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Long-term acclimation of the teleost *Oreochromis mossambicus* to various salinities: two different strategies in mastering hypertonic stress

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Abstract. Laboratory-reared tilapia (*Oreochromis mossambicus*) were long-term acclimated to freshwater (FW), brackish water (BW, 10‰ salinity), seawater (SW, 35‰ salinity) and two hypersaline media (45 and 60‰ salinity). We examined the influence of these ambient salinities on the density (D_{cc}) and diameter (d_{cc}) of DASP-MI-stained chloride cells and on the capacity for electrogenic Cl^- secretion of the in vitro opercular epithelium. To provide a characterisation of Cl^- secretion, transepithelial potential difference (PD_{te}), conductance (G_{te}) and short-circuit current (I_{sc}) were measured after mounting the respective epithelium in an Ussing-chamber. The cellular electromotive forces (E_c) and conductances (G_c) as well as the leak conductances (G_l) were obtained from G_{te}:I_{sc} plots. In the salinity range between FW and SW both D_{cc} and d_{cc} increased. All electrophysiological parameters recorded increased in parallel, indicating a strong enhancement of the capacity for Cl^- secretion on the cellular and epithelial level. In the salinity range above SW a further increase of D_{cc} was observed. However, despite a higher concentration gradient across the body surface of the tilapia during acclimation to hypersaline media, the short-circuit current (I_{sc}) was not significantly different compared to SW preparations. This reflects proportional decreases of G_c and increases of E_c, respectively. Of particular interest, we found a strong decrease of the leak conductance (G_l) in preparations from tilapia acclimated to hypersaline media compared to those from SW fish, indicating that the tight junctions become less permeable.

Introduction

Euryhaline or stenohaline marine teleosts living in seawater maintain the osmolality of their extracellular fluid hypoosmotic to the environment. They actively secrete NaCl across the gills in order to offset the salt load incurred by both passive inward diffusion and seawater ingestion (Smith 1930, Keys 1931, Motais et al. 1966). Active secretion of Cl^- is associated with the so-called chloride cells of the branchial epithelium (Keys and Willmer 1932, Foskett and Scheffey 1982). Sodium ions follow passively via the paracellular pathway (Degnan and Zadunaisky 1980b, Zadunaisky 1984). Transcellular Cl^- secretion depends on a functioning Na^+/K^+-ATPase located in the basolateral membranes and is thought to proceed via a basolateral Na^+(and K^+)-dependent entry into the cells and apical exit via ion channels (Silva et al. 1977, Epstein et al. 1980, Marshall 1981).

Due to the complex morphology of fish gills, electrophysiological investigations using an Ussing chamber seem impossible. However, the flat opercular epithelium, which functions similarly to the gills and is also rich in chloride cells, has been successfully used in Ussing chambers (Karnaky 1972, Degnan et al. 1977, Karnaky and Kinter 1977, Karnaky 1986). When mounted in an Ussing chamber, the short-circuit current, which is equal to the net flux of Cl^- from serosa to mucosa, can be measured (Karnaky et al. 1977, Foskett et al. 1981).

Oreochromis mossambicus is an extremely euryhaline teleost, which is able to tolerate salinities up to 120‰ (Stickney 1986). This species evolved in freshwater and estuarine habitats in Southeast Africa and today is widely distributed in both freshwater and seawater environments in Africa, America and Asia (Trewavas 1983). Therefore this species is a good model organism for investigations on ionic and osmotic adaptation of teleosts. The acclimatory response of tilapia to salinity changes from freshwater to seawater (35‰ S) is known to involve simultaneous increases in the density (D_{cc}) and diameter (d_{cc}) of chloride cells, as well as in the short-circuit current (I_{sc}), the transepithelial potential difference (PD_{te}) and the conductance (G_{te}) of the opercular epithelium (Foskett et al. 1981, 1982a, b).

To date, few data have been published dealing with electrophysiological or cellular parameters of branchial

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or opercular epithelia from hypersaline-acclimated teleosts, and it is of interest whether these organisms employ similar or identical means to adapt to hypersaline conditions as they do to adapt to seawater. Therefore we have studied the cellular and electrophysiological parameters of the opercular epithelium of euryhaline tilapia, which were long-term acclimated to salinities between freshwater and 60‰ S. Our results for changes in the salinity range up to seawater agree well with those cited above. However, for the salinity range above seawater our results indicate that tilapia adopt a different strategy to overcome hypertonic stress.

Materials and methods

Basic solutions and chemicals

Tilapia Ringer solution (TRS) was composed of (in mmol l^{-1}): NaCl 146, KCl 3, $CaCl_2$ 1, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 15, Tris 5, glucose 10. Chloride free saline (CFS) was prepared using gluconates. The pH of all solutions was adjusted to 7.5 with Tris directly before use. Ouabain was obtained from Merck (Darmstadt, FRG) and the fluorescence dye DASPMI was purchased from Aldrich (Milwaukee, USA).

Fish and preparation

Tilapia, *Oreochromis mossambicus* (10 to 30 g), were obtained from laboratory stock and acclimated to Rostock tapwater (FW, composition: see Vökler et al. 1987), brackish water (BW, 10‰ S), seawater (SW, 35‰ S) and hypersaline waters (45 and 60‰ S) at a temperature of 25±1 °C, as described earlier in more detail (Kültz et al. 1992). The length of the acclimation period (5 wk) was chosen so that all acclimatory responses could be considered complete. The fishes were stunned by a blow to the head and killed by cutting the spine posterior to the heart. Opercula were removed and transferred into TRS. The opercular epithelium was carefully dissected from the underlying bone and connective tissue was removed.

Determination of D_{cc} and d_{cc} of chloride cells

The opercular epithelia were incubated for 30 min at 4 °C in TRS containing 25 µmol l^{-1} DASPMI, which specifically stains the chloride cells (Bereiter-Hahn 1976, Karnaky et al. 1984). After rinsing with DASPMI-free TRS the preparations were put on slides under cover slips. A combined fluorescence and phase contrast microscope (Jenalumar, Carl Zeiss Jena) was used for examination of the tissue. As described by Foskett et al. (1981) micrographs (ORWO-NP 20, 80 ASA) were taken after visually scanning the entire epithelium. The total number of chloride cells was counted in an area of 2.14 mm^2 and then expressed as the density per cm^2 (D_{cc}). The size of chloride cells was measured on the micrographs using an objective micrometer. The maximal distance between two poles of an oval cell was defined as the cell diameter (d_{cc}). 20 cells from six fish each were measured per experimental group.

Electrophysiology of the opercular epithelia

For measurement of the electrophysiological parameters the preparation was mounted in a small, modified Ussing chamber in which 0.018 cm^2 of the epithelium was exposed to the bathing solutions. To avoid or minimise edge damage minimal amounts of "Glisseal" grease (Borer Chemie, Solothurn, Switzerland) were used. An automatic voltage-clamp device (Van Driessche and Gullentops, Leuven, Belgium) was connected to calomel electrodes (via 3 mol l^{-1} KCl/3% agar bridges to each chamber compartment), which served to measure the transepithelial potential difference (PD_{te}, reference electrode in the internal bath) and to silver wires coated with AgCl, which served to apply current to short-circuit the epithelium (measurement of I_{sc}). G_{te} was calculated from small imposed voltage pulses (5 mV) and the resulting current deflections. The conductance (G_c) and the electromotive force (E_c) associated with active Cl^- transport via chloride cells and the leak conductance (G_l) were calculated from G_{te}:I_{sc} plots according to a conventional circuit analysis, having one passive (G_l) and one active ($G_c = I_{sc}/E_c$) component in parallel. During the experiments both chamber compartments were continuously perfused with saline (3 ml min^{-1}). The internal TRS was constantly bubbled with air. The Cl^- current per chloride cell (I_{cc}) was calculated from the mean values of I_{sc} and D_{cc} of preparations from fish acclimated to identical salinities.

Statistics

Statistical evaluations were performed using the F-test followed by the Welch-test, or Student's t-test (Weber 1986). First order regression analysis was used for the conventional circuit analyses. Probability values of $p < 0.05$ were considered significant. All values in the "Results" section are given as means ± the standard error of mean ($x \pm SE$).

Results

Number and size of chloride cells

Opercular epithelia from tilapia acclimated to FW contained 2421±331 chloride cells cm^{-2} ($n=6$). Acclimation to increasing salinities resulted in increased cell densities (Fig. 1). In the range between FW and SW there was a linear increase of D_{cc} with salinity. Above SW concen-

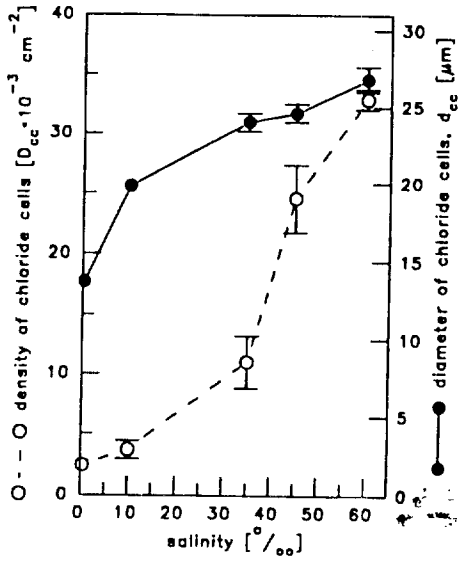

Fig. 1. *Oreochromis mossambicus*. Chloride cell densities (D_{cc}) and diameters (d_{cc}) in opercular epithelia of tilapia acclimated to various salinities. For each parameter, all differences are significant except those of cell density between freshwater and 10‰ and cell diameter above 35‰ S ($n=6$)

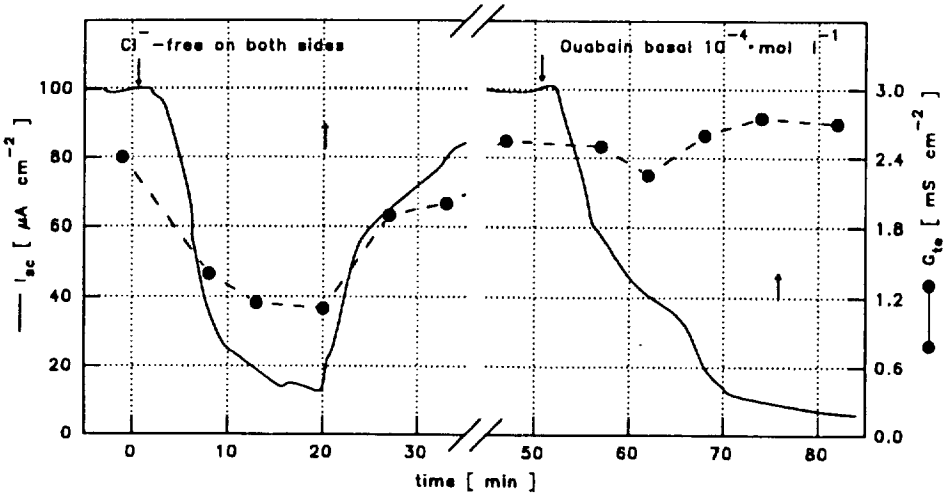

Fig. 2. *Oreochromis mossambicus.* Typical time courses of the responses of short-circuit current (I_{sc}) and transepithelial conductance (G_{te}) to Cl^- depletion and internal ouabain addition. Arrows indicate start [replacement of Tilapia Ringer solution (TRS) by special saline] and end (restoration of TRS) of manipulations. Data taken from an opercular epithelium of a tilapia acclimated to 60‰ S

trations, tilapia opercular epithelial D_{cc} values increased dramatically over those found at lower salinities. In contrast, the mean diameter of chloride cells (d_{cc}) increased only slightly in media above SW (Fig. 1).

Electrophysiological parameters

The transepithelial potential difference with TRS on either side of the opercular epithelium was negative with respect to the external side for all preparations and the polarity was independent of the acclimation medium. Thus, the I_{sc} was always positive. For salinities other than FW (no I_{sc} measurable) I_{sc} depended in each case on Cl^- ($n=6$ for each salinity) and was inhibitable with basolaterally applied ouabain (10^{-4} mol l^{-1}, Fig. 2). The magnitudes of PD_{te}, I_{sc}, the current per individual chloride cell ($I_{cc}=I_{sc}/D_{cc}$) and the G_{te} of the opercular epithelia depended strongly on the acclimation salinity of the fishes (Fig. 3). The PD_{te} increased over the whole salinity range. G_{te}, on the other hand, showed a maximum for preparations obtained from fishes acclimated to SW; opercular epithelia of tilapia acclimated to lower (FW, BW) or higher (hypersaline) salinities showed lower transepithelial conductances. I_{sc} increased only up to an acclimation salinity of 35‰. The mean I_{cc} also increased up to SW medium, but declined considerably at higher salinities. To obtain detailed information on the circuit parameters for opercular epithelia of fish acclimated to hyperosmotic salinities, we plotted the individual G_{te} values of each group against the respective I_{sc}. In Fig. 4a G_{te}: I_{sc} plot is shown for preparations obtained from tilapia acclimated to 45‰ S. As outlined by Foskett et al. (1982b), the linearity of such a plot reflects the variability in active Cl^- secretion among individuals at constant electromotive force and leak conductance, due to the individual conductance associated with the occurrence of chloride cells (G_c). According to this conventional circuit analysis the inverse of the slope of the line respresents an estimate of the mean electromotive force for active Cl^- secretion (E_c). The zero-current intercept on the G_{te}-axis gives an estimate of the mean leak conductance (G_l), which is associated with the "shunt" pathway (paracellular, nontransporting cells and perhaps some edge damage). The difference between G_{te} and G_l represents G_c, which is an estimate of the conductance associated with active Cl^- secretion via chloride cells. Except for tilapia that had

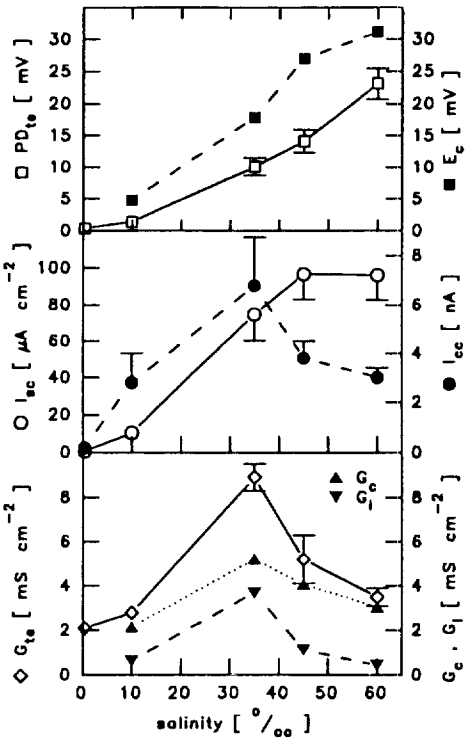

Fig. 3. *Oreochromis mossambicus.* Salinity acclimation dependence of electrophysiological parameters of tilapia opercular epithelia. Top panel: Transepithelial potential difference (PD_{te}) and cellular electromotive forces (E_c). Middle panel: Short-circuit current (I_{sc}) and current per chloride cell (I_{cc}). Bottom panel: Transepithelial conductance (G_{te}), leak conductance (G_l) and cellular conductance (G_c). Data that do not differ significantly are: I_{sc} between freshwater (FW) and 10‰ S and above 35‰ S; PD_{te} between FW and 10‰ S; G_{te} between 10 and 45‰ S, 10 and 60‰ S and 45 and 60‰ S; $n=6$ for 10 and 60‰ S, 7 for 45‰ S or 8 for FW and SW (seawater). E_c, G_l and G_c values calculated from conventional circuit analysis and therefore shown without SE

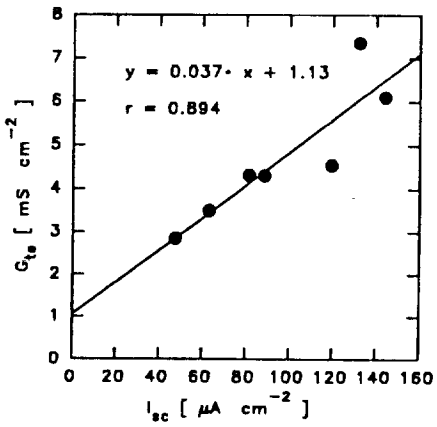

Fig. 4. *Oreochromis mossambicus.* Representative plot of short-circuit current (I_{sc}) vs transepithelial conductance (G_{te}). Regression line calculated from seven tilapia acclimated to 45‰ S. Slope of the regression line is inversely proportional to presumed common cellular electromotive forces, and its intersection with the ordinate represents the mean leak conductance

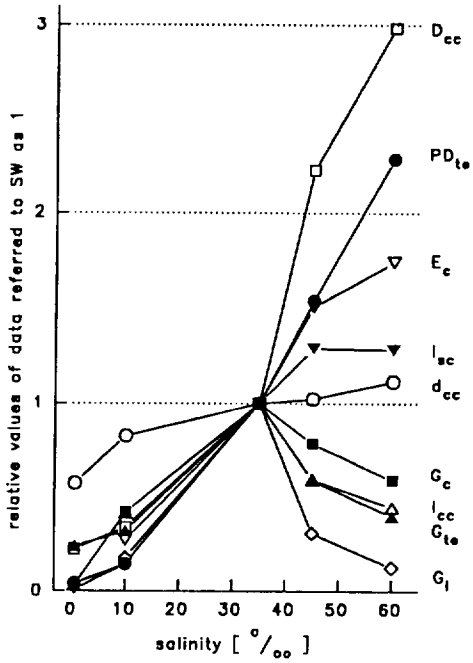

Fig. 5. *Oreochromis mossambicus.* Relative values of all data in reference to the respective data under SW (seawater) conditions, which were defined as 1. In contrast to the monotone increase of all parameters up to 35‰ S a decrease is evident in the conductances [transepithelial conductance (G_{te}), cellular conductance (G_c), leak conductance (G_i)] and the chloride cell current (I_{cc}) in hypersaline water. D_{cc}: chloride cell densities; PD_{te}: transepithelial potential difference; E_c: cellular electromotive forces; I_{sc}: short-circuit current; d_{cc}: chloride cell diameter

been acclimated to FW (I_{sc} not measurable), in all salinities linear relationships (regression coefficients above 0.8) $G_{te}:I_{sc}$ were obtained though only shown for epithelia from 45‰ S (Fig. 4). Regarding E_c, we obtained a continous increase with the salinity of the acclimation medium (see Fig. 3, top panel). Both G_i and G_c increased from FW to BW preparations, reaching a maximum in opercular epithelia of tilapia acclimated to SW (35‰ S), and decreasing again in preparations from fishes acclimated to hypersaline salinities (see Fig. 3, bottom panel).

In Fig. 5 all results are summarised and normalised to the values obtained for fishes acclimated to SW. It is obvious that the acclimatory response of tilapia changes dramatically when in hypersaline waters compared to the monotonic increase of all parameters in the range between FW and SW.

Discussion

Tilapia acclimated to FW have no need for Cl^- secretion. In fact, these fish must absorb salts from the dilute medium. The mechanism of ion uptake and the role of the gill and opercular epithelium under these conditions have been discussed previously (Karnaky 1986, Kirschner 1991), and our results indicating lowest D_{cc}, d_{cc}, I_{sc}, PD_{te} and G_{te} in FW-acclimated tilapia are consistent with these findings.

In 10‰ salinity tilapia are approximately isosmotic to the external medium. Yet despite the absence of a significant osmotic gradient across their body surface, tilapia still secrete Cl^-. This is reflected by proportional increases in cellular (D_{cc}, d_{cc}, I_{cc}, E_c, G_c) and epithelial (I_{sc}, PD_{te}, G_{te}, G_i) parameters when compared to individuals acclimated to freshwater (see Figs. 1, 3 and 5). These results are again consistent with those of Foskett et al. (1981). A possible explanation for this Cl^- secretion under isosmotic conditions may be the counteraction of active absorption of NaCl, which is needed for acid-base regulation (Evans 1984a).

Our results in the salinity range between FW and SW (Fig. 5) are consistent with previous studies by Marshall (1977), Foskett et al. (1981) and Krasny (1981). Chloride cells have been shown to be the sites of active Cl^- secretion (Foskett and Scheffey 1982). They markedly proliferate (D_{cc}) and hypertrophy (d_{cc}) with enhanced salinities, leading to an increase in cellular (I_{cc}) and epithelial (I_{sc}) ion transport capacity. As suggested by our circuit analysis, the enhanced I_{sc}, which has been shown to be equal to the net secretory flux of Cl^- ions (Degnan et al. 1977, Foskett et al. 1981), is presumably due to the parallel increases of G_c and E_c. Likewise the $G_{te}:I_{sc}$ plots indicate an increase of G_c with enhanced salinity in the range between FW to SW. This agrees well with the findings of increased passive ion permeabilities of teleost branchial and opercular epithelia following acclimation from FW to SW (cf. Evans 1984b, Karnaky 1986). The tight junctions between adjacent chloride cells are more leaky than between chloride cells and pavement cells or between adjacent pavement cells (Hwang 1987, 1988). Since the occurrence of chloride cells in multicellular complexes is a characteristic feature of teleosts acclimated to SW (cf. Karnaky 1986), the enhanced G_i could be attributed to this morphological response.

Our results, showing an I_{sc} inhibition after Cl^- depletion (>80%) or the internal application of ouabain (>90%), are nearly identical to previous reports on the opercular epithelium of *Fundulus heteroclitus* (Degnan

et al. 1977, Degnan and Zadunaisky 1980a) and on the abdominal skin of *Blennius pholis* (Williams et al. 1988). These results support the "Silva-model" (see "Introduction") for secondary active Cl^- secretion driven by the Na^+/K^+-ATPase.

Thus, the strategy of adaptation from FW to SW seems to be the development of an increasing epithelial capacity for Cl^- secretion (I_{sc}). This is due to an increase in both the number and diameter of chloride cells, each with an increased transport potential and capacity of active Cl^- transport. The increase of the leak conductance may facilitate, if paracellularly located, the passive movement of Na^+ ions following active, transcellular Cl^- extrusion. This is obligatory for active NaCl secretion under open-circuit conditions, i.e., in vivo. In this respect it should be pointed out that E_c calculated from the slope of the $G_{te}:I_{sc}$ relationship underestimates the cellular electromotive force by a factor of two due to the passive leak conductance for sodium in chloride cells. This was shown to represent 50% of the total chloride cell conductance for SW-acclimated fish (Degnan and Zadunaisky 1980a, b, Foskett et al. 1982b), and a "heterogeneous cell model" was developed in which the existence of different types of cells was considered (Foskett et al. 1982b) and the chloride cells were assumed to contribute a fixed ratio of leak to active conductances. According to this model the zero-current intercept of our $G_{te}:I_{sc}$ plots is equal to the leak conductance not associated with chloride cells (G_l) and E_c is defined by the equation $E_c = m\,(1\text{-}G_l^{cc}/G_t^{cc})$ where m is the slope of the regression line, G_l^{cc} represents the leak conductance associated with the chloride cell tight junctional pathway and G_t^{cc} is the total chloride cell conductance. Since G_l^{cc} of SW-adapted teleosts was shown to be attributed to passive sodium fluxes equal to approximately 50% of G_t^{cc}, E_c is about twice as high as predicted from the conventional circuit analysis. This E_c is thus about three times higher than the PD_{te} measured in SW preparations and should be sufficient to overcome the electrochemical gradient in vivo with SW on the external side of the epithelium. However, it is not clear whether the ratio of active versus leak conductance is indeed fixed in the chloride cells or changes during hypersaline conditions.

Our results show that I_{sc} is Cl^- dependent and generated by the Na^+/K^+-ATPase irrespective of the acclimation salinity (Fig. 2). Thus, the principal mechanisms of active Cl^- secretion are not influenced by salinity. Despite the presence of active transport at all salinities, the importance of differences in the adaptational responses of tilapia to hypersaline waters compared to salinities up to SW must be emphasised.

In Fig. 5, striking differences in the acclimatory response to salinities above SW are clearly visible. While d_{cc} is nearly constant, D_{cc} is greatly increased (Fig. 1). However, the electrophysiological parameters of these cells are very different. While Degnan et al. (1977) found a 2- to 3-fold reduction of I_{sc} on opercular epithelia of *Fundulus heteroclitus* acclimated to 200% SW compared to those acclimated to 100% SW, we observed a constancy of I_{sc} and a decrease of I_{cc} despite an increased E_c. This is most likely a consequence of the remarkable drop in the conductance of chloride cells (G_c, Fig. 5). Reductions of membrane conductances caused by suddenly enhanced extracellular osmolalities are well known (Zeiske and Van Driessche 1984, Benos and Sorscher 1992). However, whether such shock-effects are responsible for the G_c drop observed as a result of acclimation of tilapia to hypersaline media is uncertain, as the present investigation is concerned with long-term acclimation. The observed constancy of I_{sc}, despite decreasing G_c, is due to the concomitant increase in E_c. While E_c may be directly related to the increased density of chloride cells D_{cc}, the individual conductance of chloride cells ($G_c:D_{cc}$) must have fallen enormously. The need for an enhancement of E_c with increasing external salinities can simply be understood as an adaptation to the enhanced osmotic gradient across the body surface of the fishes under in vivo conditions. As in other epithelia (Yonath and Civan 1971, Isaacson 1977) the active transport processes in the tilapia opercular epithelium depend on a functioning Na^+/K^+-ATPase (see Fig. 2). E_c may therefore reflect, at least in part, the activity of this ion pump. In fact, the Na^+/K^+-ATPase activity increases in the gills of tilapia with increasing salinities over a wide range (FW to 60‰ S; Dange 1985, Kültz et al. 1992). However, the opercular membrane shows very low activity of this enzyme, which is independent of the acclimation salinity (Kültz et al. 1992).

In addition, to the cellular parameters discussed above, the very strong decrease of G_l is remarkable. This reduction of G_l may reflect a decreased permeability of the whole body surface, keeping the passive influxes of salt constant, even when the external salinity becomes more hypersaline. This allows active Cl^- secretion (as measured by I_{sc}) to remain constant. A possible increase in paracellular Na^+ secretion, at decreased G_l, may be compensated by the markedly increased PD_{te}, which represents the driving force for this process.

In summary, in the salinity range from FW up to SW, the strategy of adaptation seems to be characterised by a curative mode, i.e., an increased transport rate. In hypersaline media a more preventive mechanism is apparent, reducing the overall permeability. The results presented here raise the question of whether these alterations in the adaptational strategy are specific for tilapia or represent a common phenomenon for extremely euryhaline teleosts in general. The reduction of passive ion fluxes in hypersaline environments would keep the energetic costs of osmoregulation at a tenable level. Therefore the ability to use this strategy may represent a selective advantage during the evolution of teleosts that favours survival in waters with variable salinity such as would be encountered in pools in salty deserts, salt marshes or coastal tide pools. In particular some species of the genera *Fundulus*, *Cyprinodon* and *Rivulus*, which are known to inhabit such environments, are also known for their ability to survive in highly hypersaline waters (Parry 1966, Weisberg 1986, Abel et al. 1987). In these fishes the plasma osmolality varies only over a small range even in hypersaline waters (Maetz 1970, Feldmeth and Waggoner 1972). The size and the density of chloride cells in opercular membranes of *R. marmoratus* is not affected much by salinity in the

range between 100 to 200% SW (King et al. 1989). The short-circuit current across the opercular membrane of *F. heteroclitus* was even lower in fishes acclimated to 200% SW compared to those acclimated to SW (Degnan et al. 1977). These results indicate that not only active transport alone but also passive mechanisms could be of major importance for the maintainance of blood osmolality in hypersaline waters. On the other hand, a 3.9-fold increase of the Na^+/K^+-ATPase activity has been measured in gill homogenates of *C. variegatus* acclimated to 200% SW compared to 100% SW fish (Karnaky et al. 1976). Up to now too few data have been available to draw generalizations, but it is hoped that the present work will encourage future studies on this topic.

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