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TIME COURSE OF SALINITY ADAPTATION IN A STRONGLY EURYHALINE ESTUARINE TELEOST, *FUNDULUS HETEROCLITUS*: A MULTIVARIABLE APPROACH

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Summary

Freshwater-adapted killifish (*Fundulus heteroclitus*) were transferred directly from soft fresh water to full-strength sea water for periods of 1 h, 3 h, 8 h and 1, 2, 7, 14 and 30 days. Controls were transferred to fresh water for 24 h. Measured variables included: blood [Na^+], osmolality, glucose and cortisol levels, basal and stimulated rates of ion transport and permeability of *in vitro* opercular epithelium, gill Na^+/K^+-ATPase and citrate synthase activity and chloride cell ultrastructure. These data were compared with previously published killifish cystic fibrosis transmembrane conductance regulator (kfCFTR) expression in the gills measured over a similar time course. Plasma cortisol levels peaked at 1 h, coincident with a rise in plasma [Na^+]. At 8 h after transfer to sea water, a time at which previous work has shown kfCFTR expression to be elevated, blood osmolality and [Na^+] were high, and cortisol levels and opercular membrane short-circuit current (I_{sc}; a measure of Cl^- secretion rate) were low. The 24 h group, which showed the highest level of kfCFTR expression, had the highest plasma [Na^+] and osmolality, elevated plasma cortisol levels, significantly lower opercular membrane resistance, an increased opercular membrane ion secretion rate and collapsed tubule inclusions in mitochondria-rich cells, but no change in gill Na^+/K^+-ATPase and citrate synthase activity or plasma glucose levels. Apparently, killifish have a rapid (<1 h) cortisol response to salinity coupled to subsequent (8–48 h) expression of kfCFTR anion channel proteins in existing mitochondria-rich cells that convert transport from ion uptake to ion secretion.

Key words: chloride cell, killifish, *Fundulus heteroclitus*, cortisol, immunoassay, salinity adaptation, Ussing chamber, citrate synthase, Na^+/K^+-ATPase, cystic fibrosis transmembrane conductance regulator (CFTR).

Introduction

Estuarine euryhaline teleosts, such as the killifish *Fundulus heteroclitus*, normally experience rapid salinity changes and are therefore good models for investigations of the regulation of salt transport in teleosts (Wood and Marshall, 1994; Marshall and Bryson, 1998). Adaptation to sea water in euryhaline teleosts is thought to involve the development of ion secretion pathways in the mitochondria-rich cells of the gill epithelium under the influence of cortisol, growth hormone and insulin-like growth factors (for reviews, see Bern and Madsen, 1992; Jacob and Taylor, 1983; Mancera and McCormick, 1998). In a euryhaline freshwater teleost, the Mozambique tilapia *Oreochromis mossambicus*, cortisol and growth hormone levels are elevated 1 and 4 days after transfer to sea water but not when these animals are transferred to brackish water or fresh water (Morgan et al., 1997). Nakano et al. (1998) similarly observed an increase in growth hormone levels after transfer of tilapia to 70 % sea water, but at 12 h only. Transfer of tilapia through brackish water to full-strength sea water increases the size and number of mitochondria-rich cells in the opercular epithelium with a concomitant increase in Cl^- secretion rate (Foskett et al., 1981, 1983). Hence, freshwater-resident teleosts with euryhaline capability, such as the tilapia, readily adapt to salinity change over days if intermediate salinities are provided (Foskett et al., 1983; Nakano et al., 1998). Anadromous salmonid teleosts enter sea water once or twice in their life cycles, first as smolts and, in some species, as post-spawn kelts. Brook trout (*Salvelinus fontinalis*) show increases in plasma cortisol levels and accelerated cortisol secretion during adaptation to sea water (Nichols et al., 1985). Atlantic salmon (*Salmo salar*) kelts show acceleration of cortisol metabolism and utilization, but only subtle changes in plasma cortisol levels (Nichols and Weisbart, 1985). Cortisol,

in turn, is responsible for augmentation of Na^+/K^+-ATPase activity in salmon gills *in vivo* (McCormick, 1996) and in organ-cultured tilapia opercular epithelium (McCormick and Bern, 1989). The typical blood ion response among anadromous teleosts transferred from fresh water through brackish water to full-strength sea water is a large increase in the levels of plasma ions (Na^+ and Cl^-) that persists for at least 48 h in rainbow trout *Oncorhynchus mykiss* (Bolton et al., 1987), for 96 h in rainbow trout (Seddiki et al., 1995), for more than 6 days in Atlantic salmon kelts (Nichols and Weisbart, 1985), brown trout *Salmo trutta* (Madsen, 1990a) and rainbow trout (Madsen, 1990b) and in some cases for longer (Madsen, 1990b). Unlike anadromous fishes that change salinities a few times during their life cycle (and generally do not survive direct transfers from fresh water to full-strength sea water), estuarine-resident teleosts such as killifish must readily adapt to high and low salinity extremes and are capable of surviving direct transfer from fresh water to full-strength sea water. Killifish transferred directly to sea water have an elevated plasma osmolality (Jacob and Taylor, 1983; Zadunaisky et al., 1995), with the peak osmolality occurring 12–48 h after transfer. On the basis of the effects of manipulations of bathing solution osmolality on isolated killifish opercular epithelia, Zadunaisky et al. (1995) suggested that increases in plasma osmolality may be a direct cue for mitochondria-rich cells to increase Cl^- secretion rate.

Killifish adapt rapidly to salinity change, and the critical period for the adaptation is in the first day or two, yet few studies have focused on this period. Indeed, Jacob and Taylor (1983) call for a 'comprehensive study of the first 24 h' to reconcile the relationship between cortisol levels and Na^+/K^+-ATPase activity. The present study uses a comprehensive approach, including analysis of Cl^- secretion capacity by the opercular epithelium as well as enzyme and hormone assays. By doing this at a large number of time points, we can compare rates of change and the order of events to elucidate the mechanisms more clearly. Of particular interest are the events in the first 24 h, during which changes in blood osmolality, hormonal responses and ion transport are initiated in rapidly adapting animals. Major questions include whether new chloride cells are required or whether adaptation can occur rapidly in existing cells, what the internal cues for changes in ion transport rates are, and how these changes are produced.

Materials and methods

Animals

Adult killifish (mummichog, *Fundulus heteroclitus* L.) of both sexes were captured in Antigonish estuary in May 1997, transferred to indoor holding facilities and adapted to brackish water (salinity 3 g l^{-1}) for at least 10 days at 20–25 °C and ambient photoperiod under artificial light. Fish were fed marine fish food blend (Tetramarine, Tetra Werke, Germany) at a rate of 1.0 g 100 g^{-1} body mass day^{-1}, supplemented twice weekly with frozen brine shrimp. Experiments were carried out between June and August.

Salinity adaptation

Freshwater adaptation involved transfer of killifish from brackish water (salinity 3.0 g l^{-1}) holding facilities to dechlorinated, ultraviolet-sterilized Antigonish tap water (composition: [NaCl], 0.15–0.30 mmol l^{-1}; $[Ca^{2+}]$, 0.04–0.08 mmol l^{-1}, pH 5.5–6.5) in glass aquaria. The acclimation period was at least 2 weeks. The transfer of freshwater-adapted animals back to sea water was accomplished by placing pairs of animals in 10 l divided aquaria containing fresh water at 20 °C for 7 days (to acclimate to the new surroundings) then introducing a flow of sea water at the same temperature over a period of 10–12 min to complete the change to full-strength sea water. Animals were removed 1 h, 3 h and 8 h, and 1, 2, 7, 14 and 30 days after transfer to sea water. A transfer control group (FWC) was treated identically to the seawater transfer animals but underwent a transfer from fresh water to fresh water; these animals were killed 24 h after the mock transfer. A long-term freshwater control group (LTC) was drawn from the same group of animals and treated similarly except that they were kept as a group of 10 fish and were not transferred from fresh water.

Blood and tissue variables

Concentrations of ions, osmolality and glucose levels

Animals were anesthetized in buffered (pH 7.1) 1:5000 MS-222 in 150 mmol l^{-1} NaCl, and blood was collected from the severed caudal peduncle into heparinized hematocrit tubes. Fish were killed by decapitation, and gill tissue was placed in ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen in liquid N_2. Modal blood collection volume was 100 µl. Blood was centrifuged (1000 ***g*** for 2 min), plasma was separated, and samples for the determination of cortisol levels were heat-sealed in plastic ampoules. The balance of the plasma was diluted 1:10 with deionized water and stored in microcentrifuge tubes for analysis of ion levels and osmolality. Plasma $[Na^+]$ (±1 mmol l^{-1}) was measured by atomic absorption spectrophotometry (Varian AA375), and plasma osmolality (±1 mosmol kg^{-1}) was determined on diluted plasma by freezing point depression (Osmette A, Precision Systems). Cortland's saline (full strength) with bovine serum albumin added (20 mg ml^{-1}) was serially diluted, and it was determined that freezing point depression was a linear function of dilution down to 1:10, the dilution used for the plasma. In this way, the osmolality measured on dilute plasma could be extrapolated back to the osmolality of undiluted plasma, and pooling of blood samples (thus reducing sample numbers) could be avoided. Blood glucose concentration was determined spectrophotometrically on whole plasma using the glucose oxidase method (Sigma assay kit 510A).

Plasma cortisol immunoassay

Plasma cortisol levels were measured using a validated direct enzyme immunoassay as outlined by Carey and

McCormick (1999). Sensitivity as defined by the dose–response curve was 1–400 ng ml^{-1}. The lower detection limit was 0.30 ng ml^{-1}. Using a pooled plasma sample, the average intra-assay variation was 5.5 % (N=10) and the average inter-assay variation was 8.8 % (N=10).

Epithelial preparation

The opercular epithelium was removed and mounted in a modified Ussing chamber as described previously (Marshall et al., 1998b) except that the nerve supply was not dissected with the epithelium. The epithelium was supported by a nylon mesh and pinned out over the circular aperture (0.125 cm^2) with the rim area lightly greased and bevelled to minimize edge damage. In the membrane chambers, the following epithelial electrophysiological variables were monitored: transepithelial potential V_t (mV), transepithelial resistance R_t (Ω cm^2) and short-circuit current I_{sc} (μA cm^{-2}). I_{sc} is expressed as positive for net gain of cations (or secretion of anions). Epithelia were clamped to 0 mV except for short periods to allow recording of V_t. A current–voltage clamp (D. Lee Co., Sunnyvale, CA, USA, or WP Instruments DVC 1000) was used to measure the epithelial variables.

Bathing solutions

A modified Cortland's saline (307 mosmol kg^{-1}, pH 7.8) was used to bathe both membrane surfaces symmetrically; its composition was (in mmol l^{-1}): NaCl, 160; KCl, 2.55; $CaCl_2$, 1.56; $MgSO_4$, 0.93; $NaHCO_3$, 17.85; NaH_2PO_4, 2.97; and glucose, 5.55. The saline had a pH of 7.8 when equilibrated with ~99 % O_2/1 % CO_2 gas mixture (22 °C). To mimic the high osmolality of blood plasma in animals after transfer to sea water, NaCl was added to the Cortland's saline to bring the osmolality to 350 mosmol kg^{-1} and the NaCl content to 186 mmol l^{-1}.

Radioisotopic fluxes

Radioactive chlorine (^{36}Cl, 0.04 MBq ml^{-1} final concentration) was added to one side of the epithelium as the neutral salt, and the epithelium was left to equilibrate for 1 h. Samples from the nonradioactive side were taken at 20 min intervals, mixed with a scintillation cocktail and the radioactivity counted (Packard 2000CA) to 1 % error. Samples from the more radioactive side were taken initially and at the end of each hour of the experiment. Radioisotope fluxes are expressed as μequiv cm^{-2} h^{-1}. Fluxes were measured for two 1 h control periods, then dibutyryl cyclic adenosine monophosphate (db-cAMP; 0.5 mmol l^{-1}) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 0.1 mmol l^{-1}) were added to the serosal bath. The stimulated I_{sc} was allowed to come to a new steady state, followed by a further 1 h flux measurement period.

Gill enzyme assays

Na^+/K^+-ATPase assay

Na^+/K^+-ATPase activity was determined using a kinetic microassay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min as outlined by McCormick (1993). Gill tissue (approximately 10–15 mg, six gill filaments) was homogenized in 125 μl of SEID (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole at pH 7.3 with 0.1 % v/v deoxycholic acid) and centrifuged at 5000 ***g*** for 30 s. Samples of the supernatant (10 μl) were run in two sets of duplicates, one set containing assay mixture only and the other containing assay mixture and 0.5 mmol l^{-1} ouabain. The resulting ouabain-sensitive ATPase activity measurement is expressed in μmol ADP mg^{-1} protein h^{-1}.

Citrate synthase assay

Citrate synthase activity, an indicator of metabolic potential in mitochondria, was determined by measuring the linear rate of release of sulfhydryl-coenzyme A (CoA) with Ellman's reagents in 50 mmol l^{-1} Tris, 0.5 mmol l^{-1} oxaloacetate and 0.15 mmol l^{-1} acetyl CoA (pH 8.1) at 405 nm in a 96-well microplate as described by Leonard and McCormick (1998). Gill homogenates were prepared as described above for Na^+/K^+-ATPase measurement. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

Electron microscopy

Opercular epithelia were dissected from two animals acclimated to fresh water, from two acclimated to sea water and from two animals that had been exposed to sea water for 24 h. Tissues were pinned out flat and fixed in 2.5 % glutaraldehyde buffered with ice-cold 0.1 mol l^{-1} sodium cacodylate (pH 7.50) for 1 h followed by an ice-cold buffer rinse for 15 min. The tissue was cut into wedges and postfixed with 2 % osmium tetroxide, 3 % potassium ferricyanide (1:1 v/v) for 1 h and rinsed twice in distilled water. Tissues were dehydrated through an alcohol series to 100 %, and infiltrated and embedded in low-viscosity Spurr's resin. One block from each animal was processed, and at least two sections from each block were examined. Sections (silver to gold) were cut using a Diatome diamond knife (Switzerland), stained with lead citrate (1 min) and uranyl acetate (2 min) and examined on a Philips 300 transmission electron microscope.

Statistical analyses

Values are expressed as means ± 1 S.E.M. Time course results were analysed using single-classification analysis of variance (ANOVA) followed by *a posteriori* comparisons (Bonferroni's multiple-comparisons test); significant differences are indicated in the figures by non-matching letters adjacent to the means. Comparisons between two means, for example comparing the FWC control group with the test group, were performed using unpaired two-tailed *t*-tests. To compare results from unstimulated membranes with stimulated (db-cAMP/IBMX) membranes, paired *t*-tests were used.

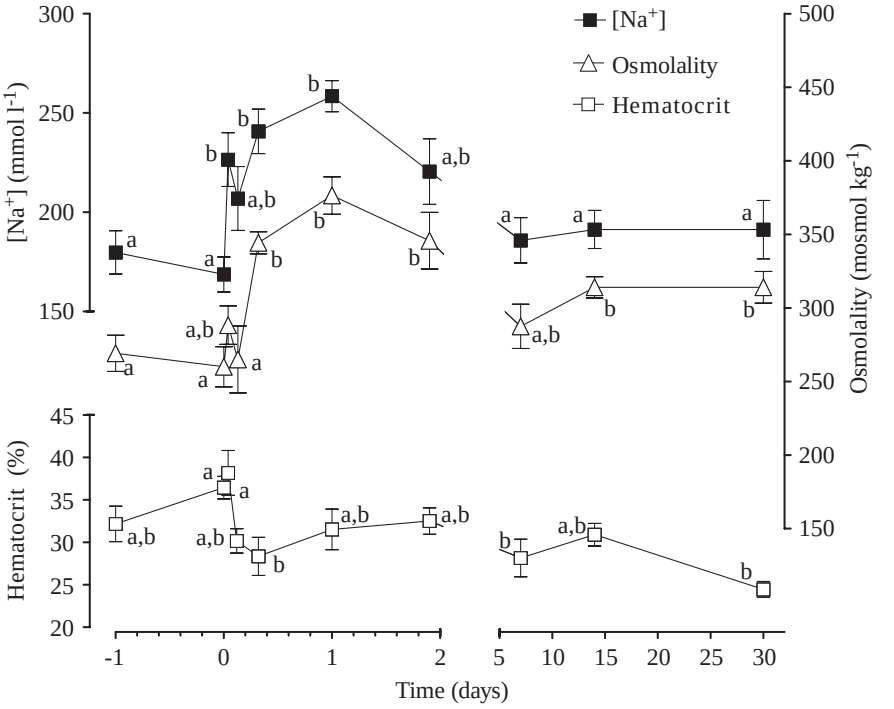

Fig. 1. Effects of rapid transfer from fresh water to sea water on plasma [Na^+], osmolality and hematocrit. On the time axis, −1.0 days indicates the long-term control (LTC) group, a group (N=12) of freshwater-adapted fish not subjected to salinity manipulation or transfer. The points at 0 h are for freshwater controls (FWC), freshwater-adapted fish held as pairs in aquaria subjected to a flow through of fresh water and sampled at 24 h. Dissimilar letters indicate a significant difference (P<0.05 or better) using Bonferroni's multiple-comparison test after one-way ANOVA. Values are means ± S.E.M. (N=10 for all time points).

Results

Plasma [Na^+] and osmolality

Direct transfer of killifish to sea water produced no mortalities, but there were clear changes in blood ion levels, osmolality and hormone levels. Plasma [Na^+] was significantly elevated at 1 h, 8 h and 24 h after transfer, compared with long-term freshwater-adapted animals. Plasma [Na^+] was higher than that of the freshwater transfer control (FWC) at 1 h, 8 h and 24 h (Fig. 1). The peak of 250 mmol l^{-1} Na^+ occurred 24 h after transfer. Plasma osmolality was elevated at 8 h, 24 h, 2 days, 14 days and 30 days compared with FWC animals (Fig. 1). The peak osmolality, also at 24 h, was 370 mosmol kg^{-1}, an increase of approximately 100 mosmol kg^{-1} (or 40 %) from control levels. Whereas the pattern for plasma [Na^+] suggests a return to control levels after full acclimation to sea water, plasma osmolality remained higher than that of the freshwater controls even after 30 days.

Hematocrit, glucose and cortisol

There was a transient increase in hematocrit, which was significantly elevated at 1 h compared with 8 h, 7 days and 30 days (Fig. 1). Plasma cortisol level was significantly elevated at 1 h and 24 h only compared with the LTC and FWC groups (Fig. 2). Plasma cortisol levels were lowest in the FWC group at 18.8±7.5 ng ml^{-1}, while the peak value occurring at 1 h was 164.7±19.8 ng ml^{-1}. The overall pattern suggests an early (1 h) sharp rise in cortisol level (1 h *versus* LTC, FWC, 14 days and

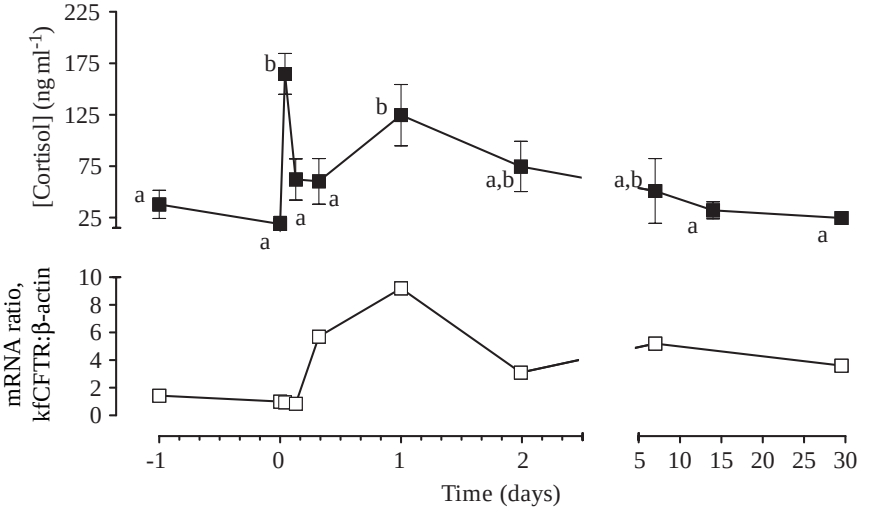

Fig. 2. Effects of rapid transfer from fresh water to sea water on plasma cortisol levels and the rate of expression of killifish cystic fibrosis transmembrane conductance regulator (kfCFTR) relative to β-actin expression, as kfCFTR:β-actin ratio (a densitometric scan of an original Northern blot by Singer et al., 1998, with permission). Dissimilar letters indicate a significant difference (P<0.05 or better) using Bonferroni's multiple-comparison test following one-way ANOVA. Values are means ± S.E.M. (N=10) for plasma cortisol levels. Other details are as in Fig. 1.

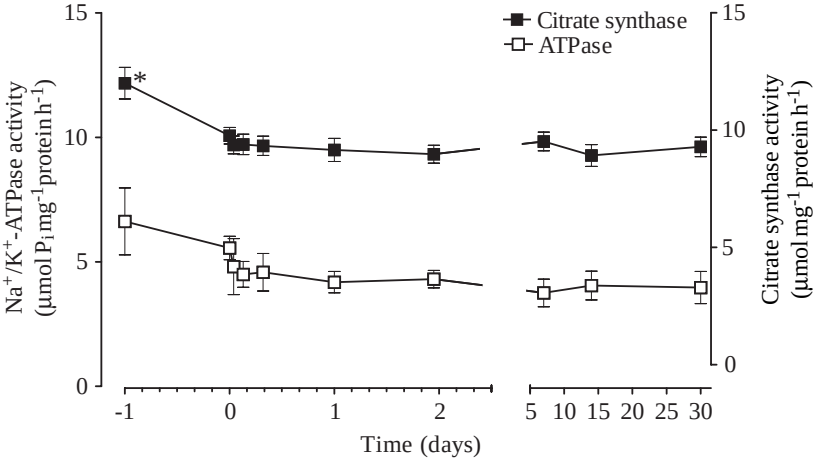

Fig. 3. Na^+/K^+-ATPase and citrate synthase activity in gill tissue. Values are means ± S.E.M. (N=10). *P<0.05, LTC *versus* FWC and P<0.01, LTC *versus* all other time periods (one-way ANOVA followed by Bonferroni's multiple-comparison test). Other details are as in Fig. 1.

30 days, P<0.001; 1 h *versus* 3 h, 8 h and 48 h, P<0.05), followed by low values at 3 and 8 h and a more protracted rise with a peak value at 24 h (24 h *versus* 14 days and 30 days, P<0.01; 24 h *versus* FWC and LTC, P<0.05). The LTC and FWC groups did not differ significantly from each other (P>0.25). Also shown in Fig. 2 are data taken from Singer et al. (1998): the ratio of kfCFTR expression to that of β-actin in killifish gill obtained by a densitometric scan of the results of a northern blot analysis. These ratios indicate elevated kfCFTR expression at 8 h, 24 h, 48 h, 7 and 28 days after transfer to sea water. Peak kfCFTR expression was at 24 h (Fig. 2). Although these expression data come from a separate experiment, the fish were taken from the same pond (in the subsequent year), the experiments were both performed in summer, the animal care procedures were the same, and the salinity transfer and tissue collection protocols were identical. Care was taken to ensure that the two experiments were comparable.

Plasma glucose level was measured on a separate group of animals to compare 24 h seawater transfer animals (3.63±0.35 mmol l^{-1}, N=6) with FWC animals (2.97±0.22 mmol l^{-1}, N=6). There was no change in plasma glucose level 24 h after transfer to sea water compared with the mock transfer controls.

Na^+/K^+-ATPase and citrate synthase activity

There was no change in Na^+/K^+-ATPase activity at any time during the transfer to sea water. Values remained at 4 μmol P_i mg^{-1} protein h^{-1} or greater throughout the experiment (Fig. 3). Values for the LTC group were most variable, but the FWC group had a similar mean (approximately 6 μmol mg^{-1} protein h^{-1}) with less variability. Citrate synthase activity is an indicator of metabolic potential in mitochondria and should increase if there is a net increase in the volume of mitochondria in the tissue. Citrate synthase activity followed approximately the same pattern as for Na^+/K^+-ATPase, i.e. no significant difference between FWC and seawater-acclimated animals. Interestingly, the LTC animals had a higher citrate synthase activity (12.0±0.67 μmol mg^{-1} protein h^{-1}, N=10) than both the FWC group (P<0.05) and the seawater transfer experimental animals (P<0.01, Fig. 3).

Opercular epithelium electrophysiology

I_{sc} for unstimulated epithelia was slightly negative in the FWC and LTC control groups and in epithelia from animals transferred to sea water for 3, 8 and 24 h (Fig. 4). Stimulation by db-cAMP/IBMX produced a positive current in all cases

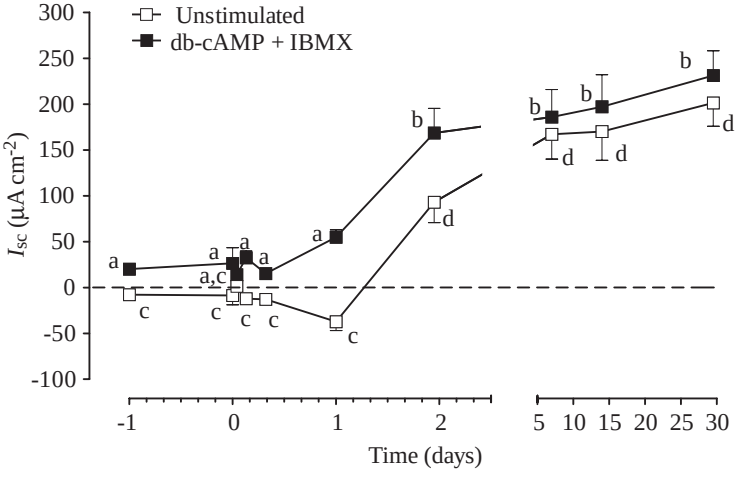

Fig. 4. Short-circuit current (I_{sc}) measured in an Ussing chamber containing opercular epithelia taken from animals transferred for various times to sea water. The unstimulated I_{sc} for each treatment is shown (open symbols) together with the I_{sc} after addition of 0.5 mmol l^{-1} db-cAMP plus 0.1 mmol l^{-1} IBMX to the serosal side (filled symbols) to stimulate maximal secretion rate. Dissimilar letters indicate a significant difference (P<0.01 or better) using Bonferroni's multiple-comparison test after one-way ANOVA. Stimulated I_{sc} was significantly greater than unstimulated (P<0.05 or better for all times except 7 days, paired t-test, two-tailed). Values are means ± S.E.M. (N=10). Other details are as in Fig. 1.

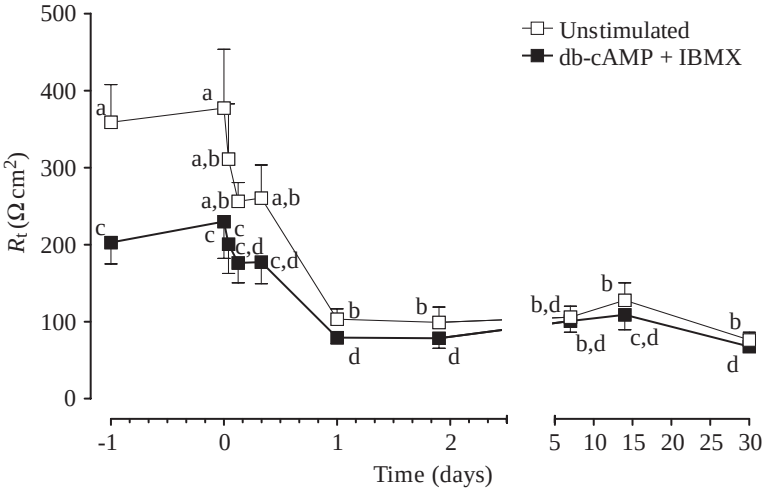

Fig. 5. Transepithelial resistance (R_t) of opercular epithelia mounted in the Ussing chamber. Values are from unstimulated epithelia (open symbols) and epithelia stimulated with 0.5 mmol l^{-1} db-cAMP plus 0.1 mmol l^{-1} IBMX (filled symbols). Dissimilar letters indicate a significant difference (P<0.01) using Bonferroni's multiple-comparison test (P<0.01 also for 1, 3 and 8 h compared with 1–30 day groups). Stimulated R_t was lower than unstimulated R_t at all time points except 7 days (P<0.05, paired t-test, two-tailed). Values are means ± S.E.M. (N=10). Other details are as in Fig. 1.

(corresponding to Cl^- secretion). The current was significantly greater than that of both controls (FWC and LTC) by 48 h and remained elevated 30 days after transfer to sea water. Resting and stimulated currents were significantly different in all groups except at 7 days (paired t-tests), and the increase in I_{sc} in the stimulated group was less than 20 % of the base current after 7, 14 and 30 days in sea water.

Transepithelial resistance (R_t) was initially high in the LTC and FWC groups, averaging 358 and 377 Ω cm^2 (Fig. 5). Stimulation with db-cAMP/IBMX markedly reduced the R_t in LTC, FWC, 1 h, 3 h and 8 h transfer groups (P<0.001, paired t-tests), whereas stimulated R_t was only marginally lower than basal R_t in the membranes from 1, 2, 14 and 30 day transfers (P<0.05, paired t-tests). Transfer to sea water produced a significant decrease in epithelial R_t (resting and stimulated) at 24 h to approximately 100 Ω cm^2. For instance, the value for the FWC group was 377.2±76.4 Ω cm^2 (N=10), while that for the 24 h group was 103.2±13.3 Ω cm^2 (N=10) (P<0.001). R_t did not change further during longer acclimation times in sea water.

The negative resting currents recorded 1–24 h after transfer to sea water (Fig. 4) were not expected, so we investigated whether these were secondary effects of the osmolality of the *in vitro* bathing solutions being lower than that of the plasma *in vivo*, because large increases in plasma osmolality occurred during acclimation (see Fig. 1). Unidirectional and net Cl^- fluxes revealed that the observed negative current could be in part the result of Cl^- uptake (Fig. 6) and that stimulation by IBMX and db-cAMP reversed this trend and produced a shift to a positive current and net Cl^- secretion. The data presented in Fig. 4 were collected from tissues bathed in Cortland's saline of osmolality 307 mosmol kg^{-1}, while the plasma osmolality of the animals 24 h after transfer to sea water was higher (Fig. 1) at approximately 350 mosmol kg^{-1}. To test whether blood osmolality was a major factor in the polarity and level of current after transfer, another group of animals was transferred to sea water for 24 h (the period corresponding to maximal plasma osmolality; Fig. 1). The opercular epithelia from this group were mounted in the Ussing chambers and bathed initially in 307 mosmol kg^{-1} Cortland's saline for 1 h, after which the osmolality was increased to 350 mosmol kg^{-1} by the addition of NaCl. The I_{sc} was initially negative (–7.0±2.2 μA cm^{-2}, N=6) in agreement with Fig. 1, but increasing the serosal osmolality produced a large positive I_{sc}

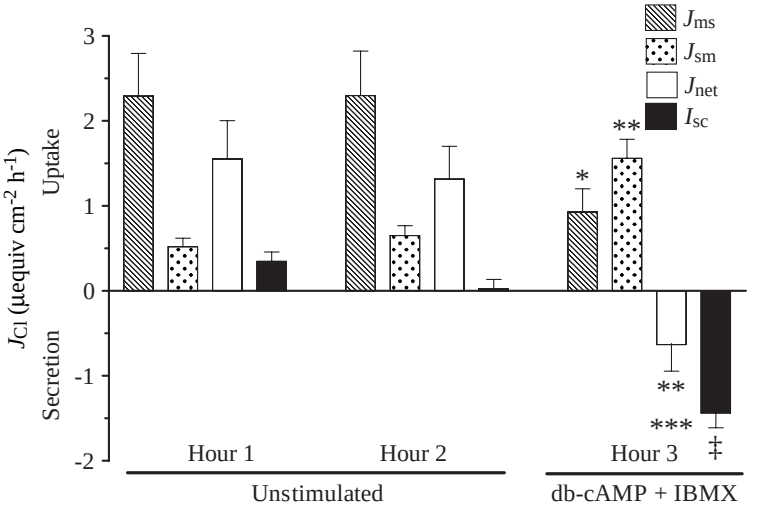

Fig. 6. Unidirectional efflux (J_{sm}), influx (J_{ms}) and net flux (J_{net}) of ^{36}Cl and short-circuit current (I_{sc}, expressed in μequiv cm^{-2} h^{-1}) across opercular epithelia transferred from fresh water to sea water for 24 h. Membranes were mounted in Ussing chambers and monitored for 2 h (unstimulated) and then for 1 h following stimulation with 0.5 mmol l^{-1} plus 0.1 mmol l^{-1} db-cAMP IBMX. I_{sc}, short-circuit current. ***P*<0.05 (paired t-test) compared with hour 2; ****P*<0.01 (paired t-test) compared with hour 2; *****P*<0.02 (unpaired t-test) for I_{sc} compared with J_{net} during hour 3. ‡P<0.0001 (paired t-test) for I_{sc} hour 3 compared with hour 2. Values are means ± S.E.M. (N=9).

of 107±19 μA cm^{-2} (P<0.05, paired t-test, N=6) which was not further altered by addition of db-cAMP/IBMX (90±12 μA cm^{-2}, N=6).

The discrepancy between net Cl^- flux (J_{net}) and I_{sc} in the first 2 h (P<0.01, unpaired t-tests, Fig. 6) suggests that Cl^- uptake occurs *via* an electrically neutral pathway. The persistent discrepancy between I_{sc} and J_{net} after stimulation with db-cAMP and IBMX suggests that the uptake pathway is still operational under these conditions, as is also indicated by the substantial mucosal-to-serosal unidirectional flux (J_{ms}) (Fig. 6).

Ultrastructural changes

Samples were taken from long-term freshwater-adapted animals, those exposed to sea water for 24 h and long-term seawater-acclimated animals. There were numerous mitochondria-rich cells in all opercular membranes regardless of acclimation salinity, but one difference was apparent among the groups. The tubular system that is clearly present in fully acclimated animals (sea water and fresh water) (Fig. 7A,C) was noticeably reduced in the cells from the 24 h transfer group (Fig. 7B). This seemed to be true for the all mitochondria-rich cells regardless of their electron density (Fig. 7B).

Discussion

Killifish held in fresh water for periods greater than 30 days retain large numbers of mitochondria-rich cells in the gills (Hossler et al., 1985; Karnaky, 1991) and opercular epithelia (Wood and Marshall, 1994; Marshall et al., 1997). This situation is clearly different from the euryhaline tilapia *Oreochromis mossambicus*, in which the gills and opercular epithelia have few, small mitochondria-rich cells (Foskett et al., 1981; McCormick et al., 1992) and levels of Na^+/K^+-ATPase activity are low in the gills (Young et al., 1988; Borski et al., 1994; Morgan et al., 1997). Many other teleosts also maintain reduced numbers of gill chloride cells and Na^+/K^+-ATPase activity in fresh water compared with sea water (Foskett et al., 1983). Killifish live in estuaries and, hence, may encounter large increases in salinity at high tides as well as rapid decreases in salinity during heavy rains. These animals appear to retain a larger number of mitochondria-rich cells when in fresh water as an adaptation to their estuarine existence, but these cells cannot be stimulated immediately to secrete Cl^-, at least to the levels normally seen in seawater-adapted animals (Fig. 4; Wood and Marshall, 1994; Marshall et al., 1997). However, the data presented here indicate that normal Cl^- secretion rates can be reached quickly, over a period of 24–48 h. The observed time course involves hormonal and osmotic stimulation of cells, *de novo* expression of important transport proteins, an increased shunt permeability of transporting epithelia and correction of elevated levels of blood ions and osmolality. The present study of these non-steady-state changes reveals important functional

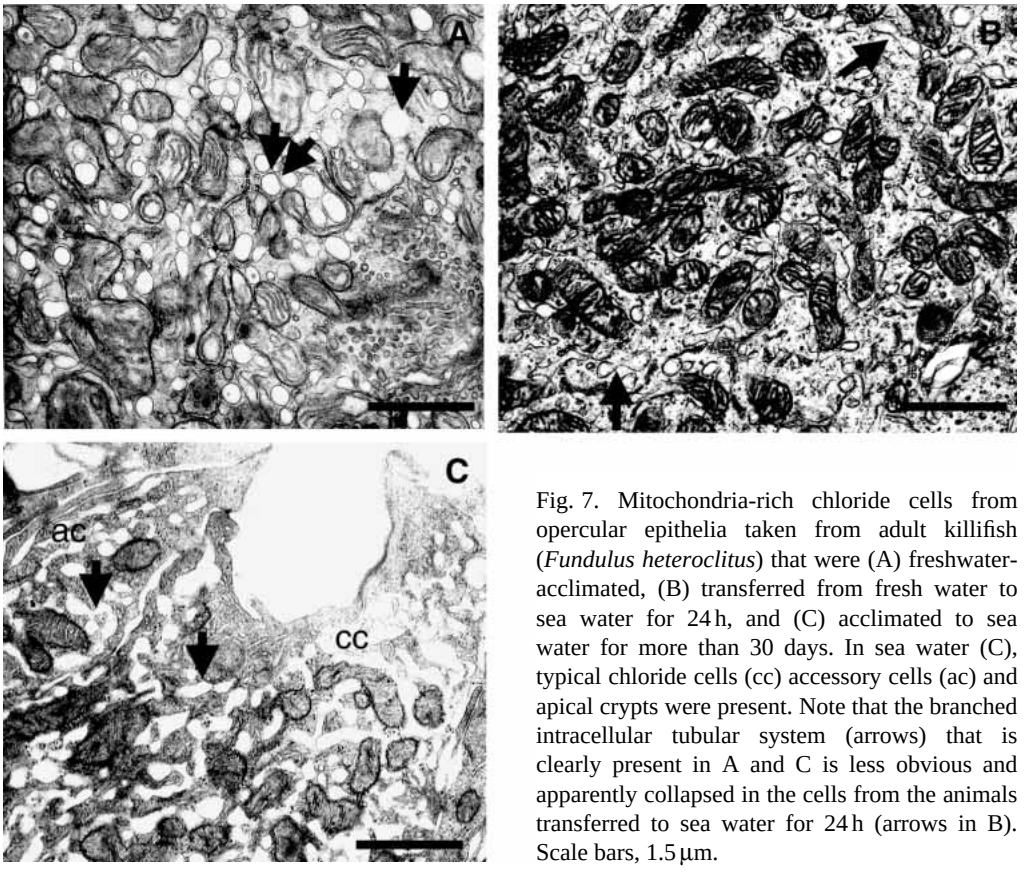

Fig. 7. Mitochondria-rich chloride cells from opercular epithelia taken from adult killifish (*Fundulus heteroclitus*) that were (A) freshwater-acclimated, (B) transferred from fresh water to sea water for 24 h, and (C) acclimated to sea water for more than 30 days. In sea water (C), typical chloride cells (cc) accessory cells (ac) and apical crypts were present. Note that the branched intracellular tubular system (arrows) that is clearly present in A and C is less obvious and apparently collapsed in the cells from the animals transferred to sea water for 24 h (arrows in B). Scale bars, 1.5 μm.

relationships among the multiple variables measured. Whereas events that follow in time are not necessarily causally related, understanding the sequence of events at least provides excellent grounds for the formation of hypotheses and the development of a model.

Killifish show a brief increase in cortisol levels on transfer to sea water (Fig. 2) and a sustained rise in cortisol at 24 h that does not appear in animals transferred from fresh water to fresh water (Fig. 2). That the animals are not overtly stressed is shown by the unchanged blood glucose level (an indirect measure of stress in teleosts; Madsen, 1990a), a level that is comparable with the normal blood plasma glucose concentration of freshwater rainbow trout (3.6–4.3 mmol l^{-1}, Madsen, 1990a; 3.8–5.2 mmol l^{-1}, McCormick et al., 1991) and killifish (3.62±0.12 mmol l^{-1}; mean ± S.E.M., N=5, Mancera and McCormick, 1998). The peak in cortisol levels occurs very early in the transfer protocol, at approximately 1 h, with cortisol returning to control levels at 3 and 8 h after transfer. These results confirm the pattern recorded after transfer of killifish to sea water by Jacob and Taylor (1983), where a pronounced but transient increase in cortisol concentration was seen despite the special precautions taken to eliminate handling stress during the salinity change (similar to the precautions used here). In both studies, cortisol levels returned to control levels after 4–6 h. Changes in cortisol dynamics have been shown to occur in salmonids entering sea water (Nichols and Weisbart, 1985; Nichols et al., 1985) and involve a greater utilization and metabolism of steroids after transfer. An increase in cortisol utilization by the target tissues would explain the low levels of plasma cortisol observed at 3 and 8 h post-transfer. The second, less-dramatic peak in cortisol at 24 h may represent a reduction in cortisol utilization, because the target tissues have effectively switched over to the expression of seawater-adaptive proteins by this time. Of particular importance is the apical membrane anion channel Cl^- secretory epithelium that has been described in killifish Cl^- cells (Marshall et al., 1995) and later cloned, sequenced and identified as a homolog of cystic fibrosis transmembrane conductance regulator, kfCFTR (Singer et al., 1998). Supporting evidence for this comes from Singer et al. (1998), who found, for the same species and transfer protocol, increased kfCFTR expression (relative to β-actin expression) at 8 h, peaking at 24 h and still remaining at levels higher than those in fresh water after 30 days in sea water. It is reasonable to suggest that the most dramatic rise in cortisol levels (at approximately 1 h) is a major cue for the initiation of kfCFTR expression (at 8 h) and that kfCFTR expression is linked to the development of the significant enhancement of Cl^- secretion that occurs 24 h after transfer.

The relationship between changes in Na^+/K^+-ATPase activity and seawater acclimation in killifish is variable. In some cases, there is a clear increase in gill Na^+/K^+-ATPase activity following acclimation to sea water (Jacob and Taylor, 1983), but in transfers from brackish water to sea water, Mancera and McCormick (1998) found no change after 24 h in sea water, nor did activity alter in the present study. This lack of change may be related to the retention of large numbers of mitochondria-rich cells in freshwater-adapted animals and a concomitant high level of Na^+/K^+-ATPase activity in these fish (5.5±0.1 μmol P_i mg^{-1} protein h^{-1}; Mancera and McCormick, 1998; 5.8±0.4 μmol P_i mg^{-1} protein h^{-1}; this study; Fig. 3) compared with other species such as tilapia in which there are few Cl^- cells and Na^+/K^+-ATPase activity is low (0.65±0.11 μmol ADP mg^{-1} protein h^{-1}; Morgan et al., 1997). Overall, our data support the suggestion that cortisol does not act alone to increase Na^+/K^+-ATPase activity; cortisol levels were clearly elevated, but Na^+/K^+-ATPase activity was unchanged. Growth hormone and insulin-like growth factor I (IGF-I) are possible candidates for hormones associated more directly with augmentation of Na^+/K^+-ATPase (Mancera and McCormick, 1998).

The role of plasma osmolality in the regulation of ion transport rates in killifish has been investigated by Zadunaisky et al. (1995), who found that a hyperosmotic shock caused rapid increases in Cl^- secretion rate. Our data confirm this finding and extend the previous work in that opercular epithelia from animals recently transferred to sea water have mitochondria-rich cells that secrete Cl^- only at the elevated plasma osmolality experienced by the animal. Reductions from the *in vivo* osmolality result in pronounced reductions in the rate of Cl^- secretion. Therefore, killifish mitochondria-rich cells appear to respond to rapid changes in plasma osmolality by increasing Cl^- secretion following cell shrinkage and decreasing secretion following cell swelling. The new higher or lower transport rate is kept constant while the cell readjusts within a few hours to the new osmolality (presumably by cell volume regulation). In preliminary work, we have shown that the reductions in Cl^- secretion resulting from cell swelling are a purely osmotic (not ionic) response that is not mediated by intracellular Ca^{2+} or by reductions in cyclic AMP levels (Marshall et al., 1998a). It is not known how widely distributed this characteristic of autoregulation of Cl^- secretion by plasma osmolality is, but potentially it may be present in many euryhaline animals.

There were no differences in gill citrate synthase activity with seawater adaptation, consistent with the relatively stable glycolytic enzyme levels measured during adaptation of tilapia to 70 % sea water (Nakano et al., 1998). The curious result of a significantly higher citrate synthase activity in the long-term freshwater controls compared with the other groups requires further examination. The only difference between the long-term controls and the freshwater transfer group was that the transfer animals were kept as pairs (for 1 week prior to the mock transfer), while the long-term animals were kept as a group of 10. Because the mock transfer produced no differences in any other variable, it is possible that this change in citrate synthase activity is related to the schooling behavior of these fish.

The ultrastructural changes observed in chloride cells 24 h after transfer suggest that the tubular system, the main location of Na^+/K^+-ATPase in chloride cells (Hootman and Philpott, 1979; Ernst and Mills, 1980), has been altered in some way.

The tubular system components, normally fluid-filled and approximately 1 μm in width, are not as clear in the 24 h transfer group as in chloride cells from seawater- or freshwater-acclimated fish (Fig. 7). This could be the result of turnover of the tubular system or the loss of the fluid in the system. It is difficult to explain an osmotically driven collapse of the tubules unless it is the result of a regulatory volume increase that drives ions and fluid from the tubular system into the cytoplasm and thus tends to collapse the tubules. It is reasonable to assume that the rise in plasma osmolality (during the first 24 h in sea water, Fig. 1) will induce osmotic shrinkage and a subsequent regulatory volume increase in the chloride cells. Alternatively, membrane trafficking processes could remove portions of the system and replace them progressively during salinity acclimation. To our knowledge, the ultrastructure of chloride cells has not been examined previously during the salinity adaptation process, but in fully acclimated animals only. The present results indicate that further examination of cell ultrastructure during the periods of greatest change could reveal functionally important information.

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