

Response of Developing Cultured Freshwater Gill Epithelia to Gradual Apical Media Dilution and Hormone Supplementation

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ABSTRACT We investigated gradual dilution of the apical medium (Leibovitz's L15 to fresh water [FW], analogous to gradual reduction in environmental salinity) and basolateral hormone support on the electrophysiological and ion-transporting properties of "developing" FW trout gill epithelia cultured on filter inserts. Epithelia were of the double-seeded type, containing both pavement cells and mitochondria-rich cells. In these experiments we were able to circumvent "symmetrical development" (typically L15 apical/L15 basolateral for 6–9 days) by commencing dilution of apical media (unchanged L15 basolateral, i.e., asymmetrical conditions) at culture-day 3, the time when transepithelial resistance (TER) and potential (TEP) would normally be increasing rapidly under symmetrical conditions. In Series 1 (without basolateral hormone support), epithelia were exposed to progressively diluted apical media (100%, 75%, 50% L15) at 24-hr intervals, thereafter cultured in 50% L15 apical media for 4 days, and then in apical FW. In Series 2, epithelia were exposed to progressively diluted apical media (100%, 75%, 50%, 25%, 12.5% L15, and FW) at 24-hr intervals with physiologically relevant doses of cortisol (500 ng ml⁻¹), prolactin (50 ng ml⁻¹), or cortisol + prolactin (500 ng ml⁻¹+50 ng ml⁻¹, respectively) added to basolateral media (100% L15). In Series 1, TER reached a plateau phase over 25 kΩ cm² under 50% L15/L15 culture conditions (after 4 days of culture) but fell to ~6 kΩ cm² after 24 hr in FW/L15 conditions. In Series 2, TER stabilized at 4–11 kΩ cm² depending on treatment. In general, apical media dilution during epithelial development was well tolerated. Preparations exhibited continued integrity right down to apical FW, indicated by only modest increases in net ion losses (i.e., basolateral to apical movement of ions), relatively stable TER values, and the expected changeover from positive to negative TEP in FW. Cortisol was clearly beneficial to FW adaptation, promoting greater TER, reduced unidirectional and net Na⁺ and Cl⁻ flux rates, and elevated Na⁺, K⁺-ATPase activity. Prolactin also offered some support, where its actions on TER were less than but additive to those of cortisol. There was no direct evidence that prolactin limited ion movements during gradual dilution. These *in vitro* studies demonstrate that "developing epithelia" were able to tolerate gradual dilution of apical media, the remarkable barrier properties of gill epithelia, and the importance of cortisol and prolactin in promoting integrity of this barrier during FW adaptation. *J. Exp. Zool.* 301A:867–881, 2004. © 2004 Wiley-Liss, Inc.

INTRODUCTION

Fish gills have a complex three-dimensional structure, comprise many different cell types (e.g., mucous cells, neuroepithelial cells, pavement cells [PVCs], mitochondria-rich cells [MRCs]), and perform a variety of physiological functions (e.g., water excretion, respiration, ion transport, acid-base regulation, and nitrogenous waste excretion; for review, see Wilson and Laurent, 2002). In seawater fish, the outer branchial epithelium actively excretes salt and limits its passive uptake

from the concentrated external environment, while in freshwater fish, the homologous layer plays a critical role in the active uptake of Na⁺ and Cl⁻ and in the limitation of diffusive ion losses to the dilute external environment. However, the

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structural and functional heterogeneity of the gill has made it very difficult to study the permeability and ion transport characteristics of the branchial epithelium in vivo. Ion-transport physiologists have overcome this problem for seawater fish by working with excised cranial epithelia (e.g., opercular epithelia from killifish and tilapia, jawskin from goby) that are flat and can be mounted in Ussing chambers in vitro, and which appear to faithfully mimic in vivo gill transport function (for reviews see Zadunaisky, '84; Marshall, '95). In addition, cultured gill epithelia from seawater fish, the sea bass (*Dicentrarchus labrax*) have been developed and actively excrete Cl^- (Avella and Ehrenfeld, '97). However, the search for comparable surrogate epithelia to mimic freshwater gill salt transport has proven disappointing, for such surrogate epithelia from freshwater fish exhibit small to negligible active uptake and large passive losses of Na^+ and Cl^- (Foskett et al., '81; Wood and Marshall, '94; Marshall et al., '92, '97; Burgess et al., '98).

In recent years, an alternative approach has been attempted, involving in vitro culture on permeable filter inserts of "reconstructed" flat branchial epithelia derived from dispersed gill cells of freshwater fish (Kelly et al., 2000). Originally developed as a layer of only PVC's (Wood and Pärt, '97), the preparation has been improved more recently by the incorporation of about 15% MRCs in vivo using a double-seeded insert (DSI) technique (Fletcher et al., 2000) and by the inclusion of hormonal support (e.g., cortisol, prolactin; Kelly and Wood, 2001a, 2001b, 2002b). In the absence of hormone support, such preparations are robust and appear to duplicate the passive permeability (ionic efflux rates) and electrical characteristics (transepithelial resistance [TER] and transepithelial potential [TEP]) of the intact freshwater gill very well, but again the situation with respect to active Na^+ and Cl^- uptake from apical freshwater has proven disappointing. At best, such preparations show very slight active Cl^- uptake, often paradoxical slight active Na^+ extrusion, and net negative overall ion balance (for review, see Wood et al., 2002). Recent studies using hormone supplements (cortisol or cortisol + prolactin) have revealed more encouraging properties (Zhou et al., 2003). That is, cortisol alone or cortisol + prolactin can promote the active uptake of both Na^+ and Cl^- by cultured epithelia when freshwater is introduced to the apical side as a single-step transition (Zhou et al., 2003). However, overall net ion balance is still

negative under these conditions, therefore it is beneficial to investigate experimental techniques that may promote an increase in the rates of "inward" ion movement while limiting passive efflux.

To date, virtually all studies using cultured branchial epithelia have been conducted after the preparation has first developed for 6–9 days to the plateau phase in TER under "symmetrical" conditions, for example, with identical isotonic media (L15 + 5% FBS) present on both surfaces. Epithelia were then exposed to a single-step change to apical freshwater (FW), resembling a transition from brackish water to FW in vivo (e.g., Wood and Pärt, '97; Wood et al., '98; Fletcher et al., 2000; Kelly and Wood, 2001a,b, 2002a,b). However, euryhaline fish moving into FW under natural conditions (e.g., in estuaries) are unlikely to undergo a single-step transition and are more likely to experience a transitional period through progressively lower environmental salt concentrations. Therefore, recently we have experimented with gradual apical dilution protocols using plateau-phase epithelia (i.e., after 6–9 days in symmetrical culture) (Kelly and Wood, 2003; Zhou et al., 2003). In these experiments, the apical media was diluted with FW over varying periods of time until 100% FW was reached. The preparations maintained integrity, but there was no evidence that ionic transport was enhanced under these FW/L15 conditions. Additionally, mild experimental dilution of the basolateral media, as might also occur in a fish moving into freshwater, did not activate transport (Kelly and Wood, 2003).

In the present study, we evaluated the responses of the cultured trout gill epithelium (DSI technique; Fletcher et al., 2000) to several untested dilution protocols. First, we reasoned that it might be necessary for the epithelium to actually experience an apical dilution during development, rather than after development, in order to activate the "freshwater pattern" of ion transport mechanisms. To our knowledge, development of a branchial epithelium in vitro during progressive dilution of the apical medium has never been attempted before. Secondly, it is known that the transition to FW in vivo is accompanied by an integrated endocrine response (McCormick, 2002). In particular, the mobilization of prolactin and cortisol have been demonstrated to significantly affect the development of gill ionoregulatory processes in vivo (Manzon, 2002; McCormick, 2002) and appear to improve the electrical and transport properties of the cultured trout gill

epithelium in vitro (Kelly and Wood, 2001a,b, 2002b; Zhou et al., 2003).

Therefore we hypothesized (1) that the pattern of regulated ion movement, both active and passive, across cultured gill preparations would benefit from gradual apical media dilution during the course of actual epithelial development and (2) that hormone supplementation would improve the development of epithelia under this dilution protocol. To examine this, two series of experiments were conducted from the point when epithelia first exhibited an indication of confluent layer formation (i.e., when TER first increased above background, approximately 24 hr after final cell seeding or day 3 after first seeding) to the final introduction of 100% apical FW (FW apical/L15 basolateral) after 5–6 days of gradual apical dilution. In Series 1, we evaluated whether developing epithelia would tolerate a graduated dilution of the apical media in the absence of hormone supplements using electrophysiological (TER, TEP) and ion transport properties (unidirectional Na^+ and Cl^- fluxes, measured radioisotopically) of the epithelia to monitor status. Building on the results of Series 1, in Series 2 we evaluated a more gradual protocol for dilution of the apical media, both alone and in combination with hormonal support (cortisol and prolactin, alone and in combination).

MATERIALS AND METHODS

Fish and preparation of double-seeded insert preparations (DSI)

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Hatchery (Orangeville, Ontario, Canada). The fish (85–150 g) were held in dechlorinated running municipal tap water (in mM: $[\text{Na}^+]=0.55$; $[\text{Cl}^-]=0.70$; $[\text{Ca}^{2+}]=1.00$; $[\text{Mg}^{2+}]=0.15$; $[\text{K}^+]=0.05$; pH 7.8–8.0) at seasonal temperatures (12–15°C). Gill cells were isolated in a laminar flow hood using sterile techniques according to the methods for DSI preparations fully detailed by Kelly et al. (2000). 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 phosphate-buffered saline [PBS], pH 7.7 with 5.5 mM EDTA). The cells were resuspended in cold culture medium (Leibovitz's L-15, supplemented with 5% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 200 $\mu\text{g ml}^{-1}$ gentamicin)

and seeded on the apical side in Falcon cell inserts (Cyclopore polyethylene terephthalate "filters," Becton-Dickinson, Franklin Lakes, NJ; pore density, 1.6×10^6 pores cm^{-2} ; pore size, 0.45 μm ; growth surface, 0.9 cm^2) at a density of 3×10^6 cells cm^{-2} . One day after seeding, each insert was rinsed with phosphate buffered saline (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 2×10^6 cells cm^{-2} (this date was termed Day 1). This is the step which allows incorporation of MRCs into the first day's layer of PVCs (Fletcher et al., 2000). After a further 24 hr, mucus and unattached cells were again removed with PBS rinsing, and 1.5 ml of culture medium with antibiotics was added to the apical side while 2 ml with antibiotics was added to the basolateral side. All procedures conformed to the guidelines of the Canadian Council of Animal Care.

Hormone treatments and apical dilution

Cortisol (hydrocortisone 21-hemisuccinate) and ovine prolactin (oPRL) were obtained from Sigma for use in Series 2. The use of oPRL to study the osmoregulatory system in fish (e.g., Kelly et al., '99; Seidelin and Madsen, '99; Mancera et al., 2002) and ion transport in cultured gill epithelia is well established (Kelly and Wood, 2002b; Zhou et al., 2003). 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 only the basolateral culture media to achieve 500 ng ml^{-1} of cortisol and/or 50 ng ml^{-1} prolactin. We selected these concentrations as realistic and potentially effective based on surveying the in vivo literature and on the results from our earlier studies (see Kelly and Wood, 2001a, 2002a,b; Zhou et al., 2003).

Series 1

In this series, progressive dilution of the apical media was carried out during development of the DSI preparations, prior to the plateau phase of TER. No hormones were used. On Day 2, 1.5 ml of 100% culture medium (L15+5% FBS) was added to the apical side and 2 ml of the same culture medium was added to the basolateral side. Electrophysiological and unidirectional ion flux measurements were first conducted with full-strength regular L15 media (100%) on both surfaces on Day 3, a time when transepithelial resistance (TER) had first exceeded 0.3 $\text{k}\Omega \text{cm}^2$.

Thereafter, the apical solution was replaced with 75% medium (regular L15 medium diluted to 75% its original strength with sterile freshwater (75% L15 + 25% FW). After 16–18 hr at 75%, flux and electrophysiological measurements were repeated (i.e., on Day 4), and then 50% medium (50% L15 + 50% FW) was substituted on the apical side. After 16–18 hr at 50%, flux and electrophysiological measurements were repeated (i.e., on Day 5). The epithelia were then maintained with 50% apical media and allowed to develop to plateau phase TER under these conditions, a process which took another 4 days. During this period, apical and basolateral media were replaced every 24 hr. At Day 9, the flux measurements at 50% apical media were conducted again. Thereafter, there was a single-step changeover from 50% media to apical freshwater, preparations were acclimated to FW/L15 conditions for 16–18 hr, and then flux measurements were conducted (4 hr).

Series 2

In this series, there were four treatments. Epithelia were allowed to develop (1) without hormonal supplementation (control) or were supplemented with either (2) 500 ng ml⁻¹ cortisol, (3) 50 ng ml⁻¹ prolactin, or (4) both 500 ng ml⁻¹ cortisol + 50 ng ml⁻¹ prolactin, added to the basolateral culture media. Hormones were not added to the apical media. The hormones were kept in the basolateral media from Day 2 onward throughout the experimental period, and these media were renewed daily. Electrophysiological and ion flux measurements were first conducted with full-strength regular L15 media (100%) on both surfaces on Day 3, the time when again the TER first exceeded 0.3 kΩ cm². Thereafter, the apical solution was replaced with 75% medium as in Series 1. After 16–18 hr at 75%, flux and electrophysiological measurements were repeated (i.e., on Day 4), and then 50% medium was substituted on the apical side. The process was sequentially repeated so measurements at 50% apical media were made on Day 5, at 25% on Day 6, at 12.5% on Day 7, and at 0% (i.e., 100% FW) on Day 8.

Electrophysiological measurements

TER across the epithelium, was monitored with STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) (Wood et al., '98). Transepithelial potential (TEP) was mea-

sured with an agar/salt bridge (3 M KCl in 4% agar) connected to Ag/AgCl electrodes (WPI) using a pH meter (Radiometer pHM 84, 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 were performed as described by Kelly and Wood (2001a).

Unidirectional ion flux measurements

Unidirectional Na⁺ and Cl⁻ fluxes across the preparations were measured by adding 0.1–0.25 μCi of the radioisotopes ²²Na and ³⁶Cl (NEN Dupont) to one side ("hot" side) and monitoring the appearance over time of radioactivity on the other side ("cold" side). After the symmetrical (100% apical L15) flux measurements were completed, both culture media were replaced by adding 75% L15 on the apical side and fresh 100% L15 media with the appropriate hormonal composition (Series 2 only) to the basolateral side. The preparation was then left for 16–18 hr to acclimate to the new apical salinity. Prior to 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 successive steps of apical dilution. For the final change to fresh water, the apical side was rinsed with fresh water three times to ensure a complete changeover of solutions and no culture medium contamination. Samples for radioactivity measurements were collected at the beginning (time 0) and at the end of a flux. The flux measurement periods were 3–6 hr; longer times were necessitated by the smaller flux rates that occur across high-TER preparations. TER and TEP were monitored at the beginning, middle, and end of each flux period, with average values reported in the text.

Each insert could be used for either influx (J_{in} , positive by convention) or efflux (J_{out} , negative) measurements, but not for both. Therefore, to calculate net flux (J_{net}), and to apply the Ussing predicted flux-ratio criterion, it was necessary to pair cultured epithelia that provided reciprocal flux measurements (Fletcher et al., 2000). Pairs were matched according to the most similar average TER (measured at T_0 , T_{middle} , and T_{final}). Unidirectional flux was calculated according the following equations (Wood et al., 1998):

$$J_{in}^{Na^+} = \Delta[{}^*X]_{BL} \times \frac{1}{(SA_{AP})} \times \frac{Volume_{BL}}{Time \times Area}, \quad (1)$$

where $\Delta[^*X]_{BL}$ is the change in ^{22}Na or ^{36}Cl radioactivity on the basolateral side ("cold" side), Volume_{BL} is the volume on the basolateral side, and SA_{AP} is the mean specific activity on the apical side ("hot" side). Conversely, for efflux (basolateral to apical flux, radioisotope placed on basolateral side):

$$J_{out}^{Na^+} = -\Delta[^*X]_{AP} \times \frac{1}{(\text{SA}_{BL})} \times \frac{\text{Volume}_{AP}}{\text{Time} \times \text{Area}}, \quad (2)$$

where $\Delta[^*X]_{AP}$ is the change in radioactivity on the apical side ("cold" side) and SA_{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 $\text{nmol cm}^{-2} \text{h}^{-1}$.

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; Kirschner, '70). The Ussing ratio was calculated as:

$$J_{in}/J_{out} = \frac{A_{AP} e^{-(ZFV/RT)}}{A_{BL}}, \quad (3)$$

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 T 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 Zhou et al., 2003).

Na⁺, K⁺ -ATPase activity

Na^+ , K^+ -ATPase activity at the end of the protocol in the epithelia of Series 2 was determined according to methods outlined by Kelly and Wood (2001a) in cells harvested after unidirectional flux experiments (after the last step of apical dilution FW was finished). 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 mean \pm 1 SEM (N), where N represents the number of electrophysiologically matched pairs of epithelia. For comparison of TER and TEP during gradual dilution, a one-way analysis of variance (ANOVA) was used.

To compare the difference between the predicted (Ussing ratio) and observed 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: TER and TEP

The developing DSI preparations tolerated progressive apical dilution well, even during the early stage of development, long before the plateau phase of TER. TER continued to increase steadily from Day 3 value of about $0.5 \text{ k}\Omega \text{ cm}^2$ value in symmetrical media (100% L15 both sides), reaching a TER in 50% L15 on Day 5 of $5.3 \text{ k}\Omega \text{ cm}^2$ (Table 1). When the preparations were held under 50% L15/L15 culture conditions for another 4 days, the TER continued to increase to a plateau value of over $25 \text{ k}\Omega \text{ cm}^2$ culture. However, upon exposure to freshwater, TER dropped to $6.14 \text{ k}\Omega \text{ cm}^2$ (Table 1). The TEP was not significantly different from 0 mV under symmetrical conditions (L15/L15) on Day 3 and 75% L15/L15 on day 4 but thereafter tended to increase to positive values as the apical media were diluted to 50% L15 (Table 1). A maximum TEP of 7.8 mV was observed in 50% L15/L15 culture conditions after 9 days of culture. As expected, TEP became negative with a value of -7.3 mV after media were replaced by freshwater (Table 1).

Series 1: Unidirectional ion flux rates and Ussing flux ratios

Na⁺ transport

The unidirectional Na^+ fluxes exhibited equal movement in both directions under symmetrical culture conditions, and net flux rates were not significantly different from zero (Table 2). During progressive apical media dilution from L15/L15 down to 50% L15/L15 and subsequent maintenance at this apical solution until Day 9 (a period during which the epithelia were also developing, as shown by the increasing TER, Table 1), both Na^+ influx rates and Na^+ efflux rates declined. The absolute value of the net flux rates actually became smaller, but because of reduced variability, the net fluxes became significantly negative (i.e., below zero) at 50% L15/L15 (Table 2). Under

TABLE 1. Electrophysiological characteristics and comparison of the observed flux ratios (influx/efflux) versus predicted flux ratios for Na⁺ and Cl⁻ across cultured epithelia of Series 1[†]

| Flux ratio | Culture conditions | | | | | | | | | | | |
|---------------------------|--------------------|-------------|---------------------|-------------|---------------------|--------------|---------------------|--------------|-----------------|---------------|-------------------------------|----------|
| | L15/L15 (Day 3) | | 75% L15/L15 (Day 4) | | 50% L15/L15 (Day 5) | | 50% L15/L15 (Day 9) | | FW/L15 (Day 10) | | Observed (×10 ⁻³) | |
| | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed |
| Na ⁺ | 1.03 ± 0.01 | 0.94 ± 0.08 | 0.75 ± 0.01 | 0.76 ± 0.04 | 0.48 ± 0.01 | 0.31 ± 0.03* | 0.37 ± 0.02 | 0.48 ± 0.08 | 14.80 ± 2.10 | 6.18 ± 0.36* | | |
| Cl ⁻ | 1.03 ± 0.01 | 1.01 ± 0.02 | 0.83 ± 0.01 | 0.86 ± 0.05 | 0.59 ± 0.01 | 0.61 ± 0.04 | 0.88 ± 0.05 | 0.52 ± 0.08* | 10.00 ± 0.56 | 11.90 ± 0.90* | | |
| TER (kΩ cm ²) | 0.53 ± 0.03 | | 1.39 ± 0.08 | | 5.30 ± 0.32 | | 25.81 ± 1.51 | | | 6.14 ± 0.06 | | |
| TEP (mV) | Zero | | Zero | | 1.1 ± 0.1 | | 7.8 ± 1.2 | | | -7.3 ± 0.4 | | |
| | (N=14) | | (N=14) | | (N=12) | | (N=11) | | | (N=10) | | |

[†]All data are expressed as mean values ± 1 SEM. An asterisk denotes a significant difference ($P < 0.05$) between observed and predicted flux ratios within a defined culture condition. Observed flux ratios higher than predicted indicate active apical-to-basolateral uptake. Observed flux ratios higher than predicted indicate active basolateral-to-apical extrusion.

FW/L15 conditions, the Na⁺ influx rates were very small, whereas the Na⁺ efflux rates increased (Table 2). The observed Na⁺ unidirectional flux ratio was not significantly different from the predicted ratio (Ussing ratio) under symmetrical conditions (L15/L15) or 75% L15/L15, indicating the transport pattern was passive. The observed flux ratio was significantly less than the predicted ratio under 50% L15/L15 (Day 5) and FW/L15 culture conditions, indicating active extrusion of Na⁺ (Table 1). There was no significant difference between the observed and predicted ratio under 50% L15/L15 after the plateau phase TER was reached (Day 9), indicating passive Na⁺ transport under this condition.

Cl⁻ transport

Unidirectional Cl⁻ fluxes exhibited very similar patterns to unidirectional Na⁺ fluxes (Table 2). Flux ratio analysis showed that there was no active transport under symmetrical, 75% L15/L15, and 50% L15/L15 culture conditions (Table 1). However, the observed flux ratio was significantly less than the predicted flux ratio under 50% L15/L15 conditions after the TER reached plateau phase (day 9), indicating active extrusion of Cl⁻ (Table 1). After a step change from 50% L15/L15 to apical FW, the observed flux ratio was significantly greater than the predicted ratio, indicating active uptake of Cl⁻ under FW/L15 conditions (Table 1).

Series 2: TER and TEP

In this series, rather than holding the epithelia at 50% L15/L15 over Days 4–8 during development until plateau TER was reached, the progressive dilution protocol was continued on a daily basis. Again, the developing epithelia tolerated apical dilution very well. Indeed, TER continued to increase steadily from the Day 3 value of about 0.3–0.4 kΩ cm² value in symmetrical media (100% L15/L15 on both sides), reaching a maximum TER at 25% L15/L15 on Day 6 in all groups (a significant trend by one way ANOVA; Fig. 1A). TER then tended to drop slightly or stabilize on Days 7 and 8 after the apical solution was changed to 12.5% L15 and finally FW, respectively. Significant differences in TER among treatment groups could first be observed when apical media were diluted to 50% L15 (Fig. 1A). These differences became particularly marked when apical media were further diluted to 25% L15 (TER in kΩ cm²; control=4.87 ± 0.18, cortisol=10.39 ± 0.95,

TABLE 2. Unidirectional flux rates (influx, efflux, and net flux) of Na⁺ and Cl⁻ in Series 1 epithelia¹

| | | Culture conditions | | | | |
|---|-----------------|------------------------|-------------------------|------------------------|-----------------------|--------------------------|
| | | L15/L15 (Day 3) | 75% L15/L15 (Day 4) | 50% L15/L15 (Day 5) | 50% L15/L15 (Day 9) | FW/L15 (Day 10) |
| Influx (nmol cm ⁻² h ⁻¹) | Na ⁺ | 2,268.5 ± 264.5 | 301.2 ± 20.2 | 51.2 ± 4.6 | 14.8 ± 4.4 | 2.5 ± 0.5 |
| | Cl ⁻ | 2,406.0 ± 244.8 | 425.2 ± 33.0 | 103.7 ± 7.2 | 16.2 ± 2.0 | 5.9 ± 1.3 |
| Efflux (nmol cm ⁻² h ⁻¹) | Na ⁺ | -2,424.1 ± 152.8 | -427.3 ± 49.2 | -179.5 ± 18.8 | -36.1 ± 5.0 | -490.5 ± 114.2 |
| | Cl ⁻ | -2,426.4 ± 254.0 | -538.4 ± 67.4 | -174.8 ± 14.9 | -36.1 ± 4.8 | -528.4 ± 126.0 |
| Net flux (nmol cm ⁻² h ⁻¹) | Na ⁺ | -155.6 ± 226.3 | -117.7 ± 37.1 | -128.2 ± 18.1 | -21.3 ± 3.8 | -488.1 ± 113.7 |
| | Cl ⁻ | -20.5 ± 50.0 (N=14) | -113.2 ± 59.0 (N=14) | -71.1 ± 11.8 (N=12) | -19.9 ± 3.5 (N=11) | -522.5 ± 125.7 (N=10) |

¹All data are expressed as mean values ± 1 SEM.

prolactin = 6.35 ± 0.76, cortisol + prolactin = 9.05 ± 1.13) and persisted under asymmetrical conditions down to 0% (apical FW; Fig. 1A). Furthermore, upon exposure to apical FW, the preparations treated with the combination of cortisol plus prolactin exhibited a significantly greater TER compared to those treated with either cortisol or prolactin alone.

The TEP was not significantly different from 0 mV in any group under symmetrical conditions (L15/L15) on Day 3 but thereafter tended to increase to positive values as the apical media were diluted, reaching a maximum on Day 6 at 25% L15/L15 (Fig. 1B). Significant differences between the TEP of hormone-treated groups (cortisol, cortisol + prolactin) and the controls could first be observed at 75% L15/L15 on Day 4 (Fig. 1B). TEP then decreased at 12.5% L15/L15 in all groups, but remained significantly higher in the cortisol group. Thereafter, TEP became negative and showed no significant differences among treatments when fresh water was placed on the apical side (Fig. 1B).

Series 2: Unidirectional ion flux rates and Ussing flux ratios

Na⁺ transport

The unidirectional Na⁺ flux measurements showed equal movement in both directions in all groups under symmetrical culture conditions, and net flux rates were not significantly different from zero (Fig. 2A). During progressive apical media dilution, efflux rates became progressively higher than influx rates on a relative basis; therefore, negative net flux rates were observed in all groups from conditions of 75% L15/L15 onward (Fig. 2B–F). Note, however, that absolute rates

tended to fall through to 50% L15/L15, and then increased again at lower apical salinities down to FW/L15. However, cortisol was effective in limiting ion flux rates (particularly in an outward direction, 75% L15/L15 down to FW/L15; Fig. 2B–F), and cortisol + prolactin had a similar effect in the later stages of dilution (50%, 12.5% L15/L15, and FW/L15; Fig. 2E,F). Prolactin exhibited no ability to limit ion efflux and net flux in very dilute conditions, although a significant reduction of efflux and net flux was observed at 50% L15/L15 only (Fig. 2C). In apical fresh water, Na⁺ influx rates were reduced in all hormonal treatments relative to the control (Fig. 2F).

The flux ratio analyses of the unidirectional flux data of Fig. 2 are shown in Table 3. There was no indication of active transport of Na⁺ under symmetrical or 75% L15 conditions. However, the observed flux ratio was lower than the predicted (Ussing) ratio in the control and cortisol treatment groups at 50% L15/L15 (indicating an active extrusion of Na⁺), but not in the prolactin and cortisol + prolactin treatment groups. The observed ratio was significantly lower than the predicted ratio with 25% L15, 12.5% L15, or FW on the apical side in all groups, indicating an active extrusion of Na⁺.

Cl⁻ transport

Similar to the Na⁺ transport pattern, the unidirectional Cl⁻ fluxes showed equal movements in both directions and no significant net Cl⁻ flux under symmetrical conditions in all groups (Fig. 3A). Relative Cl⁻ efflux, influx, and net flux rates exhibited a very similar pattern to that previously described for Na⁺, as did absolute flux

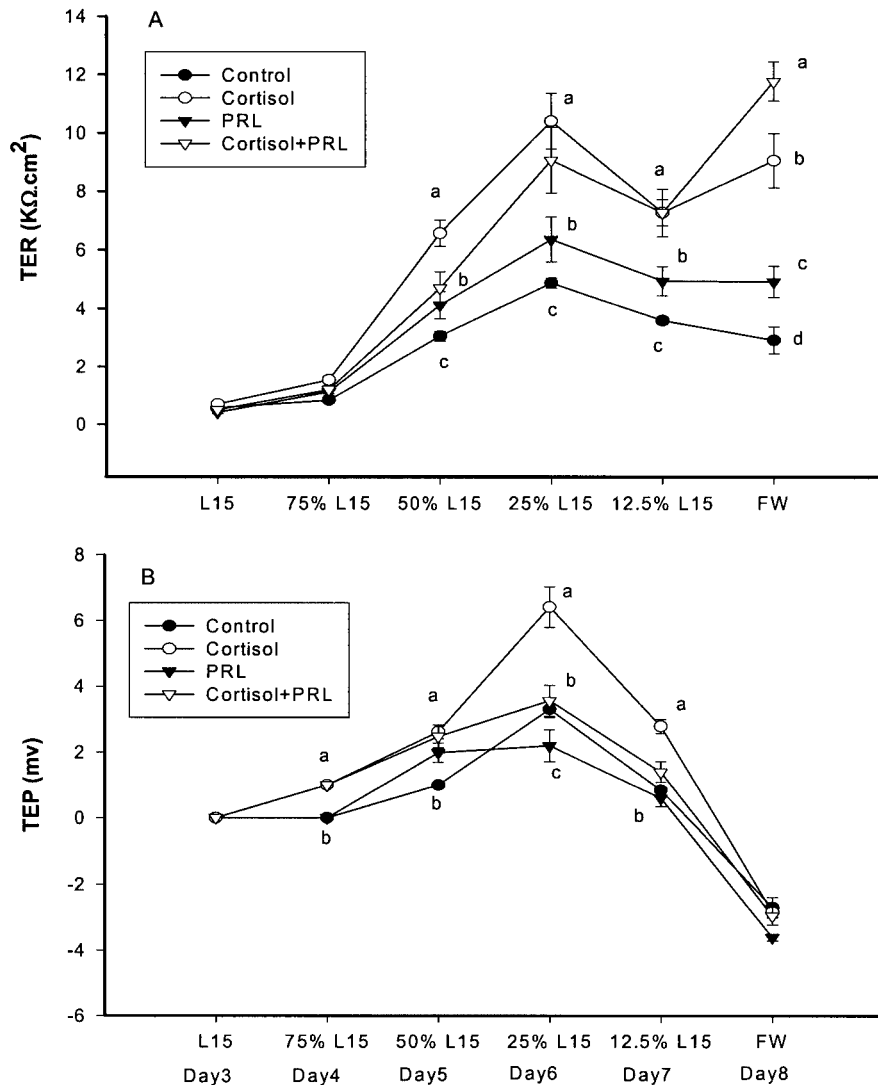


Fig. 1. Changes in (A) transepithelial resistance (TER) and (B) transepithelial potential (TEP) of cultured gill epithelia from freshwater (FW) rainbow trout of Series 2. Apical media were gradually diluted from 100% L15 to FW starting on Day 3 after seeding. Preparations were subjected to four different basolateral hormonal treatments, all in 100% L15 throughout, as shown (see text for details). Data are expressed as means \pm 1 SEM ($N=10$ matched pairs of epithelia/group). Different letters represent significant differences among treatment groups ($P<0.05$).

rates (Fig. 3B–F). Cortisol also limited the Cl^- flux rates (particularly in the outward direction, 75% L15/L15 down to FW/L15; Fig. 3B–F) as did cortisol + prolactin in the later stages of apical dilution (25% L15/L15 down to FW; Fig. 3D–F). However, prolactin alone exhibited no ability to limit ion efflux. In apical FW, a significant reduction of influx rates was observed under all hormonal treatments (Fig. 3F).

As for Na^+ , there was no difference between the observed and predicted (Ussing) flux ratios for Cl^- under symmetrical and 75% L15 conditions (Table

3). Moreover, application of the flux ratio criterion under apical 50% L15 conditions indicated an active extrusion of Cl^- in the control epithelia, whereas movement of Cl^- was passive in hormone-treated preparations (Table 3). At 25% and 12.5% L15/L15, active extrusion of Cl^- was indicated in control and cortisol-treated epithelia, whereas Cl^- movement appeared to be passive in prolactin and cortisol + prolactin treatment groups (Table 3). However, after the apical side was replaced by FW, the observed ratio was significantly greater than the predicted ratio in

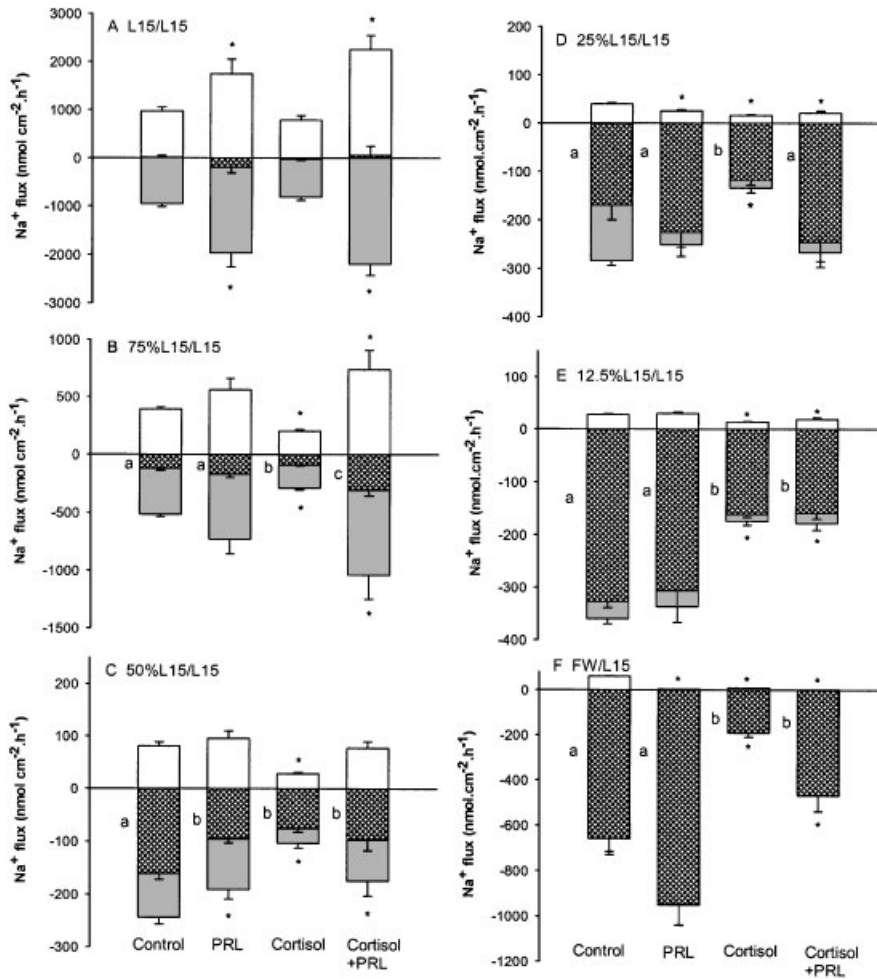


Fig. 2. Unidirectional Na⁺ flux rates across the cultured gill epithelia of Series 2 subjected to four different basolateral hormone treatments, as shown, and progressive apical dilution, 100% L15 to freshwater (FW), starting Day 3 after seeding: (A) 100% L15; (B) 75% L15; (C) 50% L15; (D) 25% L15; (E) 12.5% L15; and (F) FW. Open, gray, and hatched bars represent ion influx (apical-to-basolateral, positive), efflux (basolateral-to-apical, negative), and net flux rates, respectively. Data are expressed as means \pm 1 SEM ($N=10$ matched pairs of epithelia/group). An asterisk denotes a significant difference ($P<0.05$) between control and hormone-treated epithelia for either influx or efflux. Different letters represent significant differences among treatment groups for net flux rate ($P<0.05$).

all groups, suggesting an active uptake of Cl⁻ under these conditions.

Series 2: Na⁺, K⁺-ATPase activity

Na⁺, K⁺-ATPase activities were measured only in the epithelia of Series 2, which were harvested after the final 24-hr step of FW/L15. Significantly greater Na⁺, K⁺-ATPase activities were observed in the cortisol and cortisol + prolactin treatments compared to those in control and prolactin treatments alone (Fig. 4). There were no significant differences between cortisol and cortisol + prolactin-treated epithelia, or between control and prolactin-treated epithelia.

DISCUSSION

Overview

The current studies have demonstrated for the first time that cultured gill epithelia can tolerate gradual apical media dilution starting from just the first few days of epithelial development (i.e., at the start of the upswing on the normal sigmoidal TER curve). During the course of apical media dilution, cultured gill epithelia exhibit a normal pattern of development as temporal changes in TER were typically sigmoidal (consistent with those observed under conditions of symmetrical development; for review see Wood et al., 2002).

TABLE 3. Comparison of the observed flux ratios (influx/efflux) versus the predicted flux ratios for Na^+ and Cl^- across cultured epithelia of Series 2 where basolateral media (100% L15) were supplemented with either cortisol (500 ng ml^{-1}), prolactin (50 ng ml^{-1}), or cortisol+prolactin (500 ng ml^{-1} cortisol, 50 ng ml^{-1} prolactin) throughout¹

| Flux ratio | Hormone treatment | | | | | | | |
|---------------------|-------------------|---------------|------------------|---------------|-----------------|---------------|---------------------------|---------------|
| | Control (N=10) | | Prolactin (N=10) | | Cortisol (N=10) | | Cortisol+Prolactin (N=10) | |
| | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed |
| L15/L15 | | | | | | | | |
| Na^+ | 1.02 ± 0.01 | 0.98 ± 0.07 | 0.99 ± 0.01 | 0.90 ± 0.06 | 1.00 ± 0.01 | 0.95 ± 0.04 | 0.98 ± 0.01 | 1.07 ± 0.11 |
| Cl^- | 1.00 ± 0.01 | 1.04 ± 0.05 | 0.99 ± 0.01 | 0.90 ± 0.05 | 0.99 ± 0.01 | 1.01 ± 0.04 | 1.00 ± 0.01 | 1.12 ± 0.12 |
| 75% L15/L15 | | | | | | | | |
| Na^+ | 0.74 ± 0.01 | 0.76 ± 0.03 | 0.75 ± 0.01 | 0.76 ± 0.04 | 0.71 ± 0.01 | 0.69 ± 0.03 | 0.73 ± 0.01 | 0.69 ± 0.04 |
| Cl^- | 0.87 ± 0.01 | 0.86 ± 0.03 | 0.88 ± 0.01 | 0.85 ± 0.03 | 0.91 ± 0.01 | 0.85 ± 0.04 | 0.87 ± 0.01 | 0.82 ± 0.06 |
| 50% L15/L15 | | | | | | | | |
| Na^+ | 0.46 ± 0.01 | 0.34 ± 0.02* | 0.50 ± 0.01 | 0.49 ± 0.04 | 0.44 ± 0.01 | 0.29 ± 0.02 | 0.49 ± 0.01 | 0.47 ± 0.06 |
| Cl^- | 0.61 ± 0.01 | 0.53 ± 0.03* | 0.57 ± 0.07 | 0.54 ± 0.04 | 0.59 ± 0.01 | 0.57 ± 0.03 | 0.58 ± 0.01 | 0.54 ± 0.04 |
| 25% L15/L15 | | | | | | | | |
| Na^+ | 0.23 ± 0.01 | 0.14 ± 0.01* | 0.32 ± 0.03 | 0.12 ± 0.02 | 0.21 ± 0.01 | 0.12 ± 0.01 | 0.30 ± 0.01 | 0.13 ± 0.04 |
| Cl^- | 0.16 ± 0.01 | 0.12 ± 0.01* | 0.14 ± 0.01 | 0.12 ± 0.02 | 0.18 ± 0.01 | 0.12 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.02 |
| 12.5% L15/L15 | | | | | | | | |
| Na^+ | 0.16 ± 0.01 | 0.08 ± 0.01* | 0.16 ± 0.01 | 0.09 ± 0.01* | 0.14 ± 0.01 | 0.08 ± 0.01* | 0.16 ± 0.01 | 0.11 ± 0.01* |
| Cl^- | 0.10 ± 0.01 | 0.08 ± 0.01* | 0.09 ± 0.01 | 0.10 ± 0.01 | 0.11 ± 0.01 | 0.09 ± 0.01* | 0.09 ± 0.01 | 0.09 ± 0.01 |
| FW/L15 [†] | | | | | | | | |
| Na^+ | 45.00 ± 0.76 | 15.00 ± 1.30* | 15.11 ± 0.62 | 3.01 ± 0.21* | 35.00 ± 0.29 | 25.00 ± 2.70* | 14.40 ± 0.84 | 3.03 ± 0.14* |
| Cl^- | 45.80 ± 1.14 | 75.22 ± 9.12* | 11.90 ± 0.68 | 15.30 ± 1.25* | 39.70 ± 0.53 | 64.51 ± 5.3* | 8.10 ± 0.70 | 14.20 ± 1.32* |

¹Control preparations had no hormone supplement added. All data are expressed as mean values ± 1 SEM. An asterisk denotes a significant difference ($P < 0.05$) between observed and predicted flux ratios within a defined culture condition. A dagger indicates Ussing flux ratio $\times 10^{-3}$. Observed flux ratios higher than predicted indicate active apical-to-basolateral uptake. Observed flux ratios lower than predicted indicate active basolateral-to-apical extrusion.

In addition, a reduction in unidirectional Na^+ and Cl^- efflux rates occurs over time and TEP values switch over from positive to negative when FW is introduced to the apical compartment of the preparations. These observations illustrate the remarkable barrier properties of the FW gill epithelium: we are aware of no other cultured epithelium that has been reported to withstand such a treatment. We have previously reported that the cultured gill epithelium can tolerate gradual apical media dilution to FW after reaching plateau phase TER development (Kelly and Wood, 2003; Zhou et al., 2003). However, the high TER exhibited by DSI epithelia after 6–9 days in symmetrical culture (e.g., plateau phase TER of up to $35 \text{ k}\Omega \text{ cm}^2$) gives these preparations a robust (and stable) platform from which to acclimate to a dilution protocol. Therefore, under such conditions, media dilution may have less of an impact on epithelial function and condition than would be

the case during development when there may be some degree of “developmental plasticity.” By introducing a protocol of gradual apical media dilution almost immediately after the first indication of dispersed gill cell re-formation into a confluent layer, we reasoned that (1) the pattern of regulated ion movement across these preparations would benefit from exposure to gradual apical media dilution during development (i.e., unidirectional ion flux rates may more closely resemble those that occur in vivo) and (2) hormone supplements such as cortisol and (or) prolactin may improve the development of epithelia under such experimental conditions.

Notably, the former did not occur and in this regard we can reject our hypothesis that the pattern of regulated ion movement across these cultured preparations would benefit from the dilution protocols tested in the current studies. However, the ionoregulatory actions of cortisol, or

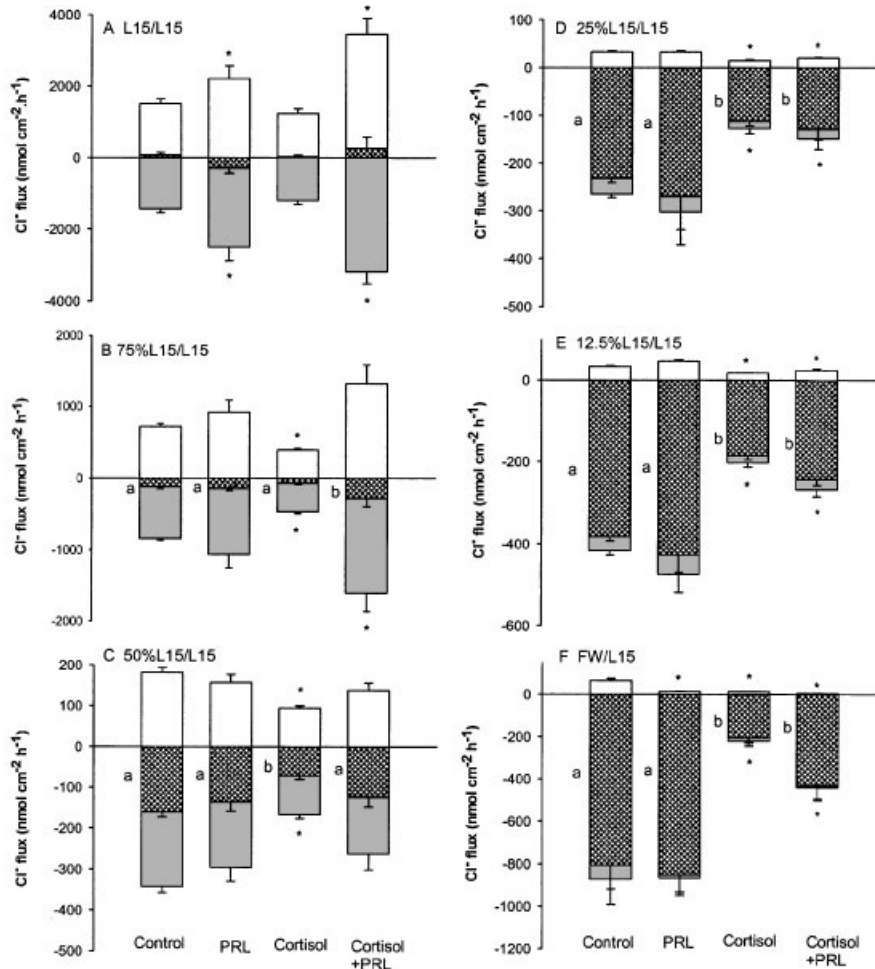


Fig. 3. Unidirectional Cl^- flux rates across the cultured gill epithelia of Series 2 subjected to four different basolateral hormone treatments, as shown, and progressive apical dilution, 100% L15 to freshwater (FW), starting Day 3 after seeding: (A) 100% L15; (B) 75% L15; (C) 50% L15; (D) 25% L15; (E) 12.5% L15; and (F) FW. Open, gray, and hatched bars represent ion influx (apical-to-basolateral, positive), efflux (basolateral-to-apical, negative), and net flux rates, respectively. Data are expressed as means \pm 1 SEM ($N=10$ matched pairs of epithelia/group). An asterisk denotes a significant difference ($P < 0.05$) between control and hormone-treated epithelia for either influx or efflux. Different letters represent significant differences among treatment groups for net flux rate ($P < 0.05$).

cortisol in combination with prolactin, certainly did benefit epithelial development during such a protocol. In particular, we found that cultured preparations were capable of tolerating apical media dilution during development without hormone support, but that treatment with these hormones (cortisol in particular) results in a substantial increase in epithelial tightness and reduction in ion efflux rates (analogous to ion loss in vivo), especially during the later stages of media dilution. This is consistent with a role for both of these hormones in mediating the development of hyperosmoregulatory mechanisms in vivo. In addition, we were also able to observe higher Na^+ , K^+ -ATPase activity in cortisol-treated pre-

parations. Previously, cortisol treatment has not increased the activity of this important ionomotive enzyme in pavement cell epithelia (Kelly and Wood, 2001a, 2002a) or DSI preparations (Zhou et al., 2003). This is in contrast to a large number of reports which have demonstrated that cortisol treatment increases the activity of this enzyme both in vivo and in vitro (for review see McCormick, '95). Hence it would appear that exposure to dilute media at an early stage of epithelial development has facilitated this response. We can therefore accept our hypothesis that the development of cultured gill epithelia under the dilution protocol used in the current study benefited from hormone supplementation.

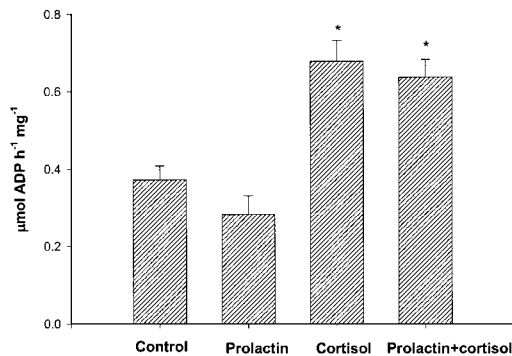


Fig. 4. Na⁺, K⁺-ATPase activities in cultured gill epithelia measured after gradual dilution from L15 to freshwater (FW) in Series 2 (dilution protocol starting on Day 3 after seeding). Epithelia were exposed to basolateral treatments of control (no hormones), cortisol (500 ng ml⁻¹), prolactin (50 ng ml⁻¹), and cortisol + prolactin (500 ng ml⁻¹ cortisol, 50 ng ml⁻¹ prolactin), all in 100% L15 throughout. Data are expressed as means ± 1 SEM (N=8). Asterisk denotes a significant difference ($P < 0.05$) between control and hormone-treated epithelia.

Changes in TER following media dilution and hormone treatment

TER measurement reflects the general “tightness” of cultured gill epithelia, and is a function of both transcellular and paracellular permeability. In the protocols of the present study, the changes in TER therefore likely reflected several events, based on previous studies (reviewed by Wood et al., 2002). In both series, TER was increasing over time (Table 1; Fig. 1A) for two probable reasons: (1) the progressive decrease in paracellular conductance caused by tightening of cell junctions as the epithelium matured, an effect expected to reach a plateau by Day 6–9, and (2) a decrease in transcellular conductance associated with each step of apical dilution. In opposition to these there would be a tendency for TER to decay slightly within each 24-hr dilution step due to some loosening of the paracellular pathway. Therefore, a TER of over 25 kΩ cm² as observed after 4 days of culture under 50% L15/L15 conditions in Series 1, indicates that TER can reach plateau phase even under asymmetrical conditions and in Series 2, physiological events appeared to allow more or less stable TER from apical 25% L15 through to FW (i.e., Days 6–8; Fig. 1A).

In earlier studies with SSI epithelia, cortisol was highly effective in increasing TER under both symmetrical and asymmetrical conditions. This effect was attributed to the tightening of the paracellular pathway (Kelly and Wood, 2001a,

2002a). In contrast, the effects of prolactin were only evident under asymmetrical conditions, acting to decrease the permeability of the transcellular pathway (Kelly and Wood, 2002b). Clearly, similar effects are seen in DSI preparations subjected to progressive apical dilution starting in the first few days of epithelial development (Series 2; Fig. 1A). The combination of cortisol + prolactin offered the best support of TER, closely followed by cortisol alone, with prolactin alone also having a small but significant positive effect (Fig. 1A). Thus the effects of the two hormones were additive, which is expected for two agents acting on different pathways. Previously, this additive effect was not observed when mature epithelia were subjected to gradual apical media dilution (Zhou et al., 2003), likely reflecting the lack of “developmental plasticity” in mature epithelia. Therefore the current protocols provided insight into the combined actions of two important ionoregulatory hormones, consistent with their actions in vivo. Of significance, however, is that cortisol, acting alone, has a marked tightening effect on the paracellular pathway (see also Kelly and Wood, 2001a, b, 2002a) and that this is likely to be an important mechanism resulting in reduced passive ion loss in vivo. This effect has been largely overlooked in vivo where research has focused on the role of cortisol in stimulating enzyme activities (particularly Na⁺, K⁺-ATPase), chloride cell presence, and active Na⁺ and Cl⁻ uptake (e.g., Laurent and Perry, '90; Perry et al., '92; McCormick, '95). In addition, while the FW-adapting role of prolactin has been known for some time and is widely accepted (e.g., Pickford and Phillips, '59; Hirano, '86), these studies suggest that there may be much more to learn about its interactions with other ionoregulatory hormones.

Changes in TEP following media dilution and hormone treatment

In general, greater absolute TEP values in cultured gill epithelia are associated with greater TER values, in accordance with Ohm's law (for review see Wood et al., 2002). Under symmetrical culture conditions, mature DSI epithelia exhibit a positive TEP in the region of +1 to +10 mV and upon exposure to apical FW exhibit a negative TEP averaging about -11 mV (Fletcher et al., 2000). Similar trends were seen in the current study. Greater absolute potentials developed in the high TER epithelia of Series 1 under 50%

L15/L15 culture conditions (Table 1) and all treatments exhibited a negative TEP when FW was present on the apical side of the preparations (Table 1; Fig. 1B).

The biphasic response in Series 2 (Fig. 1B) can be attributed to the interactive effects of increasing background TER and the influence of apical ion content. Notably, the changeover to negative TEP occurred only below an apical dilution of 12.5% L15. Positive potentials may be due to electrogenic Cl^- extrusion, as in the cultured PVC epithelium of the sea bass (Avella and Ehrenfeld, '97). Negative potentials in dilute apical media have been interpreted as mainly diffusion potentials due to the differential permeability of the epithelia to Na^+ versus Cl^- , with a very small negative electrogenic component superimposed on the latter (Wood and Pärt, '97; Wood et al., '98; Fletcher et al., 2000). These interpretations follow the generally accepted explanations for trans-gill potentials measured in fish in vivo in saline waters versus FW (Potts, '84).

One particular feature of the TEP data was the lack of hormonal influence when FW was present on the apical side in Series 2 (Fig. 1B). This differs from previous observations on SSI epithelia where cortisol treatment resulted in a more negative TEP with apical FW (Kelly and Wood, 2001a, 2002a) but agrees with previous observations that prolactin alone has no effect on the TEP of either SSI or DSI epithelia under asymmetrical conditions (Kelly and Wood, 2002b). Presumably, the hormonal treatments did not cause any changes in the differential permeability of DSI epithelia exposed to apical FW, or else the effects were outweighed by corresponding alterations in the small electrogenic component.

Unidirectional Na^+ and Cl^- flux rates following dilution and hormone treatment

In Series 2 data we can again see the dominant influence of epithelial tightness on ion movement (unidirectional Na^+ and Cl^- flux rates) in the later stages of epithelial development under more dilute apical culture conditions (Fig. 2D–F and Fig. 3D–F). Cortisol treatment reduced unidirectional and net fluxes of both ions, actions seen across the full range of apical dilution and most likely attributable to the stabilizing action of this hormone on paracellular permeability. These effects have previously been observed to occur in pavement cell epithelia of both trout and tilapia (Kelly and Wood, 2001a, 2002a), further emphasizing a key

role for cortisol in the processes of hyperosmotic regulation. The actions of prolactin were less obvious in terms of ion transport, where Na^+ and Cl^- movement across prolactin-treated epithelia tended to closely resemble ion movements measured across control preparations. This occurred despite a slight, but significant, effect of prolactin on TER. Most surprising was the action of the two hormones in combination: unidirectional ion transport rates were initially elevated and were only reduced in the later stages of dilution (e.g., Fig. 2D–F and Fig. 3D–F). This suggests that the actions of these hormones in combination may vary depending on the “external” environment.

The Ussing flux ratio analyses (Tables 1 and 3) did not indicate any marked advantage of diluting apical media or adding supplemental hormones during epithelial development in achieving the simultaneous active uptake of both Na^+ and Cl^- from dilute apical solutions. This is in contrast to our observations of simultaneous active Na^+ and Cl^- transport in the inward direction across DSI epithelia when FW is introduced acutely on the apical side (after symmetrical maturation) and the basolateral medium is supplemented with cortisol or cortisol + prolactin (Zhou et al., 2003). Both series in the current studies exhibited a pattern of active Cl^- uptake and Na^+ extrusion resembling that reported earlier in epithelia exposed acutely or gradually to apical FW after symmetrical development in the absence of hormone support (Wood et al., 2002; Kelly and Wood, 2003; Zhou et al., 2003).

Na^+ , K^+ -ATPase activity

In the epithelia of Series 2, Na^+ , K^+ -ATPase activity was significantly elevated in cortisol-treated groups, regardless of whether prolactin was present or not. Prolactin alone had no significant effect, indicating that the action was solely attributable to cortisol (Fig. 4). These results are consistent with observations in vivo that treatment with cortisol increases the activity of this enzyme in the gills of many fish species (for review, see McCormick, '95) whereas prolactin has either a negligible influence or an inhibitory effect (reviewed by Evans, 2002; Manzon, 2002). In previous studies with cultured gill epithelia (Kelly and Wood, 2001a, 2002a), cortisol did not stimulate Na^+ , K^+ -ATPase activity in either trout or tilapia SSI preparations (i.e., PVCs only present). Similarly, cortisol or cortisol and prolactin

treatment did not stimulate Na^+ , K^+ -ATPase activity after various experimental manipulations in mature DSI epithelia (Zhou et al., 2003). Therefore, cortisol appeared to promote Na^+ , K^+ -ATPase activity in cultured DSI epithelia only when the epithelia are treated to apical media dilution during the course of development, namely, when “developmental plasticity” may be greatest.

In summary, developing cultured trout DSI can maintain epithelial integrity during progressive apical media dilution to FW. Cortisol treatment is beneficial to this tolerance of dilution, tightening the epithelia and limiting ion losses. Prolactin also appears to offer some support, most clearly seen in the electrical characteristics of epithelia, where its actions on TER are additive to those of cortisol. We found no direct evidence that prolactin limits ion movement during gradual dilution in developing epithelia. However, the robust nature of developing cultured gill epithelia and the insights gained from hormone treatment using the present experimental protocol would suggest that developing epithelia may be suited to revealing the long-term effects of relevant endocrine factors under conditions of environmental change.

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LITERATURE CITED

- Avella M, Ehrenfeld J. 1997. Fish gill respiratory cells in culture: a model for Cl^- -secreting epithelia. *J Membr Biol* 156:87–97.
- Burgess DW, Marshall WS, Wood CM. 1998. Ionic transport by the opercular epithelia of freshwater acclimated tilapia (*Oreochromis niloticus*) and killifish (*Fundulus heteroclitus*). *Comp Biochem Physiol* 121A:155–164.
- Evans DH. 2002. Cell signaling and ion transport in the fish gill epithelium. *J Exp Zool* 293:336–347.
- Fletcher M, Kelly SP, Pärt P, O'Donnell MJ, Wood CM. 2000. Transport properties of cultured branchial epithelia from freshwater rainbow trout: a novel preparation with mitochondria-rich cells. *J Exp Biol* 203:1523–1537.
- Foskett JK, Logsdon DC, Turner T, Machen TE, Bern HE. 1981. Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. *J Exp Biol* 93:209–224.
- Hirano T. 1986. The spectrum of prolactin actions in teleosts. In: Ralph CL, editor. *Comparative endocrinology: developments and directions*. New York: Alan R. Liss. p 53–74.
- Kelly SP, Chow INK, Woo NYS. 1999. Effects of prolactin and growth hormone on strategies of hypoosmotic adaptation in a marine teleost, *Sparus sarba*. *Gen Comp Endocrinol* 113:9–22.
- Kelly SP, Fletcher M, Pärt P, Wood CM. 2000. Procedures for the preparation and culture of “reconstructed” rainbow trout branchial epithelia. *Methods Cell Sci* 22:153–163.
- Kelly SP, Wood CM. 2001a. Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. *Am J Physiol Regul* 281:R811–R820.
- Kelly SP, Wood CM. 2001b. The physiological effects of 3,5',3'-triiodo-L-thyronine alone or combined with cortisol on cultured pavement cell epithelia from freshwater rainbow trout gills. *Gen Comp Endocrinol* 123:280–294.
- Kelly SP, Wood CM. 2002a. Cultured gill epithelia from freshwater tilapia (*Oreochromis niloticus*): effect of cortisol and homologous serum supplements from stressed and unstressed fish. *J Membr Biol* 190:1–14.
- Kelly SP, Wood CM. 2002b. Prolactin effects on cultured pavement cell epithelia and pavement cell plus mitochondria-rich cell epithelia from freshwater rainbow trout gills. *Gen Comp Endocrinol* 128:44–56.
- Kelly SP, Wood CM. 2003. Dilute culture media as an environmental or physiological simulant in cultured gill epithelia from freshwater rainbow trout. *In Vitro Cell Dev Biol Animal* 39:21–28.
- Kirschner LB. 1970. The study of NaCl transport in aquatic animals. *Am Zool* 10:365–376.
- Laurent P, Perry SF. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell Tissue Res* 259:429–442.
- Mancera JM, Carrión RL, Martín del Río MDP. 2002. Osmoregulatory action of PRL, GH, and cortisol in the gilthead sea bream (*Sparus aurata* L.). *Gen Comp Endocrinol* 129:95–103.
- Manzon LA. 2002. The role of prolactin in fish osmoregulation: a review. *Gen Comp Endocrinol* 125:291–310.
- Marshall WS. 1995. Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. In: Wood CM, Shuttleworth TJ, editors. *Cellular and molecular approach to fish ionic regulation*. Vol 14. Fish physiology. San Diego: Academic Press. p 1–23.
- Marshall WS, Bryson SE, Wood CM. 1992. Calcium transport by isolated skin of rainbow trout. *J Exp Biol* 166:297–316.
- Marshall WS, Bryson SE, Darling P, Whitten C, Patrick M, Wilkie M, Wood CM. 1997. NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. *J Exp Zool* 277:23–37.
- McCormick SD. 1995. Hormonal control of gill Na^+ - K^+ -ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ, editors. *Cellular and molecular approach to fish ionic regulation*. Vol 14. Fish physiology. San Diego: Academic Press. p 285–315.
- McCormick SD. 2002. Endocrine control of osmoregulation in teleost fish. *Am Zool* 41:781–794.
- Perry SF, Goss GG, Laurent P. 1992. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. *Can J Zool* 70:1775–1786.
- Pickford GE, Phillips JG. 1959. Prolactin, a factor in promoting survival of hypophysectomized killifish in freshwater. *Science* 130:454.
- Potts WTW. 1984. Transepithelial potentials in fish gills. In: Hoar WS, Randall DJ, editors. *Fish physiology*. Vol. 10.

- Gills, Part B, Ion and water transfer. Orlando, FL: Academic Press. p 105–128.
- Seidelin M, Madsen SS. 1999. Endocrine control of Na⁺, K⁺-ATPase and chloride cell development in brown trout (*Salmo trutta*): interaction of insulin-like growth factor-I with prolactin and growth hormone. *J Endocrinol* 162: 127–135.
- Wilson JM, Laurent P. 2002. Fish gill morphology: inside out. *J Exp Zool* 293:192–213.
- Wood CM, Marshall WS. 1994. Ion balance, acid–base regulation and chloride cell function in the common killifish *Fundulus heteroclitus*—a euryhaline estuarine teleost. *Estuaries* 17:34–52.
- Wood CM, Gilmour KM, Pärt P. 1998. Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol* 119A:87–96.
- Wood CM, Pärt P. 1997. Cultured branchial epithelia from freshwater fish gills. *J Exp Biol* 200:1047–1059.
- Wood CM, Kelly SP, Zhou BS, Fletcher M, O'Donnell M, Eletti B, Pärt P. 2002. Cultured gill epithelia as models for the freshwater fish gill. *Biochim Biophys Acta* 1566:72–83.
- Zadunaisky JA. 1984. The chloride cell: the active transport of chloride and paracellular pathways. In: Hoar WS, Randall DJ, editors. *Fish physiology*. Vol 10. Gills, Part B, Ion and water transfer. Orlando, FL: Academic Press. p 129–176.
- Zhou BS, Kelly SP, Ianowski JP, Wood CM. 2003. Effects of cortisol and prolactin on Na⁺ and Cl[−] transport in cultured branchial epithelia from freshwater rainbow trout. *Am J Physiol Regul* 285:R1305–R1316.