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Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*)

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Abstract. Morphological features of the gill and opercular epithelia of tilapia (*Oreochromis mossambicus*) have been compared in fish acclimated to either fresh water (FW) or hypersaline water (60‰ S) by scanning electron and fluorescence microscopy. In hyperosmoregulating, i.e., FW-acclimated, tilapia only those mitochondria-rich (MR) cells present on the filament epithelium of the gill were exposed to the external medium. After acclimation of fish to hypersaline water these cells become more numerous, hypertrophy extensively, and form apical crypts not only in the gill filament but also in the opercular epithelium. Regardless of salinity, MR cells were never found to be exposed to the external medium on the secondary lamellae. In addition, two types of pavement cells were identified having distinct morphologies, which were unaffected by salinity. The gill filaments and the inner operculum were generally found to be covered by pavement cells with microridges, whereas the secondary lamellae were covered exclusively by smooth pavement cells.

Key words: Teleosts – Gill – Opercular epithelium – Mitochondria-rich cells – Pavement cell – Salinity adaptation – *Oreochromis mossambicus* (Teleostei)

Introduction

The teleost gill is the site of gas exchange, ionoregulation, acid-base regulation, and nitrogenous waste excretion (McDonald et al. 1991; Wilkie and Wood 1991). This multifunctionality requires compromises in the morphological and functional organization of the gill epithelium, in particular for facilitating diffusion of O_2/CO_2 on the one hand and maintaining ionic homeostasis in the extracellular fluids on the other hand (Randall et al. 1972; Gonzalez and McDonald 1992). The gill is composed of filaments and secondary lamellae covered by distinct epithelia (Hughes 1984), which have been shown to contribute differently to gas exchange and ion transport (Hughes and Morgan 1973; Girard and Payan 1977, 1980; Avella et al. 1987; Gardaire et al. 1991). Furthermore, in some teleosts the epithelia of the buccal cavity, and the operculum, as well as the skin, contribute substantially to ionoregulation, but not to respiratory gas exchange (Burns and Copeland 1950; Karnaky 1972; Marshall 1977).

The outer, i.e., most superficial, layer of the gill, buccal, and opercular epithelia is made up of three cell types: pavement cells, which are the most abundant; mitochondria-rich (MR) cells; and mucus cells. MR cells are the sites for active ion secretion in hypo-osmoregulating, i.e., salt water (SW)-acclimated, teleosts (Foskett and Scheffey 1982), and are also thought along with the pavement cells to be responsible for the active absorption of ions (Goss et al. 1992b; Evans 1993).

In any case, both transmission electron microscopy (TEM; Threadgold and Houston 1964; Kessel and Beams 1962; Philpott and Copeland 1963; Straus 1963; Bierther 1970; Sardet et al. 1979; Hwang 1987; Cioni et al. 1991) and scanning electron microscopy (SEM; Hossler et al. 1979, 1985; Crocker et al. 1985; Lubin et al. 1989; Franklin 1990; Maina 1990; Brown 1992; Goss et al. 1992a) studies have clearly shown that the ultrastructure of these cells is different in a variety of teleosts when exposed to different environmental salinities. In this report we examine the MR cells and the surface ultrastructure of the filament and secondary branchial epithelia as well as of the opercular epithelium of *Oreochromis mossambicus* in order to compare the adaptive changes in response to salinity of morphological features of these epithelia and discuss possible functional consequences and restraints for the opercular epithelium as a model of the gill.

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Materials and methods

Animals

Tilapia (*Oreochromis mossambicus*) were reared to sexual maturity in the laboratory (Kültz and Jürss 1993) and then acclimated to either fresh water (FW) or hypersaline water (HSW, 60‰ S) for at least 5 weeks at 25±1° C and a 12L:12D photoperiod. The acclimation to HSW was done stepwise allowing 1 week for each intermediate step (25, 40, 50‰ S). Tilapia were fed approximately 2% of their body mass once a day using commercial trout pellets (ZBO Mischfutterwerk Beeskow).

Fish were anesthetized with 0.02% MS 222 (buffered with $NaHCO_3$, pH 7.4), the pericardium was opened and the bulbus arteriosus was catheterized. The gills were perfused with a modified tilapia Ringer's solution (MTR: 146 mmol/NaCl; 3 mmol/l KCl; 1 mmol/l NaH_2PO_4; 15 mmol/l $NaHCO_3$; 20 mmol/l EDTA; 1 mmol/l epinephrine; 5000 U/ml heparin; and 5 mmol/l TRIS/HCl, pH 7.4). After exsanguination, the gill covers and individual gill arches were dissected and rinsed twice in MTR.

Scanning electron microscopy

Immediately after the final MTR rinsing, the tissue was placed in fixation buffer (0.94 mol/l NaH_2PO_4; 2.38 mol/l Na_2HPO_4; 4% glutaraldehyde; pH 7.3) for 24–48 h. Following incubation the opercular epithelium was cut into pieces approximately 25 mm^2 and the gill filaments were scraped off the underlying cartilage. Subsequently, these tissue pieces were rinsed twice in fixation buffer and incubated for 2 h in 1% OsO_4. The tissue pieces were rinsed twice in the same buffer and dehydrated in increasing concentrations of ethanol, whereafter they were placed in ethanol-ether mixtures. After evaporation of the final ether solution the tissue was mounted on a sample holder and covered with gold. Scanning electron micrographs (Conax 167, ORWO NP20 – 80 ASA film) were taken of representative areas of each sample using a Zeiss DSM 960A (Oberkochen, Germany) instrument. At least 20 different areas of gill and opercular epithelia were observed from each of six fish. The micrographs were used to measure the area of apical exposure of MR cells to the external medium. Twenty areas were measured from each of three fish from each experimental group.

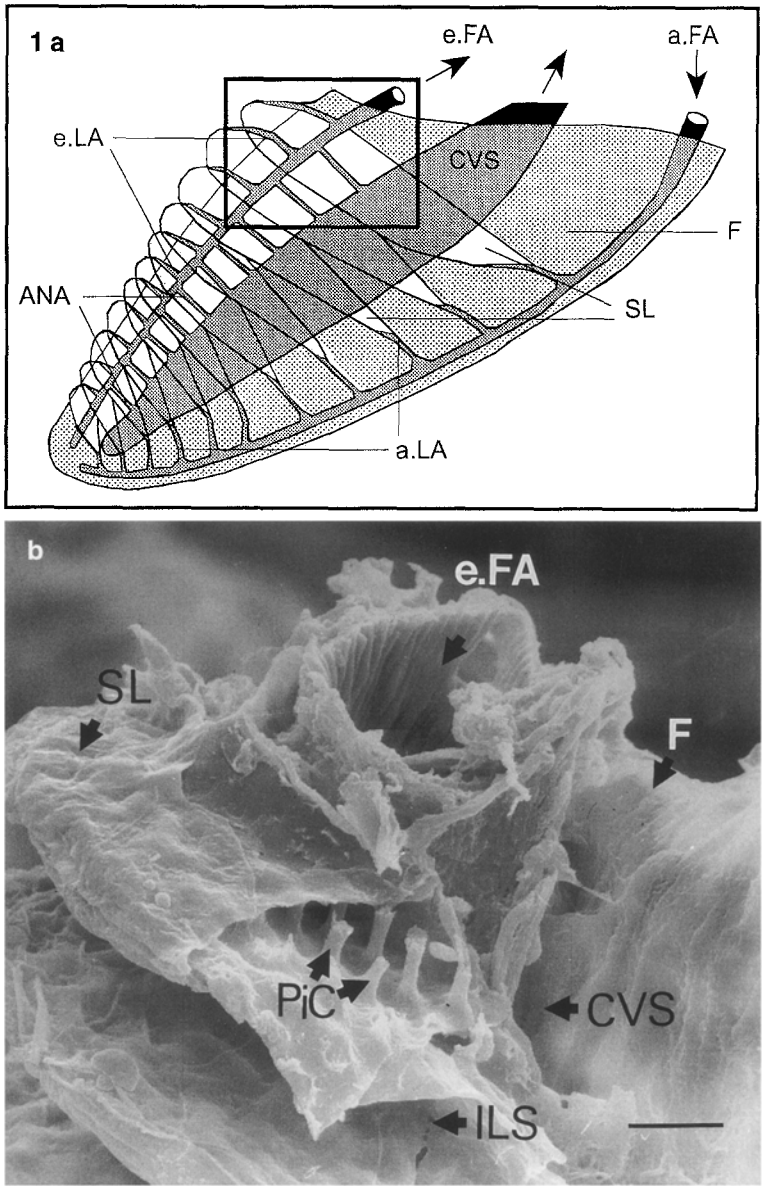

Fig. 1a, b. Organization of a gill filament of *Oreochromis mossambicus*. *a.FA* afferent filament artery; *a.LA* afferent lamellar arteriole; *ANA* arteriovenous anastomoses; *CVS* central venous sinus; *e.FA* efferent filament artery; *e.LA* efferent lamellar arteriole; *F* filament epithelium; *ILS* interlamellar space; *PiC* pillar cells; *SL* secondary lamellae. **a** Schematic illustration of basic features of organization and vascularization of the gill filament and the secondary lamellae. **b** Micrograph showing the area in the *inset* of the upper schematic. *Bar*: 10 µm

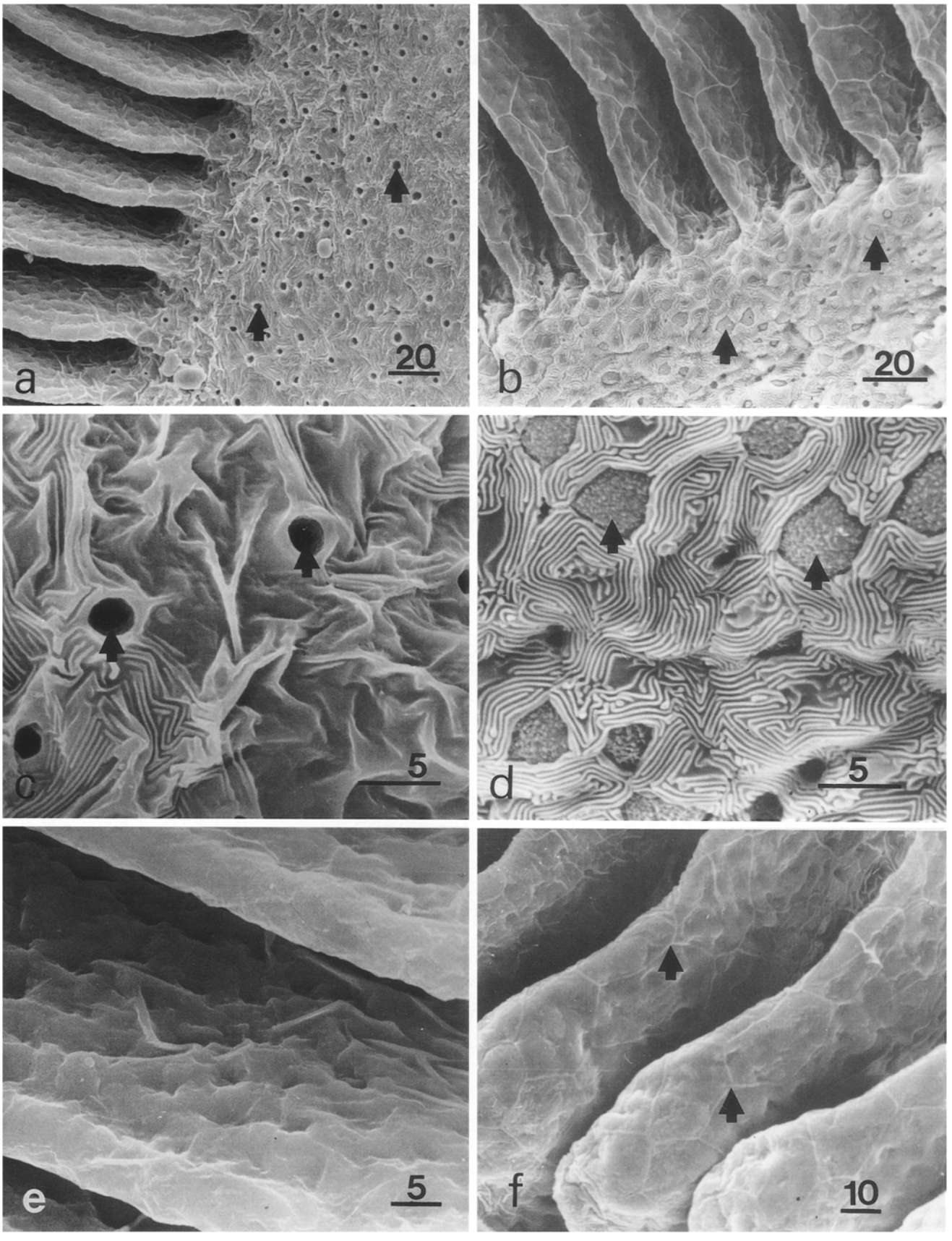

Fig. 2a–f. Gill epithelium of *Oreochromis mossambicus*. All numbers above *bars* represent distances in µm. **a** Secondary lamellae (*left*) and filament epithelium (*right*) of tilapia acclimated to HSW, *arrows* point to apical crypts of MR cells. ×500. **b** Secondary lamellae (*top*) and filament epithelium *(bottom*) of tilapia acclimated to FW, *arrows* point to apical membranes of MR cells. ×650. **c** Filament epithelium of tilapia acclimated to HSW, *arrows* point to apical crypts of MR cells, smooth pavement cells (*center*) and pavement cells with microridges (*bottom left*) are visible. ×3000. **d** Filament epithelium of tilapia acclimated to FW, *arrows* point to apical membranes of MR cells which contain numerous microvilli. × 3000. **e, f** Secondary epithelium of tilapia acclimated to HSW (**e** ×2000) and FW (**f** ×1000), which is exclusively covered by smooth pavement cells, *arrows* point to cell borders

Table 1. Number, size and apical exposure area of MR cells in the gill and opercular epithelium of *Oreochromis mossambicus* that had been acclimated to fresh water (FW) and hypersaline water (HSW). The number of MR cells is given per g of gill wet weight or per 2.14 mm² of opercular epithelium, respectively. All data shown differ significantly between FW and HSW in both the gill and the opercular epithelium ($P<0.01$, $n=6$)

Tissue	Gill epithelium		Opercular epithelium	
Salinity	FW	HSW	FW	HSW
MR cell number	$9.6\times10^5\pm 0.6\times10^5$	$2.55\times10^6\pm 3.2\times10^5$	174±14	1170±219
MR cell diameter (μm)	12.0±0.3	25.6±0.6	13.7±0.3	26.8±0.8
Area of MR cell Exposure to the External medium (μm²)	9.7±1.4	3.5±0.4	0.28±0.25[a]	2.9±0.3

[a] Rudimentary crypts only

Fluorescence microscopy

Isolation of gill epithelial cells and the preparation of the opercular epithelium were as previously described (Kültz and Jürss 1993; Kültz and Onken 1993). Cell suspensions and opercular epithelia were then stained in tilapia Ringer's solution (TRS: 146 mmol/l NaCl, 3 mmol/l KCl, 1 mmol/l NaH_2PO_4, 15 mmol/l $NaHCO_3$, 1 mmol/ $CaCl_2$, 1 mmol/l $MgSO_4$, 10 mmol/l glucose, 5 mmol/l TRIS/HCl, pH 7.4) containing 25 μmol/l of the vital mitochondria-specific fluorescence dye DASPMI [2-(*p*-dimethylaminostyryl)-1-ethyl-pyridiniumiodine, Aldrich Milwaukee, Wis.]. The cell suspensions and the epithelia were examined under a phase-contrast/epifluorescence microscope (Jenalumar, Zeiss Jena, Germany). For epifluorescence, a 470–490 nm bandpass excitation filter and a 530 nm longwave pass filter were used. Micrographs of the cell suspensions and of the area of highest density of MR cells in the opercular epithelium were taken (ORWO NP 20, 80 ASA film). The number of MR cells in a known volume of gill cell suspension was counted in the hemocytometer and then normalized to 1 g of gill epithelial tissue. Due to the heterogeneity of the MR cell density in the opercular epithelium the area of the highest density was always chosen and then the number of fluorescing cells in exactly 2.14 mm² of this area was counted. A minimum of 50 cells were counted. The diameter of the fluorescing MR cells was measured on the micrographs. Calibration was achieved using an object micrometer. Twenty cells were measured from each of three fish from each experimental group.

Statistical analysis

Results are expressed as the mean ± the standard error of the mean (SEM). Statistical comparisons for numbers, diameters, and apical exposure areas of MR cells were assessed using the *F*-test followed by the Student's *t*-test. The Welch test was used instead of the *t*-test in case of significant differences between the variances (Weber 1986). All values stated as significant had $P<0.01$.

Results

We have found significant salinity-induced changes in the ultrastructure of the branchial and opercular epithelia. However, the extent of these morphological changes differs vastly between these epithelia. In addition, we have found striking differences in the general surface structure of the filament and secondary epithelium, which are the two components of the branchial epithelium (Fig. 1).

In hypo-osmoregulating tilapia MR cells were exposed to the external medium exclusively via apical crypts, which were numerous and fully developed under these conditions (Fig. 2a). These crypts had diameters ranging from 1 to 3.5 μm (Table 1). On the other hand, apical crypts occur infrequently and appear to be rudimentary in the gill filament of hyperosmoregulating fish. Unlike hypo-osmoregulating tilapia, in the gills of hyperosmoregulating fish, most of the MR cells were directly exposed to the external medium by a large area of their apical plasma membranes (Fig. 2b), and contained many microvilli. The average exposed membrane area of MR cells of hyperosmoregulating tilapia was 9.7±1.4 μm² (Table 1). These exposed membrane patches were always surrounded by pavement cells, which were themselves covered with microridges 0.2–0.3 μm wide and 0.1–0.3 μm high (Fig. 2d). A second type of superficially located pavement cell was found in the filament epithelium of hypo-osmoregulating tilapia and consisted of a population with a smooth surface lacking microridges (Fig. 2c). However, the pavement cells possessing microridges were still largely predominant. The microridges on these cells formed a very regular pattern, which was not affected by osmotic adaptation (Fig. 2c, lower left vs. Fig. 2d).

Regardless of the external salinity, the opercular epithelium of tilapia was covered exclusively with pavement cells containing microridges (Fig. 3a, b). In hypo-osmoregulating fish, MR cells of the opercular epithelium had contact with the external medium exclusively via well-developed apical crypts (Fig. 3c), which had the same general appearance as those found in the gill filament epithelium. Very few of these crypts, which were clearly rudimentary, were detectable in the opercular epithelium of hyperosmoregulating tilapia (Fig. 3d). These crypts possessed very small openings. In sharp contrast to the morphology described in the gill epithelium, in the opercular epithelium of hyperosmoregulating tilapia the apical membranes of MR cells were never exposed directly to the external medium.

MR cells in the gill and in the opercular epithelium were found to be significantly larger and more numerous in hypo- than in hyperosmoregulating tilapia (Fig. 4; Table 1). Moreover, the large MR cells in the gill and opercular epithelium of hypo-osmoregulating tilapia had an ovoid shape in contrast to the round appearance of small MR cells in hyperosmoregulating fish (Fig. 4).

Mucus cells occured more frequently in the opercular epithelium than in the gill filament epithelium. They were covered by pavement cells and formed elabora-

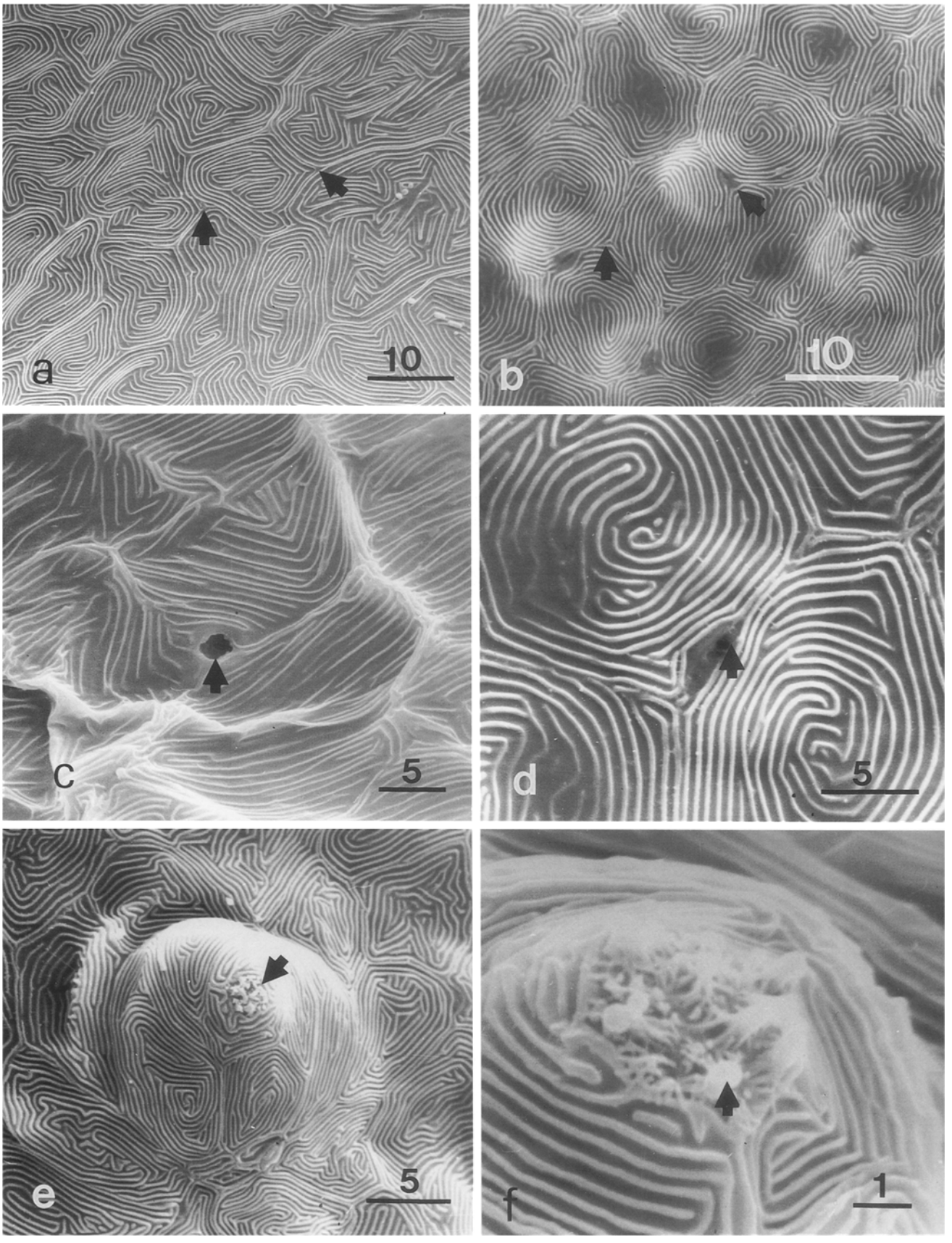

Fig. 3a–f. Opercular epithelium of *Oreochromis mossambicus*. All numbers above *bars* represent distances in µm. **a, b** Only pavement cells with microridges occur in tilapia acclimated to HSW (**a** ×1500) or FW (**b** ×1900), *arrows* point to cell borders. **c, d** Apical crypts (*arrows*) of MR cells of tilapia acclimated to HSW (**c** ×3000) or FW (**d** ×3600). **e, f** Elaboration formed by a mucus cell, which is covered by pavement cells (**e** ×2600). Note that only a small opening is left for extruding the mucus (**f** ×10000); the *arrows* point to a mucus droplet

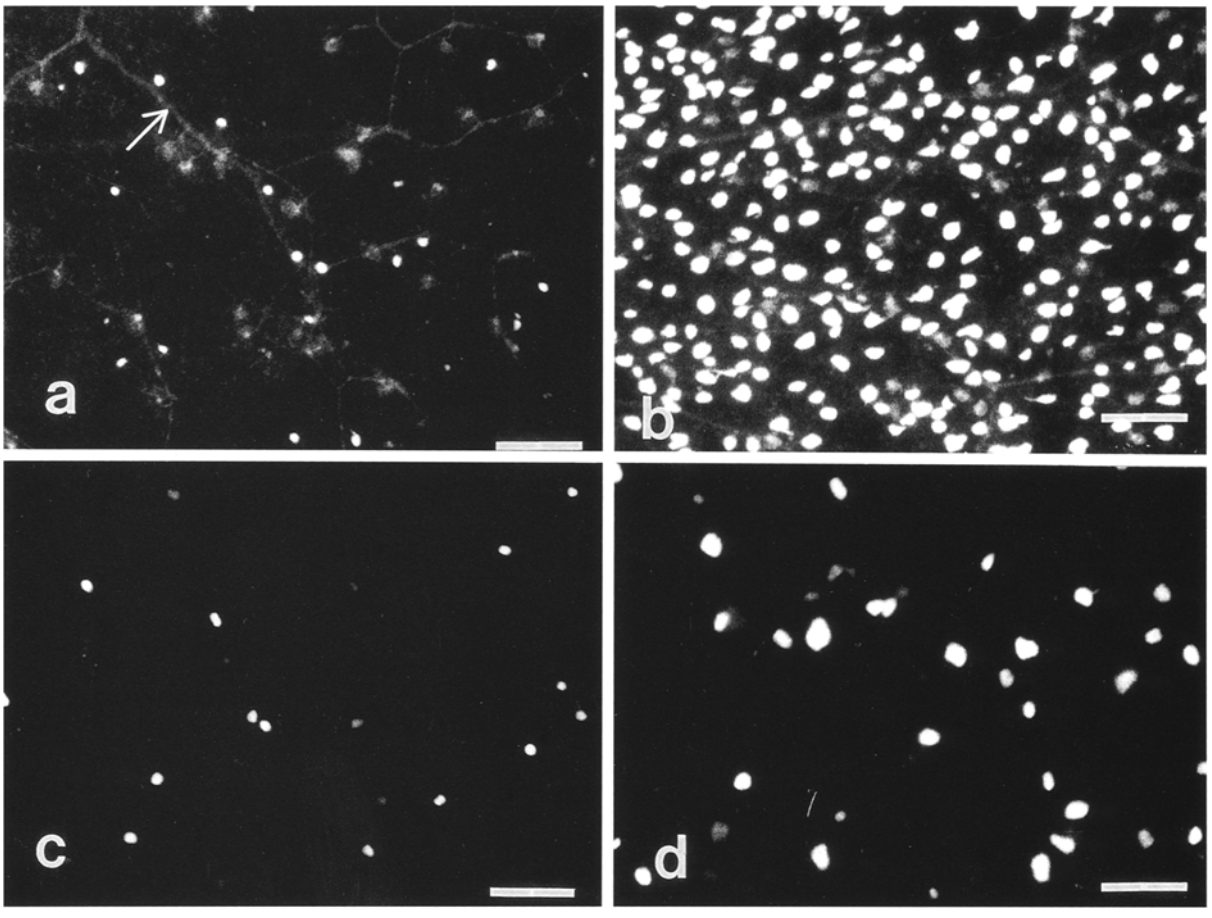

Fig. 4a–d. MR cells after staining with 25 μmol · l^{-1} DASPMI viewed under epifluorescence. All *bars* represent 60 μm. **a** Area of the highest MR cell density in an opercular epithelium of tilapia acclimated to FW. *Arrow* points to a blood vessel that has retained the fluorescent dye during the washing procedure. **b** Area of the highest MR cell density in an opercular epithelium of tilapia acclimated to HSW. **c** Cell suspension of gill cells of tilapia acclimated to FW. **d** Cell suspension of gill cells of tilapia acclimated to HSW

tions, which sometimes had an opening at the top where mucus droplets could often be seen (Fig. 3e, f). The size of elaborations formed by mucus cells varied widely, most likely due to cells being in different stages of the secretion process at the time of fixation.

Overall, the opercular epithelium resembles the surface structure of the filament epithelium much more than that of the secondary epithelium. Secondary lamellae were found to be covered exclusively by smooth pavement cells (Fig. 2e, f). Neither MR cells or mucus cells were exposed to the external medium on the secondary lamellae. The surface ultrastructure of the secondary epithelium was not influenced by salinity.

Discussion

Adaptive differences between the opercular epithelium and the gill filament epithelium

Of the three epithelia examined, salinity-induced changes in the ultrastructure have only been observed in the opercular epithelium and the gill filament epithelium but not in the secondary epithelium of the gill. Since the discovery of MR cells in the opercular epithelium of teleosts (Burns and Copeland 1950; Karnaky 1972), this tissue has been described as a continuous extension of the gill filament epithelium (Karnaky and Kinter 1977) and has been widely used as a model for the gill in electrophysiological investigations (see Pequeux et al. 1988). Although MR cells in both the opercular epithelium and the filament epithelium are most numerous and largest in hypo-osmoregulating fish, our results show that major differences in the organization of the apical membrane of MR cells exist between these epithelia after FW acclimation of tilapia. An increase in number and hypertrophy of MR cells in response to increased salinity is a common phenomenon in the teleosts (Pisam and Rambourg 1991), but it is difficult to interpret the differences in apical exposure area of MR cells caused by salinity acclimation because of the three-dimensional structure of apical crypts and microvilli. Additionally one apical crypt is generally shared by at least two MR cells to provide a "leaky" paracellular pathway for Na^+ transport (Pequeux et al. 1988). The completely different organization of the apical membrane of MR cells in hypo- or hyperosmoregulating fish might reflect the need for re-

structuring this membrane to achieve either active ion secretion or absorption.

In the gill filament epithelium of hyperosmoregulating tilapia MR cells are well exposed to the external medium. Their apical membranes, covered with microvilli, are visible as frequent patches in a considerable area of the filament surface. This is not the case in the opercular epithelium. Therefore, if MR cells are the only sites of active Na^+ or Cl^- absorption or at least an essential part of it, then it is highly unlikely that this process occurs across the opercular epithelium. Indeed, the transepithelial potential and the short-circuit current across the opercular epithelium of tilapia acclimated to FW are zero (Foskett et al. 1981; Kültz and Onken 1993), which was originally attributed to the electrically silent nature of Na^+ and Cl^- absorption. However, depletion of one of these ions from the medium in an Ussing chamber experiment causes no potential or current build up, contradicting the notion of actively independent Na^+ or Cl^- absorption across this tissue (D. Kültz and H. Onken, unpublished). On the other hand, the surface morphologies of the gill filament and opercular epithelia are very similar in hypo-osmoregulating tilapia. In both tissues many apical crypts of MR cells having similar sizes are present (Table 1). These crypts are filled with mucus, which is thought to serve as an ion trap and thereby facilitate Cl^- secretion (Philpott and Copeland 1963; Pisam et al. 1980; Pisam and Rambourg 1991). A notable difference between the opercular and filament epithelia of hypo-osmoregulating tilapia is the occasional occurrence of smooth pavement cells in the latter. Assuming that MR cells are involved in NaCl secretion as well as absorption (Goss et al. 1992b; Evans 1993; Kültz and Jürss 1993), these observations suggest that the opercular epithelium may be involved only in NaCl secretion. However, based on the fact that the area of apical exposure of individual MR cells in the filament epithelium is even larger in hyper- than in hypo-osmoregulating tilapia, it appears likely that the filament epithelium is a site for both NaCl absorption and secretion.

Differences in the ultrastructure of the gill filament and secondary epithelia

The filament and secondary epithelia of the gill are characterized by different vascularization and innervation (Laurent and Dunel 1980; Dunel-Erb et al. 1982, 1989; Laurent 1985; Bailly et al. 1989; Laurent and Hebibi 1989; see also Fig. 1). In addition, our results show that regardless of the acclimation salinity, these epithelia exhibit remarkable differences in ultrastructure. In contrast to the filament epithelium the surface ultrastructure of the secondary epithelium is not modified by salinity. Its outer layer is exclusively composed of smooth pavement cells. This might indicate a low participation of this epithelium in active ion transport across the gill of *O. mossambicus*. On the other hand, in some teleosts no difference was observed in the function and the surface ultrastructure of the filament and secondary epithelium. The secondary epithelium is considered a site for active ion absorption in salmonids (Girard and Payan 1977; Avella et al. 1987; Gardaire et al. 1991). Moreover, microridges are common on the apical membranes of pavement cells located on the secondary as well as the filament epithelia in other tilapia species (Fishelson 1980; Maina 1990, 1991) and salmonids (Olson and Fromm 1973; Laurent and Perry 1990; Crocker et al. 1985; Lubin et al. 1989, 1991; Goss et al. 1992b). MR cells were also observed on the secondary lamellae of teleost gills (Getman 1950; Copeland 1950; Dunel-Erb and Laurent 1980; Brown 1992). These species-specific differences are difficult to explain but may underlie the ability of *O. mossambicus* to tolerate a much wider range of salinity than other species of the subgenus tilapia. To our knowledge this study is the first to describe the exclusive occurrence of smooth pavement cells on the secondary lamellae of teleost gills. The occurrence of these cells and the absence of either whole MR cells or of sites of exposure of MR cells to the external medium implicates functional differences between the secondary lamellae and the filament epithelium.

It has been speculated that the conspicuous microridges of the apical surface of pavement cells arise as the result of secretory processes across these cells since they are believed to represent an extension of the apical plasma membrane formed by continuous fusion with secretory vesicles (Hughes and Wright 1970; Whitear 1990). The functional role of these structures is only poorly understood. The microridges are thought to be stabilized by elements of the cytoskeleton and may therefore have a mechanically protective function (Hawkes 1974). In this capacity, they might serve to prevent the loss of mucus from the epithelial surface. One explanation for the absence of microridges on the pavement cells located on the secondary epithelium of *O. mossambicus* gills could be the facilitation of gas exchange realized by minimizing the mucus layer between the epithelial cells and the external medium and also by enhancing the water flow along the secondary lamellae.

In conclusion, we believe the differences observed in the ultrastructure of the filament, secondary, and the opercular epithelia of *O. mossambicus* are indicative of functional differences between these epithelia. The gill filament epithelium is most likely the main site of active ion transport in both hypo- and hyperregulating tilapia. The opercular epithelium might contribute significantly only to active NaCl secretion, while the secondary epithelium seems to be specialized for gas exchange.

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