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Cultured Gill Epithelia from Freshwater Tilapia (*Oreochromis niloticus*): Effect of Cortisol and Homologous Serum Supplements from Stressed and Unstressed Fish

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Abstract. Procedures for the preparation and culture of branchial epithelia from dispersed gill cells of freshwater tilapia (*Oreochromis niloticus*) are described. Epithelia were cultured on permeable supports (terephthalate membranes, "filters") and bathed on both the apical and basolateral side with isotonic media containing 6% fetal bovine serum (FBS). When the apical medium was replaced with freshwater (pseudo in vivo asymmetrical culture conditions), transepithelial resistance (TER) increased markedly, transepithelial potential became negative, and paracellular permeability decreased. The physiological effects of cortisol and 10% homologous (tilapia) serum were investigated. Tilapia serum (TS) was prepared from unstressed and stressed fish and therefore allowed comparison between the effects of homologous serum derived from fish in differing physiological states. Under both symmetrical and asymmetrical culture conditions, cortisol significantly elevated TER across cultured tilapia gill epithelia, indicative of a significant increase in epithelial "tightness." Cortisol reduced transepithelial Na^+ and Cl^- movement and paracellular permeability. The glucocorticoid agonist dexamethasone elicited a similar response, which was inhibited by the glucocorticoid antagonist (receptor blocker) RU486. Cortisol did not stimulate active ion transport across epithelia under either symmetrical or asymmetrical culture conditions. In epithelia supplemented with TS from stressed fish, physiological changes in cultured preparations were consistent with those observed in FBS + cortisol-supplemented epithelia. Differences between the physiological status of epithelia supplemented with TS from unstressed and stressed fish could be abolished with RU486. Using TS as a medium supplement did not stimulate active ion transport under asymmetrical culture conditions, although Na^+-K^+-ATPase activity increased in TS-supplemented epithelia relative to FBS-supplemented preparations.

Key words: Cultured gill epithelia — Corticosteroids — RU486 — Permeability — Ion transport — Na^+-K^+-ATPase

Introduction

Techniques for the primary culture of branchial epithelia on permeable "filter" supports have provided promising new in vitro models for the freshwater fish gill (Wood & Pärt, 1997; Fletcher et al., 2000). Cultured gill epithelia models, derived from dispersed gill cells of freshwater rainbow trout, have been used to investigate a variety of functions attributed to the gills of freshwater fish. These include ion transport and permeability characteristics (Wood & Pärt, 1997; Wood, Gilmour & Pärt, 1998; Gilmour et al., 1998; Fletcher et al., 2000), xenobiotic handling (Carlsson & Pärt, 2001) and nitrogen elimination (Kelly & Wood, 2001a). However, although cultured gill epithelial preparations closely mimic the electrophysiological and passive transport properties of intact gills, non-diffusive ion uptake under asymmetrical culture conditions (analogous to in vivo asymmetry, freshwater on the apical surface /L-15 culture medium on the basolateral surface) is at best far less than in vivo. Unidirectional influx rates of both Na^+ and Cl^- are very low, net flux rates are highly negative, and there is evidence only for a small active uptake of Cl^-.

One possible explanation for low active ion transport rates in vitro may be that the currently used combination of L15 medium and fetal bovine serum (FBS) may not supply specific factors needed for cells to fully adapt to apical freshwater (Wood & Pärt,

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1997; Wood et al., 1998). When intact fish are exposed to a natural alteration in environmental salinity, a complex suite of hormonal factors is involved in physiological adaptation. Hormones such as cortisol, prolactin, growth hormone, insulin-like growth factors, and thyroid hormones are but a few of those implicated in maintaining homeostatic control under conditions of salinity-related environmental perturbation (McCormick, 1995). In light of this, it is encouraging that cultured gill epithelia are responsive to several of these factors—cortisol (Kelly & Wood, 2001c), 3,5′,3′-triiodo-L-thyronine (Kelly & Wood, 2001b), and prolactin (Kelly & Wood, 2002). In all cases, the effects of these hormones in vitro are, at least in part, consistent with their respective actions on ion-transporting epithelia in vivo (Kelly & Wood, 2001b,c, 2002). However, as yet, no hormone (or hormone combination) tested has been able to stimulate active ion uptake from freshwater under asymmetrical culture conditions.

Given the observations made above, several questions arise regarding the virtual lack of active ion uptake by cultured gill epithelia under pseudo in vivo conditions (freshwater apical/L15 basolateral). Firstly, are low ion uptake rates in vitro to be found exclusively in cultured gill epithelia prepared from freshwater rainbow trout (*Oncorhynchus mykiss*), so far the only freshwater species studied? In this respect, an examination of cultured gill epithelia derived from Nile tilapia (*Oreochromis niloticus*) is particularly relevant, as isolated opercular epithelia from *O. niloticus* are, to date, the only surrogate gill preparations demonstrated to exhibit nondiffusive uptake of Na^+ and Cl^- from freshwater under asymmetrical conditions in vitro (Burgess, Marshall & Wood, 1998). Secondly, would the replacement of the regular serum supplement, fetal bovine serum (FBS), with homologous fish serum further alter the physiological characteristics of cultured gill epithelia by providing elements lacking in mammalian-derived serum? Lastly, as rapid salinity transfer is a physiologically stressful condition, would serum prepared from stressed fish, in which levels of hormones such as cortisol are elevated, benefit cultured gill epithelia similarly exposed to asymmetrical conditions in vitro? In order to address these issues, we developed procedures for the preparation and culture of gill epithelia from dispersed gill cells of *O. niloticus*. The physiological characteristics of these epithelia were examined in the presence and absence of cortisol, a hormone fundamentally involved in teleost hydromineral balance (McCormick, 1995; Wendelaar Bonga, 1997) and a factor already proven to favorably alter the physiological status of cultured gill epithelia from freshwater rainbow trout (Kelly & Wood, 2001b,c). By utilizing homologous serum, prepared either from unstressed or stressed tilapia, as a medium supplement, we also examined the physiological status of cultured tilapia gill epithelia in media modified to resemble natural conditions to a higher degree.

Materials and Methods

Preparation of Cultured Branchial Epithelia

Nile tilapia (*O. niloticus*) (80–175 g) were held in flow-through dechlorinated running Hamilton tapwater (composition in mmol/l: $[Na^+]$, 0.55; $[Cl^-]$, 0.70; $[Ca^{2+}]$, 1.00; $[Mg^{2+}]$, 0.15; $[K^+]$, 0.05; pH 7.8–8.0). Photoperiod varied seasonally and temperature ranged from 22–24°C. Fish were stunned by a blow to the head and then decapitated. All procedures for gill cell isolation were conducted in a laminar flow hood using sterile techniques. Methods for initial gill cell isolation were based on those originally developed by Pärt et al. (1993), with modifications described by Wood & Pärt (1997). Briefly, gill cells were obtained from excised gill filaments by two consecutive cycles of tryptic digestion (Gibco BRL Life Technologies, 0.05% trypsin in phosphate-buffered saline, PBS, with 5.5 mmol/l EDTA) and resuspended in culture medium (Leibovitz's L15 supplemented with 2 mmol/l glutamine, 5–6% fetal bovine serum, FBS, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 200 μg/ml gentamycin). Subsequent flask cell culture and epithelial culture procedures were modified from the methods of Wood & Pärt (1997). For flask culture, cells were seeded at a density of 1,000,000–1,250,000 cells/cm^2 in culture medium into 25-cm^2 flasks (Falcon) and kept at 20–22°C in an air atmosphere. Non-adherent cells were removed by changing medium (L15 plus 2 mmol/l glutamine, 6% FBS and antibiotics, *see* above) at 24 hr. The medium was changed again at 72 hr (L15 plus 2 mmol/l glutamine, 6% FBS with antibiotics). After a further 24–48 hr in culture, the harvesting and reseeding of cells onto permeable Falcon cell-culture inserts (Cyclopore polyethylene terephthalate "filters;" pore density: 1.6×10^6 pores/cm^2, pore size: 0.45 μm, growth surface: 0.9 cm^2; (Becton Dickinson, Franklin Lakes, NJ), was conducted by the removal and replacement of medium with trypsin solution (*see* above). To facilitate the removal of cells, flasks were subjected to a mild mechanical agitation and cell detachment was confirmed by visual inspection under a phase contrast microscope (Leitz). Trypsination was terminated by the addition of a "stop" solution (10% FBS in PBS, pH 7.7). Cells were resuspended in medium (L15 plus 2 mmol/l glutamine, 6% FBS with antibiotics) and seeded onto culture inserts at a density of 800,000–900,000 cells/cm^2. Inserts were held in 12-well companion plates (Falcon) under identical incubation conditions to that stated above. Initially, inserts (apical side) and companion wells (basolateral side) contained 0.8 ml and 1.0 ml medium, respectively. After 24 hr, medium was removed along with non-adherent cells and bathing solutions were replaced with medium without antibiotics (1.5 ml on the apical side and 2.0 ml on the basolateral side). Medium (without antibiotics) was replaced every 48 hr thereafter. When asymmetrical conditions were tested, temperature-equilibrated (20–22°C) freshwater (chemical composition same as original holding water; Acrodisc®-sterilized, 0.2 μm pore size) was added to the apical side of the insert after several rinses to ensure removal of any residual medium. Additional details on the procedures for preparation and culture of gill epithelia can be found in Kelly et al. (2000).

Hormonal Treatment and Preparation of Homologous Serum

Single-use aliquots of stock cortisol or solution were prepared by dissolving either cortisol (hydrocortisone hemisuccinate, Sigma) or

dexamethasone (dexamethasone hemisuccinate, Sigma) in PBS (pH 7.7). Aliquots were stored at −20°C until use. After hormone treatment commenced, stock solutions were defrosted and diluted in L15 medium, so as to be added fresh on each medium change. Treatment of epithelia with cortisol or dexamethasone commenced 24 hr after seeding cells onto filter inserts, and this was done on the basolateral side of the insert only. At this stage in culture, a confluent epithelium appeared to have been formed. In contrast to trout gill cultures (Kelly & Wood, 2001c), if cortisol or dexamethasone was added to culture medium prior to the formation of an epithelium (i.e., after initially seeding cells into flasks or when initially seeding cells onto filter inserts), poor attachment of cells was observed. During initial experiments with cortisol, a single high dose of 1000 ng/ml was used. This dose was selected based on our previous observations of the response of trout epithelia to varying doses of cortisol (Kelly & Wood, 2001c). In experiments using the glucocorticoid agonist dexamethasone, lower concentrations (doses) of 300 nmol/l were used (approximately equivalent to 117 ng/ml dexamethasone). These were selected based on our previous observations that physiological levels of glucocorticoid (cortisol) elicit physiological changes in cultured gill epithelia, albeit at lower levels than high doses of 1000 ng/ml (Kelly & Wood, 2001c). Single-use aliquots of RU486 (mifepristone, Sigma) were prepared by dissolving RU486 in 95% ethanol. Aliquots were stored at −20°C until use. Stock solutions of RU486 were also dissolved in L15 medium, yielding a final ethanol concentration of less than 0.05%. Tests demonstrated that this level of ethanol had no effect on the performance of epithelia.

Tilapia serum (TS) was obtained from a separate stock of fish (400–600 g) held under culture conditions identical to those described above. In fish designated as "unstressed", 4 individual fish were netted from the tank simultaneously by 4 individual samplers. Blood was collected by caudal puncture. The procedure was then repeated as time allowed. All sampling took place within a 2-min period. After these initial samples were taken, remaining fish were removed from the holding tank and placed in a 40-l opaque container (water composition as above) and chased for 30 sec every 5 min during a period of 20 min. These fish were designated "stressed" and blood was collected in an identical manner to that described for unstressed fish. All blood samples were allowed to clot at room temperature for 30 min and then were centrifuged for 15 min (5,000 × g at 5°C) to obtain serum. An aliquot of serum was collected from each sample and quick-frozen in liquid nitrogen for cortisol analysis. These samples were stored at −70°C. The remaining unstressed and stressed serum samples were pooled separately and sterilized by passing twice through an Acrodisc syringe filter (0.2 μm pore size) and stored at −20°C until use. After thawing pooled serum samples for addition to L15 media, an aliquot was removed for the determination of cortisol levels in pooled samples. Cortisol was measured using a commercial ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). The RIA kit was validated for use with tilapia serum by parallelism tests where curves generated by assay standards or serial dilutions of tilapia serum ran parallel (*data not shown*). Supplementary addition-recovery tests were also conducted and were within an acceptable range, averaging 107%.

In experiments using TS, cells were initially grown in 25-cm^2 flasks using regular L15 medium supplemented with FBS (6%) as described above. TS was only presented to cultured cells 24 hr after seeding onto filter inserts. In all cases where TS was used, it was supplemented at a level of 10%, in the L15 medium, in place of FBS. The TS-supplemented medium was placed on both surfaces of the cultured epithelium. Based on our observation of serum cortisol levels, as determined in individual fish samples and in pooled samples, the use of 10% TS would result in media cortisol levels in cultured epithelia designated for "unstressed serum" and "stressed serum" treatment of >1 ng/ml and approximately 50 ng/ml, respectively (*see* Results).

Electrophysiological Measurements

Transepithelial resistance (TER) was measured using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL), whilst transepithelial potential (TEP) was measured using agar-salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrode half cells (World Precision Instruments) to a pH meter used as a high-impedance electrometer (Radiometer pHM 84, Copenhagen, Denmark). Recorded values of TER were expressed as kΩ cm^2 after correcting for TER measured across blank inserts bathed with appropriate solutions and multiplying the corrected value by filter growth area (0.9 cm^2). Chopstick electrodes were located a standardized distance from the epithelium (apical and basal electrodes 3 mm apart and apical electrode 5 mm above epithelium) during all recordings and blank determinations. All TEP measurements were expressed relative to the apical side as 0 mV after correction for junction potential according to Kelly and Wood (2001c).

Microscopy

Routine examination of cells in both flasks and inserts was conducted using a phase contrast microscope (Zeiss). The methodology used to prepare cultured epithelia in the current experiments has been demonstrated to exclude the presence of mitochondria-rich cells from trout gill preparations (Wood & Pärt; 1997; Kelly et al., 2000); however, selected preparations were stained with MitoTracker Green (Molecular Probes, Cedarlane Laboratories, Hornby, Ontario, Canada) to examine for the presence of mitochondria-rich cells. Procedures for the preparation of MitoTracker Green and staining of cultured preparations have previously been described (Kelly & Wood, 2001c).

Na^+-K^+-ATPase Activity

The activity of Na^+-K^+-ATPase was determined in individual epithelia according to the methods of McCormick (1993) with slight modifications outlined in Kelly and Wood (2001c). The protein content of supernatants used for enzyme analyses was measured according to the Bradford method (Sigma), using bovine serum albumin (Sigma) as a standard. All enzyme activities are expressed as protein-specific activities.

[^{3}H]PEG-4000 Permeability

Paracellular permeability was determined using the marker [^{3}H]polyethylene glycol (molecular mass 4000 Da; 'PEG-4000,' NEN-Dupont) according to methods previously outlined by Wood et al. (1998) and Gilmour et al. (1998). The movement of [^{3}H] PEG-4000 was determined in the efflux direction (basolateral to apical) only at appropriate time intervals after the addition of 1 μCi [^{3}H] PEG-4000 to basolateral culture medium.

Net Ion Flux

Directly measured net Na^+ and Cl^- flux rates (J_{net}) (without the use of isotopes) were determined by measuring the appearance of Na^+ or Cl^- in the apical compartment under asymmetrical culture conditions (freshwater apical/L15 basolateral), using methods previ-

ously outlined by Wood et al. (1998a). Net ion fluxes from the basolateral to apical compartments were taken as "analogous" to net loss rates from an intact animal and therefore carry a negative sign.

Unidirectional Ion Flux

Unidirectional Na^+ and Cl^- flux rates (employing radiotracers) were measured in selected series according to methods previously outlined by Wood et al. (1998). Briefly, 1 μCi of isotope ($^{22}Na^+$ or $^{36}Cl^-$) was added to either the apical side, for influx studies, or the basolateral side, for efflux studies, and the appearance monitored on the "cold" side. Inserts were used either for influx or efflux measurements only and matched for calculations of the Ussing flux ratio criterion. Insert pairs were matched based on electrophysiological similarity (TER and TEP measurements; *c.f.* Fletcher et al., 2000), and each insert was only used once in the pairing procedure. Measurements of TER and TEP were taken at the beginning, middle, and end of each flux period, and averaged.

Ussing Flux Ratio Criterion

The criterion used to detect the presence of active transport was disagreement of the measured flux ratio (J_{in}/J_{out}) with that predicted by the Ussing flux ratio equation (Kirschner, 1970). The predicted Ussing flux ratio was calculated according to the following equation:

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap}.e^{-(zFV/RT)}}{A_{Bl}} \quad (1)$$

where A_{Ap} and A_{Bl} are the activities of the ions (Na^+ and Cl^-) on the apical and basolateral sides, z is the ionic valence, V is the measured TEP in volts (average for matched inserts) and F, R, and T have their usual thermodynamic values. Under conditions of asymmetrical exposure, the activities of Na^+ and Cl^- in the apical freshwater were taken as equal to the measured concentrations. Under symmetrical conditions (or in the case of asymmetrical exposure, the basolateral side only), ion activities were 75% of the concentration as previously determined by Wood et al. (1998).

Statistical Analyses

All data are expressed as mean value ± SEM of n, where n represents the number of filter inserts. Data were either subjected to One-Way Analysis of Variance or Two-Way Analysis of Variance as appropriate, and significance of individual differences delineated using a Student Neuman-Kuels test (Sigmastat software, Jandel Scientific). When appropriate, Student's unpaired or paired t-tests were also used (Sigmastat software, Jandel Scientific). A fiducial level of $P \geq 0.05$ was used throughout.

Results

Overview of Cultured Tilapia Gill Epithelia

A typical sigmoidal pattern of transepithelial resistance (TER) developed over time after seeding tilapia gill cells on cell culture filter inserts (Fig. 1). After 6–7 days in culture, epithelial resistance tended to plateau (exhibit electrophysiological stability). All preparations used for experimental manipulation were used during this period in accordance with criteria set out by Wood et al. (1998). In epithelia grown with supplemental FBS and without the addition of hormone, epithelial resistance after 6–7 days was about 1–3 kΩ cm^2. Under these conditions, cultured tilapia epithelia did not develop a transepithelial potential (TEP) that was significantly different from 0 mV (Table 1). Unidirectional flux rates of Na^+ and Cl^- were approximately equal in the influx and efflux directions, but the net flux was slightly positive (i.e., net uptake; Fig. 2) and the measured flux ratios exceeded the Ussing flux ratios (a difference that was significant for Na^+ but not for Cl^-; Table 1), indicating nondiffusive transport in the uptake direction.

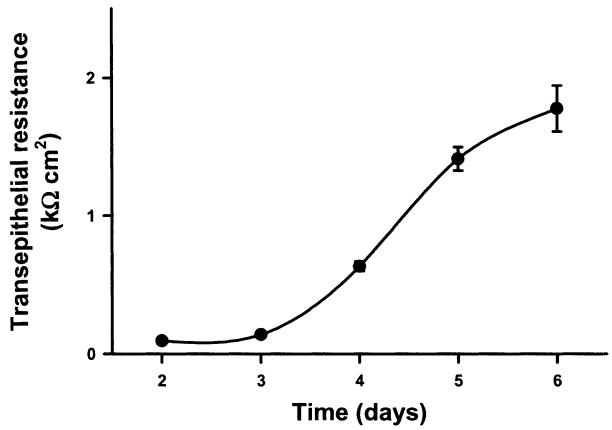

Fig. 1. Changes over time in transepithelial resistance (TER) of cultured tilapia pavement cell epithelia after first seeding (day 0) onto cell culture "filter" inserts. Epithelia were cultured under symmetrical culture conditions (L15 apical/L15 basolateral) and supplemented with 6% FBS only. Data are expressed as mean value ± SEM (n = 18). Transepithelial potential was not significantly different from 0 mV.

Upon exposure to apical freshwater (asymmetrical conditions), cultured tilapia gill epithelia exhibited responses that were qualitatively very similar to those demonstrated earlier in cultured trout gill epithelia (Wood & Pärt, 1997; Wood et al., 1998; Gilmour et al., 1998; Fletcher et al., 2000). These included greatly increased TER (up to 10-fold) and a highly negative TEP (Table 1). Unidirectional Na^+ and Cl^- influx rates decreased to just a few percent of their former values when apical L15 was replaced with freshwater (Fig. 2). Efflux rates either showed little change or decreased in some trials (Fig. 2). However, Na^+ efflux rates remained many-fold greater than influx rates, so net fluxes became highly negative (Fig. 2). The measured flux ratios decreased greatly, and the value for Na^+ was now only about half that for Cl^- (Table 1). Furthermore, the measured Na^+ ratio was significantly below the Ussing prediction, while that for Cl^- was significantly above the Ussing prediction, indicating nondiffusive transport in the efflux direction for Na^+, and in the uptake direction for Cl^- (Table

Table 1. Comparison between Ussing flux ratios (J_{in}/J_{out}) for Na^+ and Cl^- in control (0 ng/ml cortsiol) and cortisol-treated (1000 ng/ml) tilapia pavement cell epithelia under symmetrical (L15 apical/L15 basolateral) and asymmetrical (FW apical/L15 basolateral) culture conditions

	Control		Cortisol	
	Na^+	Cl^-	Na^+	Cl^-
Symmetrical				
Predicted ratio	1.00 ± 0.01	1.00 ± 0.01	0.98 ± 0.01	1.02 ± 0.01
Observed ratio	1.13 ± 0.05*	1.10 ± 0.04	1.27 ± 0.15*	2.17 ± 0.69
TER (kΩ cm^2)	1.81 ± 0.31		23.18 ± 1.16†	
TEP(mV)	Zero		0.55 ± 0.12†	
Asymmetrical				
Predicted ratio (× 10^3)	21.10 ± 0.28	8.12 ± 0.17	32.90 ± 0.60	5.54 + 0.14
Observed ratio (× 10^3)	10.10 ± 0.78*	19.70 ± 1.79*‡	21.00 + 3.65*	55.10 ± 9.84*‡
TER (kΩ cm^2)	18.58 ± 0.83		30.64 ± 0.63†	
TER (mV)	−13.42 ± 0.32		−24.36 ± 0.53†	

All data are expressed as mean values ± SEM; control epithelia, $n = 8$; cortisol-treated epithelia, $n = 5$.
* denotes significant difference ($P < 0.05$) between observed and predicted flux ratio.
‡ denotes significant difference ($P < 0.05$) between observed Na^+ and observed Cl^- flux ratio.
† denotes significant difference ($P < 0.05$) between control and cortisol-treated preparations.

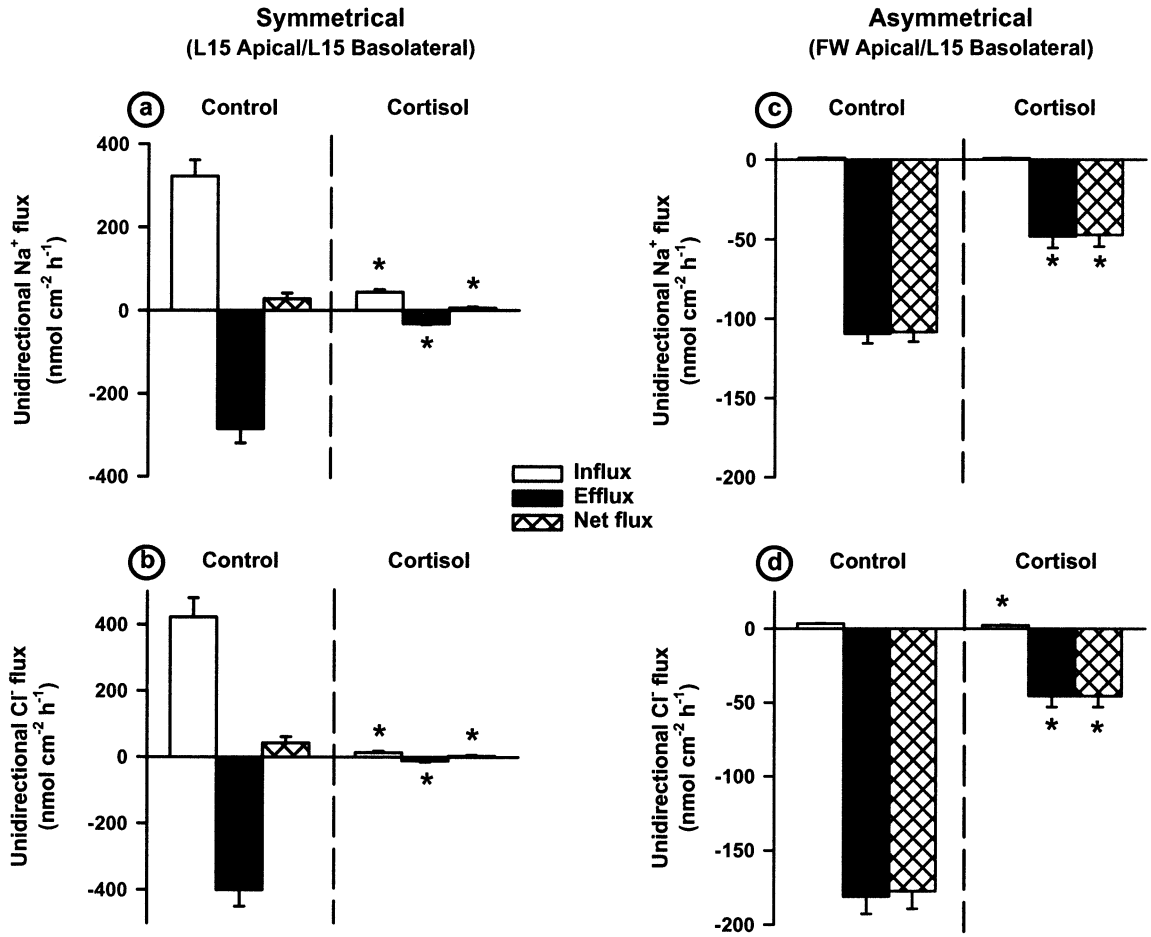

Fig. 2. Unidirectional Na^+ (*a*) and Cl^- (*b*) flux rates in control (0 ng/ml cortisol, $n = 9$) and cortisol-treated (1000 ng/ml, $n = 5$) tilapia pavement cell epithelia under symmetrical culture conditions (L15 apical/L15 basolateral). Unidirectional Na^+ (*c*) and Cl^- (*d*) flux rates in control (0 ng/ml cortisol, $n = 9$) and cortisol-treated (1000 ng/ml, $n = 5$) tilapia pavement cell epithelia under asymmetrical culture conditions (FW apical/L15 basolateral). Open, solid and cross-hatched bars represent ion influx, efflux and net flux, respectively. Data are expressed as mean values ± SEM. An asterisk denotes significant difference between control and cortisol treated preparations.

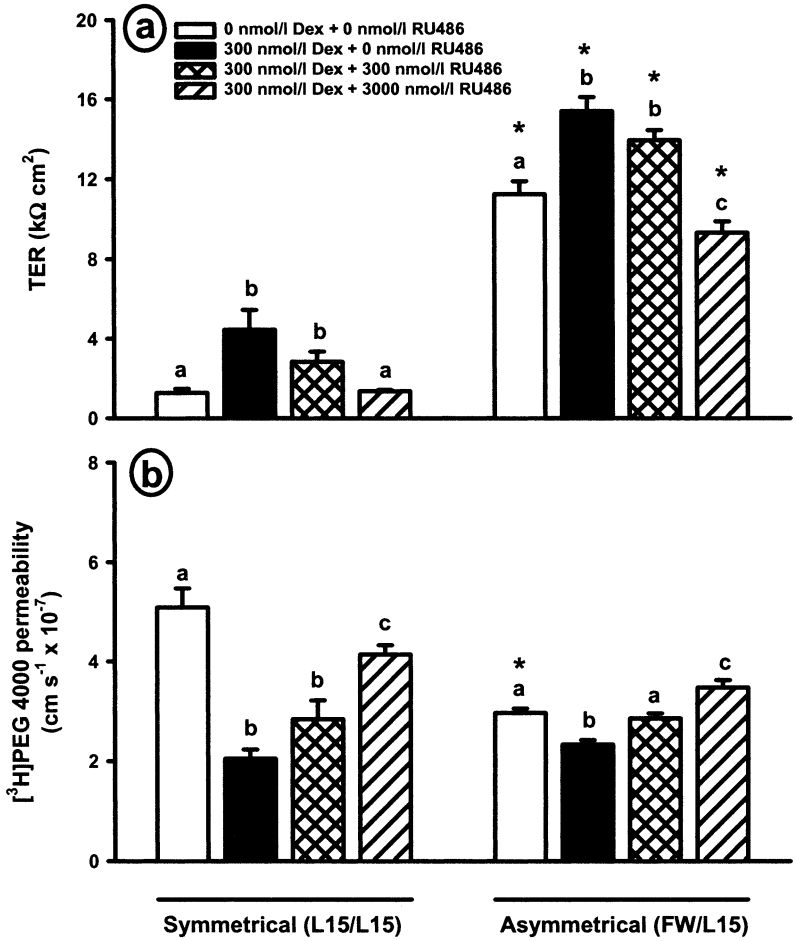

Fig. 3. Effect of dexamethasone and RU486 on (*a*) transepithelial resistance (TER) and (*b*) [^{3}H]PEG-4000 permeability (paracellular permeability) of cultured tilapia pavement cell epithelia under both symmetrical (L15 apical/L15 basolateral) and asymmetrical (FW apical/L15 basolateral) culture conditions. Data are expressed as mean value ± SEM (n = 5–6). Significant difference between culture conditions is denoted by an asterisk, while significant difference between groups within a culture condition is denoted by different letters. Note that concentrations are expressed as nmol/l where 300 nmol/l ≈ 117 ng/ml dexamethasone in L15 or 129 ng/ml RU486 in L15 respectively.

1). However, the rate of Cl^- uptake was small, so the net flux remained highly negative (e.g., Fig. 2).

Paracellular permeability, as revealed by the flux of [^{3}H]PEG-4000, generally decreased significantly upon freshwater exposure (Fig. 3; *see* also Fig. 5).

As the methods used for the preparation of cultured tilapia gill epithelia were developed by slightly modifying the methods of Wood & Pärt (1997) for the culture of rainbow trout pavement cell epithelia, we anticipated that the cultured tilapia gill epithelium would also be composed of a single pavement cell type. This appeared to be the case, as no mitochondria-rich cells could be detected in cultured tilapia gill epithelia using Mitotracker Green. Furthermore, tilapia cells and epithelia under phase contrast microscopy appeared homogeneous and did not exhibit any of the heterogeneous characteristics described by Fletcher et al. (2000) for cultured gill epithelia containing more than one cell type.

The Response of Epithelia to Cortisol Treatment

After six days in symmetrical culture, TER in control epithelia (0 ng/ml cortisol) was 1.83 ± 0.19 kΩ cm^2 while the addition of cortisol (1000 ng/ml) to culture media resulted in a highly significant elevation in TER of more than 10-fold (Table 1). When the apical solution was changed to freshwater (i.e., asymmetrical conditions), TER in cortisol-treated epithelia increased even further to around 31 kΩ cm^2, about 1.6-fold higher than the comparable value in the absence of cortisol. In control epithelia under symmetrical conditions, TEP was found to be 0 mV, whereas in cortisol-treated preparations, a very slight elevation to a positive TEP of about 0.5 mV was observed (Table 1). However, under asymmetrical conditions, TEP became even more negative when cortisol was present (−24 mV) than when it was absent (−13 mV), a highly significant difference.

In both symmetrical (L15 apical/L15 basolateral) and asymmetrical (freshwater apical/L15 basolateral) conditions, cortisol significantly ($P < 0.05$) reduced unidirectional Na^+ and Cl^- flux rates (Fig 2). Cortisol also tended to increase the measured Cl^- flux ratio under symmetrical conditions, though the changes were not significant (Table 1). Therefore, cortisol did not significantly alter the previously identified flux ratio patterns (Table 1).

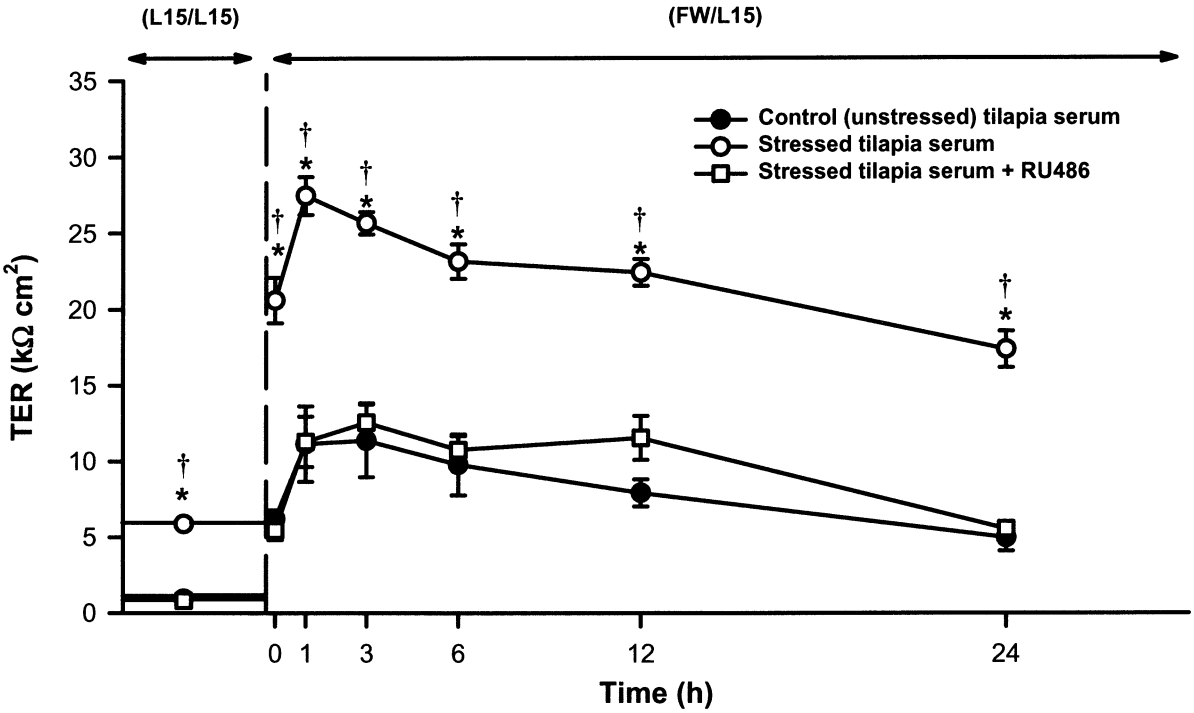

Fig. 4. Changes in transepithelial resistance (TER) of tilapia pavement cell epithelia cultured in medium supplemented with tilapia serum (TS) derived from control (unstressed) fish, stressed fish and stressed fish + RU486. Epithelia were cultured on "filter" inserts under symmetrical conditions (L15 apical/L15 basolateral) for a period of 6 days and then exposed to pseudo in vivo conditions (asymmetrical, FW apical/L15 basolateral) for a period of 24 hr. Data are expressed as mean value ± SEM (n = 8–10). An asterisk denotes significant difference ($P < 0.05$) between epithelia supplemented with control (unstressed) and stressed serum, while † denotes significant difference between epithelia supplemented with stressed serum and those supplemented with stressed serum + RU486.

The Response of Epithelia to Dexamethasone and Ru486 Treatment

Cultured tilapia gill epithelia responded to treatment with the synthetic glucocorticoid agonist dexamethasone in much the same way that they responded to cortisol. That is, treatment with 300 nmol/l dexamethasone (≈ 117 ng/ml dexamethasone in L15 medium) significantly increased TER and reduced paracellular permeability ([^{3}H]PEG-4000 permeability) under both symmetrical and asymmetrical culture conditions (Fig. 3). The TER changes (3-fold increase under symmetrical conditions, 30% increase under asymmetrical conditions) were not as large as with cortisol (*c.f.* Table 1), but the dose was 10-fold lower (approximately 100 ng/ml dexamethasone compared to 1000 ng/ml cortisol in L15 medium). These effects could be abolished by the simultaneous addition of the competitive glucocorticoid antagonist RU486, particularly at a concentration of RU486, which exceeded the dose of dexamethasone approximately 10-fold (Fig. 3). When epithelia were treated with RU486 alone, even at high doses, no significant effect on TER or [^{3}H] PEG-4000 permeability could be observed (*data not shown*).

Homologous Serum Supplements in Cultured Epithelia

Serum Cortisol Levels

Serum collected from unstressed (rapidly sampled) and stressed fish had cortisol levels (ng/ml) of 6.8 ± 1.7 (n = 19) and 439.7 ± 25.5 (n = 31), respectively. As samples from individual fish were pooled (and therefore different volumes of the above samples mixed), cortisol levels were also determined in these pooled samples prior to use as a serum supplement. Levels of cortisol were found to fall within a range similar to those determined from individual fish (unstressed = 6.9 ± 1.4 ng/ml, n = 7; stressed = 497.0 ± 62.5 ng/ml, n = 9). In both cases, differences between cortisol levels obtained from unstressed and stressed fish were highly significant. Homologous serum was subsequently added to culture medium at 10% v/v, yielding cortisol levels that would be approximately one tenth of those measured.

Alterations in TER, Paracellular Permeability, and Net Ion Flux Rates

Epithelia supplemented with homologous serum from stressed fish exhibited alterations in physiology that

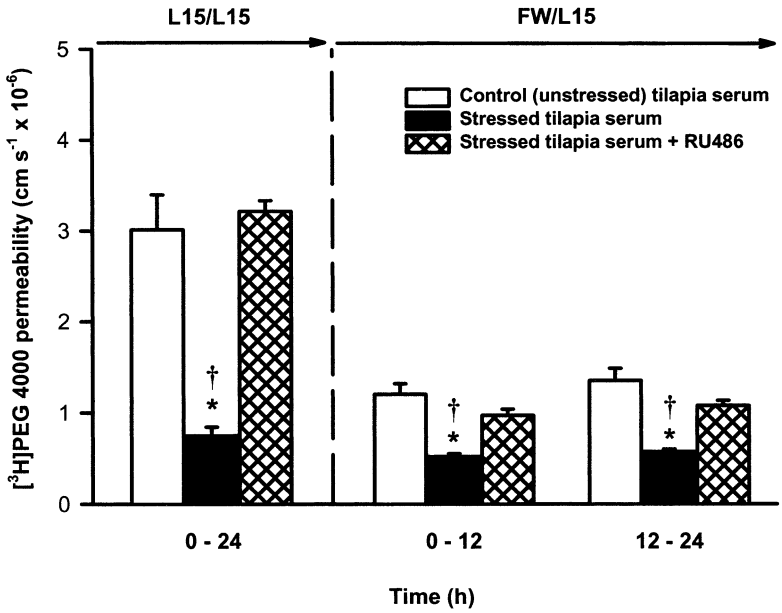

Fig. 5. Changes in [^{3}H]PEG-4000 permeability (paracellular permeability) across tilapia pavement cell epithelia cultured in medium supplemented with tilapia serum (TS) derived from control (unstressed) fish, stressed fish and stressed fish + RU486. [^{3}H]PEG-4000 fluxes were conducted over 12–24 hr periods under both symmetrical (L15 apical/L15 basolateral) and asymmetrical (pseudo in vivo, FW apical/L15 basolateral) culture conditions. Data are expressed as mean value ± SEM (n = 8–10). An asterisk denotes significant difference ($P < 0.05$) between epithelia supplemented with control (unstressed) and stressed serum, while † denotes significant difference between epithelia supplemented with stressed serum and those supplemented with stressed serum + RU486.

were consistent with the effects of cortisol or dexamethasone alone. Under both symmetrical and asymmetrical culture conditions, epithelia supplemented with TS from stressed fish exhibited an elevated TER (Fig. 4) and reduced [^{3}H]PEG-4000 permeability (Fig. 5) when compared to epithelia supplemented with TS from unstressed fish. Under asymmetrical conditions, net Na^+ and Cl^- flux rates (analogous to Na^+ and Cl^- loss rates *in vivo*) were also significantly reduced in preparations supplemented with TS from stressed fish (Fig. 6). The addition of RU486 to culture medium supplemented with TS from stressed fish completely abolished the alterations in TER (Fig. 4) and [^{3}H]PEG-4000 permeability (Fig. 5), regardless of culture condition. Similarly, under asymmetrical culture conditions, the addition of RU486 to preparations supplemented with TS from stressed fish resulted in net Na^+ and Cl^- flux rates that were not significantly different from epithelia supplemented with TS from unstressed fish (Fig. 6).

Unidirectional Ion Flux Rates

A separate series of epithelia supplemented with TS from unstressed and stressed fish were used for unidirectional flux experiments under asymmetrical conditions (FW apical/L15 basolateral) only. Unidirectional fluxes were conducted after the immediate introduction of apical FW (T 0–6 hr) and then again during the latter hours of a 24-hr experimental period (T 8–24 hr). In these preparations, TER was elevated in epithelia treated with TS prepared from stressed fish during both flux periods, however, TEP did not significantly differ at the corresponding time intervals (Table 2). Treatment with stressed TS resulted in a general reduction in both Na^+ and Cl^- movement across the epithelia, consistent with previous observations. Effects were much more prominent at the later time period (Table 2).

During the first flux period (0–6 hr), Ussing flux ratios in epithelia treated with TS from either stressed or unstressed fish suggested a pattern of nondiffusive transport similar to that found in FBS-treated epithelia (*see* above), with Na^+ being actively extruded, whilst Cl^- was actively moving in the inward direction (Table 2). After 18 hr exposure to asymmetrical culture conditions (during the 18–24 hr flux period), active Na^+ extrusion was still apparent in both unstressed and stressed serum-treated preparations. However, Cl^- movement appeared to be passive in epithelia treated with TS from unstressed fish and active in the inward direction in preparations treated with TS from stressed fish (Table 2).

Na^+-K^+-ATPase Activity

The activity of Na^+-K^+-ATPase in cultured epithelia was significantly elevated in preparations treated with TS when compared to epithelia treated with equivalent amounts (10%) of FBS in the L15 culture medium (Fig. 7a). However, treatment of epithelia with TS prepared from stressed fish, either with or without the addition of RU486, had no significant effect on Na^+-K^+-ATPase activity when compared to preparations treated with TS from unstressed fish (Fig. 7b).

Discussion

The current study illustrates that methodology originally described by Wood & Pärt (1997) for the cul-

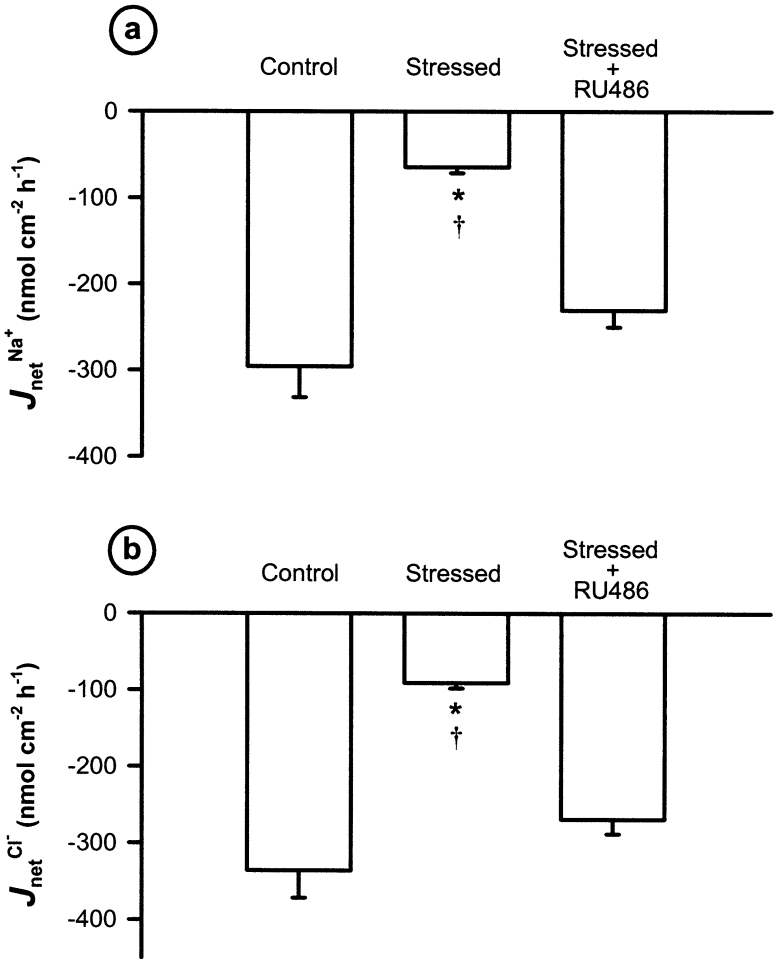

Fig. 6. Net Na^+ (*a*) and Cl^- (*b*) flux rates (J_{net}, basolateral to apical movement) across tilapia pavement cell epithelia under asymmetrical conditions (FW apical/L15 basolateral) with tilapia serum (supplementing basolateral media) derived from control (unstressed) fish, stressed fish and stressed fish + RU486 (1.5 μM). Data are expressed as mean value ± SEM (n = 8–10). An asterisk denotes significant difference between epithelia supplemented with control (unstressed) and stressed serum, while † denotes significant difference ($P < 0.05$) between epithelia supplemented with stressed serum and those supplemented with stressed serum + RU486.

Table 2. Comparison between Na^+ and Cl^- flux rates, electrical characteristics, and Ussing flux ratio analysis (J_{in}/J_{out}) of cultured tilapia (*Oreochromis niloticus*) pavement cell epithelia under asymmetrical conditions (freshwater apical/L15 basolateral) using media supplemented with tilapia serum collected from unstressed (control) and stressed (stress) fish.

		Unidirectional flux rates (nmols cm^{-2} hr^{-1})			Flux ratio (×10^3)			
		Influx	Efflux	Net flux	Predicted	Observed	TER (kΩ cm^2)	TEP (mV)
T 0–6 hr:								
Control:	Na^+	2.14 ± 0.37	−252.55 ± 53.60	−250.41 ± 53.23	16.21 ± 0.81	8.83 ± 0.47*	7.76 ± 1.15	−7.20 ± 0.61
	Cl^-	4.91 ± 1.04	−263.46 ± 58.80	−258.55 ± 57.77	11.28 ± 0.99	18.97 ± 0.76*		
Stress:	Na^+	1.30 ± 0.03	−153.36 ± 16.21	−152.06 ± 16.18	16.47 ± 1.57	8.83 ± 0.89*	14.55 ± 1.26†	−9.03 ± 0.70
	Cl^-	1.92 ± 0.19	−157.16 ± 18.29	−155.24 ± 18.23	8.76 ± 0.44	12.84 ± 1.76		
T 18–24 hr:								
Control:	Na^+	4.67 ± 0.47	−856.04 ± 103.82	−851.36 ± 103.52	23.63 ± 1.28	5.60 ± 0.45*	3.16 ± 0.31	−4.63 ± 0.34
	Cl^-	13.12 ± 1.24	−874.62 ± 106.66	−861.50 ± 105.91	18.95 ± 1.14	15.48 ± 1.28		
Stress:	Na^+	1.73 ± 0.17†	−243.14 ± 15.38†	−241.42 ± 15.35†	13.07 ± 0.62	7.1 8 ± 0.66*	9.01 ± 0.70†	−3.50 ± 0.49
	Cl^-	4.88 ± 0.59†	−247.58 ± 15.74†	−242.70 ± 15.57†	11.29 ± 0.44	19.83 ± 2.17*		

* denotes significant difference ($P < 0.05$) between predicted and observed flux ratio.
† denotes significant difference ($P < 0.05$) between control and stress epithelia.
All data are expressed as mean values ± SEM (n = 5 for all groups).

ture of gill epithelia derived from dispersed gill cells of freshwater rainbow trout, can potentially be developed for other freshwater fish species with few modifications. Similar to cultured trout gill epithelia, the cultured tilapia gill epithelia exhibit many of the characteristics expected of an in vitro branchial preparation derived from a freshwater fish (for detailed discussion, *see* Wood & Pärt, 1997; Wood et al., 1998;

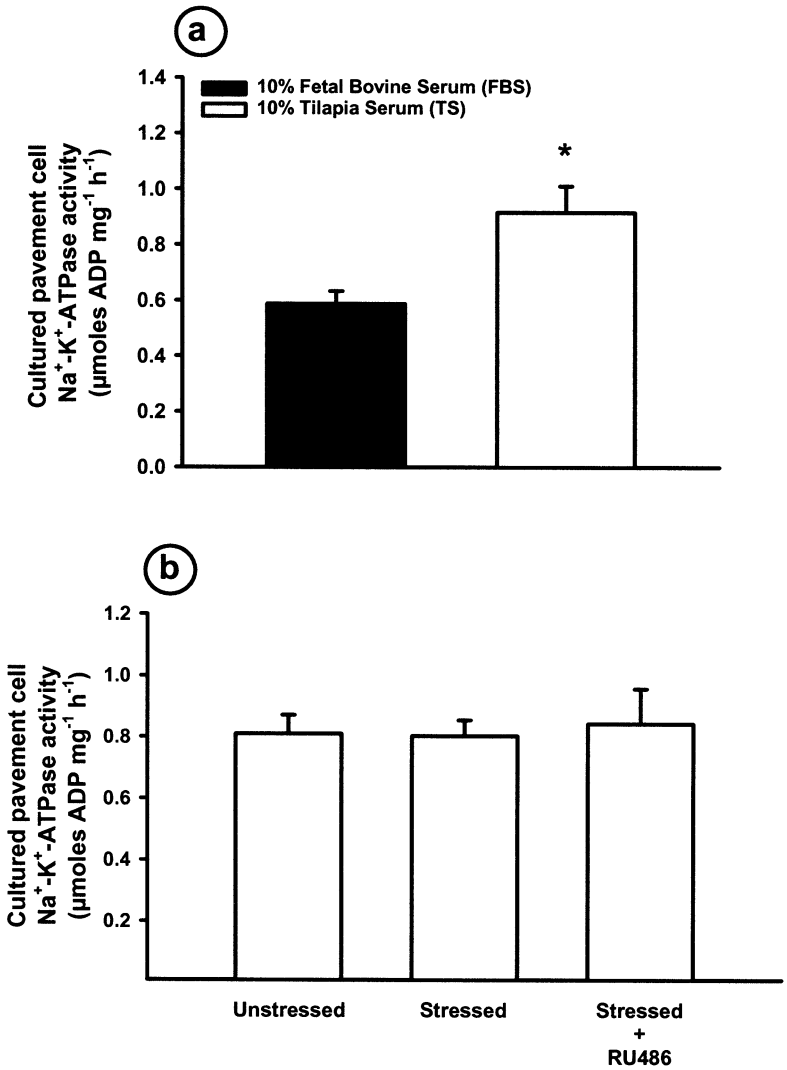

Fig. 7. Epithelial Na^+-K^+-ATPase activity in (*a*) cultured tilapia pavement cell epithelia supplemented with either fetal bovine serum (FBS, $n = 4$) or tilapia serum (TS, $n = 8$) and (*b*) cultured tilapia pavement cell epithelia supplemented with either TS derived from unstressed fish ($n = 16$), stressed fish ($n = 16$) or stressed fish + RU486 ($n = 8$). Data are expressed as mean values ± SEM. In (*a*) the asterisk denotes significant difference between epithelia supplemented with FBS and TS.

Fletcher et al., 2000; Kelly et al., 2000). It is clear, however, that cultured tilapia pavement cell epithelia, in the absence of hormone or alternative serum supplements, exhibit the same limitations as do those derived from rainbow trout. Specifically, both fail to exhibit active Na^+ and Cl^- transport rates from apical freshwater in vitro that mimic those found in vivo. This allows us to address our first line of questioning in that species differences may not be the reason for low in vitro transport rates in these model systems.

Cortisol treatment of cultured tilapia pavement cell epithelia resulted in physiological alterations similar to those observed in cultured rainbow trout pavement cell epithelia (Kelly & Wood, 2001c). Cortisol treatment increased epithelial "tightness" (observed as elevated TER and reduced paracellular permeability) and reduced transepithelial ion movement. The physiological consequences and benefits of cortisol were most clearly observed under simulated in vivo conditions (freshwater apical/L15 basolateral) where basolateral to apical ion movement (analogous to branchial ion loss in vivo) was greatly reduced. Increased epithelial "tightness" likely resulted from enhanced structural development of epithelial tight junctions, as a reduction in tight junction permeability is normally identified by elevated TER and/or reductions in paracellular movement of radiolabelled tracers (Cereijido, 1992). Dexamethasone, which in the current study elicited a response similar to that of cortisol, has been demonstrated to enhance tight junction formation and expression in cultured higher vertebrate epithelial tissues (Zettl et al., 1992; Stelwagen, McFadden & Demmer, 1999).

Tight junctions create the major barrier regulating paracellular movement of water and solutes across vertebrate epithelia (Anderson, 2001). Therefore, tight junction permeability complements transcellular transport mechanisms by defining the degree and selectivity of ion and solute movement, resulting in an important tissue-specific contribution to net transepithelial transport (Anderson, 2001). As we

have discussed previously (Kelly & Wood, 2001c), there is no current role for paracellular flux in models for ion uptake in freshwater fish and consequently, reductions in paracellular permeability would only be adaptive in minimizing diffusive ion losses in freshwater fish. Reductions in gill permeability, and diffusive ion losses, are likely to be of critical importance over a wide spectrum of physiologically challenging scenarios, in many of which there is already an acknowledged role for elevated circulating cortisol levels. For example, adaptation to very dilute freshwater (Perry & Wood, 1985; Perry & Laurent, 1989) or the presence of an environmental/physical stressor (Barton & Iwama, 1991; Wendelaar Bonga, 1997) both challenge hydromineral balance in freshwater fish. Both are documented to benefit from and/or induce elevated circulating cortisol levels (Perry & Wood, 1985; Laurent & Perry, 1990; Wendelaar Bonga, 1997). These observations are particularly interesting given that cortisol is often considered to be a seawater-adapting hormone. However, an increasing number of studies clearly suggest that cortisol plays a dual role in fish hydromineral balance, benefiting ionoregulatory homeostasis in both seawater and freshwater (for reviews *see* McCormick, 1995; Perry, 1997).

Cultured tilapia pavement cell epithelia responded to the glucocorticoid agonist dexamethasone in a manner similar to that observed for cortisol. The response of pavement cell epithelia to both cortisol and dexamethasone is consistent with the presence of gill pavement cell glucocorticoid receptors (Uchida et al., 1998). The response of pavement cell epithelia to dexamethasone is also consistent with the cortisol-like effects of dexamethasone in the branchial tissues of fish (McCormick & Bern, 1989). These effects, and also those of cortisol, are thought to be mediated via a single corticosteroid (glucocorticoid) receptor type (Mommsen, Vijayan & Moon, 1999) and ligand binding studies using the gill tissues of various fish species have demonstrated that corticosteroid potency orders generally favour synthetic glucocorticoids such as dexamethasone (for review *see* Mommsen et al., 1999). However, recent studies at both the molecular (Colombe et al., 2000) and organismal level (Sloman, Desforges & Gilmour, 2001) have suggested the presence of a putative mineralocorticoid-like receptor in fish. On the other hand, Chen, Chan & Munro (1997) have reported only a single corticosteroid receptor type in red tilapia and in our study, the glucocorticoid antagonist (receptor blocker) RU486 appeared to abolish all effects that either dexamethasone or cortisol had on the physiology of cultured tilapia pavement cell epithelia. These effects were seen at RU486 dosage levels exceeding either dexamethasone or cortisol by 10-fold and are consistent with other studies employing RU486 as an antagonist of glucocorticoid action, where similar high doses of antagonist were required to achieve complete blockade (Emilie et al., 1984). It is also notable that RU486 itself had no significant glucocorticoid effect on pavement cell epithelia, even at the highest dose used. These results, and a growing body of literature addressing corticosteroid receptor presence in teleost fish (*see* Mommsen et al., 1999), provide an impetus for further studies, in which species differences and cellular targeting should be examined.

Na^+-K^+-ATPase activity in cultured gill epithelia from tilapia was similar to previously reported activity levels for trout pavement cell epithelia in culture (Kelly & Wood, 2001b,c; 2002). These activity levels were also consistent with those expected from an epithelium lacking chloride cells and composed exclusively of pavement cells, the former of which contribute to overall gill Na^+-K^+-ATPase activity to a greater extent (Sargent, Thomson & Bornancin, 1975; Kültz & Jürss, 1993). Of further interest, however, is that Na^+-K^+-ATPase activity levels found in the cultured tilapia gill preparation were almost identical to those found in the opercular membrane of freshwater tilapia (McCormick, 1990), a surrogate model for the freshwater fish gill, which does contain chloride cells (Burgess et al., 1998). Despite these similarities, supplementing cultured tilapia pavement cell epithelia with TS in place of FBS at comparable levels resulted in an increase in Na^+-K^+-ATPase activity. The factors in TS responsible for upregulating the activity of Na^+-K^+-ATPase do not seem to be cortisol or other corticosteroids since neither elevated cortisol levels in stressed fish-derived TS nor RU486 had any effect on activity, despite marked differences in the physiological characteristics (TER, paracellular permeability, passive ion flux rates) of these epithelia. We have previously demonstrated that cortisol, when added as a sole supplement even at very high doses, has no effect on pavement cell Na^+-K^+-ATPase activity in cultured rainbow trout preparations (Kelly & Wood, 2001c). In contrast, when chloride cells are present, chronic cortisol treatment generally stimulates branchial Na^+-K^+-ATPase activity both in vivo and in vitro (for review *see* McCormick, 1995). Therefore the results of the current study, taken together with our previous observations (Kelly & Wood, 2001c), suggest that a cortisol-induced increase in Na^+-K^+-ATPase activity is likely to originate solely from chloride cell-related changes. While it is unclear what specific factor or combinations of factors in 10% TS stimulate Na^+-K^+-ATPase activity, it would be interesting to test what effects might be seen on Na^+-K^+-ATPase activities and ion transport rates if epithelia were cultured in 100% TS.

Cultured tilapia gill epithelia respond well to homologous serum as a culture medium supplement. These results are consistent with those of Avella &

Ehrenfeld (1997), who have observed a higher degree of differentiation and increased ion transporting capacity in cultured sea bass gill epithelia supplemented with 10% homologous sea bass serum. In contrast, other studies have suggested that homologous serum used at levels of 10% may not be beneficial to cultured fish cells, and possibly even toxic (Fryer, Yusha & Pilcher, 1965; Collodi & Barnes, 1990; Pärt et al., 1993). It is not clear why these differences in response occur. Interestingly, Pärt et al. (1993) made a distinction between the use of fresh serum and previously frozen serum, only the latter of which was "toxic." However, in both the current study and the study of Avella & Ehrenfeld (1997), serum was frozen after collection and prior to use as a supplement, therefore eliminating the "freezing variable" as a potential solution to contrasting results. The only distinction that is obvious between the conflicting observations is that in all the studies where homologous serum was reported to be "toxic", the cultured cells were derived from salmonid species of fish.

Certain aspects of the physiology of cultured tilapia pavement cell epithelia differ from previously observed cultured trout pavement cell epithelia. First of all, the phenomenon of poor cell attachment in tilapia gill epithelia presented with cortisol prior to the formation of a confluent epithelium does not occur in cultured trout gill cells (Kelly & Wood, 2001b,c). This apparent species-specific difference should be an important consideration when using cortisol as a supplement in primary gill cell cultures of other fish species. Similar to our observations of tilapia pavement cells, cultured mouse fibroblast cell adhesiveness is significantly impaired by the glucocorticoid agonist dexamethasone, even at levels considerably lower than those used in the current study (Jung-Testas & Baulieu, 1983). Secondly, upon exposure to asymmetrical culture conditions, both cultured tilapia and trout gill epithelia exhibit an increase in TER. However, in cultured trout gill epithelia, a parallel increase in epithelial paracellular permeability occurs and a concomitant decrease in transcellular permeability is postulated to account for the overall increase in TER (for detailed discussion *see* Wood et al., 1998; Gilmour et al., 1998). In contrast, tilapia gill epithelia exhibit a reduction in paracellular permeability under identical conditions and appear to maintain this lowered paracellular permeability for at least 24 hr. These observations suggest important differences in how the branchial epithelia of these species adapt to environmental change. Thirdly, the pattern of Na^+ and Cl^- transport across cultured tilapia pavement cell epithelia under symmetrical conditions indicated active Na^+ uptake and passive Cl^- movement. In the current study, this pattern was not significantly affected by cortisol (although there was general increase in the observed flux ratio for Cl^- under symmetrical conditions). In contrast, equivalent doses of cortisol in trout pavement cell epithelia promoted apparent active Na^+ and Cl^- uptake under identical conditions (Kelly & Wood, 2001c). These results were consistent with currently popular models of ion transport across freshwater fish gills, where Na^+ uptake is believed to occur across pavement cells (for review *see* Perry, 1997).

Under asymmetrical culture conditions, Na^+ movement appeared to be active in the outward direction, while Cl^- was generally transported in the inward direction. This phenomenon is not consistent with current models for nondiffusive ion movement across the gills of freshwater fish where Cl^- uptake is believed to occur across the chloride cell in association with Cl^-/HCO_3^- exchange (for review *see* Perry, 1997). However, Cl^- uptake across cultured pavement cell epithelia derived from freshwater rainbow trout has also been observed to occur (Wood et al., 1998; Kelly & Wood, 2001b,c, 2002). Therefore it would appear that pavement cells in primary culture, exposed to pseudo in vivo conditions, either exhibit transport characteristics that differ from those in vitro or that current models for ion transport across freshwater fish gill are as yet incomplete. In light of these observations, it is also interesting to note that Avella & Ehrenfeld (1997) observed Cl^- secretion across an analogous cultured pavement cell epithelium derived from the gills of a seawater fish, a phenomenon that contradicts current models of ion transport across the marine fish branchial epithelium where Cl^- secretion is attributed entirely to chloride cells (Zadunaisky, 1984; Wood & Marshall, 1994; Marshall, 1995).

In conclusion, it seems likely that mechanisms of ion transport across the gills of freshwater fish will remain controversial until the development of suitable in vitro models for study. While cultured gill epithelia represent a promising new technical approach, it is clear that further methodological refinement is required to achieve normal ion transport characteristics. Nevertheless, it is also clear that these models are already providing valuable insight into permeability and electrical characteristics of the gills, which were previously unknown, and interspecies differences in these characteristics. Taken together, these observations provide a firm incentive for further development and study of cultured gill models.

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References

Anderson, J.M. 2001. Molecular structure of tight junctions and their role in epithelial transport. *News Physiol. Sci.* **16:**126–130

Avella, M., Ehrenfeld, J. 1997. Fish gill respiratory cells in culture: a model for Cl^- secreting epithelia. *J. Membrane Biol.* **156:**87–97

Barton, B.A., Iwama, G.K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann. Rev. Fish Dis.* **1:**3–26

Burgess, D.W., Marshall, W.S., Wood, C.M. 1998. Ionic transport by the opercular epithelia of freshwater acclimated tilapia (*Oreochromis niloticus*) and killifish (*Fundulus heteroclitus*). *Comp. Biochem. Physiol.* **121A:**155–164

Carlsson, C. & Pärt, P. 2001. 7-Ethoxyresorufin *O*-deethylase induction in rainbow trout gill epithelium cultured on permeable supports: asymmetrical distribution of substrate metabolites. *Aquatic Toxicol.* **54:**29–38

Cereijido, M. 1992. Tight Junctions. CRC Press, Boca Raton

Chen, K.M., Chan, W.K., Munro, A.D. 1997. Dexamethasone receptors and their distribution in the brain of the red tilapia. *Fish Physiol. Biochem.* **16:**171–179

Collodi, P., Barnes, D.W. 1990. Mitogenic activity from trout embryos. *Proc. Natl. Acad. Sci.* USA **87:**3498–3502

Colombe, L., Fostier, A., Bury, N., Pakdel, F., Guiguen, Y. 2000. A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. *Steroids* **65:**319–328

Emilie, D., Galanaud, P., Baulieu, E.E., Dormont, J. 1984. Inhibition of in vitro immunosuppressive effects of glucocorticosteroids by a competitive antagonist RU-486. *Immunol. Lett.* **8:**183–186

Fletcher, M., Kelly, S.P., Pärt, P., O'Donnell, M.J., Wood, C.M. 2000. Transport properties of cultured branchial epithelia from freshwater rainbow trout: a novel preparation with mitochondria-rich cells. *J. Exp. Biol.* **203:**1523–1537

Fryer, J.L., Yusha, A., Pilcher, K.S. 1965. The in vitro cultivation of tissue and cells of Pacific salmon and steelhead trout. *Ann. N.Y. Acad. Sci.* **126:**566–586.

Gilmour, K.M., Pärt, P., Prunet, P., Pisam, M., McDonald, D.G., Wood, C.M. 1998. Permeability and morphology of a cultured epithelium from the rainbow trout during prolonged apical exposure to freshwater. *J. Exp. Zool.* **281:**531–545

Jung-Testas, I., Baulieu, E.E. 1983. Inhibition of glucocorticoid action in cultured L-929 mouse fibroblasts by RU 486, a new anti-glucocorticosteroid of high affinity for the glucocorticosteroid receptor. *Exp. Cells Res.* **147:**177–182

Kelly, S.P., Fletcher, M., Pärt, P., Wood CM. 2000. Procedures for the preparation and culture of "reconstructed" rainbow trout branchial epithelia. *Meth. Cell Sci.* **22:**153–163

Kelly, S.P., Wood, C.M. 2001a. The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J. Exp. Biol.* **204:**4115–4124

Kelly, S.P., Wood, C.M. 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, S.P., Wood, C.M. 2001c. Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. *Am. J. Physiol.* **281:**R811–R820

Kelly, S.P., Wood, C.M. 2002. Prolactin effects on cultured pavement cell epithelia and pavement cell plus mitochondria-rich cell epithelia from freshwater rainbow trout gills. *Gen. Comp. Endocrinol.* In press

Kirschner, L.B. 1970. The study of NaCl transport in aquatic animals. *Am. Zool.* **10:**365–375

Kültz, D., Jürss, K. 1993. Biochemical characterization of isolated branchial mitochondria-rich cells of *Oreochromis mossambicus* acclimated to fresh water or hyperhaline sea water. *J. Comp. Physiol.* **163B:**406–412

Laurent, P., Perry, S.F. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout *Salmo gairdneri*. *Cell Tiss. Res.* **259:**429–442

Marshall, W.S. 1995. Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. *In:* Fish Physiology, Cellular and Molecular Approaches to Fish Ionic Regulation, Vol 14. C.M. Wood, T.J. Shuttleworth editors. pp. 1–23. Academic Press, San Diego

McCormick, S.D. 1990. Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. *Am. J. Physiol.* **259:**R857–R863

McCormick, S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+,K^+-ATPase activity. *Can. J. Fish. Aquat. Sci.* **50:**656–658

McCormick, S.D. 1995. Hormonal control of gill Na^+-K^+-ATPase and chloride cell function. *In:* Fish Physiology, Cellular and Molecular Approaches to Fish Ionic Regulation, Vol 14. C.M. Wood, T.J. Shuttleworth editors. pp. 285–315. Academic Press, San Diego

McCormick, S.D., Bern, H.A. 1989. *In vitro* stimulation of Na^+-K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill. *Am. J. Physiol.* **256:**R707–R715

Mommsen, T.P., Vijayan, M.M., Moon, T.W. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fisheries* 9:211–268

Pärt, P., Norrgren, L., Bergstrom, E., Sjoberg, P. 1993. Primary cultures of epithelial cells from rainbow trout gills. *J. Exp. Biol.* **175:**219–232

Perry, S.F. 1997. The chloride cell: Structure and function in the gills of freshwater fish. *Ann. Rev. Physiol.* **59:**325–347

Perry, S.F., Laurent, P. 1989. Adaptational responses of rainbow trout to lowered external NaCl concentration: Contribution of the branchial chloride cell. *J. Exp. Biol.* **147:**147–168

Perry, S.F., Wood, C.M. 1985. Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. *J. Exp. Biol.* **116:**411–433

Sargent, J.R., Thomson, A.J., Bornancin, M. 1975. Activities and localization of succinic dehydrogenase and Na^+/K^+-activated adenosine triphosphotase in the gills of fresh water and sea water eels (*Anguilla anguilla*). *Comp. Biochem. Physiol.* **51B:**75–79

Sloman, K.A., Desforges, P.R., Gilmour, K.A. 2001. Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. *J. Exp. Biol.* **204:**3953–3961

Stelwagen, K., McFadden, H.A., Demmer, J. 1999. Prolactin, alone or in combination with glucocorticoids, enhances tight junction formation and expression of the tight junction protein occludin in mammary cells. *Mol. Cell. Endocrinol.* **156:**55–61

Uchida, K., Kaneko, T., Tagawa, M., Hirano, T. 1998. Localization of cortisol receptor in branchial chloride cells in chum salmon fry. *Gen. Comp. Endocrinol.* **109:**175–185

Wendelaar Bonga, S.E. 1997. The stress response in fish. *Physiol. Rev.* **77:**591–625

Wood, C.M., Gilmour, K.M., 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, C.M., Marshall, W.S. 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, C.M., Pärt, P. 1997. Cultured branchial epithelia from freshwater fish gills. *J. Exp. Biol.* **200:**1047–1059

Zadunaisky, J.A. 1984. The chloride cell: the active transport of chloride and paracellular pathways. *In:* Fish Physiology, Gills, part B, Ion and Water Transfer. W.S. Hoar, D.J. Randall, editors. Vol. 10. pp. 129–176. Academic Press, Orlando

Zettl, K.S., Sjaastad, M.D., Riskin, P.M., Parry, G., Machen, T.E., Firestone, G.L. 1992. Glucorticoid-induced formation of tight junctions in mouse mammary epithelia cells *in vitro*. *Proc. Natl. Acad. Sci.* USA **89:**9069–9073