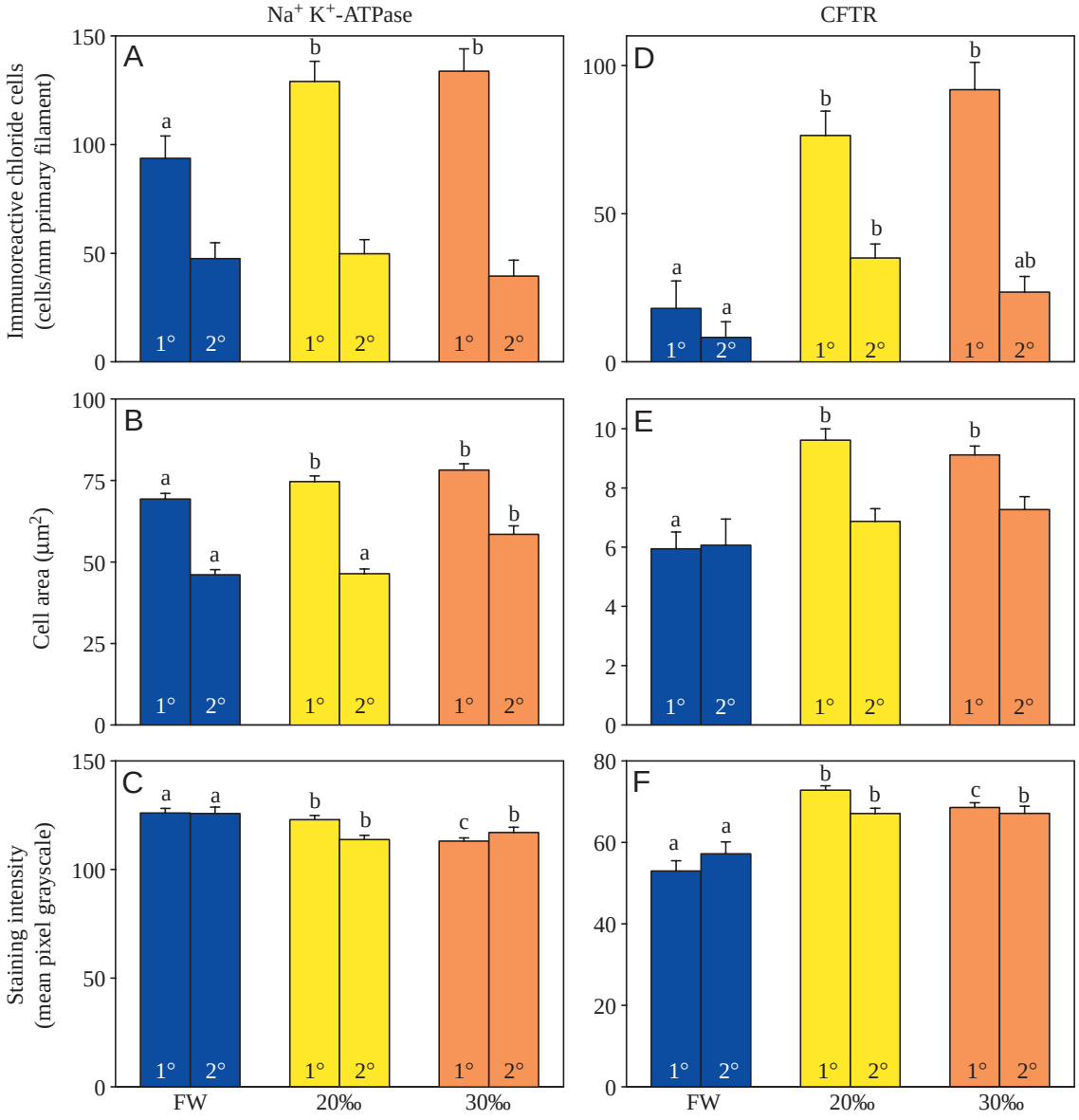

Fig. 3. Quantification of Na^+/K^+-ATPase-immunoreactive (left panels) and cystic fibrosis transmembrane conductance regulator (CFTR; right panels)-immunoreactive chloride cell number, size and staining intensity. Chloride cells on the primary filament (1°) and secondary lamellae (2°) were counted and analyzed separately. Groups with different letters were significantly different from one another (P<0.05, Student–Newman–Keuls test). Values are expressed as means ± S.E.M. At least 50 cells per individual and five individuals in each treatment group were measured.

of cells sampled in this study makes it very unlikely that the treatment differences observed were the result of this limitation caused by sectioning. It seems likely that the observed differences in the relative number of CFTR-positive chloride cells relates directly to the greater chloride secretory activity occurring in 20‰ and 30‰ salinity. The lower number of chloride cells with apical staining for CFTR in freshwater may also relate to limits of detectability; there may in fact be low staining present in the apical region of freshwater chloride cells that was not detected using the present methods. It is nonetheless clear that the intensity of staining for CFTR and the size of the CFTR-positive apical region were greatly increased by exposure to seawater. During seawater acclimation of killifish, Marshall et al. (2002) observed a 'redistribution' of CFTR from the subapical to the apical region of chloride cells. In the rectal gland of spiny dogfish (*Squalus acanthias*), peak CFTR immunofluorescence moved from the subapical to the apical region in response to vasoactive intestinal peptide, which also caused increased chloride secretion (Lehrich et al., 1998). Ernst et al. (1994) found more CFTR immunoreactivity in the apical region of the salt gland of ducks (*Anas platyrhynchos*) after salt loading. These results suggest that an increased amount or redistribution of CFTR to the apical surface is associated with increased chloride secretory activity and is consistent with the present results. Because the size, shape and intensity of staining all differed among the freshwater- and seawater-acclimated groups, it was not possible to determine whether a 'redistribution' of CFTR had occurred or whether the increase in CFTR immunofluorescence was simply due to the presence of more CFTR. More detailed studies will be necessary to examine the intracellular distribution and *de novo* synthesis of CFTR in this and other teleosts during seawater acclimation.

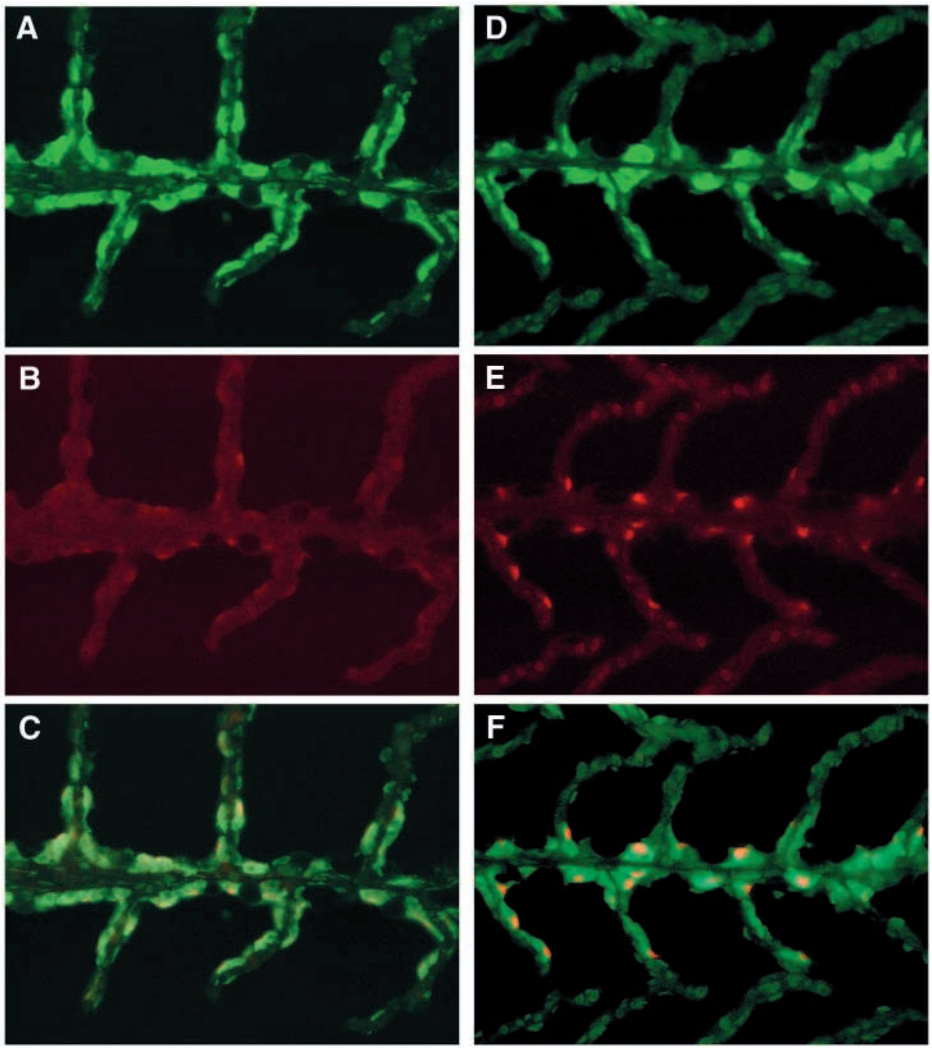

Fig. 4. Na^+/K^+-ATPase (green) and cystic fibrosis transmembrane conductance regulator (CFTR; red) immunoreactivity in gills of Hawaiian goby acclimated to freshwater (A–C) and 30‰ seawater (D–F). Double-staining was performed on the same sections, and the images combined in C and F. Images C and F were overlaid (from A,B and D,E, respectively) to highlight the colocalization of Na^+/K^+-ATPase and CFTR. The width of each image is 207 μm.

Increased salinity resulted in many more chloride cells showing positive CFTR staining, and the area and intensity of staining also increased. Based on a 5-fold increase in cell number, a 53% increase in cell area (which roughly translates to a doubling of the cell volume) and a 29% increase in staining intensity, we can roughly calculate that a 13-fold increase in CFTR occurred in the primary gill filament after seawater acclimation. The calculated increase in CFTR on the secondary lamellae was 4.4-fold, and combining the two yields an overall estimated 10-fold increase in CFTR for the entire gill. This is likely to be a minimal estimate for change, as the staining intensity is only semiquantitative and could substantially underestimate the change in amount of CFTR. It is clear from these results, however, that CFTR is upregulated during seawater acclimation, and this provides strong circumstantial evidence that CFTR is involved in chloride secretion carried out by chloride cells. Gill CFTR mRNA levels increase within 8 h of seawater exposure in killifish (Marshall et al., 1999). Two forms of CFTR mRNA have been found in Atlantic salmon, both of which increase after seawater exposure; CFTRII increases transiently, and CFTRI shows a more sustained increase (Singer et al., 2002). It will be of interest to examine the time course of changes in response to salinity to determine how quickly changes in CFTR message and protein

can occur in the Hawaiian goby and other teleosts and how it relates to temporal changes in gill morphology and ion fluxes.

Based on differences in the number, size and staining intensity of Na^+/K^+-ATPase immunoreactivity in freshwater- and seawater-acclimated fish (similar to the calculations above for CFTR), we can calculate that there was a 46% increase in the amount of Na^+/K^+-ATPase in gill tissue following seawater acclimation. Gill Na^+/K^+-ATPase activity of Hawaiian gobies increased by 24% following seawater acclimation, although there was no statistically significant difference between freshwater fish and those exposed to 30‰ salinity. We consider the measurement of activity to be more quantitative than this rough approximation based on immunoreactivity, but the two values are nonetheless similar. In many teleost species, gill Na^+/K^+-ATPase increases several fold following seawater acclimation (McCormick, 1995). This is not universally true, however, and there are many species in which Na^+/K^+-ATPase does not change or is lower following seawater acclimation, often in species of marine ancestry. The relatively moderate change in number of chloride cells and the lack of significant change in gill Na^+/K^+-ATPase activity in response to seawater is consistent with the marine ancestry of amphidromous gobies in which occurrence in freshwater is considered to be a derived trait (Chubb et al., 1998).

The $Na^+/K^+/2Cl^-$ cotransporter occurs in two major isoforms: a secretory isoform (NKCC1) and an absorptive isoform (NKCC2). The antibody used in the present study recognizes both of these isoforms in a wide variety of vertebrates. It is likely that the NKCC immunoreactivity in chloride cells of the Hawaiian goby is the secretory isoform. In most tissues, the secretory form has been found only on the basolateral membrane and tubular systems of epithelial cells, whereas the absorptive form is found only on the apical membrane. The only exception to this general rule is the choroid plexus, where both NKCC1 and Na^+/K^+-ATPase are found on the apical membrane (Haas and Forbush, 2000). The present finding of NKCC immunoreactivity throughout the chloride cells indicates a basolateral/tubular distribution and, by analogy, suggests that this is the secretory isoform. This is also suggested by the known bumetanide sensitivity of chloride secretion carried out by the chloride cell (Degnan et al., 1977). The greater number of NKCC-immunoreactive cells in seawater suggests that this transporter has increased in quantity following seawater acclimation, similar to results with other teleosts (Flik et al., 1997; Pelis et al., 2001).

Although Na^+/K^+-ATPase, NKCC and CFTR were all present in the gill chloride cells of seawater-acclimated Hawaiian goby at high concentration, there were also detectable levels of all three transporters, especially Na^+/K^+-ATPase and NKCC, in freshwater chloride cells. There are two likely explanations for this that are not mutually exclusive. First, these elevated levels in freshwater may be present to allow for the euryhalinity that is apparently characteristic of this species. By maintaining relatively high levels of Na^+/K^+-ATPase, NKCC and, to a lesser extent, CFTR, the animal has a greater capacity for moving into seawater at any time than it otherwise would; i.e. the existing transporters could be immediately activated rather than requiring *de novo* synthesis. Other teleosts that make a limited number of seasonal migrations into seawater over their lifetime, such as anadromous salmonids, can time the appearance of these transport proteins to coincide with seawater entry (see Pelis et al., 2001). Further studies are required to determine how the euryhalinity of this species changes during ontogeny and whether this is accompanied by changes in these ion transporters. Another explanation is that these transporters are involved in ion uptake. This seems quite likely in the case of Na^+/K^+-ATPase, which is involved in moving Na^+ from the interior of the chloride cell into the blood, as well as providing ionic and electrical gradients used by other transporters involved in ion uptake (Marshall, 2002). To date, there is no direct evidence that NKCC is involved in ion uptake by the fish gill. Wilson et al. (2000) hypothesized that Na^+/K^+-ATPase and/or NKCC may be involved in ammonia excretion by the mudskipper gill through substitution of NH_4^+ for K^+.

Exposure to low ion concentrations (i.e. deionized freshwater) results in a proliferation of cells on the secondary lamellae in several teleosts (Avella et al., 1987; Perry, 1997). In some species, such as American shad (*Alosa sapidissima*), the number of chloride cells on the secondary lamellae is high in freshwater fish but greatly reduced in seawater fish (Zydlewski and McCormick, 2001). Pisam et al. (1987) has described two morphologically distinct chloride cells, with the alpha-chloride cell more often in association with the circulation of the secondary lamellae. These results have led to speculation that chloride cells on the secondary lamellae are involved in ion uptake, whereas those on the primary filament are involved in salt secretion. In the Hawaiian goby, there were still a large number of chloride cells on the secondary lamellae after seawater exposure, and they had similar immunoreactivity of Na^+/K^+-ATPase, NKCC and CFTR to that of chloride cells on the primary filaments. Other than the more flattened appearance of chloride cells on the secondary lamellae, their morphological appearance and localization of transporters did not differ. Our results therefore do not provide evidence for differential function of chloride cells on the primary and secondary lamellae in the Hawaiian goby. It thus appears that among teleosts there are species-specific differences in chloride cell morphology and localization following changes in environmental ion concentrations. For instance, in the striped bass (*Morone saxatilis*), there are virtually no chloride cells on the secondary lamellae in either freshwater or seawater (Madsen et al., 1994). Varsamos et al. (2002) have recently found that the number of chloride cells on the secondary lamellae is higher in both freshwater and concentrated seawater (70‰) compared with 35‰ seawater. Findings such as these suggest that chloride cells on the secondary lamellae may relate more to increased demand for salt regulation (either uptake or secretion) than just ion uptake. Our results indicate that Na^+/K^+-ATPase, NKCC and CFTR are found specifically in chloride cells of the Hawaiian goby. Na^+/K^+-ATPase and NKCC have a basolateral/tubular

distribution, whereas CFTR is present in the apical pit region, often in a discontinuous, punctate arrangement. The number of Na^+/K^+-ATPase- and NKCC-immunoreactive chloride cells and their size increase slightly after seawater adaptation. The number of cells with CFTR-immunoreactive apical regions increases dramatically after seawater exposure, as does the area and brightness of CFTR immunoreactivity. These results verify previously proposed models for the function and localization of these transport proteins in chloride cells of teleost fish and indicate that they are all involved in chloride secretion by gill chloride cells.

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