Effects of cortisol and prolactin on Na⁺ and Cl⁻ transport in cultured branchial epithelia from FW rainbow trout

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Zhou, Bingsheng, Scott P. Kelly, Juan P. Ianowski, and Chris M. Wood. Effects of cortisol and prolactin on Na⁺ and Cl⁻ transport in cultured branchial epithelia from FW rainbow trout. Am J Physiol Regul Integr Comp Physiol 285: R1305–R1316, 2003. First published July 31, 2003; 10.1152/ajpregu.00704.2002.—The electrophysiological and ion-transporting properties of cultured gill epithelia from freshwater (FW) rainbow trout were examined in the presence of cortisol and prolactin as media supplements. Epithelia were of the double-seeded insert (DSI) type containing both pavement cells (PVCs) and mitochondria-rich cells (MRCs) and were grown in Leibovitz’s L15 media on filters allowing exposure to different apical media conditions. Experiments were carried out in two series after 7–9 days symmetrical (L15 apical–L15 basolateral) culture. In both series, 100% L15 was maintained as the basolateral medium throughout and supplemented with physiologically relevant doses of either prolactin (50 ng/ml), cortisol (500 ng/ml), or cortisol + prolactin (500 + 50 ng/ml, respectively). In series 1, epithelia were exposed to progressively diluted apical media (100, 75, 50, 25, 12.5% L15, and FW) at 24-h intervals. The preparations retained integrity [high transepithelial resistance (TER); low ion efflux rates] during this prolonged dilution protocol. Cortisol, or cortisol + prolactin, resulted in a greater TER and reduced ion efflux rates during dilution, likely an effect on junctional permeability of PVCs, but did not promote active Na⁺ and Cl⁻ uptake from apical FW. In series 2, epithelia were directly exposed to apical FW and ion fluxes measured over the first 6 h. Under these conditions, cortisol or cortisol + prolactin promoted active uptake of both Na⁺ and Cl⁻ simultaneously from apical FW, probably attributable to actions on the MRCs. However, Na⁺-K⁺-ATPase activities were not significantly altered by any of the treatments in either series. Overall, prolactin alone did not appear to promote FW adaptation but exhibited synergism with cortisol. These results provide further support for the cultured DSI epithelium as an in vitro model for ion transport in FW fish.

epithelial cell culture; double-seeded insert; pavement cells; mitochondria-rich cells

GILLS ARE THE MAJOR site for Na⁺ and Cl⁻ transport in teleost fish. In seawater fish, blood ion levels are far below those in the concentrated external environment, so the gills must actively excrete salt. In contrast, in freshwater (FW) fish, blood ion levels are far above those in the dilute external environment, so the gills must actively take up salt from FW. When environmental salinity changes, euryhaline fish physiologically adapt to the new environment. The neuroendocrine system plays a critical role in these osmoregulatory adjustments. Among the osmoregulatory hormones in teleosts, cortisol has been largely considered to be “the” seawater-adapting hormone, whereas prolactin has been regarded as “the” FW-adapting hormone (3, 7, 19, 22, 23, 28). However, studies have demonstrated that cortisol is also involved in ion uptake in FW fish species (18, 22, 27) and may interact with prolactin during the process of FW acclimation (7, 23).

It is difficult to examine the details of ion transport in vivo because of the structural and physiological complexity of the fish gill, which offers a finely divided surface composed of multiple cell types involved in a variety of functions (e.g., respiration, ion transport, acid-base regulation, and nitrogen excretion). A number of studies, mostly based on skin preparations dissected from the cephalic region of intact teleosts, have been made to develop flat epithelial preparations as surrogate models for the study of gill ion transport (reviewed in Refs. 4, 20, 36). Although such preparations have been very successful for the characterization of seawater gill transport, they have been largely unsuccessful in duplicating the active uptake processes of the FW gill (reviewed in Refs. 20, 21, 34). An entirely different approach is the primary culture, from freshly dispersed gill cells, of flat branchial epithelia on permeable filter supports in vitro. Such models have been developed for both FW (8, 13, 35) and seawater gills (1, 2). The latter vigorously transport Cl⁻ in the efflux direction. The FW models show much weaker transport, actively taking up Cl⁻, but not Na⁺, in the influx direction at low rates (8, 15, 32). They also faithfully duplicate the electrical characteristics [transepithelial resistance (TER) and potential (TEP)] and passive permeability features (Na⁺ and Cl⁻ leakage rates, paracellular permeability) of the intact gill (9, 15, 32, 35), which is advantageous, because these properties are technically difficult to monitor in vivo.

The original culture method for the FW rainbow trout (35) involved a single seeding of flask-cultured cells onto the filter inserts [single-seeded insert (SSI)] and resulted in an epithelium consisting of pavement cells (PVCs; or respiratory cells) only, whereas mito-
chondria-rich cells (MRCs; or chloride cells) were absent. An important recent advance has been the development of a procedure for double seeding of freshly dispersed gill cells directly onto the filter inserts (8). The resulting epithelium [double-seeded insert (DSI)] contains both PVCs and MRCs, the latter in a density (15% of total cells) very similar to that in the intact gill; thus, we are now beginning to “reconstruct” the gill in vitro. Fletcher et al. (8) provide additional information on the structure of the DSI preparation. Most hormonal investigations on cultured gill epithelia from FW fish examined only SSI preparations (i.e., PVCs only), to date characterizing the effects of cortisol (13, 15), 3,5′,3′-triodothyronine (14), and prolactin (16). Only in the latter case were effects in SSI and DSI epithelia compared, and the presence of MRCs clearly made a difference. Prolactin stimulated Na\(^+\)-K\(^+\)-ATPase activity in DSI epithelia, but not in SSI epithelia, while limiting permeability in both preparations during FW exposure (16).

To date, all studies using cultured branchial epithelia as a model for the FW gill have been conducted after the plateau phase in resistance development has been reached (i.e., 6–9 days after the final seeding step). At this point, the cells were exposed to a step change on the apical side from isotonic media (L15 medium on both sides, pseudo-in vivo symmetry) to FW (analogous to in vivo asymmetry), i.e., resembling a transition from brackish to FW (reviewed in Ref. 33). However, euryhaline fish are unlikely to undergo a single-step transition under natural conditions and are more likely to experience a transitional period through progressively lower environmental salt concentrations. Nothing is known about whether DSI epithelia can tolerate gradual media dilution after completion of development or about permeability and transport properties under such conditions. Furthermore, in vivo, the transition process from lower salt concentrations to FW is accompanied by an integrated endocrine response, which may include the mobilization of cortisol and prolactin, among others (23), so these hormones may well be critically important in vitro. We have yet to comprehensively address the potential use of these supplements in DSI epithelia and, more importantly, in combination with either abrupt or gradual dilution of the apical media.

With this background in mind, we examined the effects of both a gradual apical transition to FW and an abrupt single-step replacement of apical culture media with FW, alone and in combination with hormonal supplementation, on the electrophysiological and ion-transporting properties \((\text{Na}^+, \text{Cl}^-)\) of DSI epithelia. Given the important role of prolactin in the osmoregulation of FW fish, the dual function of cortisol in seawater and FW fish, and a possible synergistic effect of the two hormones during FW adaptation (see above), we evaluated the two hormones, both alone and in combination, during these two apical dilution regimes.

**MATERIALS AND METHODS**

**Fish and Preparation of DSI**

Rainbow trout \((\text{Oncorhynchus mykiss})\) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). The fish (85–150 g) were held in dechlorinated running municipal tap water (in mM: \([\text{Na}^+] = 0.55; [\text{Cl}^-] = 0.70; [\text{Ca}^{2+}] = 1.00; [\text{Mg}^{2+}] = 0.15; [\text{K}^+] = 0.05; \text{pH 7.8–8.0}) at seasonal temperatures (12–15°C). Gill cells were isolated in a laminar flow hood by using sterile techniques according to the methods originally developed by Párt et al. (25) and modified by Wood and Párt (35). The DSI epithelia were prepared following the procedure originally outlined by Fletcher et al. (8) and the full detailed methodology of Kelly et al. (12). In brief, fish were stunned by a blow to the head and then decapitated. Gill cells were excised from gill filaments by two consecutive cycles of tryptic digestion at room temperature (Gibco Life Technologies, 0.05% trypsin in PBS, pH 7.7 with 5.5 mM EDTA). The cells were resuspended in cold culture medium (Leibovitz’s L15, supplemented with 5% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 200 µg/ml gentamycin) and seeded on the apical side in Falcon cell inserts (Cyclopore polyethylene terephthalate filters, Becton-Dickinson, Franklin Lakes, NJ; pore density: 1.6 × 10⁶ pores/cm²; pore size: 0.45 µm; growth surface: 0.9 cm²) at a density of 3 × 10⁶ cells/cm². The total volume of the culture medium was initially 0.8 ml on the apical side and 1 ml on the basolateral side. One day after seeding, each insert was rinsed with PBS to remove mucus and unattached cells; new cells, freshly prepared from a second fish, were seeded onto the cell layer of each insert with a density of 2 × 10⁶ cells/cm² (this date was termed day 1). After a further 24 h, mucus and unattached cells were again removed with PBS rinsing, and 1.5 ml of culture medium with antibiotics were added to the apical side, while 2 ml with antibiotics were added to the basolateral side. The culture medium was replaced every other day and was kept free of antibiotics after day 4 at this temperature.

**Hormone Treatments and Apical Dilution**

Cortisol (hydrocortisone 21-hemisuccinate) and ovine prolactin (oPRL) were obtained from Sigma. Single-use aliquots of a stock solution were prepared with PBS, filtered through a 0.2-µm filter, and stored at −20°C. Hormones were added to basolateral culture media to achieve 500 ng/ml of cortisol and/or 50 ng/ml of prolactin. We selected these hormones and concentrations based on results from earlier studies (13, 15, 16), which had shown physiological responses to oPRL and recombinant rainbow trout prolactin in cultured epithelium (16). As noted in these earlier reports, the hormone levels are within the physiological range for rainbow trout. Hormones were not added to apical media.

**Series 1.** In this series, progressive dilution of the apical media was carried out once DSI preparations had developed to the plateau phase of TER (day 7). The DSI preparations were cultured and supplemented with hormones throughout the preceding development phases as well as during the subsequent experimental phase. In the hormone-treated groups, basolateral culture media were supplemented with either 500 ng/ml cortisol, 50 ng/ml prolactin, or both cortisol + prolactin. The hormones were kept in the basolateral media from day 2 (after first seeding) onward throughout the experimental period, and these media were renewed daily. Control media contained no hormone supplement. Once the epithelia had developed to a plateau phase of TER, electrophysiological and flux measurements (over 6–9 h) were made...
with full-strength regular apical L15 media (100%) on day 7. Thereafter, the apical solution was replaced with 75% medium (regular L15 medium diluted to 75% its original strength with sterile FW, 75% L15 + 25% FW). After 16–18 h at 75%, flux and electrophysiological measurements were repeated (i.e., on day 8), and then 50% medium (50% L15 + 50% FW) was substituted on the apical side. The process was sequentially repeated with measurements at 50% apical media on day 9, at 25% (25% L15 + 75% FW) on day 10, at 12.5% (12.5% L15 + 87.5% FW) on day 11, and at 0% (i.e., 100% FW) on day 12. Again, hormones were not added to the apical media. Mean Na⁺ and Cl⁻ concentrations measured at the start of flux periods in the various media are shown in Table 1.

**Series 2.** In this series, a single-step change from apical L15 media to FW was carried out after the plateau phase of development and subsequent experimentation, and these media were renewed daily. When epithelia exhibited a high initial number of TER, or the electrical properties of the tissues were no longer suitable for flux measurements, these media were replaced with FW (identical to the holding water, but sterilized), and electrophysiological and unidirectional ion flux measurements were conducted over a 6-h period.

**Electrophysiological Measurements**

TER, across the epithelium, was monitored with STX-2 chopping electrodes connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL; Ref. 35). TEP was measured with an agar/salt bridge (3 M KCl in 4% agar) connected to Ag/AgCl electrodes (World Precision Instruments) using a pH meter (Radiometer, Copenhagen, Denmark) as a high-impedance electrometer. All TEP measurements were expressed relative to the apical side as 0 mV. Corrections for junction potential and for blank TER of vacant inserts in each experimental condition were performed as described by Kelly and Wood (13).

**Unidirectional Ion Flux Measurements**

Unidirectional Na⁺ and Cl⁻ fluxes across the preparations were measured by adding 0.1–0.3 μCi of the radioisotopes ²²Na and ³⁶Cl⁻ (New England Nuclear DuPont) to one side (‘hot’ side) and monitoring the appearance of radioactivity, over time, on the other side (‘cold’ side). In series 1, after the symmetrical (100% apical L15) flux measurements were completed, culture media were replaced by adding 75% L15 on the apical side and fresh 100% L15 media with the appropriate hormonal composition to the basolateral side. The preparation was then left for 16–18 h to acclimate to the new apical salinity. Before the next flux measurement, the apical side was rinsed with fresh 75% L15 media. A similar protocol for changeover and flux measurements was employed for the 50, 25, 12.5, and 0% (FW) steps. For the latter, the apical side was rinsed with FW three times (2 ml each) to ensure a complete changeover and no culture medium contamination. Samples for radioactivity measurements were collected at the beginning (time 0) and at the end of a flux. TER and TEP were monitored at the beginning, middle, and end of each flux period, with average values reported in the text. The flux measurement period was 6–9 h in series 1. In series 2, the apical side was rinsed with FW four times to ensure a complete changeover to apical FW. Flux measurements were conducted immediately under asymmetrical conditions (FW/L15) for a period of 6 h.

Each insert could be used for either influx (J_{in}, positive by convention) or efflux (J_{out}, negative) measurements but not both. Therefore, J_{in} and J_{out} were measured using the Ussing predicted flux-ratio criteria, which was necessary to pair cultured epithelia that provided reciprocal flux measurements (8). Pairs were matched according to the most similar average TER (measured at T_{0}, T_{middle}, and T_{final}). Unidirectional flux was calculated according to the following equation (32)

\[ J_{in} = \Delta \left[ \text{Na}^+ \right]_{BL} \times \frac{1}{S_{AP}} \times \frac{\text{Volume}_{BL}}{\text{Time} \times \text{Area}} \]  

where \( \Delta \left[ \text{Na}^+ \right]_{BL} \) is the change in radioactivity on the basolateral side (‘cold’ side), \( \text{Volume}_{BL} \) is the volume on the basolateral side, and \( S_{AP} \) is the mean specific activity on the apical side (‘hot’ side). Conversely, for efflux (basolateral-to-apical flux, radioisotope placed on basolateral side)

\[ J_{out} = -\Delta \left[ \text{Na}^+ \right]_{AP} \times \frac{1}{S_{BL}} \times \frac{\text{Volume}_{AP}}{\text{Time} \times \text{Area}} \]  

where \( \Delta \left[ \text{Na}^+ \right]_{AP} \) is the change in radioactivity on the apical side (cold side) and \( S_{BL} \) is the mean specific activity on the basolateral side (hot side). Thus, indirectly measured net flux could be calculated from the measurements of \( J_{in} \) and \( J_{out} \) using \( J_{net} = J_{in} + J_{out} \). Ion flux rate is expressed as nanomoles per centimeter squared per hour.

The criterion used to detect the presence of active transport was the difference between the observed flux ratio (\( J_{in} / J_{out} \)) and the predicted flux ratio (Ussing ratio; Ref. 17). The Ussing ratio was calculated as

\[ J_{in} / J_{out} = \frac{A_{AP} e^{-ZFV/R_T \Theta}}{A_{BL}} \]  

where \( A_{AP} \) and \( A_{BL} \) are the activities of the ions (Na⁺ and Cl⁻) on the apical and basolateral side, Z is the ionic valence, V is the measured TEP in volts (average of the matched inserts), and F, R, and \( \Theta \) have their usual thermodynamic values. Na⁺ and Cl⁻ concentrations were converted to activities using ionic activity coefficients measured with ion-specific electrodes in representative dilutions of media (see Table 1). Na⁺-selective electrodes were calibrated on the neutral carrier ETH157 (sodium ionophore II, cocktail A, Fluka-Sigma, St. Louis, MO). The Na⁺-selective barrel was backfilled with 500 mmol/l NaCl. Na⁺-selective electrodes were calibrated in solutions of 15 mmol/l NaCl + 135 mmol/l LiCl (activity coefficient = 0.750) and 150 mmol/l NaCl (activity coefficient = 1.000) to ensure a complete changeover and no culture medium contamination.

### Table 1. Measured Na⁺ and Cl⁻ concentrations and activity coefficients employed in different concentrations of apical medium

<table>
<thead>
<tr>
<th>Apical Medium</th>
<th>Concentration, mM</th>
<th>Ion Activity Coefficients</th>
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</thead>
<tbody>
<tr>
<td>L15</td>
<td>139.1</td>
<td>146.8</td>
</tr>
<tr>
<td>75% L15</td>
<td>98.8</td>
<td>118.2</td>
</tr>
<tr>
<td>50% L15</td>
<td>62.6</td>
<td>73.4</td>
</tr>
<tr>
<td>25% L15</td>
<td>33.4</td>
<td>41.5</td>
</tr>
<tr>
<td>12.5% L15</td>
<td>13.9</td>
<td>23.1</td>
</tr>
<tr>
<td>FW</td>
<td>0.71</td>
<td>0.89</td>
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</table>

FW, freshwater.

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ponent = 0.750). Cl– activity measurements were obtained using solid-state Cl–-selective electrodes calibrated in 100 mmol/l KCl (activity coefficient = 0.770) and 10 mmol/l KCl (activity coefficient = 0.901). Potential differences between the reference (Vref) and ion-selective barrel (Vi) were measured by a high-input impedance differential electrometer (FD 223, World Precision Instruments), using an analog-to-digital converter and data-acquisition system (Axotape, Axon Instruments, Burlingame, CA).

Na+–K+–ATPase Activity

Na+–K+–ATPase activity in DSI epithelia was determined according to methods outlined by Kelly and Wood (13) in cells harvested immediately after unidirectional flux experiments were completed (in series 1 after the last step of apical dilution to FW). Results were expressed as specific activities (per unit total protein) with protein measured by the Bradford method (Sigma) using bovine serum albumin (Sigma) as a standard.

Statistical Analyses

All data are expressed as means ± SE (n), where n represents the number of electrophysiologically matched pairs of epithelia. For comparison of TER and TEP during gradual dilution in series 1, a one-way ANOVA was used. To compare the difference between the predicted and observed Ussing flux ratios, a paired Student’s t-test was used. To compare ion flux rates between different hormone treatments within a single apical media condition, ANOVA was used, followed by either Dunnett’s or Newman-Keuls multiple comparison test to delineate significance. The level of statistical significance for all analyses was P < 0.05.

RESULTS

Series 1: Gradual Apical Media Dilution

TER and TEP. In series 1, preparations exhibited plateau-phase TER values under symmetrical conditions that were very similar in all groups, ~34 kΩ cm² (Fig. 1A). Cultured epithelia tolerated progressive apical media dilution well, in a protocol that lasted to day 12 of culture (~24 h after the introduction of apical FW), maintaining epithelial integrity throughout as shown by the continuing high TER values. Significant differences between the TER values of different treatment groups could first be observed at 50% L15 (Fig. 1A). These differences became particularly marked when apical media were diluted further to 12.5% L15 (TER in kΩ cm²; control = 25.51 ± 1.07, cortisol = 31.21 ± 0.40, prolactin = 24.61 ± 1.81, cortisol + prolactin = 31.77 ± 1.81) and persisted with apical FW (Fig. 1A). Overall, TER declined with apical dilution in the control and prolactin treatments but not in the cortisol or cortisol + prolactin treatments.

A high positive TEP (+32 to +45 mV) developed in all groups after 7 days in culture under symmetrical conditions and was significantly greater in the cortisol + prolactin treatment than in any other treatment (Fig. 1B), a trend that persisted down to 50% L15. TEP gradually decreased in all groups (a significant trend by one-way ANOVA) as apical media were progressively diluted, although values remained positive down to 12.5% L15. From 50% L15 down to 12.5% L15, TEP in cortisol and cortisol + prolactin preparations was significantly greater than TEP in control and prolactin-treated groups. A negative TEP, which showed no significant variation between treatments, was observed in all groups after the apical media were replaced with FW.

Unidirectional ion flux rates and Ussing flux ratios. All groups exhibited very low Na+ flux rates under symmetrical conditions (Fig. 2A). There were no significant differences in the influx, efflux, and net flux rates among control, cortisol-, and cortisol + prolactin-treated epithelia under symmetrical conditions. However, a significantly greater influx and positive net flux were observed in the prolactin treatment. Upon progressive apical dilution, influx rates tended to fall, and net flux rates became progressively more negative, but efflux rates increased substantially only at the final step, apical FW (Fig. 2, B–F). Cortisol alone and corti-
sol + prolactin limited the Na\(^+\) influx, efflux, and net flux rates, particularly at the later stages of dilution (12.5% L15 and FW; Fig. 2, E and F). However, prolactin alone exhibited no limiting influence on Na\(^+\) efflux but rather caused small but significant increases in the efflux and net flux rates with apical FW (Fig. 2F). Absolute influx rates for Na\(^+\) from apical FW were 0.405 ± 0.030, 0.457 ± 0.045, 0.230 ± 0.030, and 0.183 ± 0.030 nmol cm\(^{-2}\) h\(^{-1}\) in the control, prolactin, cortisol, and prolactin + cortisol treatments, respectively.

Application of the flux ratio criterion indicated an active uptake of Na\(^+\) (from the apical side to the basolateral side) in all groups under both symmetrical (100% L15) and 75% L15 conditions, i.e., observed flux ratio significantly greater than Ussing predicted flux ratio (Table 2). However, after dilution to 50% L15, there appeared to be no active transport of Na\(^+\) in the control group, whereas active Na\(^+\) uptake persisted in all hormone-treated preparations in the same conditions. Although not all comparisons were significant, the same situation wherein the observed flux ratio exceeded the predicted ratio continued in all groups down to 12.5% L15. However, the situation was thereafter reversed in all groups (observed ratio less than predicted ratio) when the apical me-
were similarly caused by these hormonal treatments to the effects of prolactin on Na\(^+\). Both cortisol and cortisol + prolactin treatments, respectively. Absolute in Table 3.

Table 2. Comparison between predicted (Ussing) and observed flux ratio for Na\(^+\) and Cl\(^-\) in series 1

<table>
<thead>
<tr>
<th>Flux Conditions</th>
<th>Control Predicted</th>
<th>Control Observed</th>
<th>PRL Predicted</th>
<th>PRL Observed</th>
<th>Cortisol Predicted</th>
<th>Cortisol Observed</th>
<th>Cortisol + PRL Predicted</th>
<th>Cortisol + PRL Observed</th>
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<tbody>
<tr>
<td>L15/L15</td>
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<tr>
<td>Na(^+)</td>
<td>0.26 ± 0.05</td>
<td>0.67 ± 0.11*</td>
<td>0.29 ± 0.05</td>
<td>1.38 ± 0.29*</td>
<td>0.29 ± 0.02</td>
<td>0.82 ± 0.21*</td>
<td>0.19 ± 0.02</td>
<td>0.58 ± 0.08*</td>
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<tr>
<td>Cl(^-)</td>
<td>5.03 ± 1.23</td>
<td>1.89 ± 0.37*</td>
<td>4.04 ± 0.59</td>
<td>3.66 ± 1.56</td>
<td>3.51 ± 0.32</td>
<td>0.88 ± 0.27*</td>
<td>6.15 ± 0.58</td>
<td>1.11 ± 0.25*</td>
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<tr>
<td>75% L15/L15</td>
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<tr>
<td>Na(^+)</td>
<td>0.38 ± 0.02</td>
<td>0.91 ± 0.29*</td>
<td>0.34 ± 0.02</td>
<td>1.04 ± 0.25*</td>
<td>0.32 ± 0.02</td>
<td>0.64 ± 0.15*</td>
<td>0.29 ± 0.01</td>
<td>0.77 ± 0.10*</td>
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<tr>
<td>Cl(^-)</td>
<td>1.71 ± 0.07</td>
<td>1.46 ± 0.37*</td>
<td>1.84 ± 0.12</td>
<td>1.61 ± 0.49</td>
<td>1.71 ± 0.12</td>
<td>1.49 ± 0.42</td>
<td>2.18 ± 0.07</td>
<td>1.53 ± 0.43</td>
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<td>50% L15/L15</td>
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<tr>
<td>Na(^+)</td>
<td>0.35 ± 0.01</td>
<td>0.31 ± 0.02</td>
<td>0.33 ± 0.07</td>
<td>0.63 ± 0.14*</td>
<td>0.27 ± 0.01</td>
<td>0.53 ± 0.06*</td>
<td>0.24 ± 0.01</td>
<td>0.48 ± 0.10*</td>
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<tr>
<td>Cl(^-)</td>
<td>0.90 ± 0.03</td>
<td>1.30 ± 0.02*</td>
<td>1.00 ± 0.03</td>
<td>1.35 ± 0.58</td>
<td>1.15 ± 0.41</td>
<td>1.41 ± 0.24</td>
<td>1.21 ± 0.06</td>
<td>0.96 ± 0.40</td>
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<td>25% L15/L15</td>
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<tr>
<td>Na(^+)</td>
<td>0.24 ± 0.05</td>
<td>0.40 ± 0.03*</td>
<td>0.23 ± 0.06</td>
<td>0.32 ± 0.06</td>
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<tr>
<td>Cl(^-)</td>
<td>0.28 ± 0.01</td>
<td>0.51 ± 0.03*</td>
<td>0.51 ± 0.02</td>
<td>0.67 ± 0.12</td>
<td>0.64 ± 0.02</td>
<td>0.71 ± 0.04</td>
<td>0.75 ± 0.04</td>
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<tr>
<td>12.5% L15/L15</td>
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<tr>
<td>Na(^+)</td>
<td>0.099 ± 0.002</td>
<td>0.18 ± 0.002*</td>
<td>0.113 ± 0.003</td>
<td>0.176 ± 0.031</td>
<td>0.076 ± 0.005</td>
<td>0.15 ± 0.018*</td>
<td>0.083 ± 0.005</td>
<td>0.189 ± 0.028*</td>
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<tr>
<td>Cl(^-)</td>
<td>0.24 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>0.22 ± 0.006</td>
<td>0.24 ± 0.02</td>
<td>0.35 ± 0.02</td>
<td>0.20 ± 0.02*</td>
<td>0.34 ± 0.02</td>
<td>0.16 ± 0.02*</td>
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<td>FW/L15 (x 10(^{-5}))</td>
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<tr>
<td>Na(^+)</td>
<td>11.9 ± 0.26</td>
<td>6.07 ± 0.49*</td>
<td>12.0 ± 0.41</td>
<td>5.29 ± 0.42*</td>
<td>12.1 ± 0.44</td>
<td>6.15 ± 0.66*</td>
<td>11.6 ± 0.35</td>
<td>4.94 ± 0.62*</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>19.90 ± 0.65</td>
<td>12.70 ± 1.32*</td>
<td>10.00 ± 0.55</td>
<td>13.10 ± 2.17</td>
<td>7.65 ± 0.42</td>
<td>5.70 ± 0.96</td>
<td>8.58 ± 0.24</td>
<td>8.68 ± 2.06</td>
</tr>
</tbody>
</table>

Values are means ± 1 SE; n = 6 pairs of epithelia. *Significant difference (P<0.05) between observed and predicted flux ratios. Observed flux ratios higher than predicted indicate active apical-to-basolateral uptake. Observed flux ratios lower than predicted indicate active basolateral-to-apical extrusion. Epithelia were subjected to apical dilution to FW starting on day 7 after seeding and were exposed throughout to treatments of control (no hormones), prolactin (PRL, 50 ng/ml), cortisol (500 ng/ml), or cortisol + PRL (500 ng/ml cortisol, 50 ng/ml PRL), all in 100% L15 on the basolateral side only.

Table 3. Comparison between predicted (Ussing) and observed flux ratio for Na\(^+\) and Cl\(^-\) in series 2

<table>
<thead>
<tr>
<th>Flux Conditions</th>
<th>Control Predicted</th>
<th>Control Observed</th>
<th>PRL Predicted</th>
<th>PRL Observed</th>
<th>Cortisol Predicted</th>
<th>Cortisol Observed</th>
<th>Cortisol + PRL Predicted</th>
<th>Cortisol + PRL Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW/L15 (x 10(^{-3}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Na(^+)</td>
<td>11.3 ± 0.26</td>
<td>6.51 ± 0.64*</td>
<td>13.6 ± 0.34</td>
<td>8.70 ± 0.86*</td>
<td>8.90 ± 0.10</td>
<td>13.0 ± 1.07*</td>
<td>7.44 ± 0.18</td>
<td>14.90 ± 0.19*</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>12.5 ± 0.21</td>
<td>19.1 ± 1.76*</td>
<td>13.9 ± 0.36</td>
<td>22.8 ± 1.66*</td>
<td>12.8 ± 0.20</td>
<td>16.3 ± 0.73*</td>
<td>19.6 ± 0.94</td>
<td>34.5 ± 0.47*</td>
</tr>
<tr>
<td>TER, kΩ/cm(^2)</td>
<td>23.09 ± 0.63</td>
<td>24.05 ± 0.59</td>
<td>24.53 ± 0.49</td>
<td>26.99 ± 0.34*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEP, mV</td>
<td>-7.54 ± 0.29*</td>
<td>-8.85 ± 0.43*</td>
<td>-3.91 ± 0.25*</td>
<td>1.31 ± 0.47</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Values are means ± 1 SE; n = 7–10 pairs of epithelia. *Significant difference (P<0.05) between observed and predicted flux ratios. Different letters represent significant differences among treatment groups for transepithelial resistance (TER) and transepithelial potential (TEP) (P<0.05). Observed flux ratios higher than predicted indicate active apical-to-basolateral uptake. Observed flux ratios lower than predicted indicate active basolateral-to-apical extrusion.
the active uptake of Na\(^+\) and Cl\(^-\) from an apical composition below 25–50% and certainly not from apical FW (Table 2). Therefore, an abrupt dilution protocol was investigated in series 2.

**Series 2: Single-Step Change from Apical L15 Medium to FW**

**TER and TEP.** After 7–9 days in culture under symmetrical conditions, the cultured epithelia in this series had developed a high TER, which had reached a stable plateau in all the groups. The greatest TER (30.37 ± 0.38 kΩcm\(^2\)) was observed in the cortisol + prolactin-treated inserts, whereas there were no significant differences among the control (23.03 ± 0.58 kΩcm\(^2\)), prolactin-treated (21.19 ± 0.93 kΩcm\(^2\)), and cortisol-treated epithelia (22.13 ± 0.55 kΩcm\(^2\)). Similarly, a high TEP had developed in the cortisol + prolactin treatment (+24.86 ± 3.1 mV) relative to the control (+3.08 ± 0.28 mV), cortisol (+2.77 ± 0.30 mV), and prolactin (1.65 ± 0.19 mV) treatments.

After abrupt exposure to apical FW, TER and TEP values changed from those under symmetrical conditions, but the values remained relatively stable during the 6-h period over which unidirectional Na\(^+\) and Cl\(^-\) flux rates were measured. Mean values = \([T_{oh} + T_{sh} + T_{eh}/3]\) are tabulated in Table 3. Thus TER for
the cortisol + prolactin treatment was lower than under symmetrical conditions, although still significantly higher than in the other treatments, which had exhibited either very modest increases or no change. Upon transition to asymmetrical conditions, TEP declined markedly in all treatments, becoming highly negative in the control and prolactin groups and moderately negative in the cortisol group, but remaining positive in the cortisol + prolactin treatment (Table 3).

**Unidirectional ion flux rates and Ussing flux ratios.**

After abrupt transfer to apical FW, there were no significant differences in Na\(^+\) efflux or net flux rates among the four treatment groups (Fig. 4A). As in series 1, influx rates were much lower than efflux rates. However, in series 2, Na\(^+\) influx rates were significantly higher by 65–75% in cortisol and cortisol + prolactin groups relative to the other treatments (Fig. 4A). Na\(^+\) influx rates were 0.359 ± 0.038, 0.360 ± 0.041, 0.627 ± 0.030, and 0.596 ± 0.044 nmol cm\(^{-2}\) h\(^{-1}\) in the control, prolactin, cortisol, and prolactin + cortisol treatments, respectively. Under asymmetrical conditions (FW/L15), the observed flux ratios for Na\(^+\) were less than the predicted Ussing flux ratios in control and prolactin-treated groups, indicating active extrusion of Na\(^+\) (Table 3). However, the observed flux ratios were significantly greater than predicted ratios under identical conditions (FW/L15) in the cortisol- and cortisol + prolactin-treated groups, indicating an active uptake of Na\(^+\) from FW (Table 3). In the latter case, the difference was twofold.

Under asymmetrical conditions, cortisol + prolactin significantly reduced Cl\(^-\) influx, efflux, and net flux rates relative to the control group, whereas cortisol alone significantly reduced only Cl\(^-\) influx rates (Fig. 4B). Prolactin alone exhibited no effects on Cl\(^-\) flux rates. The Cl\(^-\) influx rates were 0.899 ± 0.057, 1.100 ± 0.084, 0.713 ± 0.044, and 0.698 ± 0.059 nmol cm\(^{-2}\) h\(^{-1}\) in the control, prolactin-, cortisol-, and prolactin + cortisol-treated epithelia, respectively (Fig. 4B). In all groups, the observed flux ratios for Cl\(^-\) were significantly greater than the predicted Ussing ratios in all groups, indicating an active uptake of Cl\(^-\) (apical to basolateral) under these conditions (Table 3). In summary, both cortisol alone and cortisol + prolactin supported the active uptake of Na\(^+\) and Cl\(^-\) from apical FW in this abrupt dilution protocol.

**Na\(^+\)-K\(^+\)-ATPase Activity in Cultured Gill Epithelia**

Within each series, there were no significant differences in Na\(^+\)-K\(^+\)-ATPase activity among different hormonal treatment groups (Table 4).

**DISCUSSION**

**Overview**

The most important finding of this study, as revealed by the data from series 2 experiments, is that cultured trout DSI epithelia are able to actively transport Na\(^+\) and Cl\(^-\) from apical FW to basolateral media under asymmetrical (FW apical/L15 basolateral) culture conditions. This occurs when epithelia composed of both PVCs and MRCs are cultured with either cortisol or prolactin + cortisol for 7–9 days and are then subjected...
to an immediate single-step introduction of apical FW. This is the first study to demonstrate simultaneous active uptake of Na\(^+\) and Cl\(^-\) in these preparations, albeit at rates much lower than those occurring in vivo. The second important finding, as revealed by series 1, is that this preparation can sustain a gradual dilution of the apical medium right down to FW over a prolonged period of time (6 days), although this regime does not promote the active uptake of Na and Cl\(^-\) from an apical medium below 25–50%. The TER values at the end of the dilution protocol remain some of the highest ever recorded for a cultured epithelium of any sort (reviewed in Ref. 33), indicating the maintenance of epithelial integrity. This illustrates the remarkable barrier properties of the FW gill epithelium; we are aware of no other cultured epithelium that could withstand such a treatment. Although the preparation will withstand these conditions in the absence of hormonal support, cortisol (or cortisol in combination with prolactin) has a substantial effect in promoting improved epithelial tightness, especially during the later stages of dilution.

**Changes in TER Following Apical Media Dilution and Hormone Treatment**

TER is an electrical indicator of the general "tightness" of epithelia, reflecting both transcellular and paracellular pathways. Recent experiments on trout DSI epithelia (see review in Ref. 33) indicate that the normal sigmoidal increase of TER to a plateau during development under symmetrical conditions does not reflect any increase in cell numbers on the filter inserts. Rather, it reflects a progressive tightening of the cell junctions (5), monitored as a reduction in paracellular permeability via PEG-4000 flux measurements (32). In mature preparations with high TER (i.e., at the plateau phase, >20 kΩcm\(^2\)) such as those studied here, the TER may increase slightly within a few minutes when the apical medium is changed acutely from L15 to FW, followed by a partial decay over the next 24 h (8). Alternately, there may be little change or a modest fall in TER on apical FW exposure (8, 33) as in series 2 (Table 3). The exact response depends on the balance between a decrease in transcellular conductance (thought to result from closure of apical channels), which increases TER, and an increase in paracellular conductance (leakiness of the tight junctions), which decreases TER (33). Because cortisol is well documented to decrease paracellular permeability in this preparation as revealed by PEG-4000 flux measurements (13–15, 33), the higher TER values during gradual apical dilution in the cortisol and cortisol + prolactin treatments of series 1 (Fig. 1A) can be attributed to this effect. However, in series 2, these same treatments did not result in a greater TER after abrupt transfer to apical FW (Table 3), suggesting that they must have caused a compensating decrease in transcellular conductance by opening apical channels. This may explain why active Na\(^+\) and Cl\(^-\) uptake from FW occurred. In general, the positive effects of cortisol + prolactin on ion transport and epithelial integrity in both series can be attributed to the cortisol component, because cortisol alone exerted similar actions, whereas prolactin alone did not. The result agrees with a similar lack of effect of prolactin on TER and ion influx in DSI epithelia under either symmetrical or asymmetrical conditions, as reported by Kelly and Wood (16). However, in a few instances, cortisol + prolactin seemed to have a greater effect than cortisol alone (e.g., on TER in series 1 and on Cl\(^-\) flux ratios in series 2), suggesting a slight synergism.

Overall, these results agree well with current ideas that both hormones contribute to osmoregulatory support during FW adaptation. Although the mobilization of prolactin and its FW-adapting roles have been known for a long time (e.g., 3, 7, 10, 19, 22, 23, 28), evidence for the mobilization of cortisol and its FW-adapting role on exposure of euryhaline fish to environmental dilution is more recent (reviewed in Refs. 7, 23). For both hormones, most of the research focus and controversy to date has been on their roles in support of Na\(^+\)-K\(^+\)-ATPase activity, chloride cell numbers and morphology, and active Na\(^+\) and Cl\(^-\) uptake (e.g., 6, 11, 18, 22, 27), although a role for prolactin in reducing passive permeability during FW adaptation has long been accepted (e.g., 3, 10). The present investigation (series 2) indicates that the same positive effects of cortisol in promoting Na\(^+\) and Cl\(^-\) influx from FW can be seen in the cultured branchial epithelium (Fig. 4 and Table 3) and that these effects can occur in the absence of stimulated Na\(^+\)-K\(^+\)-ATPase activity (Fig. 4). Furthermore, the results of series 1 (Figs. 1–3) indicate that cortisol may actually be much more important than prolactin in supporting epithelial tightness during adaptation to dilute media. Such effects of cortisol have been largely overlooked in studies on intact animals.

**Changes in TEP Following Dilution and Hormone Treatment**

In previous studies (8, 16), mature DSI epithelia at plateau TER under symmetrical conditions exhibited positive TEP values of +1 to +10 mV. On acute exposure to apical FW, these changed over to negative TEP values, averaging about −11 mV but varying in individual preparations from −1 to −35 mV (see review in Ref. 33). In general, greater absolute TEP values were associated with greater TER values, in accordance with Ohm’s law. These same trends were seen in the present experiments.

The positive TEP values (+32 to +45 mV) under symmetrical conditions of series 1 (Fig. 1B) were particularly high for cultured trout gill epithelia (cf. 33) but consistent with the very high TER recordings. Avella and Ehrenfeld (1) and Avella et al. (2) also reported high basolateral-positive TEP in a cultured branchial epithelium from seawater bass (+13 to +30 mV) under symmetrical conditions. Positive potentials may be due to electrogenic Cl\(^-\) extrusion, as in the cultured PVC epithelium of the sea bass (1, 2). Cer-
tainly, the Ussing analyses indicated active Cl\textsuperscript{−} efflux in most treatments of series 1 under symmetrical conditions as well as in control and cortisol-treated epithelia in 50–12.5% apical L15 (Table 2). These represent the treatments in which the TEP was most positive in each of the series (Table 2).

Negative potentials in dilute apical media have been interpreted as mainly diffusion potentials due to the differential permeability of the epithelia to Na\textsuperscript{+} vs. Cl\textsuperscript{−}, with a very small negative electrogenic component superimposed on the latter (8, 35). These interpretations follow the generally accepted explanations for transgill potentials measured in fish in saline waters vs. FW (29). Any differential effects of hormonal treatments on TEP were gradually lost during progressive apical dilution in series 1 (Fig. 1B) but remained intact after abrupt change to apical FW in series 2 (Table 3). Effects on TEP in dilute solution appear largely attributable to cortisol, as prolactin alone had no influence, although again there appeared to be some synergism, with cortisol + prolactin sustaining a slightly positive TEP in apical FW (Table 3). These results are in agreement with previous reports that prolactin alone has no effect on TEP of either SSI or DSI epithelia under FW/L15 conditions (16). However, the cortisol effect on TEP in the present DSI epithelia differs from previous observations on SSI epithelia in which cortisol treatment resulted in a more negative TEP under FW/L15 conditions (13, 15). Presumably, this difference reflects an effect of cortisol on the MRCs, which are present only in DSI epithelia.

Unidirectional Na\textsuperscript{+} and Cl\textsuperscript{−} Flux Rates Following Dilution and Hormone Treatment

Active uptake of both Na\textsuperscript{+} and Cl\textsuperscript{−} from apical FW was evident in both cortisol- and cortisol + prolactin-treated preparations in series 2 according to the Ussing flux ratio criterion (Table 3). Absolute Na\textsuperscript{+} and Cl\textsuperscript{−} influx rates were \( -0.6 \text{ mmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1} \) and \( -0.8 \text{ mmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1} \) for an intact rainbow trout, taking relative surface areas of the cultured epithelia and the whole gill into account (see Ref. 35 for conversion details). Absolute Na\textsuperscript{+} and Cl\textsuperscript{−} efflux rates of approximately \( -40 \text{ mmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1} \) would similarly translate to about \( -80 \text{ mmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1} \) for an intact trout. Measured in vivo rates for trout gills in vivo in FW are \( \sim 250 \text{ mmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1} \) for both influx and efflux of Na\textsuperscript{+} and Cl\textsuperscript{−} (18, 35). Clearly, there is a quantitative discrepancy, especially for influx, but the cultured branchial epithelium has a different architecture (flat) than the gill and is neither ventilated with water nor perfused with blood.

Regardless, it is highly encouraging that cortisol promotes active Na\textsuperscript{+} and Cl\textsuperscript{−} uptake from apical FW in cultured DSI epithelia. This effect of cortisol is not seen in cultured SSI epithelia composed solely of PVCs from rainbow trout (13), so it presumably reflects the action of cortisol on the MRCs in DSI preparations. This finding is consistent with current models wherein cortisol stimulates MRC function in FW fish, promoting active uptake of Na\textsuperscript{+} and Cl\textsuperscript{−} (18, 22, 23). However, to date, such models have emphasized effects on Na\textsuperscript{−}K\textsuperscript{+}-ATPase activity, which is most heavily concentrated in MRCs (31). Na\textsuperscript{−}K\textsuperscript{+}-ATPase activity did not change in the present experiments (Table 4). Rather, based on TER changes (Table 3), we argued above that cortisol may have promoted the opening of apical channels in these preparations, thereby allowing increased ion uptake from FW. Apical Na\textsuperscript{+} channels, polarized by the H\textsuperscript{+}-pumping action of proton ATPase, play a key role in current models for Na\textsuperscript{+} uptake in the FW gill (7, 20, 21, 26). The mechanism of Cl\textsuperscript{−} uptake is less well understood (26), but “maxi” Cl\textsuperscript{−} channels have been characterized in cultured gill cells from FW trout (24).

Active uptake of Na\textsuperscript{+} and Cl\textsuperscript{−} from apical FW was not seen in DSI epithelia of series 1 gradually adapted to dilute media (Table 2). Instead, active uptake stopped below an apical composition of 25–50% and changed over to an apparent active excretion of Na\textsuperscript{+} and either active or passive distribution of Cl\textsuperscript{−} in more dilute salinities. This absence of “normal” ion transport in FW is in accord with previous studies on SSI epithelia (13, 14, 16, 33) and cortisol-free DSI epithelia (8, 14; Table 3) and suggests that over the prolonged 6-day period of apical dilution, the MRCs had lost either their integrity or their ability to respond to cortisol.

In both series, cortisol or cortisol + prolactin exerted a clear effect in reducing unidirectional effluxes and net fluxes of both ions (Figs. 2–4). These actions were seen across the full range of apical dilution and are attributable to the stabilizing action of this hormone on paracellular permeability, as documented earlier for SSI preparations of both trout and tilapia (13–15). Again, this emphasizes that a key role of cortisol in FW adaptation may be in reducing passive permeability, an effect that has been overlooked in previous studies (for reviews, see Refs. 7, 23). Clearly, this responsiveness is not lost during prolonged apical dilution over 6 days and probably reflects the action of cortisol in reducing the junctional permeability of the PVCs. In tilapia SSI preparations, this action was exerted principally through glucocorticoid receptors on PVCs (15). Interestingly, Sloman et al. (30) concluded that proliferation of MRCs in the gills of intact trout transferred to very dilute FW involved mineralocorticoid rather than glucocorticoid receptors.

The actions of prolactin on ion transport were less clear-cut, tending to stimulate influxes and/or effluxes at higher apical salinities in series 1 (e.g., Fig. 2, A and C) and/or to be synergistic with cortisol in limiting efflux in series 2 (e.g., Fig. 4C and Table 3). These results were surprising, because prolactin alone is traditionally thought to reduce gill permeability during FW adaptation (7, 19, 23). However, it is still possible that water permeability, which was not measured in this study, may have been lowered by prolactin (10). The actions were also different from those seen earlier in trout SSI epithelia, where prolactin had no effect on unidirectional flux rates under symmetrical conditions yet tended to reduce efflux rates under asymmetrical conditions through a transcellular effect (16). Clearly,
more work is needed to sort out these conflicting prolactin results. Regardless, in the combined presence of cortisol + prolactin, the ionoregulatory “balance” of preparations from both series 1 and series 2 was still superior (i.e., net Na\(^+\) and Cl\(^-\) loss rates were lower in dilute apical solutions) than under hormone-free conditions, showing the advantages of hormonal supplementation (Figs. 2–4).

**Perspectives**

For the first time, we have a cultured gill epithelial preparation that actively transports both Na\(^+\) and Cl\(^-\) from apical FW to basolateral media when supplemented with cortisol (or cortisol + prolactin). Furthermore, it can tolerate progressive apical dilution to FW after reaching maturity, and hormonal supplementation is again clearly beneficial to this tolerance in limiting epithelial permeability. The cultured DSI epithelium is therefore becoming a more realistic in vitro model for ion transport and hormone effects studies. However, relative to studies on seawater gill models derived from cranial epithelia of marine teleosts (reviewed in Refs. 20, 21, 34, 36), this FW gill model is still in its infancy. In the future, a wider range of hormones (e.g., growth hormone, insulin-like growth factor, neurohypophysial and urophysial peptides, mineralocorticoids, catecholamines, atrial natriuretic peptides, angiotensin, prostaglandins, nitric oxide; cf. 7), hormone combinations, and dilution protocols should be evaluated for their potential to modify ion transport in DSI epithelia.

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**DISCLOSURES**

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