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Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water

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Summary

Cellular distribution of cystic fibrosis transmembrane conductance regulator (CFTR) immunofluorescence was detected by monoclonal antibody directed to the C terminus of killifish CFTR (kfCFTR) in chloride cells of fresh water (FW) adapted fish and animals transferred to sea water (SW) for 24 h, 48 h and 14+ days. Confocal microscopy allowed localization within mitochondria-rich (MR) cells to be determined as superficial (i.e. in the apical membrane) or deeper within the cytoplasm of the cells. In FW, 90 % of MR cells had diffuse kfCFTR immunofluorescence in the central part of the cytosol, with only 8.1 % having apical kfCFTR, which was 6.6±0.54 μm below the microridges of surrounding pavement cells. Curiously, FW but not SW pavement cells also had positive immunofluorescence to kfCFTR. After 24 h in SW, a time when kfCFTR expression is elevated, a condensed punctate immunofluorescence appeared among 18.8 % of MR cells, 13.4±0.66 μm (mean ± S.E.M.) below the surface of the cells. By 48 h, a majority (76.3 %) of MR cells had punctate kfCFTR distribution and the distance from the surface was less (7.8±0.2 μm), a distribution approaching the SW-acclimated condition (i.e. all MR cells showing kfCFTR immunofluorescence, 6.1±0.04 μm below the surface). In contrast, NKCC immunofluorescence was condensed and localized in lateral parts of MR cell complexes in FW animals and then redistributed to the whole basal cytoplasm after acclimation to SW. CFTR, the anion channel responsible for Cl^- secretion in marine teleosts, redistributes in MR cells during SW acclimation by condensation of a diffuse distribution below the apical crypt, followed by translocation and insertion in the apical membrane. NKCC, the cotransporter that translocates Cl^- across the basolateral membrane, moves from an eccentric cytosolic location in FW to a diffuse basolateral localization in SW chloride cells.

Key words: epithelial ion transport, cystic fibrosis transmembrane conductance regulator, protein trafficking, Na^+,K^+,2Cl^- cotransporter, gills, osmoregulation, euryhaline teleost, killifish, *Fundulus heteroclitus*.

Introduction

Mitochondria-rich chloride (MR) cells of the skin and gill epithelia of teleost fish are responsible for ion uptake in fresh water (FW) teleosts fish (for a review, see Perry, 1997) and ion secretion in sea water (SW) teleosts (for a review, see Marshall and Bryson, 1998). Chloride secretion in skin and gill epithelia of SW-adapted teleosts is localized to the apical crypts of exposed MR cells (Foskett and Scheffey, 1982). The transition of these cells during salinity adaptation is a model for the plasticity of ion-transporting epithelial cells, allowing examination of *de novo* generation of transporters and their insertion in polarized membrane areas, and redistribution of transporters in existing cells, as well as growth and differentiation of new ionocytes. The strongly euryhaline killifish *Fundulus heteroclitus* is a model animal for study of osmoregulation and ion balance in teleost fish and much is known about ion regulation by MR cells of their the gill and opercular epithelium (Degnan et al., 1977; Karnaky, 1998; Wood and Marshall, 1994; Marshall and Bryson, 1998; Singer et al., 1998). Killifish can live in dilute FW and at salinities up to 12 g l^{-1} (3.75× SW) (Griffith, 1974) and can rapidly adapt to large changes in salinity, including direct transfers from FW to SW and *vice versa* (e.g. Marshall et al., 1997, 1999). The CFTR anion channel has been identified electrophysiologically in the apical membranes of MR cells of the euryhaline teleost killifish (Marshall et al., 1995). This channel has been cloned and sequenced from killifish (Singer et al., 1998) and is expressed at elevated rates in the gill during adaptation of killifish to SW (Singer et al., 1998; Marshall et al., 1999). starting at approximately 8 h and reaching a maximum at approximately 24 h after transfer to SW. CFTR has been detected immunologically in SW in MR cells of a euryhaline teleost, the mudskipper *Periophthalmodon schlosseri* and is

present in the apical crypt, a cup-shaped depression in the apical portion of these ion-transporting cells (Wilson et al., 2000a). In preliminary work with killifish, antibodies to CFTR colocalize with HSP90 (a heat-shock protein that expresses in mitochondria) in cells of the gill filaments normally occupied by MR cells (Mickle et al., 2000).

Salt secretion by MR cells involves furosemide- and bumetanide-sensitive Na^+,K^+,2Cl^- cotransport at the basolateral membrane (for reviews, see Karnaky, 1998; Marshall and Bryson, 1998), a function mediated by the protein NKCC (Haas and Forbush, 1998). Na^+,K^+,2Cl^- cotransport is present in basolateral membrane vesicles from gills of FW-adapted rainbow trout *Oncorhynchus mykiss* and at higher levels in 70 % SW-adapted animals (Flik et al., 1997). The cotransport is inhibited by furosemide and bumetanide and shows appropriate kinetics with apparent control by the transmembrane K^+ activity gradient (Flik et al., 1997). NKCC cotransporter is immunocytochemically localized to the basolateral membranes of MR cells of euryhaline fish in SW (Wilson et al., 2000a) and SW salmon smolts (Pelis et al., 2001). NKCC is essential to NaCl secretion by SW animals but the gene has not yet been cloned from teleost fish. NKCC distribution in MR cells of FW-adapted Atlantic salmon parr (*Salmo salar*) colocalizes with Na^+K^+ATPase (Pelis et al., 2001), suggesting a basolateral membrane location. The function of NKCC in FW teleost fish gills remains a matter of speculation (Flik et al., 1997).

Ion transporters are known to be trafficked from staging areas in Golgi apparatus to the apical or basolateral membranes of epithelial cells. CFTR is thought to be directed to the apical membrane of cells *via* syntaxin, which binds to the carboxy terminus of human CFTR (Lehrich et al., 1998; Moyer et al., 1998; Naren et al., 2000; reviewed by Kleizen et al., 2000). Killifish CFTR has the same carboxy-terminal sequence (–dtrl) as human CFTR and thus is identifiable using a monoclonal antibody directed against this epitope; CFTR could be similarly trafficked in teleost MR cells. The aim of this study was to determine if kfCFTR distribution in MR cells was altered during adaptation to SW when Cl^- secretion rate is rising and expression of kfCFTR is high (at 24 and 48 h; Marshall et al., 1999), with the hypothesis that kfCFTR should appear in the apical crypt during this period. If kfCFTR were already in the apical crypt, it would suggest activation of kfCFTR already *in situ* in the membrane. We also predicted a redistribution during acclimation to SW of NKCC to the basolateral membrane in SW MR cells from another location.

Materials and methods

Animals

Adult killifish (mummichogs, *Fundulus heteroclitus* L.) of both genders were captured in Antigonish estuary, transferred to indoor holding facilities and adapted to brackish water (salinity 3 g l^{-1}) for at least 10 days at 20–25 °C and ambient photoperiod under artificial light. Fish were transferred to full-strength SW (salinity 32 g l^{-1}) or dechlorinated FW and acclimated for at least 2 weeks before experimentation. Fish were fed marine fish food blend (Nutrafin flakes and tubifex worms; R. C. Hagen, Montreal, Canada) at a rate of 1.0 g 100 g^{-1} body mass day^{-1}, supplemented twice weekly with frozen brine shrimp. Salinity transfers were performed as described previously (Marshall et al., 1998), where animals were adapted to FW for at least 7 days, then pairs (solitary animals are stressed by isolation) were moved to FW aquaria for a further 7 days to adapt for transfer to holding conditions. Salinity was changed by flow through of full-strength SW (32 g l^{-1}) without altering the water level or water temperature and with a minimum of disturbance to the animals. Transfer was complete in 10 min. 1 and 2 days later animals were netted and killed by decapitation; the opercular membranes were removed, pinned out flat on dental wax and bathed in a modified Cortland's saline (305 mOsm kg^{-1}, pH 7.8). Modified Cortland's saline was used to bathe both membrane surfaces symmetrically; the composition was (in mmol l^{-1}): NaCl 160, KCl 2.55, $CaCl_2$ 1.56, $MgSO_4$ 0.93, $NaHCO_3$ 17.85, NaH_2PO_4 2.97 and glucose 5.55, pH 7.8, when equilibrated with a 99 % O_2/1 % CO_2 gas mixture.

Immunocytochemistry

The primary antibody used to detect kfCFTR was mouse monoclonal anti-hCFTR (R&D Systems, Minneapolis, MN, USA) with the known epitope of (–dtrl), the carboxy terminus of hCFTR. Killifish CFTR has the same carboxy terminus (Singer et al., 1998), thus is selective for this protein. The primary antibody for detection of the Na^+,K^+,2Cl^- cotransporter (NKCC) was T4 (Lytle et al., 1992), an antibody to the carboxy region of NKCC that has been shown to bind to several isoforms of NKCC across several species (Haas and Forbush, 1998; Wilson et al., 2000a). The secondary antibody was goat polyclonal anti-mouse IgG conjugated to an Oregon Green 488 fluorophore (Molecular Probes, Eugene, OR, USA), chosen because of its stability and reliability. Opercular epithelia were dissected without the dermal chromatophore layer and pinned to modeler's wax. They were incubated in 100 nmol l^{-1} (final concentration) Mitotracker Red (Molecular Probes, Eugene, OR, USA) in phosphate-buffered saline (PBS, composition in mmol l^{-1}: NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.4) for 2 h at room temperature in the dark. Preparations were then rinsed three times in rinsing buffer comprising 0.1 % bovine serum albumin (BSA), 0.05 % Tween 20 in PBS (TPBS). The membranes were then fixed for 3 h at –20 °C in a formaldehyde-free, 80 % methanol/20 % dimethyl sulfoxide (DMSO) fixative. The methanol was used as a dehydrating agent and the DMSO as a cryoprotective agent. After three rinses the membranes were blocked with 5 % normal goat serum (NGS), 0.1 % BSA, 0.05 % TPBS, pH 7.4, for 30 min at room temperature in the dark and incubated in the primary antibody (8 μg ml^{-1} in PBS+0.5 % BSA) overnight at 4 °C. They were then rinsed three times and exposed to the secondary antibody (diluted 1:50 in PBS+0.5 % BSA) for 5 h at 4 °C. After three final rinses the membranes were mounted

in mounting medium (Geltol; Immunon Thermo Shandon, Pittsburgh, PA, USA). Slides were viewed in single blind fashion and images collected with a laser confocal microscope (Olympus FV300). In each opercular membrane, randomly selected *Z*-stack series were collected at 40×, zoom 3.0, with optical sections of 1.5±0.1 μm. The surface was identified by the microridge pattern of pavement cells and depths were measured by reference to this surface. Punctate fluorescence in MR cells was positioned for at least ten cells from at least three *Z*-stacks for each membrane, with membranes coming from at least four animals for each salinity treatment group (FW control, N=9; FW to SW for 24 h, N= 4; FW to SW for 48 h, N=6; SW, N=5).

Western immunoblots

Opercular epithelium (dissected from opercular bone), heart (dissected ventricle without conus arteriosus) and gill filaments (scraped from the arch with a razor blade) were homogenized in ice-cold SEI buffer (300 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 100 mmol l^{-1} imidazole, pH 7.4) using a homogenizer. Three SW-adapted animals were used and three separate runs performed. Homogenates were centrifuged at 2000 ***g*** for 6 min. The pellet was resuspended in 2.4 mmol l^{-1} deoxycholate in SEI buffer and centrifuged a second time at 2000 ***g***. The total protein content of the resulting supernatant was determined using the Bradford method (Bradford, 1976). Proteins were separated on a 7% polyacrylamide gel using a Mini-Protean 3 Cell system (Bio-Rad, Mississauga, ON, Canada). A total of 20 μg of protein for the opercular epithelium, heart and gill was loaded and run for 30 min at 200 V. Proteins were then transferred to a Immobilon-P membrane (Millipore, Bedford, MA, Canada) for 2.0 h using a Mini-Trans-Blot Cell (Bio-Rad, Mississauga, ON, USA). Blots were dried at 37 °C for 1.0 h, stained with Ponceau S, then visualized by destaining with 90% methanol/2.0% acetic acid. Blots were then blocked in 3% bovine serum albumin (BSA)/TTBS (0.05% Tween 20 in Tris-buffered saline: 20 mmol l^{-1} Tris-HCl, 500 mmol l^{-1} NaCl, 5.0 mmol l^{-1} KCl, pH 7.4) for 2.0 h at room temperature on a shaker. The blocking buffer was poured off and the blots incubated with the primary antibody solution 1.0 μg ml^{-1} in 1.0% BSA/TTBS) for 2.0 h at room temperature. The primary antibody used to detect CFTR was mouse anti-human CFTR antibody (R&D Systems, Minneapolis, MN, USA). The antibody used against the Na^+,K^+,$2Cl^-$ cotransporter was mouse anti human NKCC (antibody T4; Iowa Hybridoma Bank, University of Iowa, IA, USA) and the primary antibody for Na^+,K^+-ATPase was mouse anti-Na^+,K^+-ATPase α-subunit from chicken (antibody α5; Iowa Hybridoma Bank, University of Iowa, IA, USA). Following a 5.0 min wash in TTBS buffer, the membranes were incubated with the secondary antibody solution (biotin-SP-conjugated AffiniPure Goat anti-mouse IgG (Biochem Scientific, Mississauga, ON, Canada), diluted 1:8000 in 1.0% BSA/TTBS, for 1.0 h at room temperature. The blots were washed in TTBS and incubated for 1.0 h with an alkaline phosphatase-conjugated Streptavidin solution (Biochem Scientific, Mississauga, ON, Canada) diluted 1:1000 in 1% BSA/TTBS. Bands were visualized by incubating the blots in a BCIP/NBT Blue substrate development solution (Sigma, Oakville, ON, Canada).

Statistics

Values are given as means ± 1 S.E.M. The minimum level of significance is $P<0.05$ from a two-tailed test. Ratio data of fluorescence scores were analyzed by χ^2 test for k independent samples. Depth-measurement data were analyzed by single classification analysis of variance (ANOVA) with a Bonferroni post-test.

Results

Immunocytochemistry

SW control fish (5 animals) had MR cells in opercular membrane with immunoreactivity to the anti-CFTR antibody exclusively in the apical region of MR cells (Fig. 1A,B). MR cells were identified by their strong staining with Mitotracker Red, a finely granular cytoplasm, as seen in bright-field images, and their large size and centrally positioned nucleus that was Mitotracker-negative. Optical sections taken at 1.5 μm intervals identified kfCFTR immunofluorescence initially as a ring shape approximately 5–6 μm diameter which, in the succeeding deeper optical sections, became a solid punctate dot at the bottom of the apical crypt. There was no detectable kfCFTR immunofluorescence throughout the rest of the cytosol and nucleus. The surface of the epithelium was always identifiable because of the 'fingerprint' pattern of microridges of the surrounding pavement cells, and depth measurements were made with reference to this surface. Control membranes without the primary antibody had no detectable fluorescence (not shown; similar controls appear in Marshall et al., 2002). In SW MR cells, NKCC immunofluorescence was absent from the apical crypt membrane but was present throughout the basal portions of the cells excluding the nucleus (Fig. 2A).

FW control fish (9 animals) had kfCFTR immunofluorescence in MR cells that was diffuse and present throughout the basal portions of the cells but outside the nucleus (Fig. 1D,E). In a minority of MR cells (8.1%, Fig. 3) there was also strong apical crypt staining of the pattern, typical of SW (i.e. similar to Fig. 1A). However, all the cells that had apical membrane kfCFTR staining also had diffuse cytosolic kfCFTR immunostaining (unlike SW MR cells). Surprisingly, there was kfCFTR-positive immunostaining in pavement cells of FW-adapted tissues (Fig. 1D) that was completely absent from pavement cells of SW tissues (Fig. 1A). At this point it appears that the kfCFTR immunostaining is not exclusively in the apical membrane of pavement cells; rather the staining occurs at the same plane as the nucleus, indicative of cytosolic or basolateral membrane distribution. Anti-NKCC immunofluorescence was present outside the nucleus in eccentric portions of the cytosol where MR cell pairs meet (Fig. 2B). In FW opercular epithelia, MR cells usually existed in pairs or groups of three with the cells sharing a flat area of closely juxtaposed membrane. The NKCC

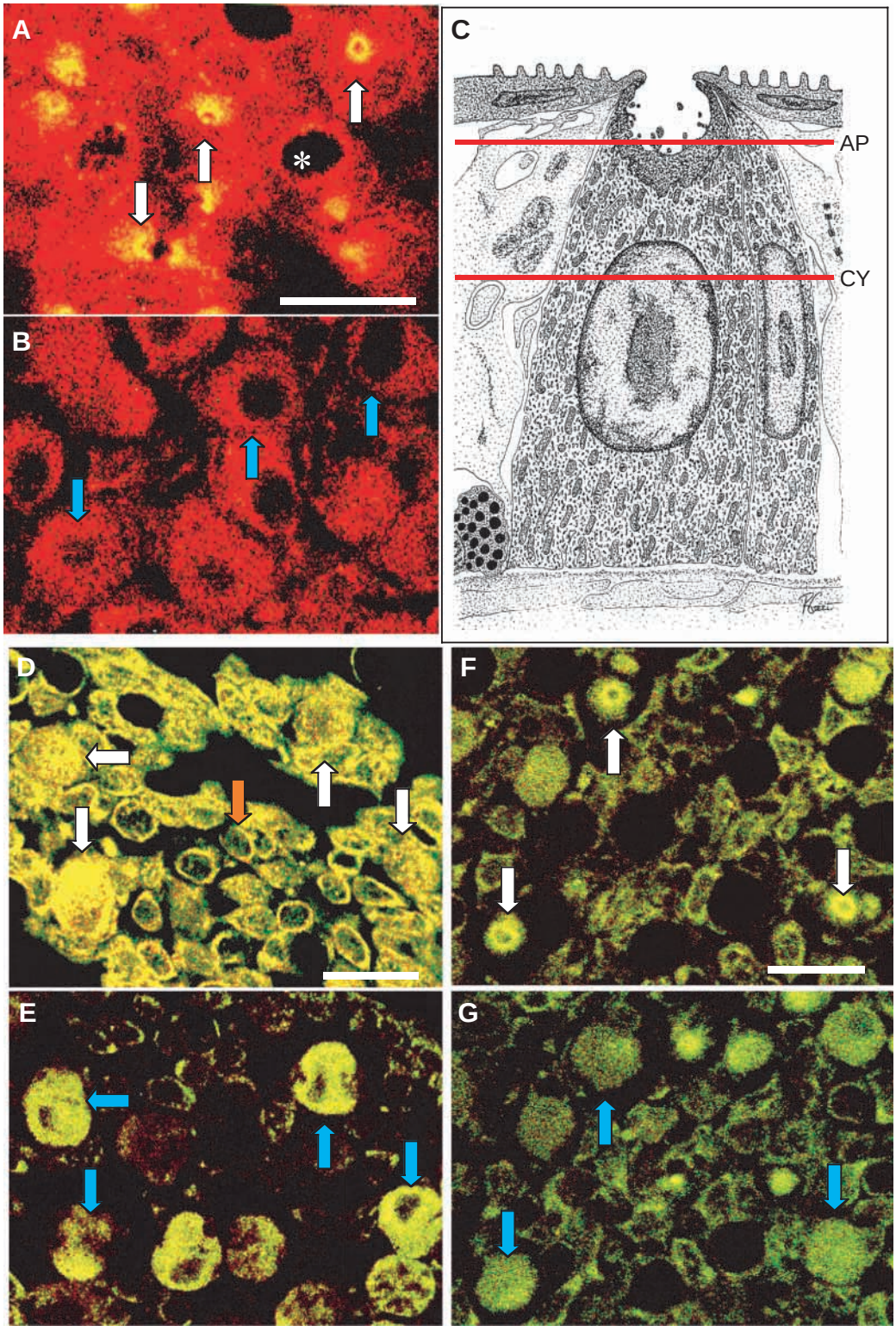

Fig. 1.

immunofluorescence was usually localized to these zones if the MR cells were in groups.

Transitional stages of MR cells were examined in animals transferred to SW for 24 h (4 animals) and 48 h (6 animals). In the 24 h transfer group there was an increasing proportion of MR cells that had punctate kfCFTR immunofluorescence in addition to the diffuse CFTR immunofluorescence (Fig. 3), but the condensed kfCFTR distribution was not superficial, but deeper into the cell, averaging 14 μm below the surface, which was significantly deeper than in FW controls, SW controls and

Fig. 1. The distribution of kfCFTR in mitochondria-rich (MR) chloride cells in killifish opercular epithelium. Images are confocal laser scans of kfCFTR revealed by immunofluorescence of mouse anti-human CFTR and goat anti-mouse IgG Oregon Green 488 and counterstained for mitochondria with Mitotracker Red (green fluorescence + red counterstain yields yellow). (A) Membrane from a SW-adapted animal has kfCFTR immunofluorescence at 4.5 μm from the surface of the chloride cells, localized to the apical crypt (white arrows). The asterisk indicates a gap in the pavement cell layer. (B) The same frame as for A but at a depth of 7.5 μm at the plane of the nucleus of the MR cell. Blue arrows indicate the mitochondria-rich chloride cells below the apical crypt. (C) Diagram of approximate depths of the optical sections, AP, apical crypt level; CY, cytosol level. (D,E) Similar kfCFTR immunostaining, but in MR cells from fish acclimated to FW. (D) An optical section at 4.5 μm from the surface has positive kfCFTR immunofluorescence evenly distributed in the chloride cells (white arrows) as well as in the pavement cells at the plane of the pavement cell nuclei (orange arrow). (E) The same frame as for D but 10.5 μm into the tissue at the level of the MR cell nuclei. Blue arrows indicate the MR cells with diffusely distributed positive immunofluorescence for kfCFTR. (F,G) Similar immunostaining but for a fish transferred from FW to SW for 48 h. (F) An optical section 9.0 μm from the surface, with ring-shaped kfCFTR-positive immunofluorescence (white arrows); (G) 13.5 μm from the surface, with kfCFTR immunofluorescence diffusely distributed in MR cells (blue arrows). Scale bars, 20 μm.

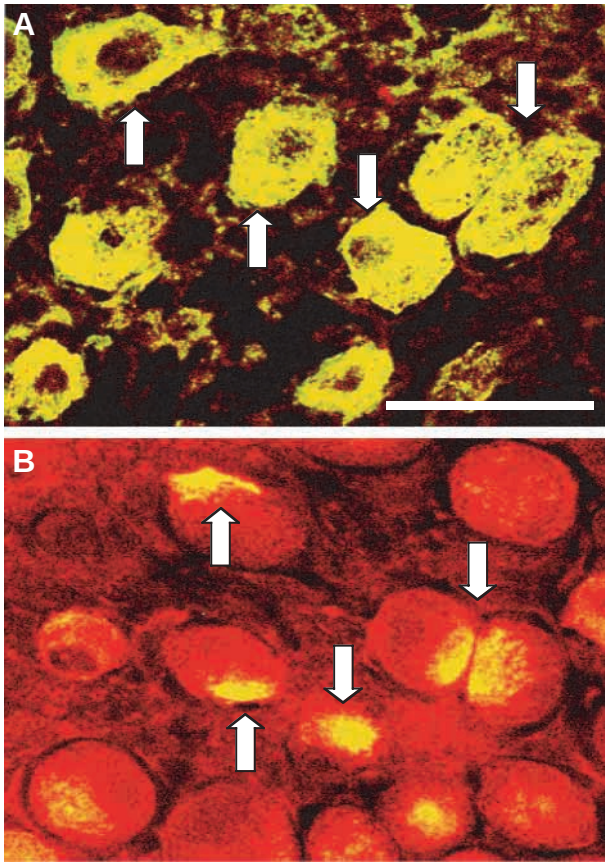

Fig. 2. The distribution of Na^+,K^+,$2Cl^-$ cotransporter in SW-adapted and FW-acclimated killifish opercular epithelium. Images are confocal laser scans of NKCC revealed by immunofluorescence of mouse anti-human NKCC and goat anti-mouse IgG Oregon Green 488 in MR cells counterstained for mitochondria with Mitotracker Red (green fluorescence with red counterstain yields yellow). (A) In SW-adapted killifish, NKCC immunofluorescence at 7.5 μm from the surface of the chloride cells is evenly distributed throughout the cytoplasm of MR cells (white arrows). (B) In FW-acclimated killifish, NKCC immunofluorescence at 9.0 μm from the surface of the chloride cells is eccentrically distributed in the chloride cells and localized between pairs of MR cells (white arrows). Scale bar, 20 μm.

FW to SW for 48 h groups (P<0.001, Bonferroni post-test following ANOVA; Fig. 4). At 48 h after transfer to SW, the majority of MR cells had both diffuse and punctate kfCFTR distribution and a distance to the surface that was intermediate but still significantly deeper (P<0.001; Bonferroni post-test following ANOVA) than in fully adapted SW animals. An example of this type of kfCFTR immunofluorescence distribution is seen in Fig. 1F,G. FW controls had few cells with punctate kfCFTR immunofluorescence compared to the fully adapted SW group (Fig. 3), but the depth from the surface was similar between the FW and SW groups (P>0.05; Bonferroni post-test following ANOVA; Fig. 4).

Western blots

The CFTR antibody was specific for a protein at 147.7±1.8 kDa molecular mass (N=3 separate animals in 3 separate runs for all bands reported) in the opercular epithelium and a 151.3±2.0 kDa protein in the gills (Fig. 5). However, two lower molecular mass additional bands were also visible, at 92.7±0.3 kDa and 88.7±0.3 kDa, respectively, in the opercular epithelium and 93.3±0.3 kDa and 89.3±0.9 kDa in the gills.

The heart (negative control) did not show any CFTR immunoreactivity. With the anti-NKCC antibody, immunoreactive bands appeared at 148.0±2.0, 92.7±0.3 and 87.8±0.3 kDa in the opercular epithelium, and at 150.0±2.0, 92.7±0.3 and 87.8±0.3 kDa in the gills. With anti-NKCC the heart also displayed three bands of similar size to those in the other tissues (149.0±1.7, 91.7±0.3 and 87.7±0.3 kDa). The Na^+K^+ATPase antibody reacted prominently with a high molecular mass protein at 119.0±1.5 kDa in the opercular epithelium and a 119.7±0.9 kDa protein in the gills. However, three additional fainter bands were also visible with lower molecular mass namely 99.7±0.7, 72.0±0.6 and 67.3±0.7 kDa in opercular epithelium and 99.3±0.3, 71.7±0.3 and 66.7±0.7 kDa in gill tissue. The heart showed four additional bands at 118.3±1.5, 102.3±3.9, 72.7±0.3 and 66.3±0.9 kDa).

Discussion

CFTR distribution in MR cells

CFTR immunofluorescence detected by an antibody homologous to the carboxy terminus of the protein identifies kfCFTR specifically, and northern analysis indicated kfCFTR expression in opercular epithelium, gill and posterior intestine of SW killifish (Singer et al., 1998). From western blots

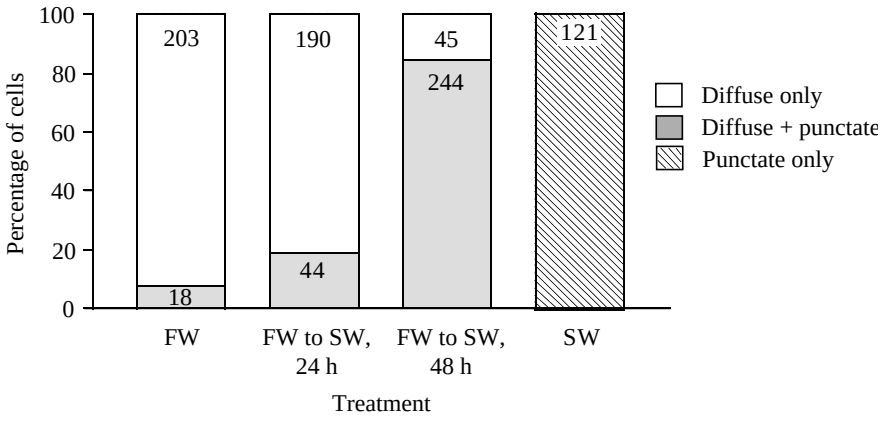

Fig. 3. Evaluation of kfCFTR immunofluorescent MR cells into those containing diffuse only, punctate only, or both diffuse and punctate distribution. Bars indicate the percentage of cells in each category; the numbers of cells measured are shown. The number of animals in each group were FW, 9; FW to SW for 24 h, 4; FW to SW for 48 h, 6; SW, 5. In FW animals, most cells had only diffuse kfCFTR immunofluorescence; 8.1 % (18 cells) had both. The percentage of cells with punctate distribution increased with acclimation to SW, and in fully adapted SW animals all cells had only apical punctate kfCFTR localization.

(Mickle et al., 2000) it appears that there are more than one isoform of the gene product, and the heavier band we observed for CFTR in the present study using a different antibody confirms the presence of the 150–160 kDa isoform. Wilson et al. (2000a) observed a single 160 kDa band with anti-CFTR in mudskipper gills. If an isoform exists without the –dtrl terminal portion, it would probably be nonfunctional in Cl^- secretion, as this terminus is a PDZ-binding domain that is associated with trafficking of the protein to the apical membrane (reviewed by Kleizen et al., 2000). It would appear that the CFTR isoform bearing the –dtrl terminus is expressed in every SW MR cell, because all MR cells in the fully adapted SW animals show the apical immunofluorescence.

The apical crypt localization is similar to that seen in another euryhaline teleost species adapted to SW, the mudskipper, and using a different antibody to CFTR (Wilson et al., 2000a). Given that two different anti-CFTR antibodies, one with a known epitope exactly the same as for the endogenous protein in killifish, yield similar localizations in two different SW-adapted teleosts, we can conclude that kfCFTR is concentrated at the apical membrane of SW MR cells. Furthermore, because kfCFTR expressed in amphibian oocytes imparts anion conductance in this system (Singer et al., 1998), and as anion channels similar in properties to hCFTR anion channels are present in the apical membrane of killifish opercular epithelium MR cells (Marshall et al., 1995), the immunofluorescence seen represents the cAMP-stimulated apical anion conductance of MR cells. The presence of the protein does not, of course, guarantee that the channels are all activated.

In opercular membranes from FW-adapted killifish, the diffuse distribution of kfCFTR immunofluorescence in the basal portion of MR cells could represent kfCFTR in the basolateral membrane (on the extensive infoldings of basolateral membrane in the tubular system of these cells), but equally possible it could be kfCFTR expressed in vesicles throughout the cytosol. A functional interpretation of the former is that basal kfCFTR could serve in anion uptake as well as transfer of acid equivalents, since CFTR anion channels are permeable to HCO_3^- (Linsdell et al., 1997) and associated with HCO_3^- secretion in mammalian duodenum (Hogan et al., 1997). The latter possibility implies expression and storage of CFTR in ‘anticipation’ of future function, once inserted into the apical membrane during SW adaptation. Other recent immunocytochemical examinations of teleost gills, did not test for CFTR antibodies in FW animals (Wilson et al., 2001a,b). Overall, the possible role of CFTR in FW ion transport is an intriguing possibility that has not been examined.

The small percentage (8.1 % of the total) of MR cells in FW-adapted animals that displayed apical crypts containing kfCFTR immunofluorescence may represent a functional subgroup of MR cells that are either involved in HCO_3^- permeation in acid–base balance (as suggested by Wilson et al., 2000a) or are preadapted for Cl^- secretion, should the animal encounter high salinity (implying that the kfCFTR is present in the apical membrane but normally inactivated). The shallow depth (as in SW membranes) of the kfCFTR distribution suggests that it is exposed to the environment

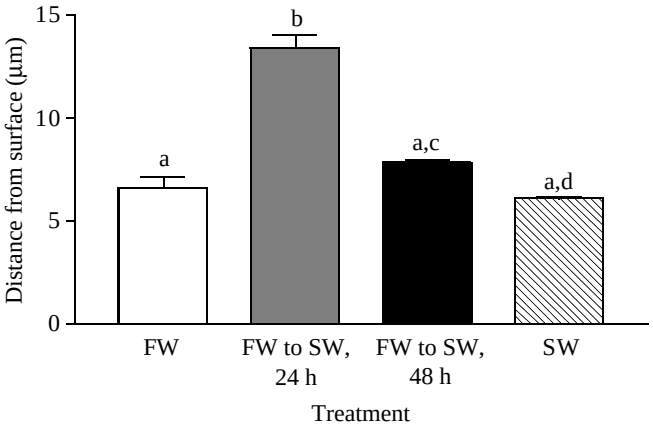

Fig. 4. The cellular location of punctate kfCFTR immunofluorescence in FW controls (a small minority of cells) and SW animals (all cells), as determined by optical sections in the confocal microscope, was in apical crypts approximately 7 μm deep. The transitional phases included many cells with punctate kfCFTR immunofluorescence well below the surface. It would appear kfCFTR distribution in FW is diffuse, then condenses in the first 24 h after salinity transfer, prior to moving kfCFTR to the apical membrane at approximately 48 h. Columns with different letters are significantly different ($P<0.05$ or better; single-classification ANOVA followed by a Bonferroni post-test).

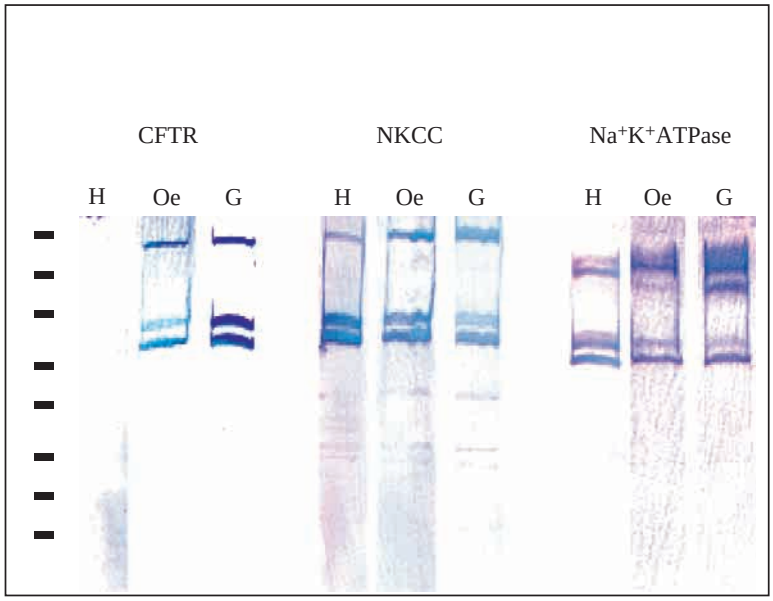

Fig. 5. Western blot analysis of CFTR, NKCC and Na^+,K^+-ATPase in opercular epithelium (20 μg) and gill tissue (20 μg for CFTR and NKCC, 10 μg for Na^+,K^+-ATPase) from SW-adapted killifish. Proteins were separated on a 7.0 % polyacrylamide gel, transferred onto an Immobilon-P membrane and probed with anti-human CFTR monoclonal antibody (1.0 μg ml–1). Proteins were visualized following incubation in BCIP/NBT Blue substrate development solution. Lanes 1, 2 and 3 show heart (H) (negative control), opercular epithelium (Oe) and gill tissue (G), respectively. The anti-hCFTR antibody is specific for a protein of approx. 150 kDa and two lower molecular mass bands in the opercular epithelium and in the gill. Lanes 4, 5 and 6 show the same tissues probed with the NKCC T4 antibody with the main band at approx. 150 kDa and two lower molecular mass bands. Lanes 7, 8 and 9 show the same tissues probed with the Na^+,K^+-ATPase α5 antibody. Visible proteins appear at approx. 120 kDa (major band) and 101, 72 and 68 kDa. Molecular mass markers (kDa) were: 205, 116, 97.4, 84, 66, 55, 45 and 33 (from top to bottom). For means and S.E.M. (N=3), see text.

rather than being covered over by pavement cells. The latter is probable because stimulation of FW opercular epithelia with cAMP produces an immediate initiation of serosa positive transepithelial potential and an I_{sc} of approximately 25 μamp cm^{-2}, 10 % of the full SW capacity of the opercular epithelium, to generate cAMP mediated Cl^- secretion (Marshall et al., 1999).

In the 24 h transfer group the kfCFTR immunofluorescence is punctate in a larger portion of MR cells (18.8 %), but the larger distance from the surface suggests that either some of the kfCFTR is in cells without apical exposure or that the apical crypts are deeper than in SW-adapted animals. Diffuse kfCFTR immunofluorescence persists in most cells and, at this same time, the generation of cAMP-stimulatable Cl^- secretion is lower than that seen in fully adapted marine killifish (approximately 23 % of SW levels; Marshall et al., 1999). At 48 h, 76.3 % of the MR cells had punctate kfCFTR immunofluorescence at a time when the cAMP-stimulatable Cl^- secretion by the opercular membrane is 71 % of that in fully adapted SW killifish (Marshall et al., 1999). There appears to be good agreement between the percentage of MR cells with punctate, SW-like, kfCFTR immunofluorescence and the capacity of the epithelium at each stage to generate cAMP-stimulatable Cl^- secretion.

The change in distribution of kfCFTR from a diffuse cytosolic pattern progressively to one of punctate apical localization suggests that kfCFTR is trafficked from subapical locations to concentrated points and inserted in the apical membrane, coinciding with the generation of cAMP-stimulatable Cl^- secretion. The process probably includes *de novo* expression of kfCFTR, as indicated by the increased level of expression, detected by northern analysis, starting at 8 h, reaching a maximum at approximately 24 h and remaining elevated during the balance of SW adaptation (Singer et al., 1998). The mechanism and regulation of this phenomenon are unknown, but presumably resemble that of higher vertebrates. In any case, it would seem to be a relatively slow process occurring over a period of days, not minutes.

Previous data reported on CFTR redistribution indicate a rapid process of exocytosis from areas immediately below the apical membrane of shark *Squalus acanthias* rectal gland salt-secreting cells (Lehrich et al., 1998). The response is rapid and mediated by vasoactive intestinal polypeptide (VIP). Such redistribution is strongly associated with elevated NaCl and fluid secretion by the rectal gland. This phenomenon has been confirmed in rat duodenum, and is also in response to VIP (Ameen et al., 1999). Hence, activation of quiescent NaCl-secreting epithelia is strongly associated with trafficking of CFTR into the apical membrane. In another system, CALU-3 airway epithelial cells, rapid CFTR activation does not appear to require exocytosis, as the channel is already present in the apical membrane (Loffing et al., 1998). The heat stress protein HSP70, which in animals is activated by a variety of stressors, promotes trafficking of CFTR, even with the ΔF508 error that in CF impedes normal trafficking (Chou-Kang and Zeitlin, 2001). Salinity change is an adaptation involving cortisol responses that occur several hours before the increase in CFTR expression (Jacob and Taylor, 1983; Marshall et al., 1999), so in teleosts we also see the association of a ‘stressor’ with augmented CFTR trafficking. The common feature shared by airway epithelia and MR cells is that the resting secretion rate is well above zero, implying that CFTR is already present in the apical membrane, in contrast to shark rectal gland and rat duodenum, where resting secretory rates are zero, obliging the cells to remove CFTR from the apical membrane in the absence of hormone.

CFTR distribution in pavement cells

Whereas kfCFTR immunostaining is absent from pavement cells of opercular epithelia from SW-adapted fish, there is

strong immunostaining of pavement cells in membranes from FW-adapted fish and in the 24 and 48 h SW-transferred animals. The exact distribution is uncertain, because of the thinness of the cells, but clear immunostaining at the plane of the nucleus indicates a cytosolic or basolateral membrane localization. The exact localization will require TEM and immunogold experiments. The presence of kfCFTR in the basolateral membranes of pavement cells in FW is consistent with the uptake of Cl^- and/or translocation of acid equivalents across the basolateral membrane in the form of HCO_3^-. It is well known that CFTR in mammalian systems has significant conductance to HCO_3^- (Linsdell et al., 1997; Hogan et al., 1997) and has been used this way in a model for fish by Wilson et al. (2000a); apical CFTR is thought to aid ammonia fluxes by moving base equivalents out across the apical membrane. When killifish are acid-loaded with injected HCl, they appear to secrete acid equivalents efficiently along with Cl^-, the acid load significantly augmenting the Cl^- efflux. In contrast, the standard FW trout response to similar treatment is to increase Na^+ efflux and inhibit Cl^- efflux while augmenting acid secretion (e.g. Wood, 1991). The killifish response might therefore be connected to activation of apical CFTR channels in FW pavement cells. Clearly closer examination of pavement cell transporters may illuminate their function in ion transport.

NKCC distribution in MR cells

NKCC immunofluorescence in Cl^--secreting epithelia is basolateral, as measured in vesicle experiments and as detected by immunocytochemistry and immunoelectron microscopy. Basolateral membrane vesicles from rainbow trout gill tissue demonstrated a bumetanide- and furosemide-sensitive Na^+,K^+,2Cl^- cotransport that was particularly sensitive to extracellular K^+ (Flik et al., 1997). Further, there was obvious expression of the cotransporter in FW-acclimated animals and an upregulation of the cotransporter on acclimation to 70 % and 100 % SW (Flik et al., 1997). NKCC has been identified immunocytochemically in basolateral surfaces of epithelial cells of shark rectal gland (Lytle et al., 1992), MR cells of mudskipper gill (Wilson et al., 2000a), MR cells of juvenile salmonid gill (Pelis et al., 2001) and human colonic epithelial cell line T84 (D'Andrea et al., 1996). In T84 cells, agonists that operate in part *via* cAMP evoke a rapid, actin-dependent, activation of NKCC and an increase in cell-surface expression of the cotransporter (D'Andrea et al., 1996). In epithelia undergoing NaCl uptake, such as the kidney, NKCC localizes to the apical membrane instead (Biemesderfer et al., 1996).

Our work suggests that a change in salinity is associated with a relatively slow redistribution of NKCC from an eccentric localized position in FW MR cells to an evenly distributed diffuse pattern in the basal portion of MR cells in SW-adapted animals. The FW immunoreactivity distribution pattern of NKCC confirms the cotransporter operation in basolateral membrane vesicles from FW rainbow trout gill (Flik et al., 1997) and extends the finding by demonstrating that NKCC is indeed restricted to MR cells of FW-adapted fish, as predicted by Flik et al. (1997). Because NKCC is apparently not present in the apical membrane area of MR cells, ion uptake observed in opercular epithelia from FW-adapted killifish (Marshall et al., 1997) probably does not involve this transporter. It is not clear how killifish differ in the handling of NKCC in FW, but the eccentric localization of NKCC in ion-transporting cells is a new finding and may be significant. Basolateral NKCC in FW could be involved in cell volume regulation of MR cells (Flik et al., 1997), but this suggestion is currently speculative. The links between MR cell volume and transport rate in SW clearly connect cell swelling with decreased salt secretion (e.g. Daborn et al., 2001), but equivalent investigations have not yet been performed in FW systems. In any case, as the NKCC operation is strongly dependent on the degree of phosphorylation of the protein (Haas and Forbush, 1998), NKCC may potentially be involved in regulation of cell volume and, secondarily, ion transport rates.

In contrast to the images seen in FW cells, the diffuse distribution in SW MR cells is similar to those seen in elasmobranch rectal gland (Lytle et al., 1992), mudskipper gill MR cells (Wilson et al., 2000a) and salmon smolt gill MR cells (Pelis et al., 2001), and is fully consistent with localization of the cotransporter to infoldings of the basolateral membrane, the tubular system of MR cells. Na^+,K^+,2Cl^- cotransport increases almost fivefold with adaptation of rainbow trout to 70 % and full-strength SW (Flik et al., 1997). NKCC abundance and the number of MR cells increased with smolting, and apparently colocalized in all cases with Na^+,K^+-ATPase immunofluorescence (Pelis et al., 2001). In FW salmon, the basal NKCC is presumably not hormonally activated because the MR cells are not actively involved in ion secretion until the animal enters SW. MR cells can rapidly close the apical crypt, allowing pavement cells to cover over MR cells, thus effectively removing the cells from contributing to NaCl secretion. This dynamic covering of MR cells has been observed in mudskipper gill when the animals are transferred to FW (Sakamoto et al., 2000) and in isolated killifish opercular epithelium exposed to basolateral hypotonic shock (Daborn and Marshall, 1999; Daborn et al., 2001).

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