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McCORMICK, STEPHEN D. *Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane.* Am. J. Physiol. 259 (Regulatory Integrative Comp. Physiol. 28): R857–R863, 1990.—Opercular membranes from freshwater tilapia (*Oreochromis mossambicus*) were maintained in vitro for 4 days and exposed to several concentrations of cortisol (0, 0.01, 0.1, 1, and 10 μg/ml). Chloride cell size, number, and Na^+-K^+-ATPase content were examined using a fluorescent mitochondrial dye (dimethylaminostyrylethylpyridiniumiodine), a fluorescent analogue of ouabain (anthroylouabain) that binds specifically to Na^+-K^+-ATPase, and a cytological stain specific for plasma and tubular membranes. In the absence of cortisol, chloride cell density of the freshwater tilapia opercular membrane decreased (from initial levels of 6,114 ± 451 to 18 ± 9 cells/cm^2) and was restored by cortisol in a dose-dependent manner. Chloride cell height (5.5 ± 0.3 μm initially and 7.8 ± 0.5 μm after 4 days in vitro) increased twofold (13.1 ± 0.7 μm) after exposure to 1 μg/ml cortisol. Initially and after 4 days in control medium, there was no detectable staining with anthroylouabain; exposure to 1 μg/ml cortisol resulted in the appearance of numerous anthroylouabain-positive chloride cells. Without cortisol, Na^+-K^+-ATPase activity of the opercular membrane remained constant through 4 days of culture (0.4–0.6 μmol ADP·mg protein^{-1}·h^{-1}); addition of cortisol caused a dose-dependent increase to a maximum of 1.2 ± 0.1 μmol P_i·mg protein^{-1}·h^{-1}. In vitro cortisol also maintained the size, density, and appearance of chloride cells from opercular membrane of seawater-adapted tilapia. The results indicate that in vitro cortisol exposure causes morphological and biochemical differentiation of the seawater form of the chloride cell.

Oreochromis mossambicus; sodium-potassium-adenosinetriphosphatase; sodium pump; anthroylouabain; osmoregulation; teleost

IN TELEOST FISHES the chloride cell is primarily responsible for net ion secretion in seawater (10). Located in gill and skin, chloride cells contain high concentrations of Na^+-K^+-ATPase that generate ionic and electrical gradients used for net ion transport (18, 28). Na^+-K^+-ATPase is located in the tubular system (continuous with the basolateral membrane), which is in turn associated with numerous mitochondria (13, 17). The latter characteristic has given rise to the term mitochondria-rich cell as a synonym for the chloride cell (see Refs. 5, 14 for review of morphology and physiology of chloride cell).

Our knowledge of the endocrine regulation of chloride cells and Na^+-K^+-ATPase has previously relied on in vivo studies. In tilapia, cortisol injections can increase chloride cell size and density (9) and gill Na^+-K^+-ATPase activity (7). Other hormones, however, including thyroid hormones [in tilapia and salmonids (7, 22)] and growth hormone [in salmonids (4, 22, 25)], can increase gill Na^+-K^+-ATPase activity and have thereby been implicated in regulating chloride cell differentiation. Prolactin may have a role in the inhibition of seawater chloride cell development (12). McCormick and Bern (21) have recently shown that in vitro cortisol treatment increases Na^+-K^+-ATPase activity and maximum ouabain binding in coho salmon gill.

To examine the direct endocrine control of chloride cell density, size, and morphology, a method for the organ culture of the opercular membrane of tilapia was developed. This membrane contains numerous chloride cells and displays ion transport characteristics that are similar to those of the gill (9, 10, 16). Chloride cells are present in both freshwater- and seawater-adapted fish but possess distinct morphologies and biochemical characteristics (9, 14, 20, 23). In the present study, the in vitro development of the seawater form of the chloride cell (in opercular membranes from freshwater tilapia) is examined in the presence and absence of cortisol. The mitochondrial stain dimethylaminostyrylethylpyridiniumiodine (DASPEI) was used to quantify chloride cell size and number. A fluorescent analogue of ouabain, anthroylouabain, was used to localize changes in Na^+-K^+-ATPase and to complement biochemical measurement of Na^+-K^+-ATPase activity of the opercular membrane. Finally, an osmium-based cytological stain was used to examine the development of the tubular system and cell height of chloride cells. The results of these experiments indicate that cortisol directly stimulates the differentiation of seawater chloride cells.

MATERIALS AND METHODS

Animals. Adult tilapia (*Oreochromis mossambicus*) of both sexes (20–50 g) that had been reared in an outdoor freshwater pond were maintained in 80-liter tanks at 27 ± 1°C and at a photoperiod of 12:12 h light-dark. Aeration was provided, and water was continuously recirculated through a charcoal and bioactive filter system with ultraviolet light. Seawater was obtained by adding appropriate amounts of Instant Ocean synthetic sea salt to achieve 33 ± 1 parts/thousand (ppt) and was checked daily. Fish were fed Hikari Cichlid Gold (Kyorin, Japan) ad libitum once daily.

Opercular membrane culture. Opercular membranes were prepared by a modification of the method of Foskett et al. (9). Animals were killed by decapitation and pithing, and the entire operculum was removed and placed in tilapia Ringer [TR, containing (in mM) 140 NaCl, 15 $NaHCO_3$, 4 KCl, 1 $MgSO_4$, 1 KH_2PO_4, 2 $CaCl_2$, and 5.6 glucose, pH 7.8]. The severed end was held stationary with alligator clips while the skin of the inner surface of the operculum was gently dissected away by reflecting the skin upward and severing the underlying connective tissue with iridectomy scissors. The basal surface of the opercular membrane was affixed to type IV collagen-coated lens paper that had been sterilized under ultraviolet light. Culture procedures and media were modified from the method of gill organ culture reported by McCormick and Bern (21). Membranes were placed in minimal essential medium [MEM; GIBCO, Hanks' salts, with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.55] with 4 mg/ml bovine serum albumin, 500 U/ml penicillin, and 250 μg/ml streptomycin in sterile 12-well culture plates and preincubated at 27°C for 1–2 h with gentle shaking. Preincubation medium was then removed and replaced with 2 ml MEM (Earle's salts, no HEPES, pH 7.55 after gassing) with 4 mg/ml bovine serum albumin, 292 μg/ml L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamycin sulfate, 5 μg/ml bovine insulin, and cortisol or cortisol vehicle (ethanol, maximum 0.1% by volume). Opercular membranes were incubated at 27°C in humidified chambers gassed with 95% O_2-5% CO_2, with gentle shaking for 4 days. The gross and histological appearance of the tissue was normal throughout the culture period. Trypan blue exclusion was >99.9% ($n = 4$). The efficacy of trypan blue exposures to detect cell damage was verified by cutting the tissue in half; trypan blue uptake occurred along the severed edges of the tissue.

Fluorescence experiments. DASPEI is a mitochondrion-specific stain that presumably stains only active mitochondria (3) and that has been widely used as a stain for teleost chloride cells (9, 15). Opercular membranes were rinsed in TR and placed in TR containing 2 μM DASPEI at room temperature for 1 h. The tissue was then rinsed twice in TR and examined using a Zeiss standard RA microscope modified for epifluorescence imaging with a Ploemopak illuminator (100 W HBO) containing a 450- to 490-nm band-pass excitation filter, a 510-nm chromatic beam splitter, and a 520-nm long-wave-pass filter.

Chloride cell density was determined in the following manner. The proportion of the membrane containing chloride cells was first estimated by determining the proportion of fields (0.46 mm^2) that contained one or more chloride cells (always >80%). The number of DASPEI-positive cells were then counted in at least 12 randomly selected nonempty fields. The average value of these measurements was multiplied by the proportion of fields containing chloride cells to yield a value for the entire membrane that was expressed as cells per square centimeter.

Cell size was calculated from micrographs as reported by McCormick (20); to compare the relative brightness of staining, the same time exposure was used for any given magnification. Cell size was measured by projecting micrographs onto a digitizing pad and tracing the cell outline from which the cross-sectional area (μm^2) was determined (Sigmascan, Jandel Scientific). Micrograph of a micrometer slide at the same magnification was used for calibration. At least 30 chloride cells were measured in each preparation to yield a single average value.

Anthroylouabain binds specifically to Na^+-K^+-ATPase, after which fluorescence increases 10-fold (8). A method for its use in fluorescent staining of teleost chloride cells was presented by McCormick (20). The opercular membrane is rinsed in 4 ml low-potassium TR (LKTR; tilapia Ringers with 0.1 mM KCl) at room temperature for 1 min and then placed in 4 ml of 2 μM anthroylouabain (Sigma) in LKTR aerated with 100% O_2 at room temperature for 1 h. The tissue was then removed and rinsed twice in 2 ml ice-cold LKTR for 3 min and examined as above with a 365-nm excitation filter, a 395-nm chromatic beam splitter, and a 420-nm long-wave-pass filter. Cell density and size were measured as described above.

Osmium staining of tubular system. Champy-Maillet's fixative, which stains phospholipids, has previously been used to stain specifically chloride cells because of their extensive tubular system that is continuous with the plasma membrane (2, 11). Opercular membranes were placed in 0.2% osmic acid solution with saturated zinc powder and 25 mg/ml iodine for 16 h. After several rinses with distilled water, the tissue was dehydrated and embedded in paraffin, using standard histological procedures. The tissue was cut in 5-μm transverse sections and mounted on glass slides; cell heights were measured directly.

Measurement of Na^+-K^+-ATPase activity. Opercular membranes were placed in 500 μl ice-cold sucrose-EDTA-imidazole buffer (SEI; 0.3 M sucrose, 0.02 M EDTA, 0.05 M imidazole, pH 7.3), frozen immediately on dry ice, and stored for up to 30 days at −80°C. Na^+-K^+-ATPase activity was measured as described previously (21). Five to 10 min before assay, the tissue was rapidly thawed, removed from SEI buffer, and homogenized in 85 μl SEI buffer with 0.1% Na deoxycholate in a 200 μl-capacity ground glass homogenizer (Wheaton no. 357848). The homogenate was centrifuged at 5,000 *g* for 30 s to remove connective tissue. The supernatant was removed and assayed for Na^+-K^+-ATPase activity and protein content.

Fifteen to 25 μl gill homogenate were placed in a final volume of 1 ml assay mixture containing 50 mM imidazole, 1 U/ml lactate dehydrogenase, 2.5 U/ml pyruvate kinase, 2 mM phospho*enol*pyruvate, 0.05 mM NADH, 0.5 mM ATP, 0.4 mM KCN, 45 mM NaCl, 2.5 mM $MgCl_2$, and 10 mM KCl (pH 7.5). A duplicate cuvette run simultaneously contained 0.5 mM ouabain. The assay mixture was quickly mixed, and the change in absorbance (340 nm) was recorded in a Perkin-Elmer recording spectrophotometer. The assay mixture was maintained at 25°C before assay, and the temperature of cuvettes was also maintained at this temperature during the assay in a water-jacketed cuvette holder.

The rate of NADH oxidation (equimolar to ATP hydrolysis and ADP production) was calculated from the linear rate between 6 and 10 min after initiation of the reaction. The absolute level of ATP hydrolysis was determined from an experimentally determined ADP standard curve. Protein content of the homogenate was measured by the method of Larson et al. (19), using bovine serum albumin as standard. Na^+-K^+-ATPase was calculated as the difference in ouabain-sensitive and -insensitive ATP hydrolysis and expressed as micromoles of ADP per milligram of protein per hour.

Statistics. Nonparametric statistics using the CRISP program (CRUNCH, Berkeley, CA) were utilized for comparing groups. When only two groups were compared, the Mann-Whitney *U*-test was used. When three or more groups were compared, the Kruskal-Wallis test was utilized; if there was a significant treatment effect, a posteriori comparisons were carried out using the Mann-Whitney *U*-test. A confidence level of 95% ($P = 0.05$) was used in all cases.

RESULTS

Initial experiments were conducted using opercular membrane of freshwater tilapia. In the absence of cortisol, DASPEI-positive cell density was reduced from initial levels of 6,114 ± 451 cells/cm^2 in opercular membrane of freshwater tilapia to 19 ± 9 cells/cm^2 after 4 days in vitro (Fig 1*A*). Addition of cortisol resulted in a substantial increase in chloride cell density relative to control levels (Fig. 1*B*); this increase was dose dependent (Fig. 2). All doses of cortisol resulted in a significant increase in chloride cell density relative to control levels ($P < 0.05$). The maximum density (7,011 ± 2,298 cells/cm^2) occurred at the highest dose of cortisol (10 μg/ml) and was not significantly different from the initial density of chloride cells in freshwater tilapia (see above) or in seawater-adapted tilapia (8,851 ± 1,139 cells/cm^2; $P > 0.3$).

DASPEI-positive cell size (cross-sectional area of chloride cells) was initially 69 ± 6 μm^2 and increased to 133 ± 8 μm^2 in the few chloride cells that appeared in the absence of cortisol (Fig. 2). In the presence of cortisol, DASPEI-positive cell size was twofold greater than initial levels but was not significantly different from in vitro controls (in the absence of cortisol; Fig. 2). DASPEI-positive cells in opercular membrane of freshwater tilapia

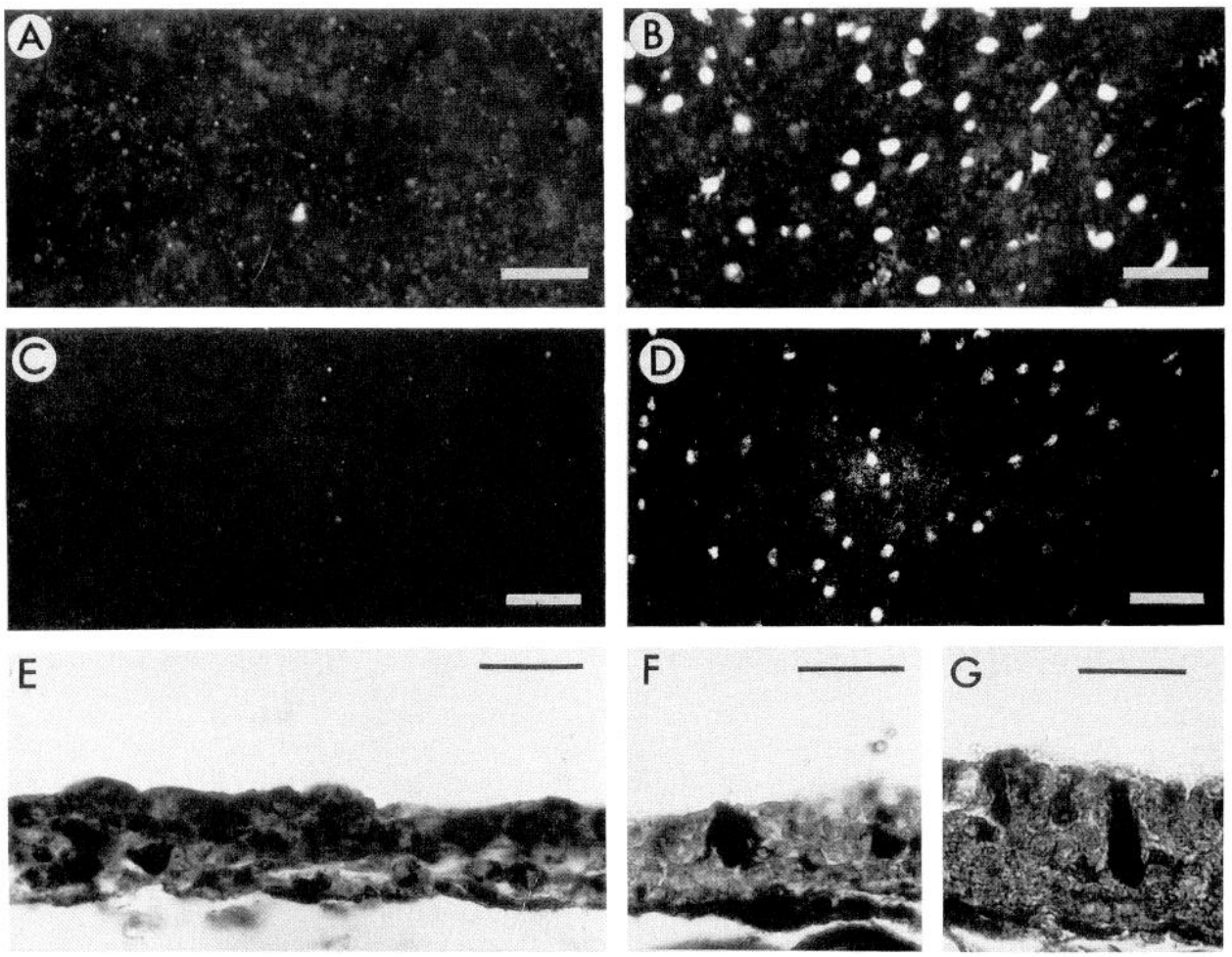

FIG. 1. Differentiation of chloride cells in response to in vitro cortisol (1 μg/ml) in opercular membrane from freshwater tilapia. Dimethylaminostyrylethylpyridiniumiodine (DASPEI) staining after 4 days in vitro in absence (*A*) and presence (*B*) of cortisol. Anthroylouabain staining after 4 days in vitro in absence (*C*) and presence (*D*) of cortisol. Osmium staining after 4 days in vitro in absence (*E*) and presence (*G, F*) of cortisol. Bar = 100 μm *A–D*; 20 μm *E–G*.

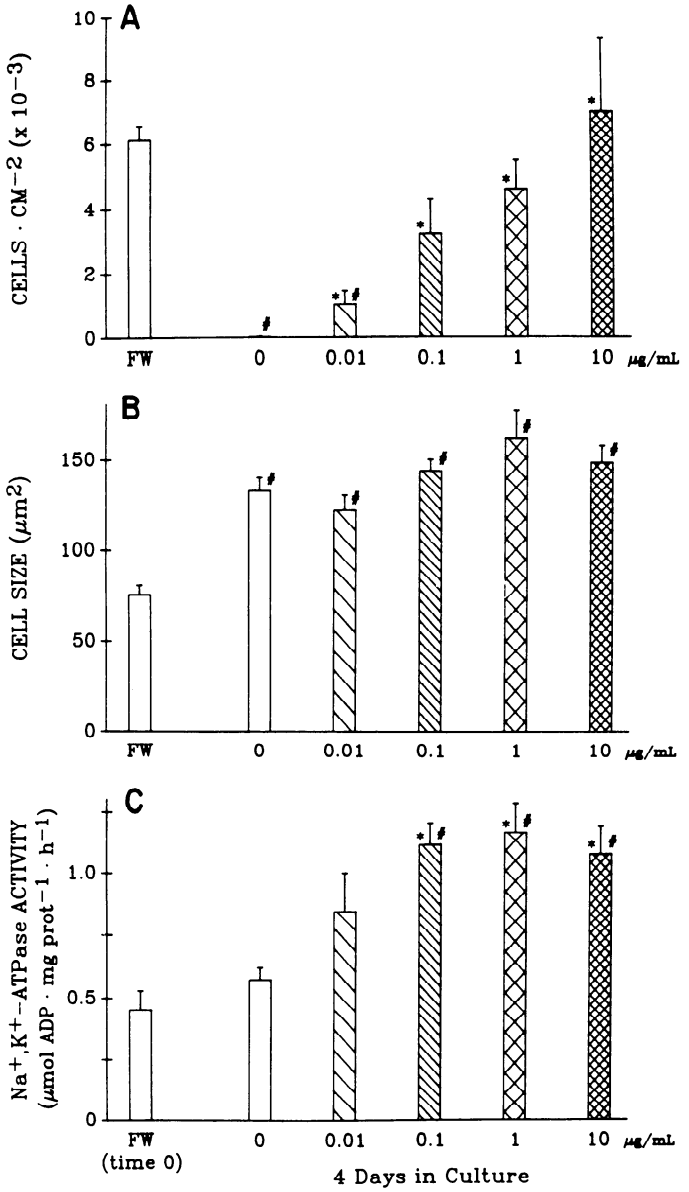

FIG. 2. Effect of cortisol (4 days in vitro) on DASPEI-positive (chloride) cell density (*A*) and size (*B*) and on Na^+-K^+-ATPase activity (*C*) in opercular membrane of freshwater tilapia. Values are means ± SE. Sample size was 5–7 membranes at each dose. Significant difference from #initial [freshwater (FW)] and *control (day 4) levels ($P < 0.05$, Kruskal-Wallis test followed by Mann-Whitney *U*-test).

before organ culture are round in appearance, as are the few chloride cells that appeared in vitro in the absence of cortisol. In the presence of cortisol, most DASPEI-positive cells were also round, although a small number (5–20%) had a "gooseneck" appearance, in that a process from the main body of the cell projected toward the apical surface of the membrane (the process was not in the same focal plane as the main body of the cell). The brightness of DASPEI staining was also affected by cortisol: chloride cells in the absence of cortisol were generally less bright than those in the presence of cortisol (Fig. 1, *A* and *B*).

Osmium staining of the opercular membrane of freshwater tilapia before and after 4 days in culture (Fig. 1*E*) revealed round chloride cells that were distinct from epithelial cells but stained more lightly than seawater

TABLE 1. *Density and size (cross-sectional area) of anthroylouabain-positive chloride cells in opercular membrane of freshwater tilapia*

	Freshwater	4 Days in Culture		Seawater
		Control	1 μg/ml Cortisol	
Cell density, cells/cm²				
Range	ND	ND	1,197–12,067	5,687–11,309
Means ± SE			4,646±2,560	9,564±1,303
Cell size, μm²				
Range	ND	ND	103–116	234–392
Means ± SE			110±3	304±40

Opercular membranes in culture were from freshwater tilapia; seawater membranes were from fish that had been adapted to 33 ppt seawater for at least 2 wk. Sample size was 1 membrane from 4 different individuals in each case. ND, fluorescence not detectable.

TABLE 2. *Effect of cortisol in vitro for 4 days on DASPEI-positive chloride cell density and size and on Na^+-K^+-ATPase activity in opercular membrane of seawater-adapted tilapia*

	Cell Density, cells/cm²	Cell Size, μm²	Na^+-K^+-ATPase Activity, μmol ADP·mg protein^{-1}·h^{-1}
Initial	8,851±1,139	333±16	1.34±0.19
In vitro			
Control	143±100†	329±87	0.97±0.16
Cortisol, μg/ml			
0.01	2,086±514*†	416±34	1.39±0.23
0.1	3,214±844*†	427±32	1.52±0.15*
1.0	2,703±568*†	387±19	1.81±0.20*

Values are means ± SE; n = 5–12 membranes for each dose. * Significantly different from control, $P < 0.05$, Kruskal-Wallis test followed by Mann-Whitney *U*-test. † Significantly different from initial, $P < 0.05$, Kruskal-Wallis test followed by Mann-Whitney *U*-test.

chloride cells. Exposure to 1 μg/ml cortisol for 4 days resulted in the appearance of numerous dark-staining chloride cells (Fig. 1, *F* and *G*), many of which were columnar in appearance. Many of these cells (16%) spanned the thickness of the membrane (Fig 1*G*); no such large columnar cells were observed in initial (freshwater) or control tissues. Osmium-positive chloride cell height was 5.5 ± 0.3 μm initially and 7.8 ± 0.5 μm after 4 days in vitro. Addition of cortisol (1 μg/ml) increased cell height twofold to 13.1 ± 0.7 μm (significantly greater than both initial and control levels, $P < 0.05$).

Initially and after 4 days in control medium, there was no detectable staining with anthroylouabain. Exposure to 1 μg/ml cortisol for 4 days resulted in the appearance of numerous anthroylouabain-positive chloride cells that ranged in density from 1,197 to 12,067 cells/cm² and in cell size from 102 to 116 μm² (Table 1). These values are comparable to the densities and cell sizes seen for DASPEI-positive chloride cells (Figs. 1*B* and 2). The slightly smaller cell size measured with anthroylouabain is apparently an inherent difference in the two vital stains, as chloride cell size in opercular membranes from seawater-adapted tilapia is slightly larger when measured after DASPEI (333 ± 16 cm²) than after anthroylouabain (304 ± 40 cm²).

Na^+-K^+-ATPase activity of the opercular membrane

of freshwater tilapia was 0.45 ± 0.08 μmol ADP·mg protein^{-1}·h^{-1} and remained constant through 4 days of culture (0.57 ± 0.05 μmol ADP·mg protein^{-1}·h^{-1}, P = 0.2). Addition of cortisol caused a dose-dependent increase in Na^+-K^+-ATPase activity to a maximum of 1.16 μmol P_i·mg protein^{-1}·h^{-1} at 1 μg/ml cortisol, nearly a threefold increase over initial levels (Fig. 2) and comparable to the activity in opercular membrane of seawater-adapted tilapia (1.34 μmol ADP·mg protein^{-1}·h^{-1}; Table 2). Na^+-K^+-ATPase activity of opercular membranes exposed to 0.1, 1, and 10 μg/ml were all significantly greater than initial and control levels ($P < 0.05$), with Na^+-K^+-ATPase activity at 0.01 μg/ml cortisol having an intermediate increase, indicating a dose-related response.

The effect of cortisol in vitro on fully differentiated chloride cells was examined by maintaining the opercular membrane of seawater-adapted tilapia in organ culture. The density of DASPEI-positive cells decreased from an initial level of 8,851 ± 1,139 to 143 ± 100 cells/cm^2 after 4 days in organ culture. Addition of cortisol (0.01, 0.1, and 1 μg/ml) increased chloride cell density significantly above control levels to a maximum of 3,214 ± 844 cells/cm^2 at 0.1 μg/ml cortisol (Table 2). However, there was no apparent dose dependence, and the density at all doses was significantly lower than that seen initially. DASPEI-positive chloride cell size was not significantly changed in vitro. Na^+-K^+-ATPase activity of the opercular membrane was 1.34 ± 0.19 μmol ADP·mg protein^{-1}·h^{-1} initially and declined to 0.97 ± 0.16 μmol ADP·mg protein^{-1}·h^{-1} after 4 days in organ culture. Addition of cortisol to the medium increased Na^+-K^+-ATPase activity in a dose-dependent manner to a maximum of 1.81 ± 0.20 μmol ADP·mg protein^{-1}·h^{-1} at 1 μg/ml cortisol (Table 2).

DISCUSSION

The methods used for identification of chloride cells in the present study have been used previously in several teleost species (2, 9, 11, 15, 20); their specific targets represent different characteristics of this highly specialized cell and may not necessarily be altered simultaneously. In particular, the two fluorescent dyes are at least partially dependent on the current activity of the cell; activity and number of mitochondria in the case of DASPEI (3) and turnover and amount of Na^+-K^+-ATPase in the case of anthroylouabain (8, 20). Staining with osmium is dependent solely on the presence of a tubular system (11); the intensity of staining apparently depends on its increase and extension throughout the cell (2).

In the present study, the number of DASPEI-positive chloride cells was greatly reduced through 4 days of organ culture in the absence of cortisol (Figs. 1 and 2). Most of these cells were less bright than those seen initially and in the presence of cortisol. Although we did not attempt to quantify this difference, it probably reflects a reduction of mitochondrial activity and/or number in these cells. It is interesting to note that the few DASPEI-positive cells present in the absence of cortisol have a larger cross-sectional area (Fig. 2). These cells may represent a small number of cells that have been stimulated in vivo [perhaps by cortisol (9)] and have hypertrophied in vitro. These apparently are not differentiating, however, as cell height was not increased markedly and there was no detectable anthroylouabain staining in the absence of cortisol.

The density of chloride cells was clearly affected by cortisol treatment in vitro. From the present study we cannot determine whether this is the result of the maintenance and differentiation of existing (freshwater) chloride cells or the de novo production of chloride cells. At a dose of 1 μg/ml, the density of DASPEI-positive chloride cells was restored to that seen initially in freshwater tilapia. Intermediate doses resulted in intermediate increases in chloride cell density (Fig. 2). These intermediate doses can be considered "physiological" in that they are similar in magnitude to circulating levels for this species. Values in freshwater range between 5 and 40 ng/ml; exposure to seawater results in a transient increase to 100–160 ng/ml (1). Acute and chronic stress can increase circulating cortisol up to 350 ng/ml in some teleosts (24).

The effects of cortisol on chloride cell density in the present study differs from the in vivo cortisol treatment reported by Foskett et al. (9). In the latter experiments, cortisol injections in freshwater tilapia increased cell density threefold relative to saline-injected animals. In the present study, although cortisol clearly maintained the number of chloride cells seen initially, it did not increase the density above that seen initially. It is possible that the capacity of cortisol in vivo is the result of a longer period of treatment. Alternatively, there may be a limitation in the organ culture system or missing endocrine factors that do not permit hyperplasia of chloride cells in response to cortisol in vitro. The physiological importance of this difference is unclear, as chloride cell density of the opercular membrane increases only transiently after transfer to seawater [2-fold increase after 3 days in seawater (9)] and does not differ significantly after ≥2 wk in seawater (Fig. 1 and Table 2; Ref. 9).

The increase in cross-sectional area of DASPEI-positive chloride cells in response to cortisol is problematical in that cell size also increased in the few DASPEI-positive cells that appeared in the absence of cortisol. More clear are the changes in cell height, which doubled in the presence of cortisol. A significant proportion of these cells completely spanned the opercular membrane, thereby achieving contact with the serosal and external fluids. [The inability to always observe chloride cells completely spanning the membrane is the result of the relatively thick sections (5 μm) relative to the width of the chloride cell (10–15 μm); only 25% of chloride cells from seawater-adapted tilapia were seen to completely span the membrane.] Many DASPEI-positive chloride cells in the presence of cortisol had an armlike projection from the main body of the cell in an apparent attempt to span the width of the membrane. These cells and others spanning the membrane were observed after osmium staining and were never observed in the absence of cortisol. Serosal-external contact is an important functional characteristic of epithelial transport cells (26) and

of seawater chloride cells in particular (14, 23). The ability of in vitro cortisol treatment to induce increases in cell height and to cause serosal-mucosal contact is a strong indication that cortisol alters the morphology and function of the chloride cell.

In most euryhaline teleosts, gill Na^+-K^+-ATPase activity increases three- to fivefold after adaptation to seawater (18). The increase in Na^+-K^+-ATPase can be localized primarily to the chloride cells (20, 27) and more specifically to the extensive tubular system of the chloride cell (13, 17). Karnaky et al. (14) estimates that there are up to 10^8 Na^+-K^+-ATPase molecules/chloride cell in seawater-adapted *Fundulus heteroclitus*. Na^+-K^+-ATPase activity of the entire opercular membrane of tilapia increases from 0.45 to 1.34 μmol ADP·mg protein^{-1}·h^{-1} after transfer from freshwater to seawater (Fig. 2 and Table 2).

Using organ cultures of gill filaments, McCormick and Bern (21) demonstrated a highly specific, direct stimulatory effect of cortisol on gill Na^+-K^+-ATPase activity and on the number of ouabain-binding sites in coho salmon (*Oncorhynchus kisutch*). They observed a 50% increase in gill Na^+-K^+-ATPase activity after 4 days in vitro with 10 μg/ml cortisol at 14°C; 1 μg/ml resulted in intermediate increases, and significant effects were not observed at 0.1 μg/ml. In the present study conducted at 25°C, threefold increases in Na^+-K^+-ATPase activity were observed with 1 μg/ml cortisol (from 0.45 initially to 1.20 μmol ADP·mg protein^{-1}·h^{-1}), and intermediate increases in Na^+-K^+-ATPase activity were observed with a dose as low as 0.01 μg/ml (Fig. 2).

With the use of a fluorescent derivative of ouabain, changes in Na^+-K^+-ATPase activity can be localized to the chloride cell (20; present study); there is no detectable staining with 2 μM anthroylouabain in the opercular membrane of freshwater tilapia nor after 4 days in vitro in the absence of cortisol (Table 1). Exposure to 1 μg/ml cortisol for 4 days resulted in the appearance of numerous anthroylouabain-positive cells, demonstrating that the observed increases in Na^+-K^+-ATPase activity are located primarily in chloride cells. Although the intensity of fluorescence was not quantified, these cells appeared less bright than those in the opercular membrane of seawater-adapted tilapia. There was a wide range in the density of anthroylouabain-positive cells in response to cortisol (Table 2), and the mean value was lower than that for DASPEI-positive cells in response to the same dose of cortisol (Fig. 2). It is possible that most but not all chloride cells are expressing and activating Na^+-K^+-ATPase after in vitro cortisol treatment.

In the present study, insulin was included under all in vitro conditions (5 μg/ml). McCormick and Bern (21) observed that insulin in vitro (0.1–10 μg/ml) had no effect on gill Na^+-K^+-ATPase activity in the presence or absence of cortisol (0.1–10 μg/ml). However, subsequent studies (McCormick, unpublished observations) have shown that insulin can slightly but significantly decrease the in vitro decline in gill Na^+-K^+-ATPase activity that occurs when initial levels of Na^+-K^+-ATPase are high, such as in seawater-adapted fish. Studies with mammalian tissue have shown insulin to be required for eliciting many endocrine responses in vitro (29). It is unlikely that insulin is having an important direct effect on Na^+-K^+-ATPase in the present study, as Na^+-K^+-ATPase activity of initial and control membranes in the presence of insulin for 4 days were not significantly different (Fig. 2) nor could insulin by itself prevent the disappearance of DASPEI-positive chloride cells that occurred in the absence of cortisol.

Although no attempt to record the time course of changes in vitro was made in the present study, subsequent studies have shown that a dramatic decline in DASPEI-positive cell density also occurs after 2 days in vitro and that this decline can be restored by cortisol (S. D. McCormick and T. Herndon, unpublished results). The response to cortisol after 4 days in vitro is comparable to that seen in vivo during seawater adaptation. Foskett et al. (9) found that changes in DASPEI-positive cell size were detectable after 3 days in seawater; chloride cell size and development of short-circuit current (chloride secretion) did not reach peak levels until 3 wk after transfer. In light of this extended period of in vivo development, the observation that opercular membrane Na^+-K^+-ATPase activity reaches normal seawater levels within 4 days of in vitro cortisol treatment is of interest. Unfortunately, there is no information on temporal changes in opercular membrane Na^+-K^+-ATPase activity in response to seawater exposure. Dange (6) found that gill Na^+-K^+-ATPase activity of tilapia reached peak levels within 1 wk of transfer to 26 ppt seawater.

In addition to its differentiative effects, cortisol had an apparent maintenance effect on the chloride cells in the opercular membrane from seawater-adapted tilapia. Cell density was significantly increased at all doses of cortisol (0.01, 0.1, and 1 μg/ml) relative to controls, although not to the levels seen initially (Table 2). There was some indication that in vitro cortisol may cause hypertrophy of seawater chloride cells and increased Na^+-K^+-ATPase of the opercular membrane (Table 2), although the changes were not statistically significant.

The present study demonstrates that in vitro cortisol induces increased chloride cell size and serosal external contact, maintains chloride cell density, and increases Na^+-K^+-ATPase activity that can be localized specifically to chloride cells. These observations are consistent with the hypothesis that cortisol directly stimulates the differentiation of seawater chloride cells. Further studies are necessary to determine whether cortisol can induce all of the morphological and biochemical characteristics of seawater chloride cells and whether cortisol can directly increase the total capacity or rate of ion transport.

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