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Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills

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Received 18 October 2000; accepted in final form 8 May 2001

Kelly, Scott P., and Chris M. Wood. Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. Am J Physiol Regulatory Integrative Comp Physiol 281: R811–R820, 2001.—Cortisol had dose-dependent effects on the electrophysiological, permeability, and ion-transporting properties of cultured pavement cell epithelia derived from freshwater rainbow trout gills and grown on cell culture filter supports. Under both symmetrical (L15 media apical/L15 media basolateral) and asymmetrical (freshwater apical/L15 media basolateral) culture conditions, cortisol treatment elevated transepithelial resistance, whereas permeability of epithelia to a paracellular permeability marker (polyethylene glycol-4000) decreased. Cortisol did not alter the Na
- K
-ATPase activity or the total protein content of the cultured preparations. During 24-h exposure to asymmetrical conditions, the net loss rates of both Na
+ and Cl
- to the water decreased with increasing cortisol dose, an important adaptation to dilute media. Unidirectional Na
+ and Cl
- flux measurements and the application of the Ussing flux-ratio criterion revealed cortisol-induced active uptake of both Na
+ and Cl
- under symmetrical culture conditions together with an increase in transepithelial potential (positive on the basolateral side). Under asymmetrical conditions, cortisol did not promote active ion transport across the epithelium. These experiments provide evidence for the direct action of cortisol on cultured pavement cell epithelia and, in particular, emphasize the importance of cortisol for limiting epithelial permeability.

Until now, the majority of reconstructed epithelium characterization has been conducted without the addition of hormonal support (7, 41, 43). The issue of hormonal support was recently addressed (11), but no beneficial effects were demonstrated. However, only single low doses of either teleost prolactin or teleost growth hormone were used, and the majority of work was done after the abrupt addition of the hormone supplement. It is possible that a single low dose of hormone and/or the abrupt addition of hormonal supplements to cultured pavement cell epithelia are insufficient to stimulate changes in epithelial physiology.

Cortisol plays an important role in the hydromineral balance of fish (for reviews, see Refs. 28 and 40). In branchial tissue, the mechanisms of action include alterations in gill chloride cell morphology and development and regulation of key ion-transporting enzymes, all of which generally result in increased salinity tolerance during seawater entry (for review, see Ref. 28) or increased ion uptake in freshwater fish species (17, 34, 36). Similar morphological and biochemical effects have been reported to occur in vitro both in branchial tissue (29) and opercular epithelial tissue in organ culture (26), a strong indication of the direct action of cortisol on teleostean ion-transporting tissues.

With the use of an established technique for the primary culture of an epithelium composed exclusively of gill pavement cells (43), we investigated the effects of cortisol on a cell type that, in vivo, covers up to 90% of the gill surface. This contrasts with virtually all other studies that have focused exclusively on potential effects of cortisol on chloride cells. This is particularly relevant in light of the recent reports of cellular gene expression and localization of cortisol receptors in pavement cells of chum salmon (Oncorhynchus keta) (39). Recent evidence has demonstrated that cortisol may play an important role in the modulation of ion-transporting enzymes related to hyperosmoregulation, namely the H
+ -ATPase (20) and the Na
+ -K
+ -ATPase (28) with associated effects on branchial ionic influx in freshwater fish (17, 34). This seems to indicate a functional role for cortisol in the ionoregulatory physiology of freshwater fish. Although much of this work has
been interpreted in light of altered chloride cell numbers and exposure, currently, popular models of ionic uptake in freshwater fish place Na\(^+\) influx, at least in part, across the pavement cell (33). We therefore investigated whether cortisol would promote active Na\(^+\) and/or Cl\(^-\) uptake from the apical medium by this pure pavement cell epithelium. Lastly, the cultured pavement cell epithelium is a very suitable preparation for studies on passive transport and electrical characteristics of the gill (11, 41, 43). In the ionoregulatory physiology of fish adapted to dilute media, changes in gill permeability may be just as important as changes in active transport. We therefore investigated the effects of cortisol on these passive properties of the gill, a potential mechanism(s) of action that, to date, has received little or no attention.

**MATERIALS AND METHODS**

**Preparation of cultured branchial epithelia.** Rainbow trout (*Oncorhynchus mykiss*; 80–175 g) were held in dechlorinated running tap water (composition: [Na\(^+\) = 0.55, [Cl\(^-\) = 0.70, [Ca\(^2+\) = 1.00, [Mg\(^2+\) = 0.15, [K\(^+\) = 0.05 mM, pH 7.8–8.0]. Photoperiod and temperature varied seasonally (13–17°C). Fish were stunned by a blow to the head and then decapitated. All procedures for gill cell isolation were conducted in a laminar flow hood using sterile techniques. Methods for initial gill cell isolation have previously been described (32, 43). Briefly, gill cells were obtained from excised gill filaments by two consecutive cycles of trypsin digestion (Gibco Life Technologies, 0.05% trypsin in PBS with 5.5 mM EDTA) and resuspended in culture medium [Leibovitz’s L-15 supplements by two consecutive cycles of tryptic digestion (Gibco 43). Briefly, gill cells were obtained from excised gill filaments by two consecutive cycles of trypsin digestion (Gibco Life Technologies, 0.05% trypsin in PBS with 5.5 mM EDTA) and resuspended in culture medium [Leibovitz’s L-15 supplemented with 2 mM glutamine, 5–6% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml gentamycin]. Subsequent flask cell culture and epithelial culture procedures were based on previously established methods (43). For flask culture, cells were seeded at a density of 500,000 to 800,000 cells/cm\(^2\) in culture medium into 25-cm\(^2\) flasks (Falcon) and kept at 18°C in an air atmosphere. Nonadherent cells were removed by changing media [L-15 + 2 mM glutamine, 6% FBS, and antibiotics (see above)] at 24 h. From this point forward, all media used in flasks and inserts were antibiotic-free. The media were changed again at 96 h (L-15 with antibiotics [see above]) at 24 h. From this point forward, all media used in flasks and inserts were antibiotic-free. The media were changed again at 96 h (L-15 + 2 mM glutamine, 6% FBS without antibiotics). After a further 48–72 h in culture, the harvesting and reseeding of cells onto permeable Falcon culture inserts (Cyclopore polyethylene terephthalate “filters”; Becton Dickinson, Franklin Lakes, NJ; pore density: 1.6 x 10\(^6\) pores/cm\(^2\), pore size: 0.45 μm, growth surface: 0.9 cm\(^2\)], were conducted by the removal and replacement of media with trypsin solution (see above). To facilitate the removal of cells, flasks were subjected to a mild mechanical agitation, and cell detachment was confirmed via visual inspection under a phase-contrast microscope (Leitz). Trypsination was terminated by the addition of a “stop” solution (10% FBS in PBS, pH 7.7). Cells were resuspended in media and seeded onto culture inserts at a density of 700,000–800,000 cells/cm\(^2\). Inserts were held in 12-Well companion plates (Falcon) under identical incubation conditions to those stated above. Initially, inserts (apical side) and companion wells (basolateral side) contained 0.8 and 1.0 ml media, respectively. Bathing solutions were topped up to 1.5 and 2.0 ml at 24 h and replaced every 48 h thereafter. When asymmetrical conditions were tested, temperature equilibrated (18°C) freshwater (Acrodisc sterilized, 0.2-μm pore size, chemical composition same as original holding water) was added to the apical side of the insert after several rinses to remove any residual media. Additional details on the procedures for preparation and culture of rainbow trout epithelia have been described (14).

**Hormonal treatment.** Single-use aliquots of stock cortisol solution were prepared by dissolving cortisol (hydrocortisone hemisuccinate, Sigma) in PBS (pH 7.7) to get a final concentration of 0.5 mg/ml. Aliquots were stored at −20°C until use. The stock solution was defrosted and diluted in L-15 media [L-15 + 2 mM glutamine, 6% FBS with or without antibiotics as appropriate (see above)] so as to be added fresh on each media change. Three concentrations of cortisol were used (10, 100, and 1,000 ng/ml), the lower two of which are within the physiological range for rainbow trout. Treatment of the cells with each appropriate dose commenced immediately after first seeding the cells into culture flasks. After the cells were reseded onto cell culture inserts, cortisol was supplemented on the basolateral side of the insert only. Cells and epithelia bathed in media without the addition of supplemental cortisol were treated as controls (0 ng/ml). In one experiment, where the time course of cortisol action was assessed, cortisol (1,000 ng/ml) was added to the basolateral Adx-established established inserts grown under control conditions (0 ng/ml).

**Electrophysiological measurements.** Transepithelial resistance (TER) was monitored using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmometer (World Precision Instruments, Sarasota, FL). Transepithelial potential (TEP) was measured using agar-salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes (World Precision Instruments) to a pH meter used as a high-impedence electrometer (Radiometer pHM 84, Copenhagen, Denmark). All TEP measurements were expressed relative to the apical side as 0 mV after correction for junction potential. The reported membrane voltage was corrected for junction potential by measuring junction potential with electrodes placed in identical solutions (symmetrical = basolateral L15/apical L15; asymmetrical = basolateral L15/apical water) in blank inserts. Additionally, measurement of junction potential in the same solutions linked via a KCl agar bridge gave an identical result. All values for TER were expressed relative to blank corrections using vacant inserts bathed apically and basolaterally with appropriate solutions. Daily measurements of TER across filter inserts under symmetrical conditions (i.e., with culture media on both sides) were made 48 h after the initial seeding of inserts. Under asymmetrical conditions (i.e., fresh water added to the apical side) and, in the case of a time-course experiment, symmetrical conditions (after the addition of cortisol to established epithelia) TEP and/or TER measurements were conducted in an identical manner at appropriate time intervals.

**Microscopy.** Routine examination of cells in both flasks and inserts was conducted using a phase-contrast microscope (Zeiss). A single series of inserts was stained with Mitotracker Green (Molecular Probes, Cedarlane Laboratories, Hornby, Ontario, Canada) to examine the epithelium for the presence of mitochondria-rich (MR) cells. Mitotracker was dissolved in DMSO to make a stock solution of 200 μM and stored in light-protected conditions at 0–4°C. Staining medium was prepared immediately before use by adding the dye stock solution to culture media to obtain the desired final dye concentration (500 nM). Cells were incubated in situ for 30 min at 18°C and then rinsed three times with L-15 media before observation with an epifluorescence microscope (Leitz).

**Na\(^+\)K\(^-\)ATPase activity and epithelium protein content.** The activity of Na\(^+\)-K\(^+\)-ATPase was determined for individual inserts treated with varying doses of cortisol. Epithelia were subjected to a mild trypsination (∼2 min) to obtain a
cell suspension. Trypsination was terminated by the addition of the resulting cell suspension to a stop solution (10% PBS in PBS, pH 7.7) and centrifuged (Beckman J-21C, 0–4°C) for 10 min at 500 g. Cells were then washed in PBS (pH 7.7) and centrifuged (Beckman J-21C, 0–4°C) for a further 10 min at 500 g. Ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3) was added to the cell pellet, and the preparation was quick-frozen in liquid nitrogen. Cells were stored at −70°C until further analysis.

Before analysis, samples stored in SEI buffer were defrosted and a volume of SEID buffer (0.5 g sodium deoxycholate in 100 ml of SEI) was added, resulting in a final SEI-to-SEID ratio of 4:1. Cells were sonicated (Branson, Sonifier 450) on ice for 30 s, centrifuged (Eppendorf 5415C, 0–4°C) at 5 000 g for 1 min, and resulting supernatant was immediately analyzed for Na\(^{+}\)-K\(^{+}\)-ATPase activity using previously established methods (27). Because of the low amount of protein available, the assay was run for 40 min at 24°C. The activity rate of the enzyme was linear throughout this period. The protein content of supernatants was measured according to the Bradford method (Sigma) using bovine serum albumin (Sigma) as a standard. All enzyme activities are expressed as protein specific activities. Protein content for individual epithelia (μg epithelia) was determined after correcting for the volume of homogenizing buffer used for epithelia/cell sonication.

\[^{3}H\]polyethylene glycol-4000 permeability. The permeability of the cultured epithelium preparation to the paracellular permeability marker, \[^{3}H\]polyethylene glycol-4000 (PEG-4000; molecular mass 4,000 Da; New England Nuclear-DuPont) was measured under both symmetrical and asymmetrical conditions using methods previously described (11, 41). Permeability was determined in the efflux direction (basolateral to apical) after the addition of 1 μCi PEG-4000 to the basolateral culture media. The appearance of PEG-4000 in the apical compartment was determined at 12 (symmetrical)–24 h (asymmetrical) consecutive intervals. During this period, TER was closely monitored at time intervals ranging from 3 to 6 h.

\[^{3}H\]PEG permeability was calculated according to

\[
P (\text{cm/s}) = \left( \frac{\Delta \text{PEG}^*}{\text{PEG}^*} \right)_{\text{Ap}} \cdot \left( \frac{\text{Volume}_{\text{Ap}}}{\text{PEG}^*} \right)_{\text{Bl}} \cdot \left( \frac{\text{Area}}{\text{Time}} \right)
\]

where \(\Delta \text{PEG}^*\) is the change in radioactivity on the apical side, [PEG\(^*\)]\(_{\text{Bl}}\) is the mean radioactivity on the basolateral side, and Volume\(_{\text{Ap}}\) defines the area of epithelial growth in the insert (0.9 cm\(^2\)).

Net and unidirectional ion flux measurements. To establish the effect of cortisol on ion flux rates over prolonged exposure to asymmetrical conditions, directly measured net Na\(^{+}\) and Cl\(^{−}\) flux rates (Jout, without the use of isotopes) were conducted on a single series of epithelia over a 24-h period. The net Na\(^{+}\) flux, for example, was calculated using the following equation

\[
J_{\text{out}}^{\text{Na}^+} (\text{nmol cm}^{-2} \cdot \text{h}^{-1}) = \Delta \text{Na}^+ \cdot \text{Area} \cdot \left( \frac{\text{Volume}_{\text{Ap}}}{\text{Time}} \right)
\]

where \(\Delta \text{Na}^+\) is the change in total Na\(^{+}\) concentration on the apical side, and Time is 24 h.

Unidirectional ion flux rates (employing radiotracers) were measured in several series of control (0 ng/ml cortisol) and “high-dose” treated (1,000 ng/ml) epithelia only. Measurement of unidirectional Na\(^{+}\) and Cl\(^{−}\) flux was performed according to methods previously described (41, 43). Briefly, 1 μCi of isotopes (\(^{22}\)Na\(^{+}\) or \(^{36}\)Cl\(^{−}\)) was added to either the apical side, for influx studies, or the basolateral side, for efflux studies, and the appearance was monitored on the “cold” side. For unidirectional ion flux rates determined under symmetrical conditions, ion flux rates were first recorded in the basolateral-to-apical direction across the inserts after an incubation period of 6 h. The inserts were then washed out for a period of 2–3 h with cold media followed by the measurement of ion flux rates in the apical-to-basolateral direction over a second 6-h incubation period. Under symmetrical conditions, measurements of TER and TEP were recorded at time 0 and at the end of the flux period (6 h). With the use of this approach, each insert could be used as a single individual for calculations of the Ussing flux-ratio criterion (see below). Under asymmetrical conditions, a similar 6-h flux period was adopted; however, inserts were either used for influx or efflux measurements only and matched for calculations of the Ussing flux-ratio criterion. Insert pairs were matched based on electrophysiological similarity (TER and TEP measurements; see Ref. 7) and each insert was only used once in the pairing procedure. Measurements of TER and TEP were taken at the beginning, middle (3 h), and end of each flux period (6 h). Unidirectional influxes (Jin, positive sign) and effuxes (Jout, negative sign) were calculated according to the following example equations:

\[
J_{\text{in}}^{\text{Na}^+} (\text{nmol cm}^{-2} \cdot \text{h}^{-1}) = \Delta \text{Na}^+ \cdot \text{Area} \cdot \left( \frac{\text{Volume}_{\text{Bl}}}{\text{Time}} \right)
\]

where \(\Delta \text{Na}^+\) is the change in radioactivity on the basolateral side, and SA\(_{\text{Ap}}\) is the mean specific activity on the apical side. Conversely, for efflux

\[
J_{\text{out}}^{\text{Na}^+} (\text{nmol cm}^{-2} \cdot \text{h}^{-1}) = \Delta \text{Na}^+ \cdot \text{Area} \cdot \left( \frac{\text{Volume}_{\text{Ap}}}{\text{Time}} \right)
\]

where SA\(_{\text{Bl}}\) is the mean specific activity on the basolateral side. Thus “indirectly measured” net flux could be calculated from the simultaneous measurements of Jin and Jout using the conservation equation

\[
J_{\text{in}} = J_{\text{out}}
\]

Ussing flux-ratio criterion. The criterion used to detect the presence of active transport was disagreement of the measured flux ratio (Jin/Jout) with that predicted by the Ussing flux-ratio equation (16). The predicted Ussing flux ratio was calculated according to

\[
\frac{J_{\text{in}}}{J_{\text{out}}} = \frac{A_{\text{Bl}} \cdot e^{-zF_{\text{V/RT}}}}{A_{\text{Ap}}}
\]

where Ap and Bl are the activities of the ions (Na\(^{+}\) and Cl\(^{−}\)) on the apical and basolateral sides, respectively, \(z\) is the ionic valence, \(V\) is the measured TEP in volts (average for matched inserts), and \(F\), \(R\), and \(T\) have their usual thermodynamic values. Under conditions of asymmetrical exposure, the activities of Na\(^{+}\) and Cl\(^{−}\) in the apical freshwater were taken as equal to the measured concentrations. Under symmetrical conditions (or in the case of asymmetrical exposure, the basolateral side only), ion activities were 75% of the concentration, as previously determined (41).

Statistical analysis. All data are expressed as means ± SE, where \(n\) represents the number of filter inserts. For comparisons between varying cortisol doses, data were either subjected to repeated one- or two-way analysis of variance (Sigmastat software, Jandel Scientific). Subsequent significance between groups was delineated using Student’s unpaired or paired t-tests where appropriate (Sigmastat software, Jandel Scientific).
RESULTS

**TER during growth of the epithelium.** TER increased over time after seeding cells into inserts. The typical sigmoidal pattern of this curve was unaltered by the addition of cortisol (Fig. 1). All epithelia approached a plateau in TER ~6 days after seeding, and experimental manipulation commenced at this time. The final resting resistance of control epithelia (0 ng/ml cortisol) was ~1.3 kΩ cm², thus meeting previously outlined resistance criteria (see Ref. 41). The addition of 10 ng/ml cortisol to the culture medium had little effect on the final resting resistance of the epithelium (~1.5 kΩ cm²); however, higher cortisol doses of 100 and 1,000 ng/ml significantly ($P < 0.0001$) elevated the final resting resistance to ~7.5 and 26.3 kΩ cm², respectively (Fig. 1). Significant differences between TER measured across control epithelia and those treated with 100 ng/ml cortisol occurred after 5 days, whereas significant differences between control values and those found in epithelia treated with 1,000 ng/ml occurred after 4 days. In epithelia (control and 1,000 ng/ml cortisol treated only) used for radiotracer studies under symmetrical conditions, cortisol had similar effects on TER. The plateau TER of control and cortisol-treated inserts was 3.79 ± 0.35 ($n = 10$) and 24.38 ± 0.70 kΩ cm² ($n = 10$), respectively.

In a single series of established control epithelia exhibiting a plateau in TER of 6.37 ± 0.51 kΩ cm² ($n = 14$), the addition of cortisol (1,000 ng/ml) to the basolateral media of randomly selected preparations (with a mean TER of 6.59 ± 0.66 kΩ cm², $n = 7$) resulted in a significant increase in TER 48 h after the addition of the hormone (Fig. 2). After 84 h, the TER of control and cortisol-treated epithelia was 11.58 ± 1.03 ($n = 7$) and 20.42 ± 0.95 kΩ cm² ($n = 7$), respectively (Fig. 2).

**Epithelial protein content.** The soluble protein content of epithelia treated with varying doses of cortisol did not significantly differ between groups. Epithelial protein content was 50.6 ± 4.1, 52.3 ± 1.2, 48.7 ± 1.33, and 53.1 ± 1.5 µg/epithelia for preparations treated with 0, 10, 100, and 1,000 ng/ml cortisol, respectively. This indicates that cortisol likely did not increase the mass of cells in the epithelia but rather specifically acted to increase the TER of the epithelia.

**Microscopy.** Examination of cells, in flasks and inserts, by phase-contrast microscopy did not reveal any evidence of MR cells in culture (see Refs. 7 and 14). In addition, MitoTracker staining revealed no intensely fluorescent cells.

**TER measurements under asymmetrical conditions.** The replacement of media with freshwater in the apical compartment of the insert resulted in an immediate increase in the TER in the 0, 10, and 100 ng/ml cortisol treatments followed by a further rise to a peak at 3 h. At 1,000 ng/ml cortisol, TER acutely dropped initially, and the increase at 3 h was marginal. In control epithelia, resistance increased ~4.5 fold from 1.3 kΩ cm² under symmetrical conditions to 5.8 kΩ cm² at 3 h (Fig. 3). Peak TER values at 3 h were ~8.5, 20.6, and 32.8 kΩ cm² for epithelia treated with 10, 100, and 1,000 ng/ml cortisol, respectively (Fig. 3). These values represented increases of 5.7 (10 ng/ml cortisol)-, 2.7 (100 ng/ml cortisol)-, and 1.2-fold (1,000 ng/ml cortisol) from starting TERs under symmetrical conditions. After the 3-h peak, all preparations exhibited a general decline in TER over the 24-h exposure period. This decrease in TER was greater during 3–12 h postfreshwater exposure than during the 12–24 h postfreshwater-exposure period. After 24 h of asymmetrical conditions, only inserts treated with 1,000 ng/ml cortisol exhibited a TER that was significantly lower than the respective TER measured under symmetrical conditions before the addition of freshwater, although this treatment...
still demonstrated the highest absolute TER (Fig. 3). In epithelia (control and 1,000 ng/ml cortisol treated only) used for radiotracer studies under asymmetrical conditions, inserts exhibited an average TER of 7.09 ± 0.46 (n = 4) and 23.44 ± 1.29 kΩ cm² (n = 10) over the 6- to 9-h flux period for control and cortisol-treated preparations, respectively.

[^H]PEG-4000 permeability. Under symmetrical conditions, the paracellular permeability of the cultured epithelium decreased in parallel with increasing doses of cortisol (Fig. 4). Significant differences were found between all groups (P < 0.0001). Under asymmetrical conditions (24-h flux period), the cultured epithelia exhibited a similar trend (P < 0.0001), however, no statistical significance could be detected between groups treated with 100 and 1,000 ng/ml cortisol (Fig. 4).

Direct measurement of net Na⁺ and Cl⁻ flux rates under asymmetrical conditions. Net Na⁺ and Cl⁻ flux rates directly measured over the initial 24 h of apical freshwater exposure, from the basolateral media to the apical freshwater, exhibited a marked decline in response to increasing cortisol dose. Flux rates of 450 and 500 nmol·cm⁻²·h⁻¹ under control conditions for Na⁺ and Cl⁻, respectively, were reduced to 87 and 131 nmol·cm⁻²·h⁻¹ at the highest dose of 1,000 ng/ml cortisol (Fig. 5). Significant differences between groups were found for all treatments (P < 0.05).

Na⁺-K⁺-ATPase activity. The activity of Na⁺-K⁺-ATPase in cultured epithelia was not significantly (P > 0.05) affected by either abrupt or chronic cortisol treatment. In epithelia grown without the addition of cortisol, some of which were subsequently treated with...
cortisol (1,000 ng/ml) for 84 h, Na\(^+\)-K\(^+\)-ATPase activity was 0.52 ± 0.02 (\(n = 7\)) and 0.47 ± 0.02 \(\mu\)mol ADP-mg protein\(^{-1}\)h\(^{-1}\) (\(n = 7\)) for control and cortisol-treated groups, respectively (\(P > 0.11\)). In epithelia grown in the presence of varying doses of cortisol, Na\(^+\)-K\(^+\)-ATPase activity averaged 0.45 \(\mu\)mol ADP-mg\(^{-1}\)h\(^{-1}\) in all groups (Fig. 6) after 24 h under asymmetrical conditions.

**DISCUSSION**

The in vivo and in vitro effects of cortisol on branchial and opercular tissues have been the focal point of numerous studies (for review, see Ref. 28); however, no previous study has attempted to delineate the effects of cortisol on branchial pavement cells only. In this respect, the results of the current study are...
unique. Although we are presently dealing with an in vitro situation, there are several aspects of the current study that emphasize the physiological relevance of observations made herein. First, the actions of cortisol abruptly added to the culture medium of "established" epithelia are evident after 48 h. This time frame is consistent with the in vivo actions of cortisol on fish gills (8). Second, all but the highest dose of cortisol used in the present study are physiologically relevant, because reported pre- and poststress levels in rainbow trout are 2–76 and 30–480 ng/ml, respectively (3). Last, recent studies have demonstrated that cortisol receptors in gill tissues are almost as abundant in the pavement cells as they are in the chloride cells (39).

The effect of cortisol on TER measured across the cultured pavement cell epithelium was markedly dose dependent. The origins of a cortisol-induced increase in TER seemed likely to relate to changes in the junctional relationships between pavement cells, because the number of tight junctions between cells in culture correlates well with TER (5) and, particularly, paracellular resistance (19). With the use of [3H]PEG-4000 as a paracellular permeability marker, we were able to establish that a cortisol-induced elevation in TER was, at least partly, attributable to a reduction in paracellular permeability; however, at this point it is unclear to what degree changes in transcellular permeability may also contribute to this phenomenon. It seems unlikely that a cortisol-induced increase in TER and reduction in paracellular permeability are simply related to changes in epithelial "thickness" (increased cell mass), because there were no differences in protein content of epithelia treated with varying doses of cortisol.

On exposure to freshwater, the TER of the cultured epithelium increased severalfold, an electrophysiological phenomenon that has been previously described for this preparation (11, 41, 43) and for tested surrogate gill models for the branchial epithelium (22). In previous studies, this increase in TER has been attributed to increased transcellular permeability, because paracellular permeability increased after exposure to asymmetrical conditions (41). This can also be observed in the current study. However, at the highest cortisol dose (1000 ng/ml), differences between paracellular permeability of epithelia under either symmetrical or asymmetrical conditions are less obvious. This occurs despite an ~55% reduction in TER from initial values in the region of 26 kΩ cm² (under symmetrical conditions) to ~12 kΩ cm² after 24 h freshwater exposure. This would suggest that in contrast to the control and low-dose cortisol-treated inserts, an initially reduced transcellular permeability in high-dose cortisol-treated inserts is increasing during the 24 h of freshwater exposure, whereas paracellular permeability remains low.

Currently, there is no role for paracellular flux in models for ion uptake in freshwater fish, and reductions in paracellular permeability only appear to be adaptive in minimizing diffusive ion losses. In light of the latter, cortisol-induced changes in paracellular permeability are likely to be highly relevant, particularly from an ecophysiological standpoint in which environmental perturbation may result in ionic disequilibrium (6) or adaptation to very dilute freshwater may limit ion uptake (18, 35). An elevation in plasma cortisol is the most widely used indicator of stress in fish, and typically, plasma cortisol levels will rise within minutes after exposure to an acute stressor (3, 40). In freshwater fish, stressors cause increased passive ion loss and water uptake (6); however, the role that cortisol may play during such conditions is confounded by the simultaneous mobilization of catecholamines. Indeed, it is generally accepted that increased diffusive ion loss in stressed freshwater fish can be attributed to elevated catecholamine-induced vasodilation of the gills would result in increased branchial blood flow and surface area, thereby accelerating diffusive ion losses (37, 40). Furthermore, under such conditions, increased permeability of paracellular "tight" junctions themselves has also been implicated in diffusive ion loss (12, 31). In light of these reported effects, elevated levels of cortisol may play a significant compensatory role in reducing passive ion loss by reducing the paracellular permeability of pavement cells. This argument is further strengthened by

Table 1. Comparison between Ussing flux ratios ($J_{in}$/$J_{out}$) for Na$^+$ and Cl$^-$ in control (0 ng/ml cortisol) and cortisol-treated (1,000 ng/ml) epithelia under symmetrical (L15 apical/L15 basolateral) and asymmetrical (FW apical/L15 basolateral) culture conditions

<table>
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<tr>
<th>Symmetrical</th>
<th>Control</th>
<th>Cortisol</th>
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<tbody>
<tr>
<td></td>
<td>Predicted ratio</td>
<td>Observed ratio</td>
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<tr>
<td>Na$^+$</td>
<td>0.969 ± 0.005</td>
<td>0.804 ± 0.023*</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>1.033 ± 0.005</td>
<td>0.914 ± 0.016*</td>
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<td>(n = 10)</td>
<td>(n = 10)</td>
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<table>
<thead>
<tr>
<th>Asymmetrical</th>
<th>Predicted ratio ($×10^{-3}$)</th>
<th>Observed ratio ($×10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>32.78 ± 0.67</td>
<td>14.64 ± 0.88*</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>13.50 ± 1.57</td>
<td>38.20 ± 4.80*</td>
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</tbody>
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Values are means ± SE; n, no. of filter inserts. *Significant difference (P < 0.05) between observed and predicted flux ratios.
Cortisol greatly reduced the influx and efflux components of both Na$^+$ and Cl$^-$ under symmetrical conditions. In cortisol-treated epithelia, no significant difference could be detected between the influx and efflux components of Na$^+$; however, unidirectional efflux of Cl$^-$ was significantly lower than influx, resulting in a positive inward net flux. In contrast to the control epithelia, the flux ratios for cortisol-treated epithelia under symmetrical conditions indicated active uptake of both Na$^+$ and Cl$^-$. With regard to active Na$^+$ uptake, this phenomenon is in general accord with currently popular models of ion transport across freshwater fish gills, where Na$^+$ uptake is believed to occur across the pavement cells and is driven by the electrogenic actions of a vacuolar-type H$^+$ ATPase. Furthermore, elevation of the specific activity of this key enzyme has been demonstrated in freshwater fish treated with cortisol (20). However, in current models, there is no place for active Cl$^-$ uptake across the pavement cells, and active Cl$^-$ uptake is believed to occur across the chloride cells in association with Cl$^-$/HCO$_3$$^-$ exchange (33). Because the current preparation lacks chloride cells, the results suggest that either current theories on Cl$^-$ transport across fish gills are, as yet, incomplete or pavement cells in primary culture exhibit transport characteristics that are not indicative of pavement cells in intact gills.

In defense of the former suggestion, one of the underlying principles for the use of gill cells in primary culture is that they will have little time to alter cellular function, as opposed to the use of immortal cell lines that may dedifferentiate over time. Indeed, the addition of cortisol to primary cultured fish gill cells greatly reduced the influx and efflux components of both Na$^+$ and Cl$^-$. This is in accord with the Ussing flux-ratio criterion, applied to control epithelia under symmetrical conditions, detected slight but significant active transport of both Na$^+$ and Cl$^-$. Under symmetrical conditions, the tendency toward active Na$^+$ transport in the outward direction has been observed in our earlier studies (43); however, Cl$^-$ movement under symmetrical conditions was not previously associated with active transport (41, 43). In control epithelia exposed to asymmetrical conditions (apical freshwater), the Ussing flux-ratio analysis indicated active transport of Cl$^-$ in the inward direction and Na$^+$ in the outward direction. Similar patterns have previously been observed in our earlier studies (7, 41).

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CORTISOL AND A CULTURED BRANCHIAL EPITHELIUM

Perspectives

Mechanisms of ion transport across freshwater fish gills remain highly controversial, in part, due to the absence of a suitable in vitro model. The primary culture of gill cells on filter supports represents a promising direction in the development of a freshwater gill model. Current methodology allows for the generation and study of epithelia comprising either pavement cells only, as in the present study, or both pavement and MR cells (7). The heterogeneity of the intact branchial epithelium (or appropriate surrogate model) would not normally allow for the study of chronic hormonal effects on pavement cell physiology. Cultured branchial epithelia are ideally suited for assessing the actions of key osmoregulatory hormones on gill cells, and the current study is the first to address the direct effects of cortisol solely on gill pavement cell function. Clearly, the next stage is to assess the effects of cortisol on branchial epithelia, which incorporate both MR and pavement cells, and thereby deduce effects when the two cell types commonly thought to participate in active ion transport are present simultaneously.

We express our appreciation to M. Grosell for helpful comments. This work was supported by a National Science and Engineering Research Council research grant to C. M. Wood. All procedures conformed to the guidelines of the Canadian Council of Animal Care.

REFERENCES

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