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Excellent Salinity Tolerance of Mozambique Tilapia (*Oreochromis mossambicus*): Elevated Chloride Cell Activity in the Branchial and Opercular Epithelia of the Fish Adapted to Concentrated Seawater

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ABSTRACT—Changes in morphology and cellular activity of the chloride cells in branchial and opercular epithelia were examined in tilapia, *Oreochromis mossambicus*, adapted to fresh water (FW), seawater (SW) and concentrated SW (180% SW). The tilapia are adaptable to a wide range of salinity, maintaining the plasma osmolality within physiological levels. Gill Na^+, K^+-ATPase activity was remarkably increased in response to elevated environmental salinity. Using immunocytochemical staining with an antiserum specific for α-subunit of Na^+, K^+-ATPase, chloride cells were detected on the afferent half of the filament epithelia. The size of immunoreactive chloride cells was twice larger in SW and four times larger in 180% SW than in FW. Confocal laser scanning microscopic observations revealed the frequent occurrence of chloride cell complexes under hypersaline conditions. By electron microscopy, a deeply invaginated apical crypt and well-developed tubular network were observed in chloride cells of SW- and 180% SW-adapted fish, indicating enhanced cellular activity. Chloride cells present in the opercular membrane were also developed in response to increased salinity. These findings suggest that highly activated chloride cells in branchial and opercular epithelia may be responsible for salt secretion in hyperosmotic environments. The excellent salinity tolerance of tilapia appears to be attributed to their ability to develop chloride cells in response to increased environmental salinity.

INTRODUCTION

Teleost fishes inhabiting both fresh water (FW) and seawater (SW) maintain the osmolality of their body fluid at a relatively constant level. Plasma osmolality is usually maintained around 300 mOsm kg^{-1}, which is about one third of SW osmolality and is still higher than the FW level (Evans, 1993). Regulation of water and ions in fish takes place mainly in the gills, kidney and intestine, creating ionic and osmotic gradients between the body fluid and external environments.

Chloride cells in the gill epithelium and opercular membrane are important osmoregulatory sites in maintaining ionic balance in fish (Marshall, 1995; McCormick, 1995). The cells are characterized by the presence of a rich population of mitochondria and an extensive tubular system in the cytoplasm. The tubular system is continuous with the basolateral membrane, resulting in a large surface area for the placement of ion transporting proteins such as sodium-potassium adenosinetriphosphatase (Na^+, K^+-ATPase), a key enzyme for chloride cell activities (Hootman and Philpott, 1979). The chloride cells have been implicated in ion secretion in SW (Marshall, 1995; Zadunaisky, 1997) and possibly in ion uptake in FW (Perry, 1997).

Morphological changes in chloride cells in response to environmental salinity have been observed in several teleost species. In general, when euryhaline teleosts are transferred from FW to SW, chloride cells are increased in number and size, along with an increase in Na^+, K^+-ATPase activity (McCormick, 1995). Furthermore, on the basis of their location and response to SW transfer, two different types of chloride cells were identified in the gills of chum salmon (*Oncorhynchus keta*) and Japanese eel (*Anguilla japonica*) (Uchida *et al.*, 1996, 1997; Sasai *et al.*, 1999). Filament chloride cells activated in SW are likely to play a central role in ion secretion in SW, and lamellar chloride cells that disappear during SW adaptation seem to be involved in ion uptake in FW.

Tilapia, the genus *Oreochromis*, are widely distributed in the wild and cultivated in fish farms owing to their hardy nature, rapid growth rates and tolerance of varied environmental salinity (Pullin, 1991). Among these tilapia species,

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the Mozambique tilapia (*O. mossambicus*) is a suitable model for studies on osmoregulatory mechanisms, because this euryhaline tilapia is adaptable to a wide range of salinity from FW to SW (Suresh and Lin, 1992; Vonck *et al.*, 1998). Furthermore, the tilapia possesses excellent salinity tolerance for surviving even in concentrated SW (Dange, 1985; Suresh and Lin, 1992; Kültz *et al.*, 1995; Nakano *et al.*, 1997). Such strong euryhalinity of the tilapia intrigues us to explore the mechanisms of osmoregulation and salinity tolerance. In the present study, we investigated changes in morphology and cellular activity of chloride cells in the gills and opercular membrane in FW-, SW- and concentrated SW-adapted tilapia. Our findings indicate that excellent salinity tolerance of the tilapia is attributable to their ability to develop chloride cells in response to increased environmental salinity.

MATERIALS AND METHODS

Fish

Adult tilapia (*Oreochromis mossambicus*) were collected from a FW pond in northern Okinawa Island, Japan, and were maintained in tanks (200-*l*) with recirculating FW at 25°C under a natural photo period. They were fed on artificial tilapia pellets, "Tilapia 41M" (Shikoku Kumiai Shiryo, Tokushima, Japan), once a day. FW-acclimated male tilapia, weighing 130–150 g, were separated into two groups: one maintained in FW (n=9) and the other transferred to half strength SW (50% SW, n=17). The latter fish were kept in 50% SW for 10 days and then adapted gradually to full strength SW (100% SW, Cl^-: 548 mM) for 4 days. SW-acclimated fish were subdivided into two groups: one maintained in SW (n=7) and the other exposed to concentrated SW (180% SW, Cl^-: 935 mM, n=10). To prepare the concentrated SW, artificial SW powder, "Aqua salz" (Nissei Sangyo, Tokyo, Japan) was gradually added to normal SW during 2 weeks. For full acclimation to concentrated SW, the fish were kept in 180% SW for 2 more weeks. Fish of the three experimental groups were killed after completion of acclimation to 180% SW. No fish died during the acclimation period in any experimental group.

Plasma osmolality

To evaluate the adaptability of the tilapia to different environmental salinities, plasma osmolality was measured. After anesthetized with 0.05% 2-phenoxyethanol, blood was collected from the caudal vessels with a syringe. The blood plasma was immediately separated by centrifugation at 10,000 rpm for 5 min. Osmolality was measured with a vapor pressure osmometer (Wescor 5500, Logan, UT).

Gill Na^+, K^+-ATPase activity

Gill Na^+, K^+-ATPase activity was examined in FW-, SW- and 180% SW-adapted fish. Gill filaments were dissected out and stored in 200 μl SEI buffer (150 mM sucrose, 10 mM Na_2EDTA and 50 mM imidazole) at –80°C until analyses. Na^+, K^+-ATPase activity was measured as described in Uchida *et al.* (1996).

Fluorescent staining of chloride cells in the opercular membrane

The appearance and size of chloride cells in the opercular membrane were examined in tilapia adapted to FW, SW and 180% SW. Chloride cells in whole-mount preparations of the opercular membrane were localized using 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI, Sigma, St. Louis, MO), a fluorescent probe specific for mitochondria (Bereiter-Hahn, 1976). The opercular membrane was carefully removed from the operculum and incubated for 1 hr in 5 ml Ringer solution (concentrations in mM: Na^+, 122; Cl^-, 124; K^+, 3; Mg^{2+}, 1.25; Ca^{2+}, 1; SO_4^{2-}, 1.25; PO_4^{3-}, 0.4; CO_3^{2-}, 2, pH 7.4) containing 2 μM DASPEI. The membrane was then rinsed with Ringer and mounted on glass slides, coverslipped and examined with a fluorescence microscope (Nikon X2F-EFD2, Tokyo). Excitation and emission filters used were an EX 450-490 and a BA 520-560, respectively. Photomicrographs were taken on three fields for each experimental fish (n=3) with slide film (Fujichrome 400D, Fuji Film, Tokyo).

Chloride cell size was measured from slides magnified by a profile projector. The outlines of 10 chloride cells per slide were traced on transparent plastic, and the size was measured using a digitizer (KD 4600, Graphtec, Tokyo). The actual cell size was corrected using a micrograph of an objective micrometer slide taken at the same magnification.

Western blot analysis

A specific polyclonal antibody against the α-subunit of Na^+, K^+-ATPase was raised in a rabbit by Sawady Technology Co. Ltd. (Tokyo). The antigen designed was Cys-Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys-Ser. The amino acid sequence of the synthetic peptide was based on sequences of high homology and the areas of hydrophilicity of Na^+, K^+-ATPase α-subunit as described in Ura *et al.* (1996). The antigen was emulsified with complete Freund's Adjuvant, and immunization was performed in a New Zealand white rabbit.

The specificity of the raised antiserum, named NAK 121, was confirmed using Western blot analysis. Membrane fractions were prepared from the gills of 180% SW-adapted tilapia. The gills were homogenized on ice in homogenization buffer consisting of 25 mM Tris (pH 7.4), 0.25 M sucrose and a pellet of Complete Protein Inhibitor (Boehringer Mannheim, Mannheim, Germany). The homogenate was first centrifuged at 4,500×g for 15 min, and the supernatant was subjected to 200,000×g for 1 hr. The pellet was resuspended in the homogenization buffer. All above procedures were performed at 4°C. Protein content of the sample was quantified by the BCA Protein Assay kit (Pierce, Rockford, IL). The samples (12 μg) were solubilized in a sample loading buffer (0.25 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% βmercaptoethanol, 30% glycerol, 0.01% bromophenol) and heated at 70°C for 15 min. They were separated by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels. As a molecular length marker, Prestained SDS-PAGE Standards (188–7.3 kDa, BIO-RAD, Hercules, CA) was electrophoresed in parallel. After electrophoresis, the protein was transferred from gel to a polyviniliden difluoride (PVDF) membrane (ATTO Co. Tokyo).

The membranes were preincubated in 50 mM Tris-buffered saline (TBS, pH 7.6) containing 0.05% Triton X 100 and 2% skim milk at 4°C overnight and were then incubated with the primary antiserum for 1 hr at room temperature. The primary antiserum was diluted at 1: 400 with 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 10% normal goat serum, 0.1% bovine serum albumin, 0.1% keyhole limpets hemocyanin (Sigma), 0.05% Triton X-100 and 0.01% sodium azide (NB-PBS). The specificity of the immunoreaction was also confirmed by incubating the membranes with normal rabbit serum at the same dilution. After rinsing in washing buffer (TBS, 0.05% Triton X-100, 0.2% skim milk), the membranes were stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981), using commercial reagents (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Briefly, the blots were incubated sequentially with biotinylated anti-rabbit IgG for 1 hr and ABC for 1 hr at room temperature. The blots were finally incubated with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 3 min to visualize the immunoreactive bands.

Immunocytochemical detection of gill chloride cells

Branchial chloride cells were detected immunocytochemically using the antiserum NAK 121. Gills were dissected out and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 20 hr at 4°C. The second gill arch was removed, dehydrated in

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ethanol, and embedded in paraplast. Serial sagittal and cross sections (4 μm) were cut and mounted on gelatin-coated slides. The sections were immunocytochemically stained using Vectastain ABC kit (Vector Laboratories) as described in Uchida *et al.* (1996). The primary antiserum at 1: 4000 dilution was applied to the sections overnight at 4°C. The specificity of the immunoreaction was confirmed by incubating the section with normal rabbit serum at the same dilution. The sections were observed under a microscope (Nikon) equipped with a differential interference contrast device.

For quantitative analyses, the size of chloride cells was used as a criterion of chloride cell activity. The cell size was measured using an image analyzer (Argus-20, Hamamatsu Photonics, Hamamatsu, Japan). From each experimental fish (n=3), about 10 cells sectioned near the center and including the nucleus were randomly selected. The cell size was expressed as sectional area in μm^2.

Confocal laser scanning microscopic observations on gill chloride cells

To further investigate the localization and cellular activity of the chloride cells in the gill epithelium, a whole mount preparation of the gill filament was immunocytochemically stained with the antiserum and examined by confocal laser scanning microscopy (LSM). Prior to the whole-mount immunostaining, the antiserum was affinity-purified and labeled with fluorescein isothiocyanate (FITC, Sawady Technology Co. Ltd).

For the detection of chloride cells, gills were fixed as described above. The filaments were removed from the second gill arch, washed in PBS, and incubated with FITC-labeled anti-Na^+, K^+-ATPase diluted 1: 500 with NB-PBS at 4°C overnight. After rinsing in PBS, the samples were mounted on glass slides and examined with a confocal laser scanning microscope (LSM310, Zeiss, Oberkohen, Germany). The 488 nm line of an argon ion laser was used as the excitation wavelength, and the emission was collected at 515–565 nm. Digital images (512×512) of serial optical sections were obtained at intervals of 20 μm and overlaid to produce an image of great depth of focus.

Transmission-electron microscopy

Small pieces of the second gill arch were fixed in 2% PFA-2% glutaraldehyde (GA) in 0.1 M PB (pH 7.4) for 6 hr at 4°C. The tissues were then post-fixed in 1% osmium tetroxide in the same buffer for 1 hr. After dehydration in ethanol, the tissues were transferred to propylene oxide and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife and mounted on grids. The sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Hitachi H-7100, Tokyo).

Scanning-electron microscopy

Gills were fixed in 2% PFA-2% GA in 0.1 M PB (pH 7.4) for 6 hr at 4°C and postfixed in 1% osmium tetroxide in the same buffer for 1 hr at room temperature. The samples were dehydrated in ethanol, immersed in 2-methyl-2-propanol, and dried using a freeze-drying device (JEOL JFD-300, Tokyo). Dried samples were mounted on specimen stubs, coated with gold in an ion sputter (JEOL JFC-1100) and examined with a Hitachi S-2150 scanning electron microscope.

Statistical analysis

All data are presented as means±S.E.M. Significance of differences was determined by Student's *t*-test or the Cochran-Cox test after variance analysis by *F*-test.

RESULTS

Plasma osmolality and gill Na^+, K^+-ATPase activity

Plasma osmolality of the tilapia adapted to FW, SW and 180% SW was between 310–350 mOsm kg^{-1} (Fig. 1A). The levels in SW- and 180% SW-adapted fish were significantly ($P<0.001$) higher than those in FW-adapted groups. There was no significant difference between SW and 180% SW groups. The gill Na^+, K^+-ATPase activity was increased in accordance with elevated environmental salinity (Fig. 1B). The activities were twice higher in SW-adapted fish and 5 times higher in 180% SW-adapted fish than that in FW-adapted fish.

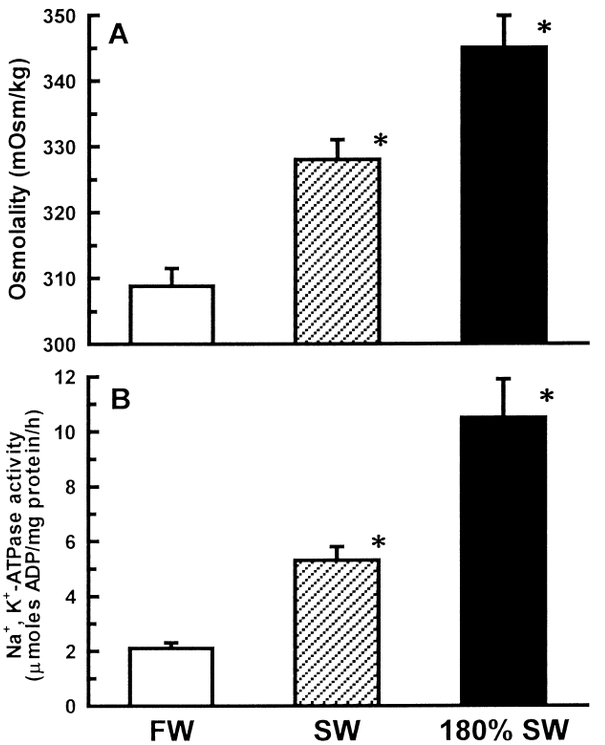


Fig. 1. Plasma osmolality (A) and gill Na^+, K^+-ATPase activity (B) in tilapia adapted to fresh water (FW), seawater (SW) and concentrated SW (180% SW). Data are expressed as mean±S.E.M. FW, n=9; SW, n=7; 180% SW, n=10. *$P<0.001$, significantly different from the value of FW-adapted group.

Chloride cells in the opercular membrane

A considerable number of chloride cells were present in the opercular membrane, with which the operculum is lined, in FW-, SW- and 180% SW-adapted tilapia (Fig. 2). DASPEI-positive chloride cells in the opercular membrane were developed with increasing salinity. Compared with FW-adapted fish, the sectional area of the opercular chloride cells was significantly larger in both SW- and 180% SW-adapted fish ($P<0.001$). The size was three times larger in 100% SW, and five times larger in 180% SW than that in FW (Fig. 3A).

Western blot analysis

Membrane fractions were prepared from the gills of 180% SW-adapted tilapia and analyzed by Western blot using the antibody against the synthetic peptide based on sequences of high homology of Na^+, K^+-ATPase α-subunit (Fig. 4). The antibody recognized a major protein band with an approxi-

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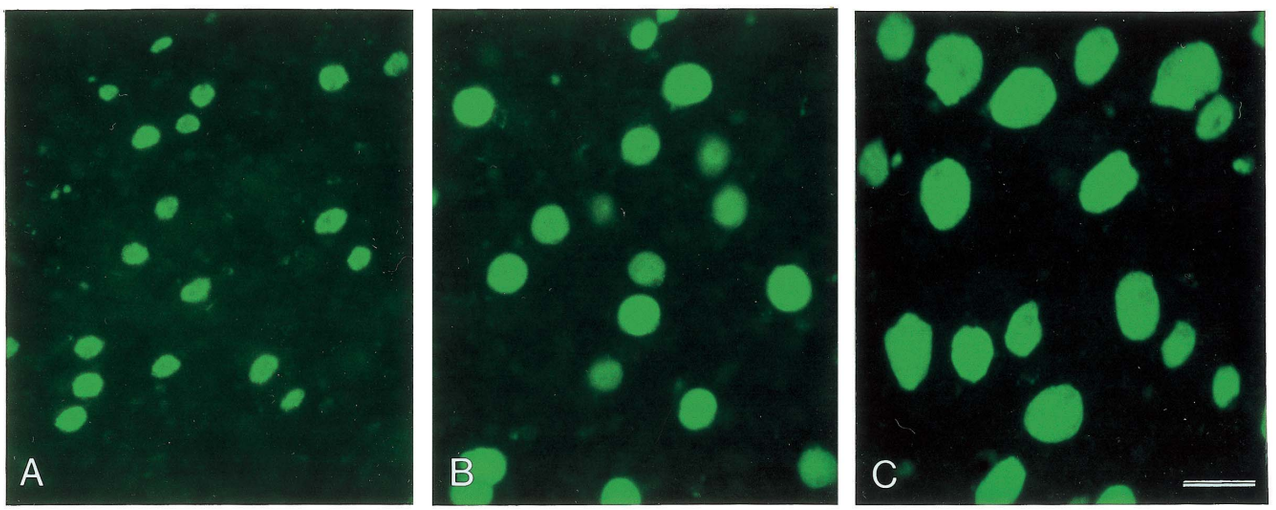


Fig. 2. DASPEI-positive chloride cells in the opercular membrane in tilapia adapted to fresh water (A), seawater (B) and concentrated SW (C). Chloride cells in the opercular membrane became larger with increasing salinity. Bar: 20 μm.

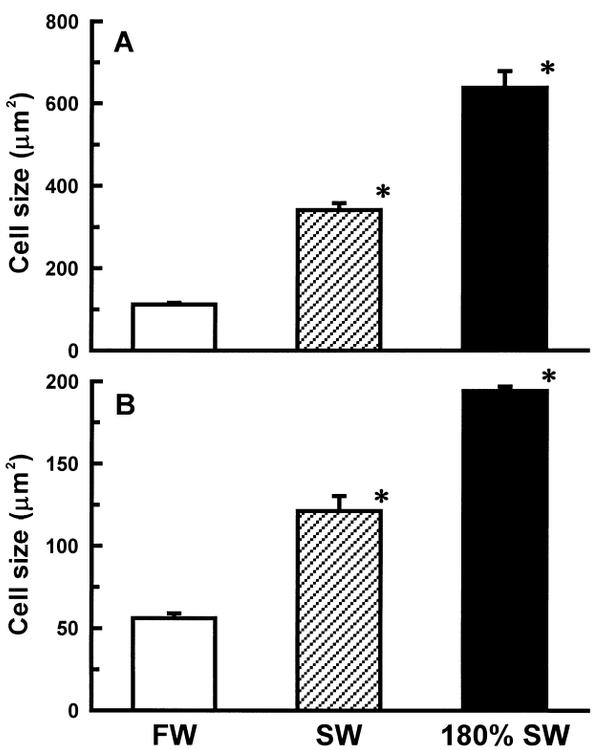


Fig. 3. Size of chloride cells in opercular membrane (A) and gills (B) of tilapia adapted to fresh water (FW), seawater (SW) and concentrated SW (180% SW). Data are expressed as mean±S.E.M. of 30 cells from 3 individuals. *P<0.001, significantly different from the value of FW-adapted group.

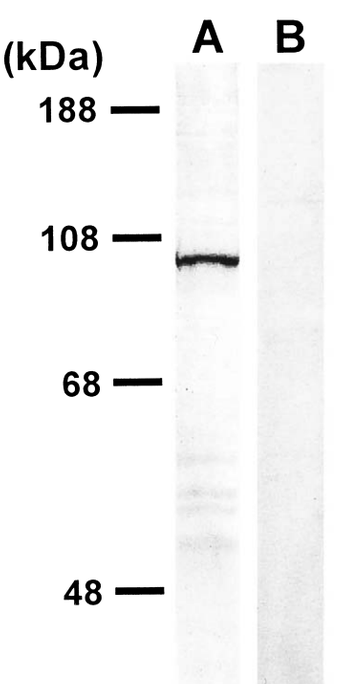


Fig. 4. Western blot analysis for Na^+, K^+-ATPase protein expressed in tilapia adapted to concentrated seawater. The membranes were incubated with an antiserum specific for Na^+, K^+-ATPase (lane A) and with normal rabbit serum (lane B). Note a single band of approximately 100 kDa, corresponding to a predicted molecular mass of Na^+, K^+-ATPase protein. Positions of molecular weight markers, expressed in kDa, are indicated on the left side of the figure.

mate size of 100 kDa. No band appeared when membranes were incubated with normal rabbit serum.

Light microscopic observations on gill chloride cells

To determine morphological changes in branchial chloride cells, the gills were immunocytochemically stained using the antiserum specific for Na^+, K^+-ATPase. Na^+, K^+-ATPase-immunoreactive (ir) chloride cells were detectable mainly in the filament epithelia (Fig. 5). They were usually round or

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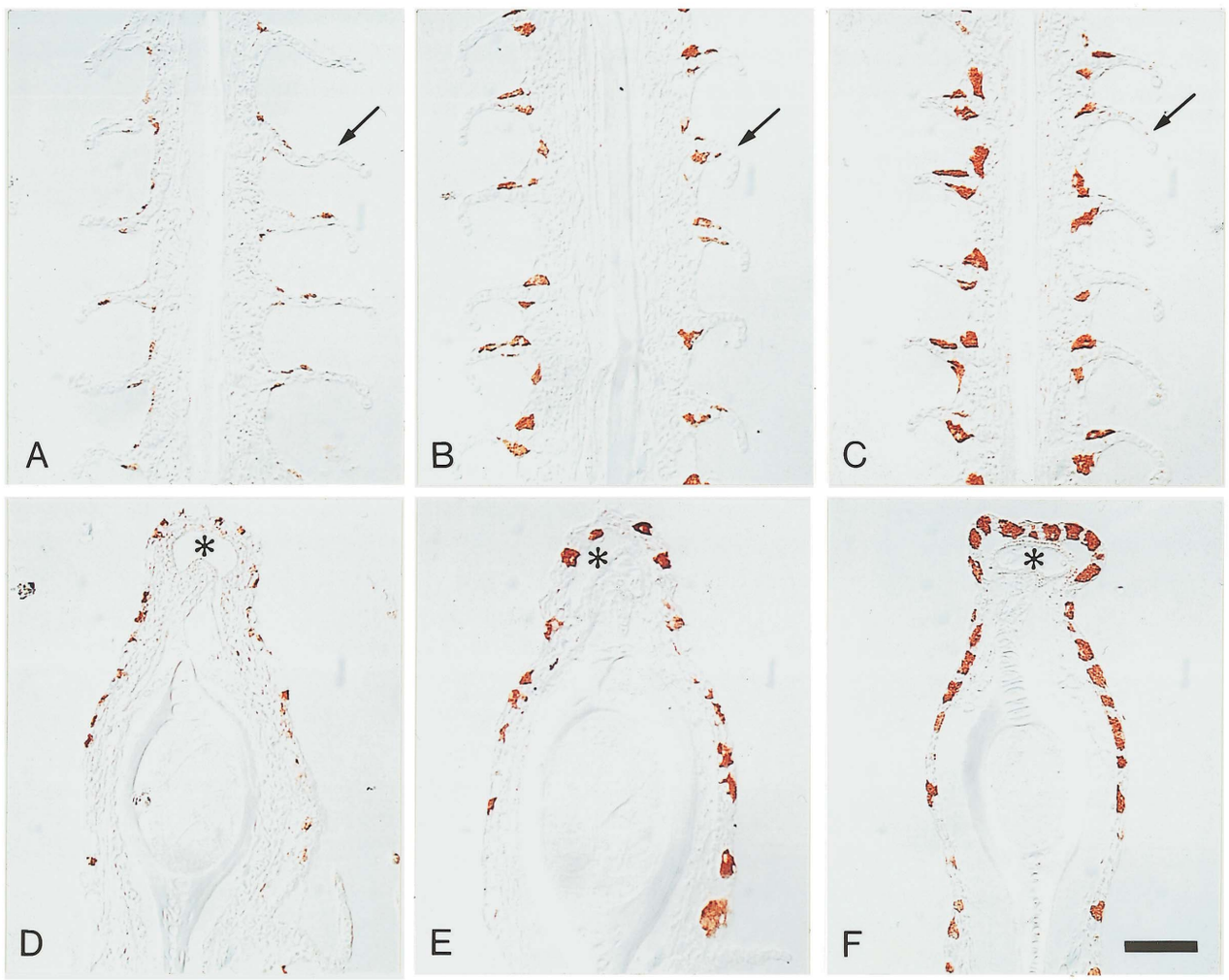


Fig. 5. Immunocytochemical localization of chloride cells in the gills of tilapia adapted to fresh water (A and D), seawater (B and E) and concentrated SW (C and F). The sagittal (A–C) and cross sections (D–F) of the gill filament were incubated with an antiserum specific for Na^+, K^+-ATPase. Immunoreactive chloride cells are localized on the afferent side of the filament epithelia and developed with increasing environmental salinity. Arrows, gill lamella. Asterisks, afferent artery. Bar: 30 μm.

columnar in shape. Sagittal sections stained with anti-Na^+, K^+-ATPase showed that chloride cells were mainly present at the base of lamellae, the interlamellar region and the edge of the afferent vascular side, whereas chloride cells were scarcely observed on the lamellar epithelia in any fish (Figs. 5A–C). Furthermore, cross-sectional observations clearly showed that chloride cells were localized on the afferent half of the filament, particularly at the flat region of the afferent edge where lamellae were absent (Figs. 5D–F). When the sections were incubated with normal rabbit serum, no ir-cells were observed (data not shown). The quantitative analyses were made on the afferent side of the filament, since chloride cells were more abundant on the afferent half than on the efferent. Chloride cells were significantly larger well in accordance with increased environmental salinity (Fig. 3B).

Confocal laser scanning microscopic observations on gill chloride cells

To further reveal the localization and cellular activity of the chloride cells in the gill epithelium, a whole-mount preparation of the gill filament was stained with anti-Na^+, K^+-ATPase serum and observed by confocal laser scanning microscopy. In the three experimental groups, chloride cells were localized mostly at the flat region of the afferent side and interlamellar regions of the filament (Figs. 6A–C), as shown in the light microscopic observations. Ir-chloride cells were scarcely observed on the lamellar epithelia. Three dimensional analysis of corresponding fluorescent images showed increased intensity of the immunoreaction for Na^+, K^+-ATPase and density of the chloride cells with increased environmental salinity (Figs. 6D–F). As evidenced by the presence of more than one immunonegative nucleus, chloride cells in both SW- and 180% SW-adapted tilapia often formed a

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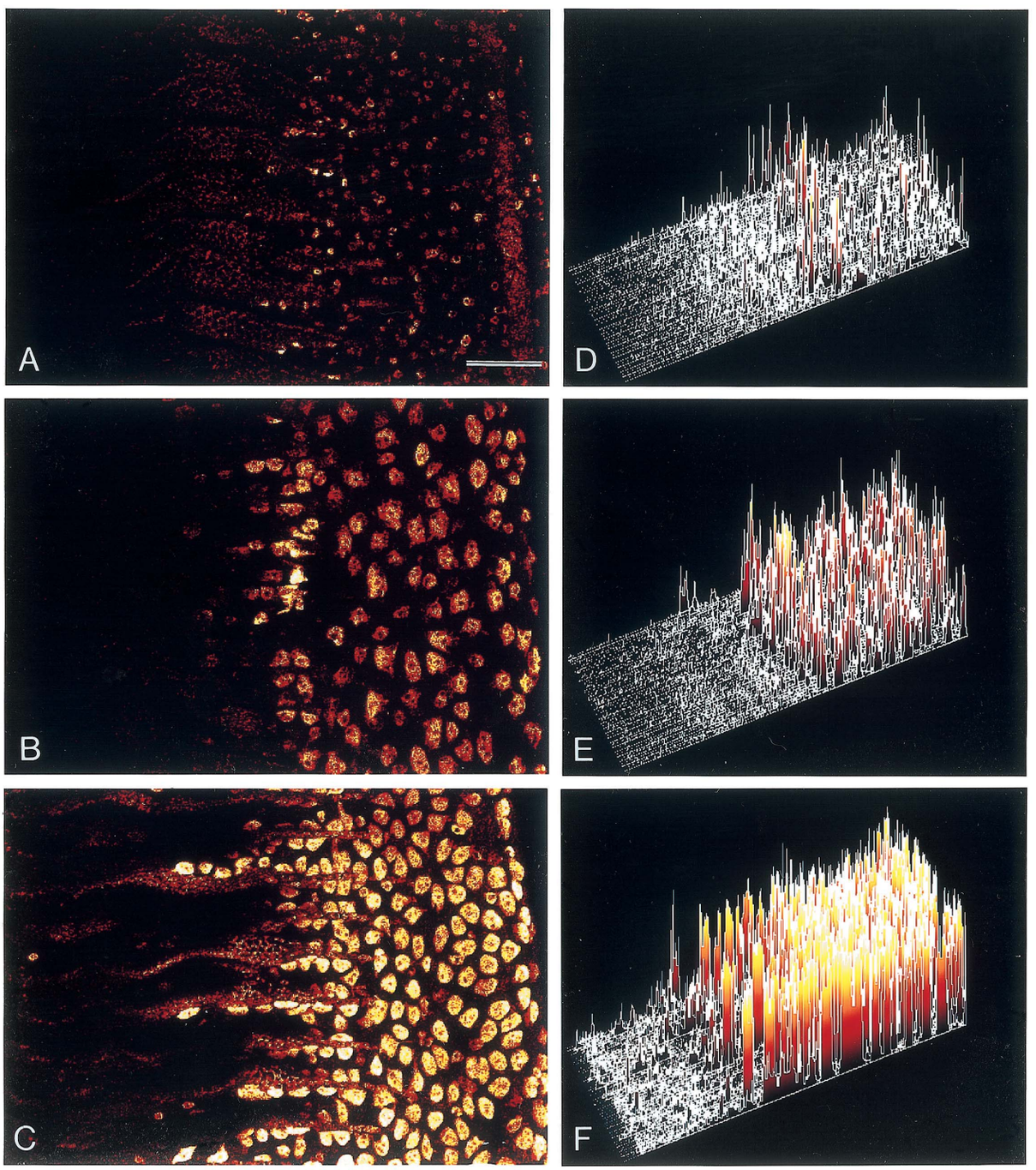


Fig. 6. Confocal laser scanning micrographs of the whole mount preparations of gill filaments stained with anti-Na^+, K^+-ATPase in tilapia adapted to fresh water (FW, A), seawater (SW, B) and concentrated SW (180% SW, C). Three dimensional profiles of corresponding fluorescent images represent intensity of the immunoreaction for Na^+, K^+-ATPase and density of chloride cells in the gills of FW (D)-, SW (E)- and 180% SW (F)-adapted tilapia. Note that immunoreactive chloride cells are observed mainly on the afferent side of the filament and well developed with increasing salinity. Bar: 80 μm.

multicellular complex (Fig. 7B, C), whereas such structures were rarely observed in FW fish (Fig. 7A).

Electron-microscopic observations on gill chloride cells

By transmission-electron microscopy, the chloride cells in the filament epithelia were readily identified by their typical morphological features: the presence of numerous mitochondria and an extensive tubular system in the cytoplasm (Fig. 8). The cells were round and columnar in shape with a basally-located nucleus, and were in contact with external

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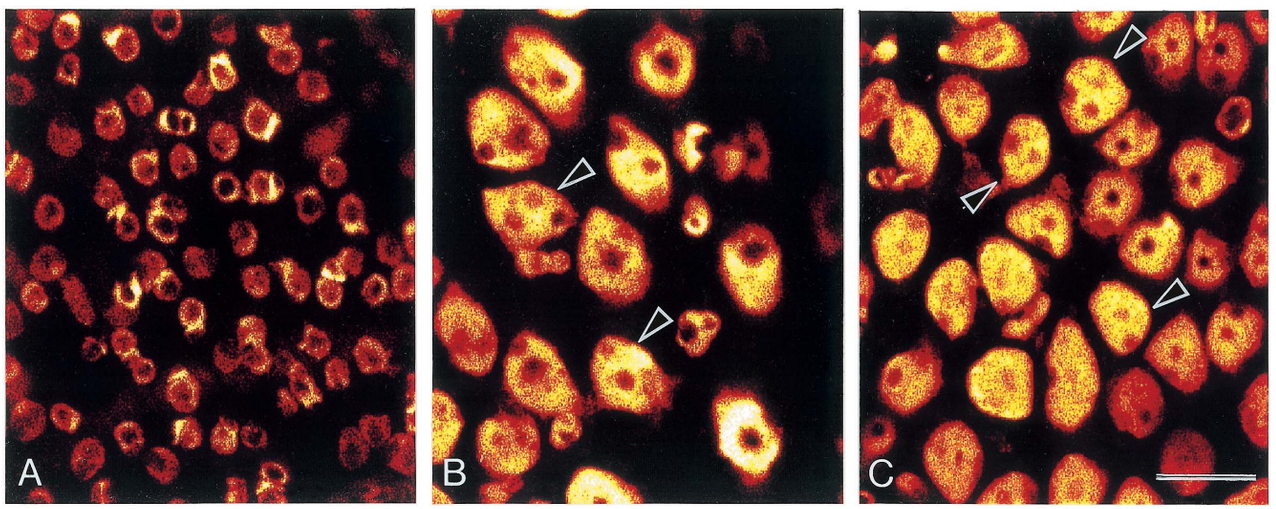


Fig. 7. Magnified views of gill chloride cells in tilapia adapted to fresh water (FW, A), seawater (SW, B) and concentrated SW (180% SW, C) by confocal laser scanning microscopy. The whole mount preparations were stained with anti-Na^+, K^+-ATPase. Chloride cells in both SW- and 180% SW-adapted tilapia frequently form multicellular complexes (arrowheads), whereas such a multicellular complex was rarely observed in FW fish. Bar: 20 μm.

environments via their apical membrane on the mucosal side, and with internal environments via the basolateral membrane on the serosal side (Figs. 8A–C). Their ultrastructure showed marked differences among the three experimental groups. In FW fish, the chloride cells with a shallow apical pit were small and contained moderately developed tubular systems in the cytoplasm (Fig. 8D). The cells were well developed in the SW-adapted fish: the cells were larger than in FW fish, often extending to the basal membrane, and the tubular network in the cytoplasm was more dense than that in FW fish (Fig. 8E). The chloride cells in 180% SW-adapted fish were most developed: the cells showed higher electron density with numerous mitochondria and an expanded tubular system in the cytoplasm (Fig. 8F). The tubular system also formed the densest network of anastomosed tubules, compared with other experimental groups. In both SW and 180% SW fish, the apical membrane was deeply invaginated to form an enlarged apical pit, and two or three cells congregated and shared a common apical pit, forming a multicellular complex, as was also evident in whole mount immunocytochemistry (Fig. 8B, C).

Scanning-electron microscopic observations revealed that the chloride cells had numerous apical openings in contact with external environments (Fig. 9). They were located at the boundary of the pavement cells. The pavement cells covered a large part of the chloride cells except for the apical openings. The apical openings were frequently observed on the afferent edge of the filament epithelia, whereas the openings were rarely observed on the lamellar epithelia (Fig. 9A). The openings were more numerous in SW-adapted fish than in FW-adapted fish, and they were most abundant in 180% SW-adapted fish (Figs. 9B–D). The size of the openings also became larger in hypersaline conditions.

DISCUSSION

Several tilapia species have been widely used as models in studies on fish osmoregulation because of their excellent euryhalinity. Among tilapia species, *O. mossambicus* seems to be most tolerant of high salinity (Suresh and Lin, 1992). In the present study, FW-acclimated tilapia were well adapted to full-strength SW, and even to 180%-concentrated SW. The plasma osmolality of those fish was 310–350 mOsm kg^{-1}, which is a typical plasma osmolality level in teleost fish, indicating their successful adaptation to these salinities. Similar results were obtained in tilapia adapted to FW, SW and 160% SW (Nakano *et al*., 1997).

Gill Na^+, K^+-ATPase plays a central role in secreting excess monovalent ions in SW and its activity serves as a reliable indicator of SW adaptability in various fish species (McCormick, 1995). The elevation of gill Na^+, K^+-ATPase activity and capability of fish to tolerate hyperosmotic environments are well correlated not only in migratory salmonids (Boeuf, 1993; Uchida *et al*., 1996) but also in tilapia (Dange, 1985; Young *et al*., 1988; Kültz *et al*., 1992). The enzyme activity obtained in the present study was remarkably increased with increasing salinity, as reported in a previous study (Dange, 1985).

Na^+, K^+-ATPase is composed of two different protein subunits, a catalytic α-subunit with a molecular weight of about 100 kDa and a glycosylated β subunit with a molecular weight of about 50 kDa. Molecular approaches have revealed the genes of Na^+, K^+-ATPase α and β subunits and expression of these genes in fish osmoregulatory organs (Kisen *et al*., 1994; Cutler *et al*., 1995a, b; Hwang *et al*., 1998). In the present study, a specific antibody was raised against a synthetic peptide, which was based on a highly conserved sequence of the α subunit in different vertebrates and invertebrates, as

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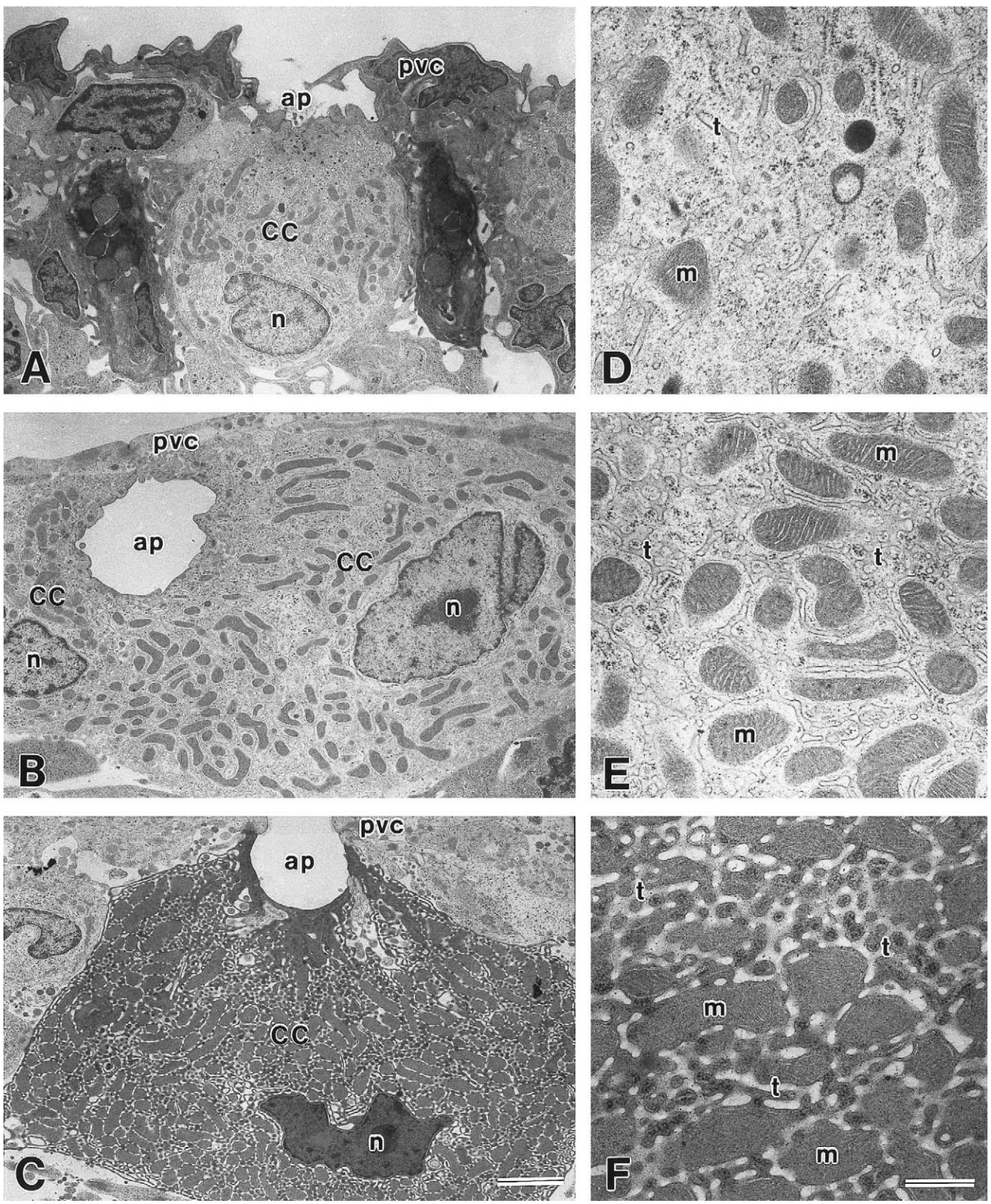


Fig. 8. Transmission electron micrographs of chloride cells in the gills of tilapia adapted to fresh water (FW, A), seawater (SW, B) and concentrated SW (180% SW, C). D, E, F: Magnified views of chloride cells in FW (D)-, SW (E)- and 180% SW (F)-adapted fish. Note the numerous mitochondria (m) and well-developed tubular system (t) of the chloride cells in 180% SW adapted fish compared with those in FW- and SW-adapted fish. cc: chloride cell, pvc: pavement cell, ap: apical pit, n: nucleus. Bars: 2 μm in A–C; 0.5 μm in D–F.

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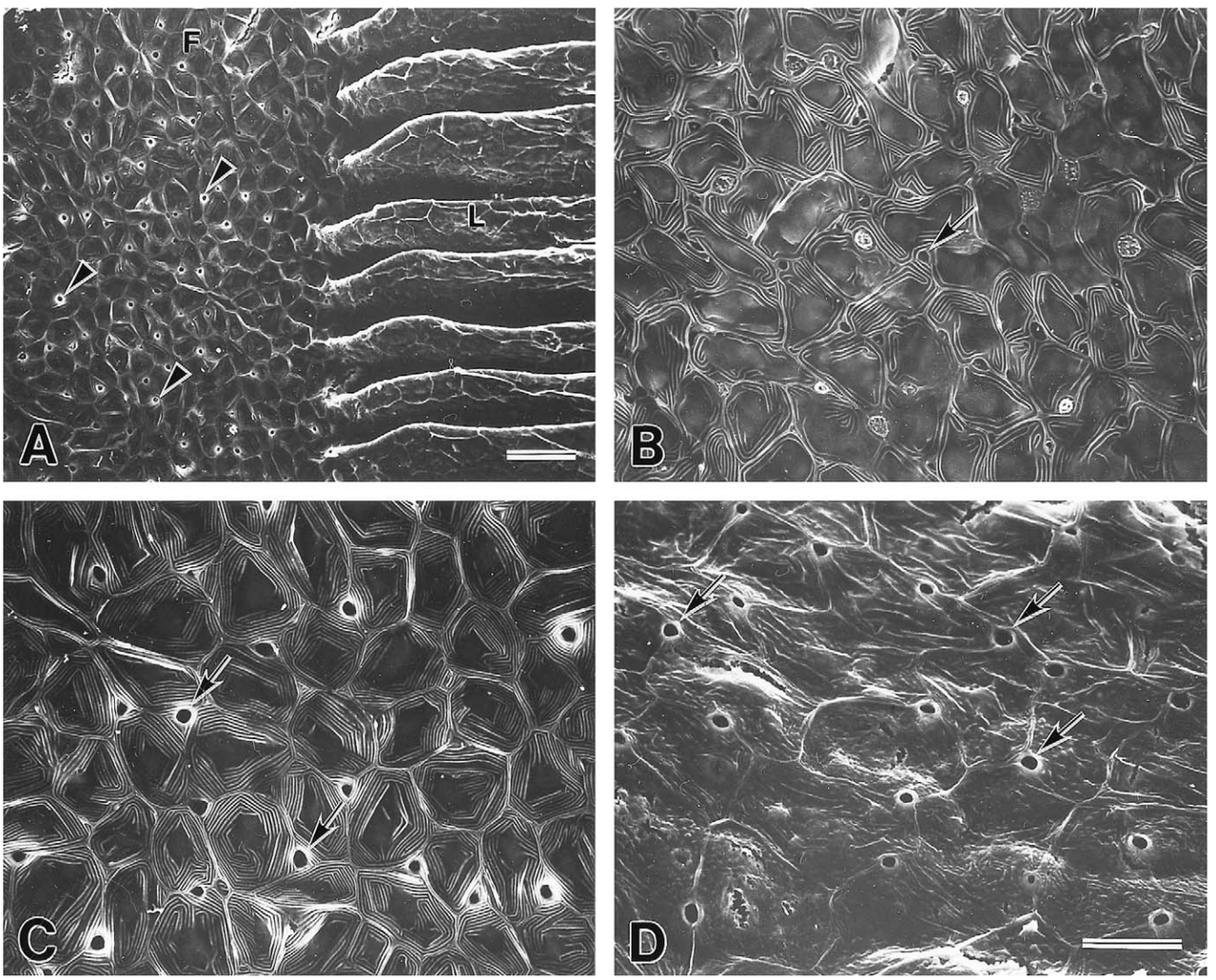


Fig. 9. A: Scanning electron micrograph of the gills in tilapia adapted to seawater (SW). Apical openings (arrowheads) are frequently observed on the afferent side of the filament epithelia. B, C, D: Magnified views of afferent side of the filament epithelia in fresh water (B)-, SW (C)- and concentrated SW (D)-adapted tilapia. Apical openings (arrows) are well developed with increasing salinity. F: filament epithelia, L: lamellar epithelia. Bars: 20 μm in A; 10 μm in B–D.

described in Ura *et al*. (1996). By Western blot analysis, the antibody reacted with only a single protein band of about 100 kDa, corresponding to a predicted molecular mass of Na^+, K^+-ATPase α subunit (Hwang *et al*., 1998). Therefore, the result strongly indicates the high specificity of the antiserum to Na^+, K^+-ATPase α subunit.

The antibody specifically recognized large spherical cells located in the filament epithelia, as observed in other immunocytochemical studies (Ura *et al*., 1996; Uchida *et al*., 1996, 1997; Witters *et al*., 1996; Shikano and Fujio, 1998). Na^+, K^+-ATPase is located in the tubular membrane, which is continuos with the basolateral membrane, in branchial chloride cells (Karnaky *et al*., 1976; Hootman and Philpott, 1979). In our electron-microscopic observations, the chloride cells in the filament epithelia were readily identified by the presence of numerous mitochondria and an extensive tubular system in the cytoplasm. The extensive distribution of the tubular system in the cytoplasm may result in labeling over the cell with the nucleus unstained. On the basis of their morphology and location, Na^+, K^+-ATPase-ir cells observed in tilapia gills coincide well with mitochondria-rich chloride cells. The antiserum used here is also applicable to the detection of chloride cells in chum salmon, Japanese eel and killifish (our unpublished observations). Therefore, the antiserum raised in the present study appears to be an excellent marker for the detection of chloride cells by immunocytochemical staining.

Na^+, K^+-ATPase-ir cells in FW-, SW- and 180% SW-adapted tilapia were generally localized on the afferent half of the filament epithelia, whereas chloride cells were rarely observed on the lamellar epithelia. These findings are consistent with previous reports in the tilapia (Kültz *et al*., 1995; Lee *et al*., 1996; Van der Heijden, 1997). In contrast, our previous studies demonstrated two different types of chloride cells in the filament and lamellar epithelia of chum salmon (Uchida *et al*., 1996, 1997). Filament chloride cells were activated in SW and lamellar chloride cells were mainly observed in FW, sug-

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gesting that they may act as sites responsible for salt secretion in SW and ion uptake in FW, respectively. Similar responses of filament and lamellar chloride cells to environmental salinities were also observed in sea bass and Japanese eel (Hirai *et al.*, 1999; Sasai *et al.*, 1999). There might be a considerable difference in chloride cell distribution in different species.

The osmoregulatory function of branchial chloride cells in SW is to secrete excess monovalent ions (McCormick, 1995). Activation of chloride cells was observed, when several species of teleosts were transferred from FW to SW (Pisam and Rambourg, 1991; Yoshikawa *et al.*, 1993; Kültz *et al.*, 1995; Uchida *et al.*, 1996; Van der Heijden *et al.*, 1997). In smolting salmonids, chloride cell activity was gradually enhanced in association with increased gill Na^+, K^+-ATPase activity, resulting in successful adaptation to SW (see reviews by Boeuf, 1993; Clarke and Hirano, 1995). In the present study, the chloride cell size was markedly increased well in accordance with elevated environmental salinity. Furthermore, according to LSM observations, the density and Na^+, K^+-ATPase immunoreactivity of the chloride cells were highly enhanced in response to increased salinity. Thus, observed increases in size and density, as well as intensity of the immunoreaction, seem to reflect increases not only in whole gill Na^+, K^+-ATPase activity but also in the enzyme content in chloride cells.

LSM observations revealed the frequent occurrence of chloride cell complexes in the gills of SW- and 180% SW-adapted tilapia, whereas such a multicellular complex was rarely observed in FW. The multicellular complex, which usually consists of chloride cells and adjacent accessory cells, has been described in several teleost species (Hwang, 1987, 1988; Pisam *et al.*,1989; Wendelaar Bonga *et al.*, 1990). Shiraishi *et al.* (1997) have demonstrated that the complex in the yolk sac membrane of tilapia larvae adapted to SW possesses multiple shallow junctions on the cytoplasmic processes of the accessory cells extending to the apex of the main cell. Such structures of the multicellular complex could be advantageous to sodium ion extrusion, since sodium ion is probably secreted through a paracellular pathway down its electrical gradient in SW (Marshall, 1995; McCormick, 1995). The occurrence of the multicellular complex in adult tilapia adapted to hypersaline conditions also suggests enhanced chloride cell activity and its significant function as the site for salt secretion.

Accessory cells have been considered to be a population of partially differentiated chloride cells, since mitochondria and tubular system are moderately developed in the cytoplasm (Hootman and Philpott, 1980; Pisam, 1981; Wendelaar Bonga *et al.*, 1990). In chum salmon fry, filament chloride cells are replaced continuously by newly-differentiated cells in both FW and SW (Uchida and Kaneko, 1996). The turnover of these cells is much greater in SW than in FW. Considering the strong euryhalinity of the tilapia, the formation of chloride cell complexes may also reflect accelerated cellular turnover in hyperosmotic environments.

Mature chloride cells involved in ion transport are in contact with the external environmental water via the apical pit, and with internal environments via the basolateral membrane (Wendelaar Bonga and Van der Meij, 1989; Wendelaar Bonga *et al.*, 1990). In our electron-microscopic studies, the apical openings became numerous in response to increased salinity. The apical membrane was deeply invaginated and the tubular network was highly developed in hypersaline conditions, resulting in large surface areas for the placement of ion transporting proteins such as apical chloride channel and basolateral Na^+, K^+-ATPase. These results also suggest that the excellent salinity tolerance of tilapia is attributed to well-developed chloride cells in association with increased activities of various ion transporting proteins.

In the present study, chloride cells were also present on the afferent side of the filament in FW-adapted tilapia, although they seemed less active, compared with those in hypersaline-adapted fish. Recent studies have demonstrated that chloride cells have diverse functions in FW, including calcium ion uptake (Flik *et al.*, 1995), sodium and chloride ion uptake (Perry, 1997) and acid-base regulation (Goss *et al.*, 1995; Lin and Randall, 1995). Although definite functions of chloride cells in FW have yet to be demonstrated, less active chloride cells observed in FW-adapted tilapia are the possible sites for ion uptake. Exploring the multifunction of the chloride cells in ion transport would be an interesting area for future investigations.

In some teleosts, the epithelium of the operculum contributes substantially to ionoregulation (Marshall, 1995). Similar to branchial epithelia, the opercular membrane contains a high density of chloride cells (Karnaky *et al.*, 1984; Kültz, 1995; Marshall *et al.*, 1997). Opercular chloride cells have been used in many studies on chloride cell functions as a model for branchial chloride cells. Our results clearly showed that DASPEI positive chloride cells in the opercular membrane became larger with increasing salinity. Kültz *et al.* (1995) also reported that opercular chloride cells were more numerous and activated extensively in tilapia acclimated to a hypersaline condition. Using a vibrating probe technique, Foskett and Scheffy (1982) demonstrated that opercular chloride cells are definitively the site of active chloride secretion in SW-adapted tilapia. These findings clearly indicate a significant implication of opercular chloride cells in ion secretion to maintain hydromineral balance in hypersaline conditions. In contrast, morphological and electrophysiological evidence has revealed a correlation between opercular chloride cells and sodium, chloride and calcium uptake in FW-adapted fish (McCormick *et al.*, 1992; Li *et al.*, 1997; Marshall *et al.*, 1997; Verbost *et al.*, 1997). Similar to branchial chloride cells, opercular chloride cells present in FW-adapted tilapia presumably act as sites for ion uptake in hypoosmotic environments.

In summary, on the basis of their morphology and Na^+, K^+-ATPase activities, chloride cells in the gills and opercular membrane of the tilapia became activated with increasing environmental salinity. Those activated chloride cells may function as sites responsible for salt secretion in hyperosmotic

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environments. Exceptionally strong salinity tolerance of the tilapia may be attributed to their excellent ability to develop chloride cell activity in response to increased environmental salinity.

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