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Cortisol stimulates calcium transport across cultured gill epithelia from freshwater rainbow trout

Scott P. Kelly · Chris M. Wood

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Abstract
Summary The effect of cortisol on calcium (Ca^{2+}) transport across cultured rainbow trout gill epithelia composed of both pavement cells (PVCs) and mitochondria-rich cells (MRCs) was examined. Under symmetrical culture conditions (L15 media apical/L15 media basolateral), cortisol had subtle effects on gill epithelial preparations. Both control and cortisol treated epithelia exhibited Ca^{2+} influx and efflux rates (measured radioisotopically using ^{45}Ca) that were approximately balanced, with a slight inwardly directed net Ca^{2+} flux. Ussing flux ratio analysis indicated active Ca^{2+} transport in the inward direction across epithelia bathed symmetrically regardless of hormone treatment. In contrast, under asymmetrical conditions (freshwater apical/L15 media basolateral) control epithelia exhibited active Ca^{2+} transport in the outward direction (basolateral to apical) throughout experiments conducted over a 24-h period, whereas cortisol-treated preparations exhibited active transport in the inward direction (apical to basolateral) during the early stages of an asymmetrical culture period (e.g., *T*0–6 h) and passive transport during the later stages (e.g., *T*18–24 h). When soft freshwater (with tenfold lower [Ca^{2+}]) was used for asymmetrical culture instead of freshwater, control epithelia developed outwardly directed active Ca^{2+} transport properties, whereas cortisol-treated preparations did not. The results of this study support a hypercalcemic role for cortisol in rainbow trout and demonstrate that treating cultured gill epithelia composed of both PVCs and MRCs with cortisol can stimulate active Ca^{2+} uptake under circumstances that more closely resemble natural conditions for fish gills (i.e., freshwater bathing the apical side of the epithelium).

Keywords Cortisol · Mitochondria-rich cells · Pavement cells · Ion transport

S. P. Kelly (✉)
Department of Biology, Farquharson Life Science Building,
York University,
4700 Keele Street,
Toronto, ON, Canada M3J 1P3
e-mail: spk@yorku.ca

C. M. Wood
Department of Biology, McMaster University,
Hamilton, ON, Canada L8S 4K1

Introduction

Calcium (Ca^{2+}) homeostasis in freshwater (FW) teleost fishes occurs, in part, through Ca^{2+} acquisition from the surrounding environment. Of particular importance is transbranchial Ca^{2+} movement, which is a well-studied phenomenon believed to take place primarily across the mitochondria-rich cells (MRCs) of fish gills (for reviews, see Perry 1997; Marshall 2002; Evans et al. 2005). Ca^{2+} influx rates have been demonstrated to alter as a result of various environmental perturbations, with a particularly effective stimulus being acclimatization of fishes to Ca^{2+} poor water (e.g., Perry and Wood 1985; McCormick et al. 1992). Diminishing the environmental Ca^{2+} pool increases MRC abundance and apical exposure (Perry and Wood 1985; McCormick et al. 1992), a response that seems to be at least partly mediated by elevated circulating cortisol levels (Perry and Wood 1985). Since these early observations, the hypercalcemic properties of cortisol have been

variously exploited to examine, among other things, intraspecific relationships between Ca^{2+} uptake and MRC exposure at the surface of the gill as well as the regulation of epithelial proteins believed to be involved in Ca^{2+} movement across the apical membrane of the gill (Perry et al. 1992; Shahsavarani and Perry 2006). To the best of our knowledge, the direct effects of cortisol on Ca^{2+} transport across gill epithelia have yet to be explored.

Recently developed techniques for the preparation and culture of FW fish gill epithelia on permeable filter supports have provided new opportunities for the examination of gill epithelia properties *in vitro* (Wood and Pärt 1997; Fletcher et al. 2000; Kelly and Wood 2002a). Cultured freshwater gill preparations can be "reconstructed" either as pavement cell (PVC) epithelia (Wood and Pärt 1997; Kelly and Wood 2002a) or as epithelia containing both PVCs and MRCs (Fletcher et al. 2000). The latter of these preparations, thus far developed only for rainbow trout gills, has been demonstrated capable of actively transporting Ca^{2+} in the correct vectorial direction (i.e., apical to basolateral uptake) under symmetrical culture conditions (L15 media bathing both the apical and basolateral side of the epithelium). Trout gill preparations containing PVCs only do not exhibit the same properties (Fletcher et al. 2000). It would, therefore, appear that the presence of MRCs in cultured gill epithelia confers Ca^{2+} transporting properties, and this observation is consistent with current models of Ca^{2+} movement across the FW gill (Perry 1997; Marshall 2002; Evans et al. 2005). However, cultured preparations do not actively transport Ca^{2+} in the inward direction when epithelia are exposed to more "natural" conditions—i.e., bathed on the apical side with FW (Fletcher et al. 2000). We have recently reported the sensitivity of trout epithelia to various hormone treatments (Kelly and Wood 2001a, b; 2002a, b; Zhou et al. 2003, 2004), including cortisol, which has marked and dose-dependent effects on the electrophysiological properties and ion movement across cultured preparations (Kelly and Wood 2001a, 2002a). The aforementioned studies not only offer us insight into the role of these various signaling factors on gill epithelial characteristics, but also allow us to augment the properties of cultured gill preparations by stimulating ion transport and enhancing epithelia integrity under both symmetrical and, importantly, asymmetrical culture conditions (for review, *see* Wood et al. 2002).

Therefore, the objectives of these studies were to examine the Ca^{2+}-transporting properties of cultured gill epithelia composed of both PVCs and MRCs in the presence of cortisol, a hypercalcemic hormone, under several culture conditions as follows: (1) symmetrical conditions (L15 apical/L15 basolateral); (2) regular asymmetrical (FW apical/L15 basolateral); and (3) soft freshwater (SFW) asymmetrical (SFW apical/L15 basolateral).

Materials and Methods

Preparation of cultured rainbow trout gill epithelia. Cultured gill epithelia were prepared using stock rainbow trout held in flow-through Hamilton dechlorinated tap water (approximate composition in mM: $[Na^+]$=0.55, $[Cl^-]$=0.70, $[Ca^{2+}]$=0.90, $[Mg^{2+}]$=0.15, $[K^+]$=0.05, pH range 7.8–8.0) at ~12° C. Fish size ranged from 200 to 250 g. The epithelia prepared and used in these studies were of the variety originally developed and described by Fletcher et al. (2000). These preparations are composed of both PVCs and MRCs. Full details for the preparation of gill epithelia have been outlined in Kelly et al. (2000). Falcon cell culture inserts, incorporating track-etched polyethylene terephthalate membranes with a pore size of 0.4 μm and a pore density of 1.6×10^6 pores/cm^2, of 0.9 cm^2 surface area were employed. Cortisol treatment of cultured gill epithelia has previously been outlined by Kelly and Wood (2001a). A single concentration of 500 ng/ml cortisol (hydrocortisone hemisuccinate, Sigma, on the basolateral side only) was used for all experiments. This dose was selected based on previous observations of the response of cultured trout epithelia treated with cortisol (see Kelly and Wood, 2001a; Zhou et al. 2003). Epithelia were cultured under symmetrical culture conditions (i.e., Leibovitz's L-15 supplemented with 2 mmol l^{-1} glutamine and 6% fetal bovine serum bathing both apical and basolateral surfaces of the preparation) for ~ 6 d before experimental manipulation, and experiments were conducted under symmetrical (L15 apical/L15 basolateral) and asymmetrical (sterile FW apical/L15 basolateral) conditions. Cortisol was present in culture media from the beginning of the 6-d culture period and throughout all experiments. Total $[Ca^{2+}]$ in supplemented L15 media was 1.37±0.02 mM (n=80). In addition to "regular" asymmetrical conditions with FW bathing the apical side of epithelia, a second set of asymmetrical experiments were run using soft FW (SFW) so that the conditions were SFW apical/L15 basolateral. Sterile SFW was prepared by mixing regular FW (composition as above) with reverse osmosis water in a 95:5 ratio (i.e., 95% reverse osmosis water and 5% regular freshwater). The resulting $[Ca^{2+}]$ was ~89 μM.

Electrophysiological measurements of cultured rainbow trout gill epithelia. A custom modified voltohmmeter (World Precision Instruments, Sarasota, FL) fitted with chopstick electrodes (STX-2) were used to measure transepithelial resistance (TER) across trout epithelia. All reported TER measurements were background-corrected for the resistance of the cell culture insert and appropriate solutions alone, following which TER was expressed as a function of epithelial (insert cup) surface area (cm^2). To monitor the development of cultured epithelia, TER

measurements are recorded daily from 24 h after the last seeding of cells in to the insert cups. Unidirectional Ca^{2+} flux experiments were initiated to coincide with a plateau of TER that regularly occurs 6–8 d after a final seeding of cells (for details, *see* Kelly et al. 2000). Transepithelial potential (TEP) measurements across cultured trout epithelia were performed using agar-salt (3 M KCl in 4% agar) bridges connected to a high-impedance electrometer (Radiometer pHM 84, Copenhagen, Denmark) through Ag/AgCl electrodes (World Precision Instruments). TEP measurements were expressed relative to the apical side as 0 mV after correction for junction potential.

Unidirectional Ca^{2+} flux measurements across cultured rainbow trout gill epithelia. Unidirectional flux measurements using a ^{45}Ca radiotracer (NEN-PerkinElmer—Waltham, MA) were conducted according to methods previously outlined for unidirectional Na^+ and Cl^- flux measurements by Wood et al. (1998). Measurements worked on the principle of radioisotope appearance from a "hot" to a "cold" side of the cultured epithelial preparation. For influx (apical to basolateral isotope movement: positive sign) and efflux (basolateral to apical isotope movement: negative sign) studies, 0.5–1.0 μCi of ^{45}Ca was added to a solution designated the "hot" side. As with previous studies, epithelial preparations used for unidirectional flux measurements were employed for influx or efflux study only and matched for calculations of the Ussing flux ratio (*see* next section) based on electrophysiological similarity. Under symmetrical culture conditions (L15 apical/L15 basolateral) TER and TEP measurements were recorded at the beginning and at the end of the flux period. Under asymmetrical conditions, when FW or SFW was bathing the apical side of epithelia, TER and TEP measurements were recorded at the beginning (0 h), middle (3 h), and end (6 h) of each flux period. Unidirectional Ca^{2+} flux rates were examined under three experimental conditions as follows: (1) control *versus* cortisol-treated epithelia, symmetrical (L15 apical/L15 basolateral); (2) control *versus* cortisol-treated epithelia, regular asymmetrical (FW apical/L15 basolateral) culture conditions; and (3) control *versus* cortisol-treated epithelia, SFW asymmetrical (SFW apical/L15 basolateral) culture conditions. Ca^{2+} fluxes under asymmetrical conditions were first examined immediately after the introduction of FW or SFW (i.e., *T*0–6 h). A second flux was subsequently run 18 h after initial exposure to asymmetrical conditions (i.e., *T*18–24 h).

Ussing flux ratio criterion and active Ca^{2+} transport across cultured trout gill epithelia. As an indication of active Ca^{2+} transport across cultured trout epithelia, we used the Ussing flux ratio criterion, which examined for disagreement between the passive flux ratio predicted from the measured electrochemical gradient and the actual measured flux ratio (J_{in}/J_{out}), as outlined by Kirschner (1970). The predicted flux ratio was calculated as a function of ionic activities, valence, measured TEP, and several thermodynamic constants as follows:

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap} \cdot e^{-(zFV/RT)}}{A_{Bl}}$$

where A_{Ap} and A_{Bl} are Ca^{2+} ion activities on the apical and basolateral side of the epithelium respectively, z is Ca^{2+} valence, V is the measured TEP (average for matched inserts, see above) and the usual thermodynamic values are given to F, R, and T. In media, A_{Ca} was 86% of measured concentrations and under asymmetrical conditions A_{Ca} in apical freshwater (both regular and soft freshwater) was equal to the measured concentrations.

Statistical analysis. All data are expressed as mean values±SEM (n), where n represents the number of filter inserts. Significant differences ($P \leq 0.05$) between groups were determined using either a one-way analysis of variance (ANOVA) followed by a Student–Newman–Kuels test or Student's unpaired or paired t tests as appropriate (Sigmastat Software, Jandel Scientific-San Rafael, CA).

Results

Symmetrical (L15 apical/L15 basolateral) culture conditions. At the outset of the flux period, the TER values of control and cortisol-treated epithelia were 31.15±0.73 and 33.04±0.23 kΩ cm^2, respectively. These values were not significantly different. In contrast, there was a significant difference ($P \leq 0.05$) between TEP values of control (+20.47±2.55 mV) and cortisol-treated (+30.20±0.95 mV) epithelia. With the exception of an ~5-mV increase in the TEP measured across both treatment groups, the electrophysiological characteristics of epithelia for the duration of the flux period varied little from values recorded at the outset of the experiment (Table 1).

In control epithelia, radioisotopically determined unidirectional Ca^{2+} flux measurements under symmetrical culture conditions revealed influx rates (apical to basolateral) that were slightly greater than efflux rates (basolateral to apical), resulting in a net flux rate that was in the inward direction (Fig. 1). Epithelia that had been treated with cortisol exhibited approximately equal movement of ions in both directions. A slight observable difference between the net flux rates of control and cortisol-treated preparations can be attributed to decreased Ca^{2+} influx across cortisol-treated epithelia as a significant ($P \leq 0.05$) decline in Ca^{2+} influx rate was evident (Fig. 1). A significant ($P \leq 0.05$) and robust disagreement between

Table 1. Comparison between predicted and observed flux ratios for Ca^{2+} and electrophysiological characteristics of cultured epithelial preparations treated with cortisol (500 ng/ml) under symmetrical culture conditions (L15 apical/L15 basolateral)

	Flux ratio			
	Predicted	Observed	TER (kΩ cm^2)	TEP (mV)
Control (n = 7)	0.16±0.03	1.58±0.34*	32.85±0.60	+25.14±3.04
Cortisol (n = 7)	0.06±0.01	1.66±0.54*	32.89±0.34	+35.32±0.97†

Data are expressed as mean values±SEM (n=number of matched epithelia).
Abbreviations: *TER* transepithelial resistance, *TEP* transepithelial potential, *L15* Leibovitz's *L-15* media.
*Denotes significant difference ($P \leq 0.05$) between predicted and observed flux ratio within a treatment. Predicted and observed flux ratios were compared by paired t test.
†Denotes significant difference, as compared by a t test, between TEP values.

the predicted and observed Ussing flux ratio criterion for both control and cortisol-treated epithelia was evident (Table 1), strongly suggesting active Ca^{2+} uptake across these preparations.

Asymmetrical (FW apical/L15 basolateral) culture conditions. Unidirectional Ca^{2+} flux experiments under asymmetrical culture conditions were conducted twice over a 24-h period using the same insert preparations. During the first flux period (T0–6 h), TER was slightly higher in control preparations when compared to cortisol-treated epithelia (Table 2). The TEP across control epithelia was −1.36±0.48 mV. The TEP across cortisol-treated epithelia was measured at +8.33±2.12 mV (Table 2). Relative to symmetrical culture conditions, efflux rates across cultured epithelia under asymmetrical conditions (T0–6 h) were approximately tenfold greater, whereas influx rates were approximately sixfold greater (Fig. 2). Therefore, unidirectional influx rates were approximately 60% of the efflux rates, resulting in a net flux in the outward (basolateral to apical) direction (Fig. 2). No significant differences between efflux, influx, or net flux rates between control and cortisol-treated epithelia during the *T*0–6 h asymmetrical flux period were observed. Statistically significant disagreements with the relevant Ussing flux ratio criteria indicated active Ca^{2+} transport in the outward direction (basolateral to apical) across control preparations and active Ca^{2+} transport in the inward direction (apical to basolateral) across cortisol-treated epithelia (Table 2). During the second asymmetrical flux period (*T*18–24 h), TER had declined relative to *T*0–6 h and no significant difference was observed between control and cortisol-treated epithelia (Table 2). In contrast, a significant difference between TEP values between treatments persisted, with both treatments now exhibiting a negative TEP relative to the apical side (control=−6.24±0.30 mV and cortisol-treated=−1.83±0.66 mV). Unidirectional Ca^{2+} influx rates across control epithelia moderately, but not significantly, increased after an 18-h culture under asymmetrical conditions (Fig. 2). However, Ca^{2+} efflux rates significantly increased ($P \leq 0.05$) and, accordingly, net flux rates significantly increased in the outward (basolateral to apical) direction. In cortisol- treated preparations, a significant increase ($P \leq 0.05$) in Ca^{2+} influx rates was observed relative to control values during the same time period and relative to influx rates during the first 6 h of asymmetrical culture conditions (Fig. 2). Although Ca^{2+} efflux also increased in cortisol-treated preparations, the extent of the change was not as substantial as that observed in control epithelia, resulting in a net flux rate that was not significantly different from the *T*0–6 h net flux rate (Fig. 2). Disagreement between the predicted and observed flux ratio continued to suggest that active Ca^{2+} transport was occurring in the outward direction across control epithelia. No significant difference between the observed and predicted flux ratio was evident in cortisol-treated epithelia, indicating passive movement of Ca^{2+} (Table 2).

Asymmetrical (SFW apical/L15 basolateral) culture conditions. Before the start of unidirectional Ca^{2+} flux experiments under asymmetrical conditions with SFW bathing the apical side of the preparations, control and

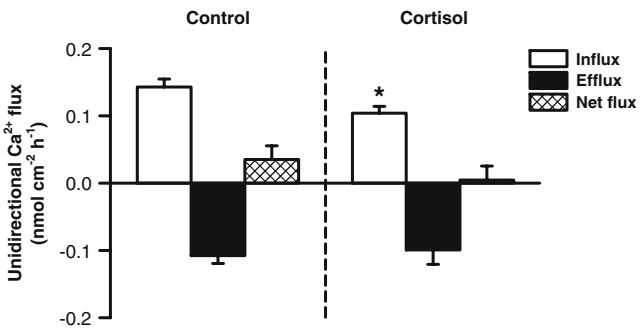

Figure 1. Effect of cortisol (500 ng/ml, basolateral side only) on unidirectional Ca^{2+} flux across cultured gill epithelia under symmetrical culture conditions (L15 apical/L15 basolateral). Data are expressed as mean values ± SEM (n=7). An *asterisk* denotes significant difference ($P \leq 0.05$) between flux rates of control and cortisol treated preparations.

Table 2. Comparison between predicted and observed flux ratios for Ca^{2+} and electrophysiological characteristics of cultured epithelial preparations treated with cortisol (500 ng/ml) and exposed to asymmetrical culture conditions of either apical freshwater/basolateral L15 OR apical soft freshwater/basolateral L15 for a 24-h period

	Flux ratio			
	Predicted	Observed	TER (kΩ cm^2)	TEP (mV)
Apical FW/Basolateral L15				
*T*0 – 6 h:				
Control (*n*=7)	0.938±0.033	0.658±0.104*	34.43±0.48	−1.36±0.48
Cortisol (*n*=7)	0.344±0.083	0.581±0.099*	30.83±1.28	+ 8.33±2.12†
T18–24 h**:**				
Control (*n*=7)	1.318±0.033	0.462±0.061*	19.96±1.10	−6.24±0.30
Cortisol (*n*=7)	0.857±0.072	0.693±0.075	23.69±2.27	−1.83±0.66†
Apical SFW/Basolateral L15				
*T*0–6 h				
Control (*n*=7)	0.145±0.008	0.096±0.022	35.21±1.78	−9.29±0.73
Cortisol (*n*=5)	0.059±0.004	0.102±0.023	30.97±3.57	+0.63±0.80†
*T*18–24 h				
Control (*n*=7)	0.099±0.003	0.075±0.005*	28.05±1.58	−6.45±0.38
Cortisol (*n*=5)	0.081±0.006	0.132±0.028	38.07±3.37‡	−3.60±0.96†

Data are expressed as mean values±SEM (*n*=number of matched epithelia).
Abbreviations: *TER* transepithelial resistance, *TEP* transepithelial potential, *FW* freshwater, *SFW* soft freshwater, *L15* Leibovitz's L-15 media.
*Denotes significant difference ($P \leq 0.05$) between predicted and observed flux ratio within a treatment and time period. Predicted and observed flux ratios were compared by paired *t* test.
†Denotes significant difference, as compared by a *t* test, between control and cortisol TEP values within a time period.
‡ Denotes significant difference, as compared by a *t* test, between control and cortisol TER values within a time period

cortisol-treated epithelia exhibited TER values of 27.33± 2.49 kΩ cm^2 and 24.82±2.79 kΩ cm^2, respectively. These values were not significantly different. TEP across the same preparations did significantly differ ($P \leq 0.05$) with control epithelia exhibiting a TEP of +15.67±2.04 mV and cortisol-treated epithelia exhibiting a greater TEP of +25.60± 4.98 mV. During the first flux period (*T*0–6 h) under asymmetrical conditions (SFW apical/L15 basolateral), TER values increased across both treatment groups, but were not significantly different between groups. TEP in

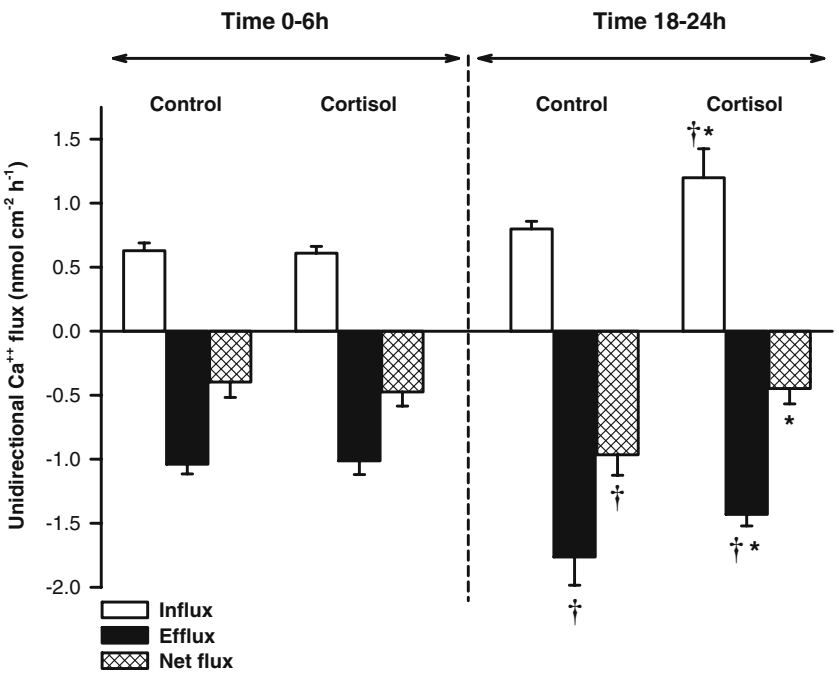

Figure 2. Unidirectional Ca^{2+} flux across control (0 ng/ml cortisol) and cortisol treated (500 ng/ml, basolateral side only) cultured gill epithelia under asymmetrical culture conditions (freshwater apical/L15 basolateral). Data are expressed as mean values±SEM (*n*=7). An *asterisk* denotes significant difference ($P \leq 0.05$) between flux rates of control and cortisol treated preparations within a specific time period. Dagger denotes a significant difference ($P \leq 0.05$) between either control or cortisol-treated preparations between different time periods.

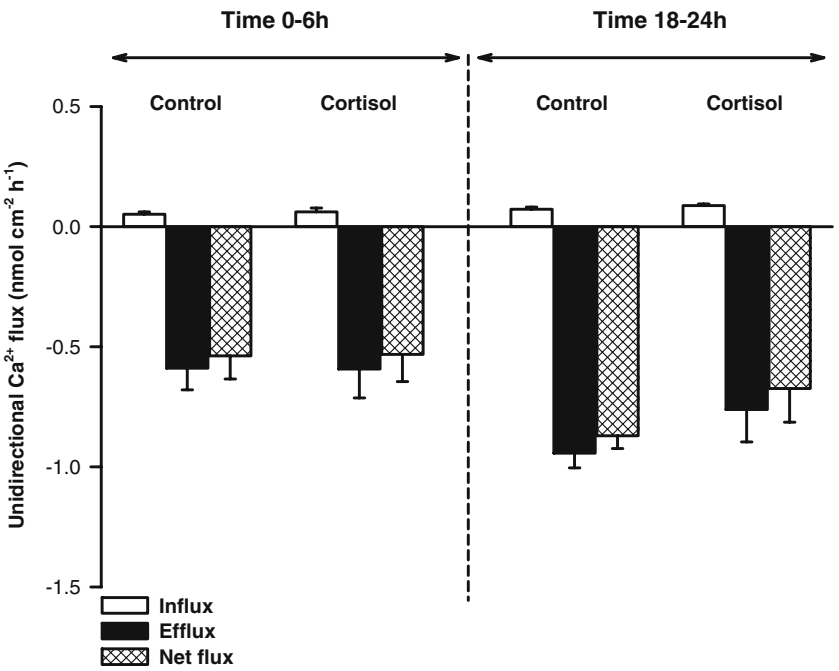

Figure 3. The effect of soft freshwater (SFW) asymmetrical culture conditions (SFW apical/L15 basolateral) on unidirectional Ca^{2+} flux across control (0 ng/ml cortisol) and cortisol-treated (500 ng/ml, basolateral side only) cultured gill epithelia. Data are expressed as mean values±SEM (n=5–7). No significant differences ($P \geq 0.05$) were found between groups or time periods.

control epithelia exhibited a negative value of −9.29± 0.73 mV. The TEP of cortisol-treated epithelia was marginally positive at +0.63±0.80 mV (Table 2). These values significantly differed ($P \leq 0.05$). During the $T0$- to $T6$-h flux period, Ca^{2+} influx across both preparations was considerably lower than that found across preparations in regular freshwater, with influx rates from SFW ranging from ~ 0.050 to 0.060 nmol cm^{-2} h^{-1}. Cortisol treatment had no significant effect on Ca^{2+} influx measured at this time period under these conditions (Fig. 3). Cortisol also had no significant effect on efflux or net flux rates during the $T0$- to $T6$-h flux period. There was no statistically significant disagreement between the predicted and observed flux ratios for either treatment, suggesting passive Ca^{2+} movement across the preparations (Table 2). However, it should be noted that there was an obvious trend that paralleled the results observed at $T0$–6 h under regular FW apical/L15 basolateral conditions. That is, the observed flux ratios suggested active Ca^{2+} extrusion across control epithelia and active Ca^{2+} uptake across cortisol-treated epithelia (Table 2).

During the second flux period ($T18$–24 h), TER remained high across epithelia (Table 2) and cortisol-treated inserts exhibited a significantly ($P \leq 0.05$) elevated TER relative to control epithelia. TEP values were also significantly different (Table 2). Statistically significant disagreement between the predicted and observed flux ratios for control preparations indicated active Ca^{2+} transport in the outward direction. There was no such disagreement between predicted and observed flux ratios for cortisol-treated epithelia (Table 2).

Discussion

Overview. The results of this study confirm the ability of cultured trout gill preparations composed of a heterogeneous cell population, i.e., preparations composed of both pavement cells (PVCs) and mitochondria rich cells (MRCs), to actively transport Ca^{2+} in the inward direction under symmetrical culture conditions (L15 apical/L15 basolateral) (see Fletcher et al. 2000). The current study also confirms the curious observation that active Ca^{2+} transport occurs in the outward direction under asymmetrical (FW apical/L15 basolateral) conditions (see Fletcher et al. 2000). The differences observed in absolute Ca^{2+} flux rates between the present study and that of Fletcher et al. (2000) likely originates from differences in epithelial "tightness"—~ 10 kΩ cm^2 in Fletcher et al. (2000) versus ~32 kΩ cm^2 in the present study. Under asymmetrical conditions, epithelial tightness (as measured by TER) did not exhibit the same disparity between the two studies.

To date, we have not tested the potential modulatory effects of any hormone or hormone combination on Ca^{2+} movement across cultured gill preparations, although we have examined the effects of cortisol, prolactin, and triiodo-L-thyronine (either alone or in combination) on Na^+ and Cl^- transporting properties of cultured gill epithelia (Kelly and Wood 2001a, b; Kelly and Wood 2002a, b; Zhou et al. 2003, 2004). Of the hormone treatments already tested in isolation, cortisol elicits the most robust response from cultured gill epithelia, regardless of epithelial cellular composition (Kelly and Wood 2001a, b; Kelly and Wood 2002a, b; Zhou et al. 2003, 2004). Under symmetrical

culture conditions, cortisol treatment has been demonstrated to significantly tighten cultured PVC epithelial preparations, at least in part by reducing paracellular permeability, and stimulate active, inwardly directed Na^+ and Cl^- transport (Kelly and Wood 2001a, 2002a). Cortisol treatment has also been demonstrated to stimulate active Na^+ uptake under asymmetrical culture conditions in preparations composed of both PVCs and MRCs (Zhou et al. 2003). Here we demonstrate for the first time that cortisol treatment of cultured trout epithelia stimulates Ca^{2+} uptake under asymmetrical culture conditions (i.e., FW apical/L15 basolateral). These observations are consistent with the hypercalcemic properties of cortisol in rainbow trout (Perry and Wood 1985; Flik and Perry 1989; Laurent and Perry 1990; Shahsavarani and Perry 2006).

Cortisol and Ca^{2+} transport across cultured trout epithelia—symmetrical and asymmetrical (FW apical/L15 basolateral) culture conditions. The current working model for Ca^{2+} movement across the freshwater fish branchial epithelium involves apical entry of the cation through an epithelial Ca^{2+} channel (ECaC) and basolateral extrusion by either a Ca^{2+}-ATPase and/or a Na^+ /Ca^{2+} exchanger (*see* Shahsavarani et al. 2006). It is presumed that apical entry occurs down an electrochemical diffusion gradient and correlative evidence suggests that movement across the apical membrane is the rate limiting step in transbranchial uptake (for review, *see* Evans et al. 2005). In addition to the mechanistic details, the favored model for Ca^{2+} transport also recognizes the branchial MRC as being primarily responsible for Ca^{2+} transport. There is a substantial amount of support for this contention. Ishihara and Mugiya (1987) have demonstrated the localization of electron-opaque calcium salt precipitates to MRCs, and in more recent experiments utilizing isolated gill MRCs and PVCs, Galvez et al. (2006) reported Ca^{2+} uptake rates in MRCs that were threefold higher than those found in PVCs. However, the bulk of the evidence that supports the localization of Ca^{2+} transport to MRCs comes from the close relationship between Ca^{2+} uptake and MRC density/exposure (e.g., Perry and Wood 1985; Marshall et al. 1992, 1995; McCormick et al. 1992; Perry et al. 1992). Nevertheless, both the study of Galvez et al. (2006) and immunohistochemical work of Shahsavarani et al. (2006) on the cell-specific localization of ECaC suggest that the PVCs may also contribute to Ca^{2+} uptake to a lesser degree.

Several of the studies reporting a relationship between Ca^{2+} uptake and MRC density/exposure utilize cortisol treatment as a means to alter MRC abundance in the gill epithelium (Perry and Wood 1985; Marshall et al. 1992, 1995; Perry et al. 1992). As Fletcher et al. (2000) have demonstrated that cultured gill epithelia composed of both PVCs and MRCs exhibit active Ca^{2+} uptake (a phenomenon confirmed in this study), whereas preparations composed of PVCs alone do not, it would be expected that treatment of the former with cortisol would positively alter the dynamics of Ca^{2+} transport. Under symmetrical culture conditions, the effects of cortisol on Ca^{2+} transport are subtle and both control and cortisol-treated epithelia exhibit qualitatively similar Ca^{2+} transport properties. However, under asymmetrical culture conditions, when FW is first introduced to the apical side of the culture preparations (i.e., *T*0–6 h), cortisol reverses the direction of active Ca^{2+} transport, counteracting a "maladaptive" property of cultured preparations whereby control epithelia exhibit outwardly directed (basolateral to apical) active Ca^{2+} transport when exposed to conditions that more closely resemble a natural environment (i.e., FW bathing the apical side of the gill epithelium). Over a more prolonged period of asymmetrical culture (i.e., between 18 and 24 h), Ca^{2+} influx rates across control epithelia did not significantly alter, whereas efflux rates significantly increased resulting in net flux rates approximately doubling in the outward direction. Qualitatively similar results across "untreated" epithelia composed of both PVCs and MRCs can be observed when examining temporal alterations in Na^+ and Cl^- movement (Kelly and Wood 2002b). That is, a disproportionate increase in ion efflux rate relative to influx rate results in an increased, outwardly directed, net flux rate. However, in the current experiments, Ca^{2+} influx rates across cortisol-treated preparations during the *T*18- to *T*24-h flux period were approximately double those observed at *T*0–6 h, whereas Ca^{2+} efflux rates increased only 1.4-fold. The overall result was that net flux rates did not appreciably change over the 24-h exposure period. Despite these observations, the flux ratio analysis indicated that inwardly directed active Ca^{2+} transport had now been replaced with passive transport mechanisms. Nevertheless, Ca^{2+} transport was not active in the outward direction. Taken together, the above results support the contention that the hypercalcemic properties of cortisol positively influence Ca^{2+} transport across cultured epithelia, especially under asymmetrical culture conditions.

Cortisol and Ca^{2+} transport across cultured trout epithelia—asymmetrical (SFW apical/L15 basolateral) culture conditions. Exposure to lowered environmental Ca^{2+} has been demonstrated to increase Ca^{2+} transport capacity in rainbow trout (Perry and Wood 1985) and other fishes (McCormick et al. 1992). Furthermore, lowering environmental ion content has also been demonstrated to increase circulating cortisol levels (Perry and Wood 1985, Perry and Laurent 1989). It is generally accepted that these two phenomena are linked, in that cortisol contributes to the maintenance of Ca^{2+} balance by mediating, at least in part, an upregulation of transport mechanisms required for Ca^{2+}

homeostasis. Given the close relationship between Ca^{2+} balance and cortisol in fish acclimated to ion-poor water, we were interested in examining this association *in vitro*. An ideal situation would be to culture gill epithelial cells that exhibit an ion poor water phenotype; however, after isolation of gill cells from fish acclimated to ion-poor water, the required extensive culture period in apical L15 media under conditions resembling the mineral content of blood would likely diminish the desired transport characteristics. Instead, we rationalized that treatment with cortisol and subsequent exposure to ion-poor conditions (soft freshwater, SFW) would offer insight into the benefits of cortisol in such an environment.

When epithelia were initially exposed to SFW, Ca^{2+} influx rates ranged from 0.05 to 0.06 nmol cm^{-2} h^{-1}. These values were approximately tenfold lower than influx rates across epithelia exposed to regular FW (i.e., 0.06 nmol cm^{-2} h^{-1} in SFW versus ~0.60 nmol cm^{-2} h^{-1} in regular FW) and are in accordance with an approximate tenfold reduction in water [Ca^{2+}] (i.e. ~90 μM versus ~900 μM in SFW and FW, respectively). Ca^{2+} efflux rates across epithelia exposed to apical SFW were approximately ~56% of those observed across epithelia exposed to apical FW, and due to the low Ca^{2+} influx rates, net Ca^{2+} flux rates were not found to be that different when comparing regular FW- and SFW-exposed preparations. When compared to control epithelia, cortisol had no significant effect on flux rates measured across epithelia exposed to apical SFW either during the *T*0- to *T*6-h or *T*18- to *T*24-h time periods. The flux ratio analysis also indicated passive movement of Ca^{2+} across both control and cortisol-treated preparations during the *T*0- to *T*6-h flux period, although the numbers exhibited a trend that mimicked the flux ratio characteristics observed under normal FW conditions. In contrast, during the *T*18- to *T* 24-h flux period, the flux ratio analysis indicated active Ca^{2+} transport in the outward direction across control epithelia and passive transport in cortisol-treated preparations.

A particularly interesting observation was an ~8 kΩ cm^2 increase in the TER of cortisol-treated preparations between the first (*T*0–6 h) and second (*T*18–24 h) flux periods. This increase in TER contrasts with the ~7 kΩ cm^2 decrease observed in control epithelia. This is the first time we have observed an increase in TER across cultured gill epithelia exposed to asymmetrical culture conditions for an extended period of time. Under normal circumstances, the TER of epithelial preparations will decrease to varying degrees over an asymmetrical culture period of this duration (*see* Kelly and Wood 2002b; Wood et al. 2002; this study), presumably as epithelial integrity is slightly compromised. Cortisol generally attenuates a decline in TER across cultured epithelia under asymmetrical conditions (Kelly and Wood 2001a; Wood et al. 2002), and has been shown to reduce diffusive Na^+ and Cl^- movement in the outward direction across epithelia (Kelly and Wood 2001a). In cortisol-treated epithelia exhibiting, an increased TER during *T*18–24 h, net Ca^{2+} flux is reduced relative to control preparations, but not significantly. Nevertheless, electrophysiological data suggest that the integrity of cortisol-treated epithelia is enhanced and in a manner consistent with acclimation to the asymmetrical conditions rather than simple tolerance.

Conclusion. The current study confirms the ability of cultured gill epithelia composed of both PVCs and MRCs to actively transport Ca^{2+} in the inward direction (apical to basolateral) under symmetrical culture conditions (L15 apical/L15 basolateral). We also report, for the first time, that cortisol can stimulate active Ca^{2+} uptake under asymmetrical conditions, and thus reverse the outwardly directed active Ca^{2+} transport that is seen in the absence of hormone supplementation. These observations are consistent with the hypercalcemic actions of cortisol and the general consensus that cortisol helps mediate Ca^{2+} acquisition across freshwater fish gill epithelia. These observations are also consistent with the presence of membrane proteins such as ECaC in cultured gill epithelia, the dominant localization of such proteins to MRCs in cultured preparations (Shahsavarani et al. 2006), and the modulation of ECaC by cortisol treatment *in vivo* (Shahsavarani and Perry 2006). However, it is noteworthy that ECaC has also been localized to PVCs both *in vivo* and *in vitro* (e.g., in cultured gill preparations containing PVCs only) which, together with the isolated cell results of Galvez et al. (2006), challenges the generally accepted gill Ca^{2+} transport paradigm, at least in terms of the exclusivity of Ca^{2+} transport across MR cells (Shahsavarani et al. 2006). It is currently assumed that active Ca^{2+} uptake does not occur across cultured PVC (see Fletcher et al. 2000). However, given the above outlined observations, a reevaluation of the Ca^{2+} transport capacity of PVCs (PVC epithelia) in the presence of hormones such as cortisol is warranted.

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