The Journal of Experimental Biology 206, 4113-4123

doi:10.1242/jeb.00659

Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique

Fumi Katoh* and Toyoji Kaneko

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

*Author for correspondence (e-mail: fkatoh@marine.fs.a.u-tokyo.ac.jp)

Accepted 11 August 2003

Summary

Short- and long-term responses to direct transfer from seawater to freshwater were examined in gill chloride cells of killifish, which developed distinct freshwater- and seawater-type chloride cells in the respective environments. In a short-term response within 24 h after transfer, seawater-type chloride cells forming a pit structure on the apical surface were transformed into freshwater-type cells equipped with developed microvilli on the flat or projecting apical membrane, *via* the intermediate type. The transformation process was accompanied by the disappearance of apically located Cl^- channel (cystic fibrosis transmembrane conductance regulator) and neighboring accessory cells. Chloride cell replacement was also examined as a long-term adaptation to freshwater transfer, using a newly established 'time-differential double fluorescent staining (TDS)' technique. In the TDS technique, *in vivo* labeling of chloride cells was performed on two separate days, using two distinguishable mitochondria-specific fluorescent probes. For 3 days after freshwater transfer, 14.7% of seawater-type cells were replaced with newly differentiated freshwater-type cells, whereas these ratios of chloride cell replacement were much lower (1.2% and 1.8%) in seawater- and freshwater-maintained groups, respectively. In consequence, following direct transfer of killifish from seawater to freshwater, seawater-type chloride cells were transformed morphologically and functionally into freshwater-type cells as a short-term response, followed by the promotion of chloride cell replacement as a long-term response.

Key words: branchial chloride cell, killifish, *Fundulus heteroclitus*, time-differential double fluorescent staining.

Introduction

Teleost fish maintain their ion concentration and osmolality of the body fluid at levels that are different from those in the external environment. In adult teleosts, chloride cells in the gill epithelia are the major site of ion absorption and secretion, and are thus important in both freshwater and seawater adaptation. Although chloride cells are generally characterized by having numerous mitochondria and an extensive tubular system in the cytoplasm, the morphology and function of chloride cells differ greatly between freshwater- and seawater-adapted fishes. Based on their location and response to environmental salinity, distinct freshwater- and seawater-type chloride cells have been described in euryhaline and migratory species that are adaptable to both environments. For example, in chum salmon *Oncorhynchus keta* (Uchida et al., 1996), Japanese eel *Anguilla japonica* (Sasai et al., 1998) and Japanese sea bass *Latealabrax japonicus* (Hirai et al., 1999), chloride cells located in the gill lamellar epithelium are considered to be freshwater-type cells that appear in hyposmotic environments, whereas those in the gill filament are seawater-type cells that are activated in hyperosmotic environments. Pisam et al. (1987, 1990, 1993) also described two types of chloride cells (α and β) in the gill filament of tilapia *Oreochromis niloticus*, guppy *Lebistes reticulatus*, gudgeon *Gobio gobio*, loach *Cobitis taenia* and turbot *Scophthalmus maximus*; α cells are transformed into seawater-type chloride cells during seawater adaptation, whereas β cells are involved in freshwater adaptation.

Recent studies have shown that chloride cells in some euryhaline fishes alternate their morphology and ion-transporting functions to meet abrupt environmental osmotic changes. In the tilapia yolk-sac membrane, a sequential observation technique revealed that single freshwater-type chloride cells were transformed into seawater-type multicellular complexes during seawater adaptation (Hiroi et al., 1999). Sakamoto et al. (2000) also showed that mudskipper *Periophthalmus modestus* closed the apical pit of chloride cells following transfer from seawater to freshwater. In seawater-

adapted killifish, the cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+,K^+,2Cl^- cotransporter have been demonstrated in the apical pit and cytoplasm of MR cells, respectively. These ion transporters are redistributed during freshwater adaptation (Marshall et al., 2002).

In most teleosts examined so far, chloride cells became larger and denser when the fish were transferred from freshwater to seawater, concomitant with increases in gill Na^+/K^+-ATPase activity (Langdon and Thorpe, 1985; Richman et al., 1987; McCormick, 1995; Uchida et al., 1996, 2000; Shiraishi et al., 1997). This ion-transporting enzyme is located in the tubular system that is continuous with the basolateral membrane, and plays a key role in ion-transporting functions of chloride cells (McCormick, 1995). Thus, chloride cell morphology and gill Na^+/K^+-ATPase activity were thought to be reliable indices of seawater adaptability. However, this is not the case with killifish *Fundulus heteroclitus*, which also exhibits morphologically distinct freshwater- and seawater-type chloride cells in the respective media (Marshall et al., 1997; Katoh et al., 2001). In killifish, chloride cells are mostly located in a flat region of the afferent-vascular edge (the trailing edge, in terms of water flow) of the gill filament, and the chloride cell size is generally larger in freshwater-adapted than in seawater-adapted fish. Furthermore, gill Na^+/K^+-ATPase activity, as well as oxygen consumption, does not differ between freshwater- and seawater-adapted fish (Marshall et al., 1999; Katoh et al., 2001), suggesting that both types of chloride cells are equally active in the two environments but exhibit different ion-transporting functions.

According to the current model for transcellular ion transport by chloride cells, various ion-transporting proteins are placed in either the apical or basolateral membrane. In seawater-type chloride cells, for example, Cl^- enters the cell, together with Na^+ and K^+, across the basolateral membrane *via* the Na^+,K^+,2Cl^- cotransporter, and accumulates intracellularly so that Cl^- exit occurs down its electrochemical gradient through Cl^- channels in the apical membrane. The driving force for Cl^- secretion is the Na^+ electrochemical gradient established by Na^+/K^+-ATPase in the basolateral membrane. Meanwhile, Na^+ secretion occurs down its electrochemical gradient *via* a cation-selective paracellular pathway. In killifish, the CFTR has been identified as the apically located Cl^- channel (Marshall et al., 1995; Singer et al., 1988), and thus the existence of CFTR in the apical membrane could provide evidence for functional seawater-type chloride cells.

In seawater-type chloride cells in the killifish gills, the apical membrane invaginates to form a pit, and the cells often interdigitate with neighboring accessory cells. In freshwater-type cells, on the other hand, the apical membrane is flat or protrusive, being equipped with microvilli. Although the structural difference is evident between freshwater- and seawater-type chloride cells in killifish, it is not clear whether one cell type is replaced by another cell type, or whether one cell type changes its function and morphology into another type, following transfer between freshwater and seawater.

In this study, we aimed to clarify both the short- and long-term responses of gill chloride cells to direct transfer from seawater to freshwater in killifish. To visualize the chloride cell replacement, or cell turnover, following freshwater transfer, we developed a 'time-differential double fluorescent staining (TDS) technique', in which *in vivo* labeling of chloride cells was performed on two separate days, using two distinguishable mitochondria-specific fluorescent probes. Our findings revealed functional and morphological transformation of chloride cells from seawater- to freshwater-type in a short term, which was followed by recruitment of newly differentiated, freshwater-type cells as a long-term response to freshwater transfer.

Materials and methods

Fish

Experimental fish were offspring of killifish *Fundulus heteroclitus* L. obtained from National Research Institute of Fisheries Science, Fisheries Research Agency, Kanagawa, Japan (Shimizu, 1997). They were kept in 50% seawater at Ocean Research Institute, University of Tokyo, Japan, and fed tilapia pellets. Prior to experiments, the fish were acclimated to full-strength seawater (559 mmol l^{-1} Na^+, 13 mmol l^{-1} Ca^{2+}) or freshwater (1.26 mmol l^{-1} Na^+, 0.55 mmol l^{-1} Ca^{2+}) in 250 liter indoor tanks at 25°C at least for 1 month. The fish were fed during the acclimation period, but feeding was stopped for 1 day before transfer and during the experiments.

Time-course observations following transfer from seawater to freshwater

Forty seawater-acclimated fish weighing 2.8–7.8 g were transferred directly to freshwater in 40 liter tanks at 25°C, and removed at 0 h (initial controls), 3 h, 12 h, 1 day, 3 days, 7 days, 14 days and 30 days after transfer. The fish (N=5 each) were anesthetized with 0.05% 2-phenoxyethanol, and blood was collected from the caudal vessels into capillary tubes. The plasma was separated by centrifugation at 4000 ***g*** for 5 min. Plasma Na^+ concentrations were measured using an atomic absorption spectrophotometer (Hitachi Z-5300, Japan). The gills were removed and fixed in 4% paraformaldehyde (PFA) in 0.1 mol l^{-1} phosphate buffer (PB, pH 7.4) for 24 h for whole-mount immunocytochemistry, and in 2% PFA, 0.2% glutaraldehyde (GA) in the same buffer for 3 h for CFTR immunocytochemistry. For transmission (TEM) and scanning (SEM) electron microscopy, the gills were fixed in 2% PFA, 2% GA in PB for 24 h. The fixed gill samples were stored in 70% ethanol. For histological observations, we examined three animals in each experimental group.

Antibodies

For the detection of chloride cells in the gill filaments, we used an antibody specific for Na^+/K^+-ATPase. The antiserum (NAK121) was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the Na^+/K^+-ATPase α-subunit, and the specific antibody was affinity-purified (Katoh et al., 2000; Uchida et al., 2000). The

specificity was confirmed by western blot analysis (Katoh et al., 2000).

The antibody used to detect killifish CFTR was a mouse monoclonal antibody to human CFTR (R&D Systems, MN, USA). This antibody was raised against a carboxy-terminal sequence of human CFTR, which was identical to that of killifish CFTR (Singer et al., 1998).

Western blot analysis for antibody to CFTR

Since the CFTR content in the gill was thought to be too small for detection by western-blot analysis, the sample was subjected to immunoprecipitation prior to western blotting. The gill filaments were scraped in 1 ml of lysis buffer consisting of IP buffer (pH 7.4; 140 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes and 5 mmol l^{-1} EDTA), inhibitors (10 mmol l^{-1} benzamidine, 1 μg ml^{-1} Pepstatin A and 2 mmol l^{-1} phenyl methyl sulfonyl fluoride) and 1% Triton-X 100, and left on ice for 20 min to lyse the cells. The lysate was centrifuged at 5000 ***g*** for 5 min at 4°C, and the supernatant was incubated with 1 μl of the CFTR antibody at room temperature for 3 h. Slurry (20 μl) containing 50% protein A sepharose beads (Amersham Biosciences, Uppsala, Sweden) blocked overnight with 1% bovine serum albumin (BSA), was added to the sample, and the mixture was incubated for 1 h at 4°C. After washing five times with IP buffer containing inhibitors and centrifugation at 10 000 ***g*** for 30 s, 30 μl of hot Laemmli buffer (Laemmli, 1970) containing 5% β-mercaptoethanol was added to beads binding to the antibody, and the mixture was incubated at 65°C for 15 min. The sample was centrifuged at 10 000 ***g*** for 2 min, and the supernatant was frozen for later western blotting. The supernatant was separated by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels (Atto, Japan). After electrophoresis, the protein was transferred from the gel to a polyvinylidene difluoride membrane (Atto). The specific protein band on the membrane was detected with anti-CFTR diluted 1:100, according to the method described in Katoh et al. (2000).

Whole-mount immunocytochemistry

The gill filaments were removed from gill samples fixed in 4% PFA. After washing in 0.01 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Triton X-100, whole-mount preparations of the gill filaments were incubated overnight at 4°C with a mixture of anti-human CFTR at a final dilution of 1:1000 and anti-Na^+/K^+-ATPase (NAK121) labeled with Alexa Fluor 546 (Molecular Probes, OR, USA) diluted 1:500 with PBS containing 0.05% Triton X-100, 10% normal goat serum (NGS), 0.1% BSA, 0.02% keyhole limpet hemocyanin (KLH) and 0.01% sodium azide. The samples were washed for 1 h with PBS containing 0.05% Triton X-100, and then incubated with anti-mouse rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes) overnight at 4°C. The samples were then washed for 1 h with PBS, placed in a chamber slide closed with a coverslip, and observed with a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). The 488 nm argon–ion laser was used for Alexa Fluor 488 and the 543 nm helium–neon laser for Alexa Fluor 546, to give the appropriate excitation wavelengths. The sizes of chloride cells stained with Alexa Fluor 546-labeled anti-Na^+/K^+-ATPase were measured on stored LSM images by means of an internal program. The chloride cell area was obtained from 20 cells per gill filament. Three gill filaments were examined per individual (N=3).

Fluorescence microscopy

The gills fixed in 2% PFA, 0.2% GA in PB were immersed in 30% sucrose in PBS for 1 h, and embedded in Tissue-Tek OCT compound (Sakura Finetek, Japan) at –20°C. Cryosections (2 μm thick) were cut on a cryostat (CM 1100, Leica, Germany) at –20°C, and collected onto amino propyl-triethoxy silane (APS)-coated slides (Matsunami, Japan). The sections were then incubated with a mixture of anti-human CFTR at a final dilution of 1:1000 and anti-Na^+/K^+-ATPase (NAK121) labeled with Alexa Fluor 546 diluted 1:1000 with PBS containing 2% NGS, 0.1% BSA, 0.02% KLH and 0.01% sodium azide (NB-PBS) overnight at 4°C, and then with anti-mouse rabbit IgG labeled with Alexa Fluor 488 for 2 h at room temperature. The sections were observed under a fluorescence microscope with blue (excitation, 450–490 nm; emission, 520–560 nm) and green (excitation, 510–560 nm; emission, >590 nm) excitation filter blocks for Alexa Fluor 488 and for Alexa Fluor 546, respectively (Nikon E800, Japan).

Scanning electron microscopy

Gill filaments fixed for electron microscopy were dehydrated in ethanol, immersed in 2-methyl-2-propanol, and dried using a freeze-drying device (JEOL JFD-300, Japan). Dried samples were mounted on specimen stubs, and coated with platinum palladium in an ion sputter (Hitachi E-1030, Japan), before examination using a SEM (Hitachi S-4500).

Transmission electron microscopy

Tissues fixed for electron microscopy were postfixed in 1% osmium tetroxide in PB for 1 h at room temperature. After dehydration in ethanol, the gill tissues were transferred to propylene oxide and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife, mounted on grids, stained with uranyl acetate and lead citrate, and observed using a TEM (Hitachi H-7100).

Chloride cell replacement during freshwater adaptation

To examine chloride cell replacement in response to salinity change, seawater-acclimated killifish weighing 15.8–27.8 g were transferred to freshwater. For the purpose of comparison, the fish of similar size (19.0–36.9 g) acclimated to seawater or freshwater were also examined for chloride cell replacement under constant environmental salinity. Experimental fish were fin-clipped for individual discrimination. Chloride cell replacement was examined using a newly developed time-differential double fluorescent staining technique (see below). During the experiment, the fish were kept in the dark to prevent

the fluorescent probes from fading. The water temperature was maintained at 25°C throughout the experiment.

Time-differential double fluorescent staining

To examine chloride cell replacement, we developed a time-differential double fluorescent staining (TDS) technique, in which *in vivo* vital staining for chloride cells was performed just before transfer (day 0) and 3 days after transfer (day 3), using two distinguishable mitochondria-specific fluorescent probes.

On day 0, the fish were immersed one by one in 200 ml of 50 μmol l^{-1} Rhodamine 123 (Molecular Probes) dissolved in the respective environmental waters for 3 h to label chloride cells (pre-existing chloride cells). To assess the chloride density on day 0, biopsy samples of gill filaments were removed immediately after the first labeling under anesthesia with 0.05% 2-phenoxyethanol. The samples were placed in a chamber slide with PBS topped by a coverslip, and observed under a fluorescence microscope with the blue-excitation filter block (excitation, 450–490 nm; emission, 520–560 nm; Nikon). The fish were allowed to recover in their respective environmental waters for 30 min after the biopsy sampling, and then transferred from seawater to freshwater, whereas those in control groups were maintained in their respective environments throughout the experiment. On day 3, for the second labeling, the fish were incubated for 3 h as described above, in 1 μmol l^{-1} MitoTracker Red CM-H_2XR_{os} (Molecular Probes) in their respective environmental waters. MitoTracker was first dissolved in dimethyl sulfoxide at a concentration of 1 mmol l^{-1}, and then diluted to the final concentration with environmental waters. After 3 h incubation, gill filaments were removed again from the same individuals under anesthesia. The filament samples were prepared as stated above, and observed under the fluorescence microscope with blue- and green- (excitation, 540–580 nm; emission, 600–660 nm, Nikon) excitation filter blocks. Consequently, the pre-existing cells labeled on day 0 were recognized as Rhodamine 123-positive cells stained in green, and newly differentiated cells during the last 3 days, as well as the pre-existing cells, were stained with MitoTracker in red on day 3. Therefore, newly differentiated cells were identified as Rhodamine 123-negative, MitoTracker-positive cells on day 3.

The fluorescence-microscopic images were recorded with a digital camera (DXM1200, Nikon) attached to the microscope. To determine the chloride cell turnover, the numbers of Rhodamine 123-positive cells on days 0 and 3, and MitoTracker-positive cells on day 3, were counted in the afferent-vascular edge of 15 filaments from 3 individuals in each experimental group.

Statistics

Data are presented as mean ± standard error of the mean (S.E.M.). The significant differences in the plasma Na^+ concentration and chloride cell size were determined by Games Howell's test after analysis of variance (ANOVA) by Bartlett's test. Significant differences in the density of Rhodamine 123-positive chloride cells (pre-existing cells) between days 0 and 3 and the total cell density between days 0 and 3 were examined using a two-sample *t*-test. The significance of difference in frequencies of MitoTracker-positive cells (newly differentiated cells) on day 3 between three experimental groups was determined by the χ^2-test for independence after ANOVA among individuals in each group.

Results

Plasma Na^+ concentration

The plasma Na^+ concentration showed a transient decrease ($P<0.01$) at 12 h after transfer to freshwater, but was restored by day 3 to the level that was not significantly different from the level before transfer. Thereafter, a constant level was maintained up to day 30 (Fig. 1A).

Western blot analysis for CFTR

In western blot analysis, the anti-CFTR recognized one specific protein band with a molecular mass of about 150 kDa (Fig. 2A). No band was detected in the control (Fig. 2B), in which the membrane was incubated with NB-PBS in place of the antibody.

Immunocytochemistry for Na^+/K^+-ATPase and CFTR

A large number of Na^+/K^+-ATPase immunoreactive chloride cells were detected in both the whole-mount preparations and cryosections of the gill filaments in all

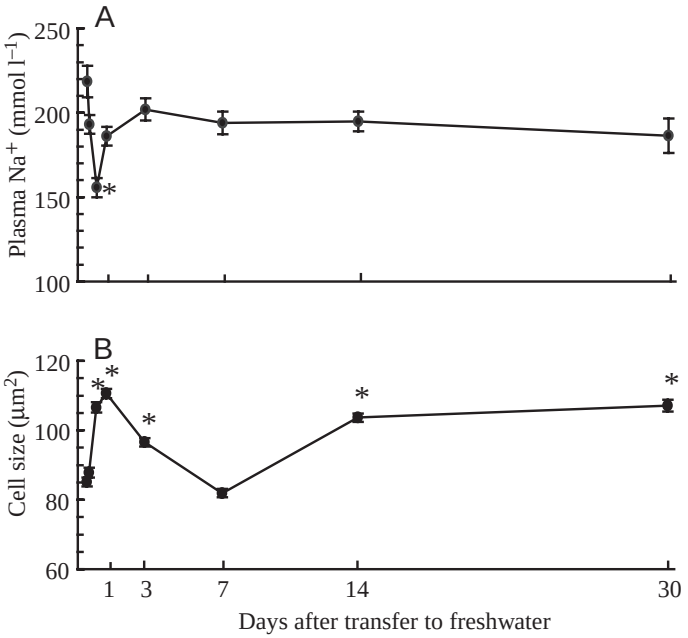

Fig. 1. Changes in plasma Na^+ concentration (A) and branchial chloride cell size (B) following direct transfer from seawater to freshwater. Values are means ± S.E.M. (N=5). Asterisks indicate significant differences compared with the values of seawater-adapted fish (0 h).

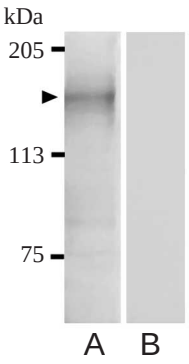

Fig. 2. Western blot analysis of the cystic fibrosis transmembrane conductance regulator (CFTR) protein expressed in the gills of seawater-adapted killifish. The membrane was incubated with (A) or without (B) anti-CFTR. One specific protein band (arrowhead) was detected in lane A. The positions of molecular markers (kDa) are indicated on the left side of the figure.

experimental fish (Fig. 3). Fig. 1B shows the changes in the average size of chloride cells following transfer. The chloride cells became significantly larger at 12 h, but were decreased on day 7 to a size equivalent to that before transfer. The decreased chloride cell size on day 7 was significantly increased again on day 14, and the increased cell size was maintained thereafter.

CFTR immunoreactivity was detected in the apical region of Na^+/K^+-ATPase-immunoreactive chloride cells in seawater-acclimated fish before transfer in both whole-mount preparations and cryosections (Fig. 3A,B). The immunoreactivity appeared along the apical membrane, which formed a pit in seawater, and was rarely observed in the other part of the cell (Fig. 3A,B). The apically located CFTR immunoreactivity was also present in fish at 3 h after transfer to freshwater (Fig. 3C,D), but the intensity was apparently reduced at 12 h (Fig. 3E,F). The signal completely disappeared on day 1 and was not observed thereafter (Fig. 3G–L).

Scanning electron-microscopic observations

Chloride cells are in contact with the external environment through their apical surface. The apical membranes of chloride cells were located at the boundary of pavement cells, and most frequently observed on the afferent edge of gill filament epithelia. In seawater-acclimated fish before freshwater transfer, the apical membrane of most chloride cells invaginated to form a pit (Fig. 4A,B). At 3 h after transfer, the apical pits of chloride cells became shallow and the openings were enlarged to a varying extent, whereas the apical structure of some chloride cells was similar to that observed at 0 h (Fig. 4C,D). Through the enlarged apical opening, poorly developed microvilli were observed on the apical membrane, and a small apical surface of an accessory cell

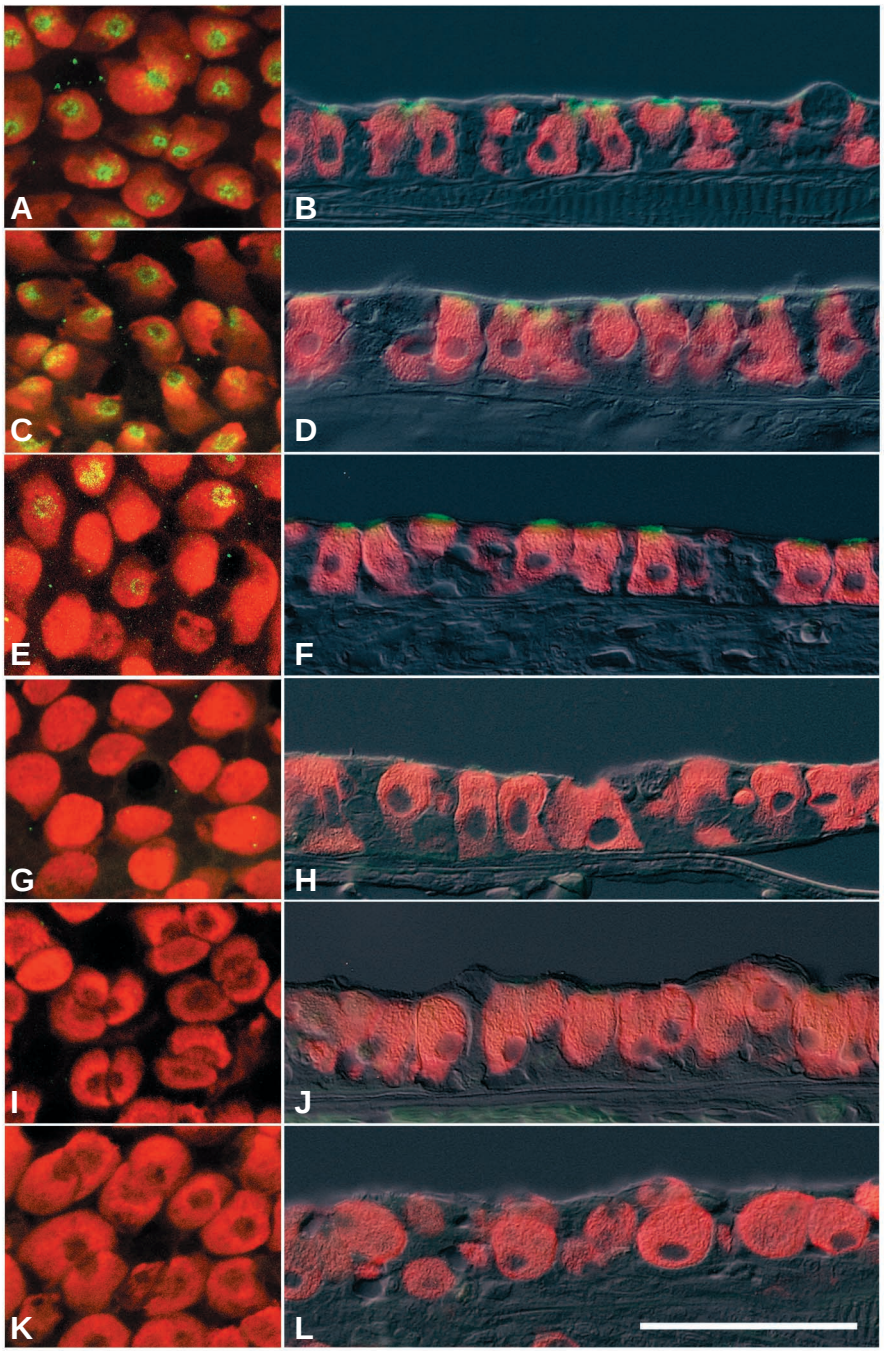

Fig. 3. Confocal laser scanning micrographs of whole-mount preparations of the gill filaments (A,C,E,G,I,K) and fluorescence microscope images of gill cryosections (B,D,F,H,J,L) in killifish at 0 h (A,B), 3 h (C,D), 12 h (E,F), 1 day (G,H), 7 days (I,J) and 30 days (K,L) after transfer from seawater to freshwater. Gill filaments were double stained with anti-cystic fibrosis transmembrane conductance regulator (green) and anti-Na^+/K^+-ATPase (red). Scale bar, 50 μm.

occasionally appeared next to the chloride cells (Fig. 4D). At 12 and 24 h (day 1), the pit structures typically seen at 0 h were rarely observed, but the apical membrane of most chloride cells was flat and equipped with microvilli (Fig. 4E–G). On day 3, however, some shallow pits with an enlarged opening appeared again among the flat apical membranes of chloride cells (Fig. 4H,I). On day 7 and later, the apical membranes of chloride cells were flat or even protrusive, and the microvilli

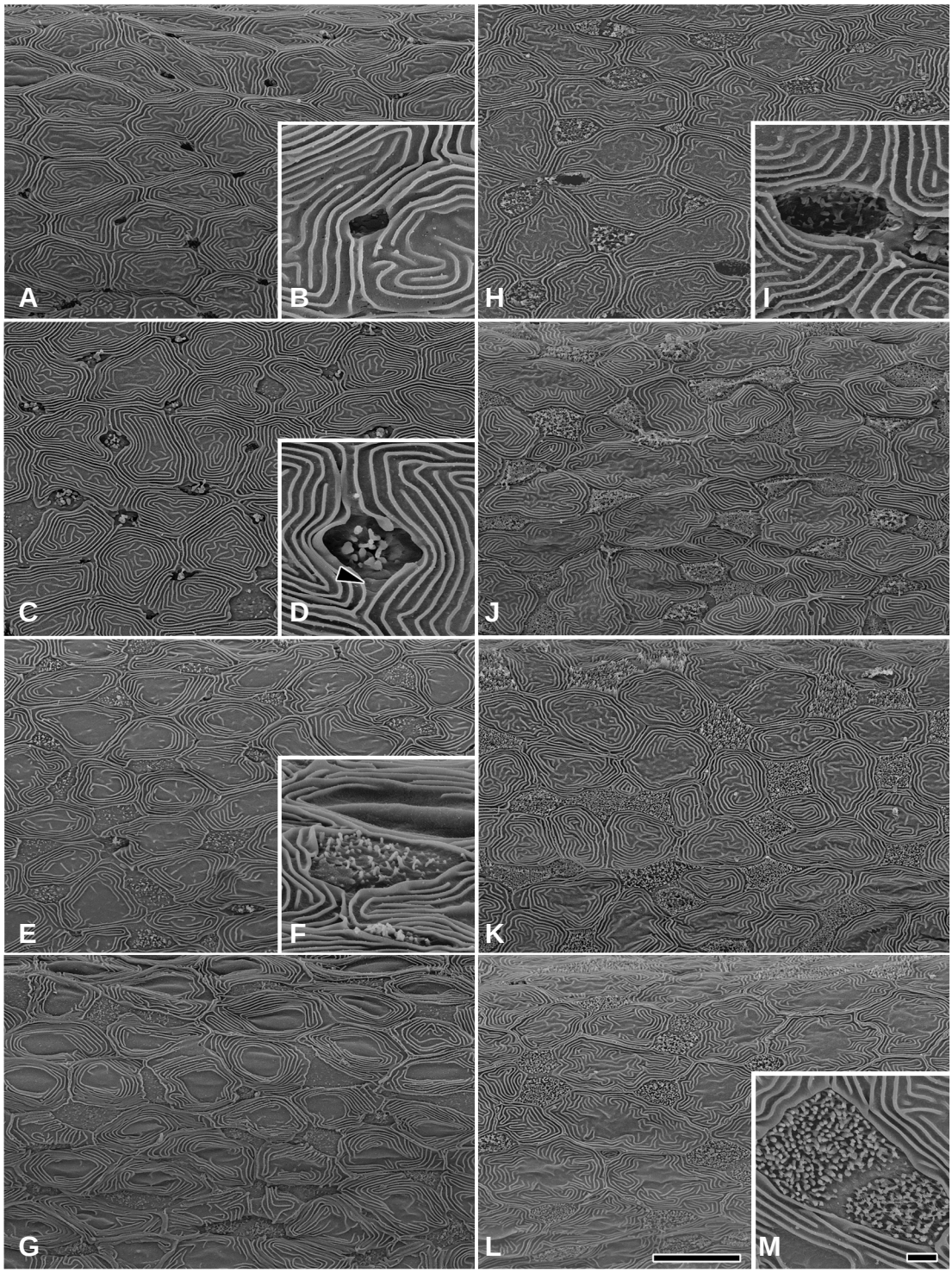

Fig. 4. Scanning electron micrographs of gill filaments in killifish at 0 h (A,B), 3 h (C,D), 12 h (E,F), 1 day (G), 3 days (H,I), 7 days (J), 14 days (K) and 30 days (L,M) after transfer from seawater to freshwater. (B,D,F,I,M) Enlarged views of the apical region of chloride cells, showing an apical surface of an accessory cell next to the chloride cell (arrowhead in D), which is characteristic of the seawater-type chloride cell. Scale bars, 10 μm (A,C,E,G,H,J–L); 1 μm (B,D,F,I,M).

on the surface were well developed (Fig. 4J–M).

Transmission electron-microscopic observations

Chloride cells are generally characterized by a rich population of mitochondria in the cytoplasm, and thus readily identified by TEM (Fig. 5). In seawater-acclimated fish, the apical membrane invaginated to form a pit, as seen with SEM. The chloride cells often interdigitated with neighboring accessory cells, forming multicellular complexes. The chloride and accessory cells shared an apical pit (Fig. 5A). At 3 h after transfer, the pit became shallow and in some cases, the apical region of chloride cells rose to the external environment. The chloride cell and neighboring accessory cell still formed a cellular complex (Fig. 5B). At 12 h, the pit structures of chloride cells were rarely observed, and the apical membranes were flat or slightly projecting. At the same time, accessory cells were no longer observed at 12 h (Fig. 5C). The microvilli on the apical membrane were increasingly developed after 12 h (Fig. 5D–H), and the apical membrane was covered with dense and elongated microvilli on day 30 (Fig. 5H). As seen with SEM, exceptionally some chloride cells with a shallow pit and an enlarged apical opening appeared on day 3, but were not observed afterward. Moreover, numerous small electro-dense vesicles were evident below the apical membrane in the cytoplasm of chloride cells on days 7–30 (Fig. 5F–H).

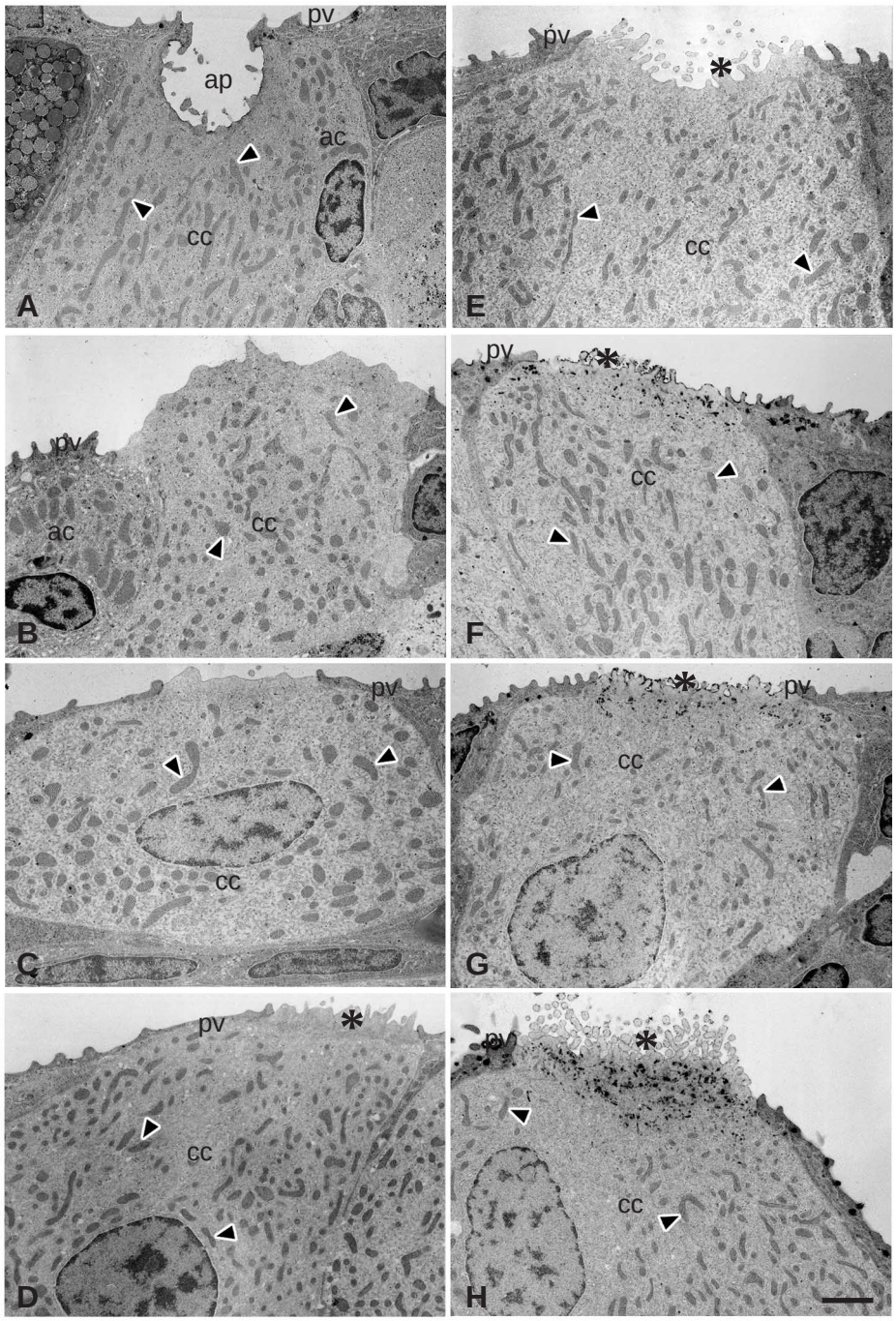

Fig. 5. Transmission electron micrographs of branchial chloride cells (cc) in killifish at 0 h (A), 3 h (B), 12 h (C), 1 day (D), 3 days (E), 7 days (F), 14 days (G) and 30 days (H) after transfer from seawater to freshwater. Multicellular complexes were observed at 0 h and 3 h after transfer. Arrowheads indicate numerous mitochondria. ap, apical pit; pv, pavement cell; ac, accessory cell; *, microvilli. Scale bar, 1 μm.

Chloride cell replacement following transfer from seawater to freshwater

Many Rhodamine 123-positive chloride cells were detected in the afferent-vascular edge of gill filaments in both seawater- and freshwater-acclimated fish at the beginning of the experiment (day 0). After the second staining with MitoTracker on day 3, Rhodamine 123-positive (pre-existing) chloride cells and MitoTracker-positive (pre-existing and newly differentiated) chloride cells were observed under a fluorescence microscope as green and red cells, respectively (Fig. 6A,B,D,E,G,H).

There were no significant differences in number between Rhodamine 123-positive cells on day 0 and MitoTracker-positive cells on day 3 in the three experimental groups (Fig. 6C,F,I). In other words, the total number of chloride cells did not change in any experimental condition. Following transfer from seawater to freshwater, however, the number of Rhodamine 123-positive (pre-existing) cells decreased

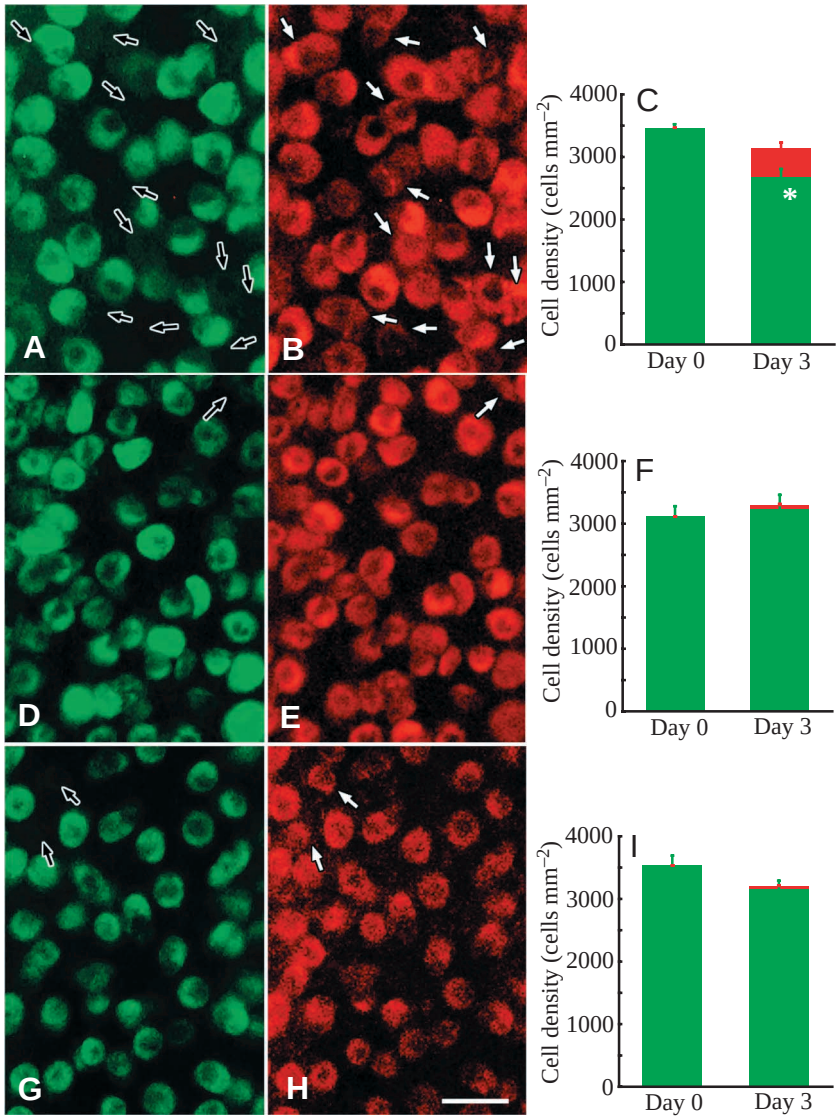

Fig. 6. Time-differential double fluorescent staining of branchial chloride cells in killifish transferred from seawater to freshwater (A,B), or maintained in freshwater (D,E) or in seawater (G,H). Gill chloride cells were double labeled *in vivo* with Rhodamine 123 (A,D,G) just before transfer (day 0) and MitoTracker (B,E,H) on day 3. Cells positive for both Rhodamine 123 and MitoTracker were pre-existing. Arrows indicate newly differentiated chloride cells, which are Rhodamine 123-negative (black arrows)/MitoTracker positive (white arrows). (C,F,I) Changes in chloride cell density on day 0 and day 3 in fish transferred from seawater to freshwater (C), and fish maintained in freshwater (F) or in seawater (I). Values are means ± S.E.M. Green and red portions of the columns indicate the density of pre-existing and newly differentiated chloride cells, respectively. Significant differences in frequency of newly differentiated chloride cells were detected between freshwater-transferred and two control groups (P<0.01). An asterisk indicates significant difference from the density of Rhodamine 123-positive, pre-existing cells on day 0 (P<0.01). Scale bar, 20 μm.

significantly (P<0.01) on day 3 (Fig. 6C), whereas there was no difference in the number of pre-existing cells between days 0 and 3 in the freshwater- or seawater-maintained groups (Fig. 6F,I). On day 3, the ratios of newly differentiated (Rhodamine-negative, MitoTracker-positive) chloride cells were 14.7%, 1.2% and 1.8% in transferred, seawater and freshwater groups, respectively. Significant differences in frequency of newly differentiated chloride cells were detected between freshwater-transferred and the two control groups (P<0.01), but not between seawater and freshwater control groups (Fig. 6C,F,I).

Discussion

Following transfer of seawater-acclimated killifish to freshwater, plasma Na^+ levels showed a transient decrease at 12 h, which was restored by day 3. The drastic decrease following transfer is considered to be the result of diffusional ion loss through the body surfaces in fish exposed to the hyposmotic environment. The quick recovery in Na^+ levels implies active ion absorption, presumably in the gill epithelia, in response to decreased ion levels. Conversely, Jacob and Taylor (1983) reported that Na^+ levels were elevated at 24 and 48 h in killifish transferred from freshwater to seawater, but recovered on 3 days after transfer. These findings indicate the excellent osmoregulatory ability of killifish to adapt to both hyper- and hyposmotic environments.

Chloride cells were detected in the whole-mount preparations of the gill filaments by LSM. The mean size of chloride cells became significantly larger just after transfer, when plasma Na^+ levels showed a transient decrease. The increase in the chloride cell size might be caused by the sudden decrease in plasma ion concentration following direct transfer to the hyposmotic environment. Most probably, enlarged chloride cells in freshwater are in charge of ion uptake to compensate for the ion loss. Enlargement of chloride cells has been reported in both gills and opercular membrane of freshwater-adapted killifish (Katoh et al., 2001), whereas chloride cells are larger in seawater than in freshwater in other euryhaline species (Langdon and Thorpe, 1985; Richman et al., 1987; Uchida et al., 1996, 2000; Shiraishi et al., 1997; Hiroi et al., 1999). In killifish gills, seawater-type chloride cells have an apical pit and interdigitate with neighboring accessory cells to form multicellular complexes, whereas the apical membrane is flat or protrusive with developed microvilli on it in freshwater-type cells (Katoh et al., 2001). A similar apical membrane structure has been observed in several species adapted to freshwater (Hossler et al., 1985; Laurent and Hebibi, 1988; Perry et al., 1992; Shawn et al., 1993; Greco et al., 1996; Perry, 1997, 1998; Kelly et al., 1999).

In spite of the occurrence of distinct freshwater- and seawater-type chloride cells, whether one cell type degenerates and is replaced by newly differentiated cells of another type, or whether chloride cells alter their functions in response to environmental salinity change, is still controversial. In addition to typical seawater- and freshwater-type cells, our SEM and TEM observations revealed the occurrence the intermediate type, which was most frequently observed at 3 h after transfer. The intermediate-type cells were accompanied by accessory cells, which is characteristic of the seawater type. However, the apical openings were larger and the pits were shallower than those observed in the typical seawater type, and more like those in the freshwater type. Through the enlarged opening, poorly developed microvilli were observed on the apical membrane, which was not evident in the seawater type. Considering that the intermediate-type cells appeared within 3 h after the transfer into freshwater, it is most probable that seawater-type cells were transformed into the intermediate type. Since freshwater-type cells, similar to those in fish fully acclimated to freshwater, were frequently observed at 12 h, the intermediate-type cells appear to be further transformed into the freshwater type. Thus, our observations indicate a plasticity of chloride cells, whereby in killifish the seawater type is transformed into the freshwater type following transfer to freshwater.

It has been demonstrated in some teleosts that chloride cells alternate their morphology and ion-transporting functions to meet abrupt environmental osmotic changes. Using a sequential observation technique, Hiroi et al. (1999) revealed that single freshwater-type chloride cells in the yolk-sac membrane of Mozambique tilapia *Oreochromis mossambicus* are transformed into seawater-type multicellular complexes during seawater adaptation. Mudskipper *Periophthalmus modestus* tolerates hypotonicity by closing the apical pit of existing seawater-type chloride cells on exposure to freshwater (Sakamoto et al., 2000). The apical pit density in the opercular membrane decreases in seawater-adapted killifish subjected to a hyposmotic shock on the basolateral surface (Daborn et al., 2001). All these findings are interpreted as an acute adaptive response of chloride cells to environmental changes.

The CFTR has been identified electrophysiologically in the apical membranes of killifish chloride cells (Marshall et al., 1995), and has been cloned and sequenced from the gills of seawater-adapted killifish (Singer et al., 1998). According to the sequence analysis, killifish CFTR has the same carboxy-terminal sequence as human CFTR (Singer et al., 1998), and thus a monoclonal antibody to human CFTR directed against this epitope is applicable to killifish. In our western blot analysis, the antibody recognized one specific protein band, of approx. 150 kDa, in agreement with the expected size of killifish CFTR (Singer et al., 1998). The result therefore confirmed the high specificity and availability of the antibody to killifish CFTR.

Marshall et al. (1999) reported that killifish CFTR expression increases at 8 h after transfer from freshwater to seawater, peaking at 24 h and remaining at levels higher than those in freshwater after 30 days in seawater. The CFTR expression was linked to the enhancement of Cl^- secretion that occurred at 24 h after transfer to seawater. In the present study, the CFTR immunoreactivity was detected in the apical membrane of chloride cells at 0, 3 and 12 h after transfer from seawater to freshwater, but disappeared at 24 h. This result is consistent with the observation by Marshall et al. (1999), in the sense that CFTR expression in chloride cells is more closely related to seawater adaptation, suggesting the involvement of CFTR-immunoreactive chloride cells in Cl^- secretion in seawater. This also indicates that pre-existing seawater-type chloride cells are able to change their function, as well as their morphology, to those of the freshwater-type.

The recovery of plasma Na^+ levels from a sharp decrease just after transfer to freshwater could be partly accounted for by the functional and morphological alteration of chloride cells from seawater to freshwater type. In addition to such an acute response, we examined chloride cell replacement as a long-term effect of freshwater transfer. In the present study, we developed the time-differential double fluorescent staining (TDS) technique, which made it possible to visualize chloride cell replacement more directly and easily than by conventional methods. Fluorescent labeling techniques provide a fast and valuable method for qualitatively and quantitatively assessing chloride cells, and mitochondria-specific probes enabled the vital staining of chloride cells (Ayson et al., 1994; Hiroi et al., 1999; Li et al., 1995; Sakamoto et al., 2000; Marshall et al., 2002). For the TDS, we adopted two specific mitochondrial probes, Rhodamine 123 and MitoTracker (MitoTracker Red CM-H_2XR_{os}), to track the turnover of branchial chloride cells.

Although the total number of chloride cells did not change in any group during the experimental 3 day period, the ratio of newly differentiated chloride cells to the total cells at the end of the experiment was markedly higher in the freshwater-transferred group (14.7%) than the freshwater- or seawater-maintained group (1.8% and 1.2%, respectively). Meanwhile, there was no significant difference between numbers in seawater- and freshwater-maintained groups. These results indicate that the turnover rate of chloride cells is relatively low under a constant osmotic environment, but is accelerated after transfer to freshwater. Under constant osmotic conditions, a small proportion of chloride cells are continuously replaced with the same type of cells. In contrast, the enhanced chloride cell turnover after transfer seems to be the result of replacement of pre-existing seawater-type chloride cells with newly differentiated, freshwater-type cells. Thus, the replacement of chloride cells after transfer not only sustains the chloride cell population, but also contributes to the alteration in ion-transporting function of the gills. This may serve as a long-term adaptative response to a different osmotic environment, together with a short-term response of morphological and functional transformation of pre-existing chloride cells. During the time-course observations on chloride cells after transfer into freshwater, a rapid increase in the chloride cell size at 12 and 24 h was followed by a gradual decrease over 1 to 3 days after transfer. In our SEM and

TEM observations, developing freshwater-type cells were occasionally observed on day 3. Such transient inactivation of chloride cells can be explained by the recruitment of newly differentiated, small freshwater-type cells, following the short-term adaptive response.

Our results contrast sharply with those from some other teleosts where the chloride cell turnover was not different between freshwater and seawater killifish. In guppy, cell differentiation and renewal of the gill epithelium were three times faster in 50% seawater-adapted fish than in freshwater-adapted fish (Chretien and Pisam, 1986). Using 5-bromo-2′-deoxyuridine (BrdU) incorporated into nuclei during DNA synthesis, Uchida and Kaneko (1996) revealed that the chloride cell turnover of chum salmon was about three times greater in seawater than in freshwater. This discrepancy between killifish and other fishes may reflect the preference of killifish for seawater over freshwater. The Na^+/K^+-ATPase activity and oxygen consumption are generally greater in seawater than in freshwater in most euryhaline teleosts, such as tilapia, salmonids and eels (Morgan et al., 1997; Uchida et al., 1996, 2000; Richman et al., 1987; Sasai et al., 1998), suggesting their freshwater preference. In these species, the chloride cell turnover seems greater in seawater, in which chloride cells consume more energy for adaptation to the less favorable environment. In contrast, there was no difference in either Na^+/K^+-ATPase activity or oxygen consumption between freshwater- and seawater-adapted killifish (Katoh et al., 2001). Since chloride cells are equally active in both media, the turnover rates may not be different between freshwater- and seawater-acclimated killifish.

In this study, we focused on the short- and long-term adaptive responses of killifish following direct transfer from seawater to freshwater. Just after transfer, when plasma Na^+ levels showed an abrupt decrease, the transformation of chloride cells occurred from the pre-existing seawater type to freshwater type *via* the intermediate type. This transformation process is accompanied by the disappearance of apically located Cl^- channel (CFTR) and neighboring accessory cells. Such morphological and functional changes are interpreted as an acute adaptive response of chloride cells to cope with the unexpected decrease in plasma ion levels following direct transfer to freshwater. On the other hand, the chloride cell turnover was enhanced after transfer, and pre-existing seawater-type chloride cells are replaced with newly differentiated, freshwater-type cells. This is considered a long-term adaptive response to the hyposmotic environment. Taken together, these results clearly indicate that the branchial chloride cells show a two-phase response in adapting to freshwater environment: the short-term functional and morphological transformation of pre-existing cells, and the long-term cell replacement by newly differentiated cells. Moreover, for examination of the chloride cell replacement, we have established the TDS technique. Compared with conventional methods using ^{3}H-thymidine or BrdU, the newly developed TDS technique provides a more convenient and precise method for assessing chloride cell turnover. Since limited information is available on chloride cell turnover, this new technique will be an excellent experimental tool for further studies on chloride cell differentiation.

We are grateful to Dr Akio Shimizu, National Research Institute of Fisheries Science, Fisheries Research Agency, for supplying the killifish. We are also grateful to Dr Junya Hiroi, St Marianna University School of Medicine, for his technical guidance and facility for CLSM. We thank Prof. Greg G. Goss, University of Alberta, for the protocol of immunoprecipitation. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. F.K. was supported by a Research Fellowship awarded by the Japan Society for the Promotion of Science for Young Scientists.

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