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Cortisol reduces paracellular permeability and increases occludin abundance in cultured trout gill epithelia

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ABSTRACT

A role for the tight junction (TJ) protein occludin in the regulation of gill paracellular permeability was investigated using primary cultured "reconstructed" freshwater (FW) rainbow trout gill epithelia composed solely of pavement cells. Cortisol treatment reduced epithelial permeability characteristics, measured as changes in transepithelial resistance (TER) and paracellular [^{3}H]PEG-4000 flux. Cortisol also reduced net Na^+ flux rates when epithelia were exposed to apical FW. cDNA encoding for the TJ protein occludin was cloned from rainbow trout and found to be particularly abundant in gill tissue. In cultured gill preparations, occludin immunolocalized to the TJ complex and transcript abundance dose-dependently increased in response to cortisol treatment in association with reduced paracellular permeability. Occludin protein abundance also increased in response to cortisol treatment. However, occludin mRNA levels did not change in response to apical FW exposure, and [^{3}H]PEG-4000 permeability did not decrease. These data support a role for occludin in the endocrine regulation of paracellular permeability across gill epithelia of fishes.

1. Introduction

The tight junction (TJ) complex plays an important role in the regulation of epithelial permeability in vertebrates. It is composed of a number of transmembrane and cortical proteins and the presence, as well as abundance, of different TJ proteins appears to be a key element in TJ heterogeneity between and within tissues. Occludin is a tetraspan transmembrane TJ protein that is broadly expressed in vertebrate epithelia (Feldman et al., 2005). Since the initial discovery of occludin (Furuse et al., 1993), numerous studies have suggested an important role for this TJ protein in the regulation of epithelial permeability (Feldman et al., 2005). More specifically, an increase in occludin abundance is most often associated with reductions in paracellular permeability across diverse epithelia and endothelia (Feldman et al., 2005). However, the majority of work conducted on the physiological function of occludin in vertebrate epithelia has been accomplished using mammalian models. Recently it has been proposed that occludin may contribute to the regulation of epithelial permeability in aquatic vertebrates under conditions of altered hydromineral status (Chasiotis and Kelly, 2008, 2009; Chasiotis et al., 2009). In this regard, occludin has been found to be abundant in epithelia that regulate salt and water balance in fishes, such as the gill, kidney and gastrointestinal (GI) tract (Chasiotis and Kelly, 2008; Chasiotis et al., 2009). In the freshwater (FW) goldfish kidney, a spatially distinct distribution pattern of occludin can be observed along the nephron (Chasiotis and Kelly, 2008). The "tight" distal tubules and collecting ducts of the nephron exhibit robust occludin immunoreactivity (occludin-ir), while the "leakier" proximal regions of the nephron exhibit little or no occludin-ir. Furthermore, in the gill tissue of goldfish, occludin protein abundance significantly increased when fish were acclimated to ion-poor water (Chasiotis et al., 2009). This has been proposed to contribute to a reduction in the permeability of the paracellular pathway across the gill (Chasiotis et al., 2009) and is consistent with the observations of Cuthbert and Maetz (1972) who reported that the gill epithelium of goldfish exposed to ion-poor conditions exhibits a considerable reduction in outwardly directed ion movement. This presumably results in a beneficial reduction in passive ion loss in an environment where limitations are set on active ion acquisition.

Despite the above observations, and to the best of our knowledge, there are no studies that have related alterations in the specific machinery of the TJ complex in fishes with measured changes in epithelial permeability. Primary cultured gill epithelial models that allow for the "reconstruction" of FW gill epithelia *in vitro* present an appropriate tool for such studies (see Wood and Pärt, 1997; Fletcher et al., 2000; Kelly et al., 2000; Kelly and Wood, 2002). These models exhibit passive transport and perme-

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ability characteristics that closely mimic the *in vivo* characteristics of the FW gill epithelium (Wood and Pärt, 1997; Fletcher et al., 2000; Kelly et al., 2000; Kelly and Wood, 2002). Furthermore, corticosteroid (cortisol) treatment of cultured gill epithelia results in a distinct epithelial tightening effect which is driven at least in part by reduced paracellular permeability properties (Kelly and Wood, 2001, 2002). This sensitivity to cortisol provides a simple means by which to manipulate transepithelial as well as paracellular permeability characteristics and these observations in fishes are in accord with the tightening effects of corticosteroids on other vertebrate epithelia and endothelia (Zettl et al., 1992; Stelwagen et al., 1999; Antonetti et al., 2002; Förster et al., 2005).

Based on this knowledge, the objectives of the current study were to examine cortisol-induced alterations in the permeability characteristics of a cultured gill epithelium prepared from FW rainbow trout and relate alterations in paracellular permeability to modifications in occludin abundance. We hypothesized that if occludin is involved in regulating the barrier properties of gill epithelia in fishes, occludin abundance should increase in association with reductions in paracellular permeability.

2. Materials and methods

2.1. Cultured rainbow trout gill epithelia

The preparation and culture of gill epithelia from FW rainbow trout was carried out in order to produce preparations composed of gill pavement cells only. Methods have been detailed by Kelly et al. (2000) and were originally developed by Wood and Pärt (1997). Briefly, cultured epithelia were prepared using stock rainbow trout (200–450 g) held in flow-through dechlorinated tap water (approximate composition in mmol l^{-1}: $[Na^+]$ = 0.55–0.59, $[Cl^-]$ = 0.70–0.92, $[Ca^{2+}]$ = 0.76–0.90, $[K^+]$ = 0.04–0.05, pH range 7.4–8.0) at 10–12 °C. Cells were initially cultured in 25 cm^2 flasks in Leibovitz's L-15 media supplemented with 2 mmol l^{-1} glutamine and 6% foetal bovine serum (L15). At confluence (~4–5 days), cells were harvested and seeded into cell culture inserts (0.9 cm^2 growth area, 0.4 μm pore size, 1.6×10^6 pores/cm^2 pore density; Falcon BD, Mississauga, ON, Canada). Culture inserts were housed in companion cell culture plates (Falcon BD) and after cell seeding (at a density of 700,000 cells/culture insert), epithelia were allowed to develop a stable transepithelial resistance (TER) (over ~5 days) with L15 culture media present on both apical and basolateral surfaces of the preparation (i.e. symmetrical culture conditions). The treatment of epithelia with cortisol was conducted according to methods previously outlined by Kelly and Wood (2001). Two physiologically relevant doses of cortisol were selected (50 and 500 ng/ml) based on the aforementioned study as well as observations made by Kelly and Wood (2002). Cortisol was added to culture media in flasks 24 h after first seeding and when epithelia were cultured in inserts, cortisol was added to the basolateral media only. Therefore, epithelia cultured in flasks and subsequently in inserts were exposed to cortisol for a total of 9–10 days. In a separate set of experiments which were conducted in order to determine whether alterations in occludin transcript abundance translated into alterations in occludin protein abundance, only flask-cultured epithelia were used to harvest tissue. The rationale for this was that inserts did not provide enough protein for Western blot analysis after conducting the extraction protocol used in these studies (see Section 2.6). Therefore, in these experiments cultured epithelial cells were exposed to a single dose of cortisol (500 ng/ml) for 5 days. This period of time and dose of cortisol is sufficient to elicit a significant increases in TER and accompanying decrease in [^{3}H]PEG-4000 permeability (see Section 2.2) in cultured epithelia (data not shown). Finally, in experiments where FW was added to the apical side of cultured preparations (i.e. asymmetrical culture conditions), temperature-equilibrated sterile dechlorinated FW (composition as detailed above) was used.

2.2. Electrophysiological, [^{3}H]PEG-4000 and net Na^+ flux measurements

Measurements of TER were conducted using chopstick electrodes (STX-2) fitted to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL, USA). TER was recorded every 24 h after seeding cells onto culture inserts to monitor epithelial development. Under asymmetrical conditions, TER was monitored at 3 h intervals. All measurements of TER are reported as background-corrected values taking into account the resistance measured across a "vacant" culture insert containing appropriate solutions.

Paracellular permeability across cultured epithelia was examined using the paracellular permeability marker, [^{3}H]polyethylene glycol (molecular mass 4000 Da; 'PEG-4000'; NEN-Dupont, Mississauga, ON, Canada) according to previously detailed methods and calculations (Kelly and Wood, 2001). [^{3}H]PEG-4000 (1 μCi) was added to the basolateral compartment of culture preparations and its appearance in the apical compartment monitored as a function of time and epithelial surface area. Net Na^+ flux rates from basolateral to apical compartments under asymmetrical culture conditions were measured and calculated according to methods detailed by Kelly and Wood (2001).

2.3. Cloning and qRT-PCR analysis of rainbow trout occludin cDNA

Total RNA was isolated from trout gill tissue using TRIzol® Reagent (Invitrogen Canada Inc., Burlington, ON, Canada), according to manufacturer's instructions. Gill RNA was then treated with DNase I (Amplification Grade; Invitrogen Canada Inc.) and first-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase and Oligo(dT)$_{12-18}$ primers (Invitrogen Canada Inc.).

Using a ClustalX multiple sequence alignment of occludin coding sequences from 9 different species (human [NM_002538]; mouse [NM_008756]; rat [NM_031329]; cow [NM_001082433]; dog [NM_001003195]; platypus [XM_001510548]; opossum [XM_001380557]; frog [NM_001088474]; zebrafish [NM_212832]), degenerate primers were designed based on highly conserved regions. A partial rainbow trout cDNA fragment was amplified by reverse transcriptase PCR (RT-PCR) using occludin degenerate primers under the following reaction conditions: 1 cycle of denaturation (95 °C, 4 min), 40 cycles of denaturation (95 °C, 30 s), annealing (53 °C, 30 s) and extension (72 °C, 30 s), respectively, final single extension cycle (72 °C, 5 min) (0.2 μM dNTP, 2 μM forward and reverse primers, 1× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase) (Invitrogen Canada Inc.). Gel electrophoresis (1% agarose for ~90 min at 5 V/cm) verified a PCR product at the predicted amplicon size of ~796 bp. The DNA fragment was excised from the gel and purified using a QIAquick Gel Extraction Kit (QIAGEN Inc., Mississauga, ON, Canada). The purified amplicon was sequenced in the York University Core Molecular Biology and DNA Sequencing Facility (Department of Biology, York University, Toronto, ON, Canada). A partial coding sequence (CDS) of trout occludin was confirmed using a Basic Local Alignment Search Tool (BLAST) search.

To obtain the complete rainbow trout occludin CDS, both 5′- and 3′-rapid amplification of cDNA ends (RACE) PCR was performed using a SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA) as per manufacturer's instructions. RACE-PCR products were resolved by electrophoresis, purified and sequenced as described above in order to complete the trout occludin CDS, GenBank accession number GQ476574.

2.4. Occludin expression profile and qRT-PCR analysis of occludin mRNA abundance in rainbow trout tissues

Quantitative real-time PCR (qRT-PCR) was used to examine occludin mRNA distribution and abundance in discrete rainbow trout tissues, as well as occludin transcript abundance in cultured epithelia from flasks and cell culture inserts. For expression profile studies, total RNA was extracted from the following tissues: brain, eye, gill, bulbus arteriosus, atrium, ventricle, esophagus, anterior and posterior stomach, pyloric ceca, anterior intestine, middle intestine and posterior intestine, liver, gallbladder, spleen, swimbladder, kidney, muscle, adipose tissue and blood. The extraction of RNA and synthesis of cDNA from all tissues was conducted as outlined in the previous section. Primers for trout occludin (forward: 5′ CAGCCCAGTTCCTCCAGTAG 3′ and reverse: 5′ GCTCATCCAGCTCTCTGTCC 3′, predicted amplicon size ~340 bp) were designed using the CDS generated by 5′- and 3′-RACE-PCR described above. β-Actin was used as an internal control (forward: 5′ GGACTTTGAGCAGGAGATGG 3′ and reverse: 5′ GACGGAGTATTTACGCTCTGG 3′, predicted amplicon size ~354 bp). β-Actin primers were designed based on GenBank accession number AF157514.

qRT-PCR analysis of occludin and β-actin was conducted using SYBR Green I Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and a Chromo4™ Detection System (CFB-3240, Bio-Rad Laboratories Canada Ltd.) under the following conditions: 1 cycle denaturation (95 °C, 4 min) followed by 40 cycles of denaturation (95 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 30 s), respectively. To ensure that no primer–dimers or other non-specific products were synthesized during reactions, a melting curve analysis was carried out after each qRT-PCR run.

2.5. Immunolocalization of rainbow trout occludin

Trout gill epithelia cultured in inserts were allowed to develop a stable TER under symmetrical culture conditions. Epithelia were briefly rinsed with phosphate-buffered saline (PBS, pH 7.7) and fixed for 20 min at room temperature (RT) with 3% paraformaldehyde. Fixed epithelia were then permeabilized with ice-cold methanol for 5 min at −20 °C, washed with 0.01% Triton X-100 in PBS for 10 min and blocked for 1 h at RT with antibody dilution buffer (ADB; 10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS). Epithelia were incubated overnight at RT with a custom-synthesized polyclonal antibody raised in rabbit against a synthetic peptide (CHIKKMVGDYDRRA) corresponding to a 14-amino acid region of rainbow trout occludin (1:100 dilution in ADB; New England Peptide, LLC, Gardner, MA, USA). For a negative control, epithelia were also incubated overnight with ADB lacking primary antibody. Epithelia were then washed with PBS and incubated for 1 h at RT with TRITC-labeled goat anti-rabbit antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After a final wash with PBS, epithelia were excised from the insert housings using a scalpel and mounted on glass microscopy slides with Molecular Probes ProLong Antifade (Invitrogen Canada

Inc.) containing 5 μg ml^{-1} DAPI (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada). Fluorescence images were captured using a Reichert Polyvar microscope (Reichert Microscope Services, Depew, NY, USA) and an Olympus DP70 camera (Olympus Canada, Markham, ON, Canada). Adobe Photoshop CS2 software was used for contrast and brightness adjustment of entire images (Adobe Systems Canada, Toronto, ON, Canada).

2.6. Western blotting and protein quantification of rainbow trout occludin

Trout gill epithelia cultured in flasks were briefly rinsed with ice-cold PBS, scraped using a plastic cell scraper into a lysis buffer (10 mmol l^{-1} Tris–HCl, pH 7.5, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} NaCl, 1 mmol l^{-1} PMSF) containing 1:200 protease inhibitor cocktail (Sigma–Aldrich Canada Ltd.) and then homogenized by repeatedly passing through a 26G needle. Homogenates were centrifuged at 20,000 × *g* for 25 min at 4 °C to remove cell debris and the resultant supernatant was collected. To separate cytosolic and membrane protein fractions, supernatant was further centrifuged at 54,000 × *g* for 90 min at 4 °C and the remaining pellet (membrane fraction) was resuspended in a solubilizing buffer (50 mmol l^{-1} Tris–HCl, pH 7.5, 1 mmol l^{-1} EDTA, 1% Triton-X-100, 0.5% SDS, 1 mmol l^{-1} PMSF) containing 1:200 protease inhibitor cocktail. Pellet protein concentrations were determined using the Bradford assay (Sigma–Aldrich Canada Ltd.) according to the manufacturer's guidelines. Samples (2 μg) were boiled at 100 °C in 6X sample buffer (360 mmol l^{-1} Tris–HCl, 30% glycerol, 12% SDS, 600 mmol l^{-1} DTT, 0.03% Bromophenol Blue) and subjected to SDS-PAGE on 12% acrylamide gels followed by semi-dry transfer to polyvinylidene difluoride (PVDF) membranes for 2 h. Membranes were blocked for 1 h at RT in blocking solution (Tris-buffered saline (TBST; 10 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 0.05% Tween-20, pH 7.4) containing 5% nonfat dry skim milk powder), incubated overnight at 4 °C with the custom rabbit polyclonal anti-trout occludin antibody described above (1:1000 dilution in blocking solution), washed with TBST, incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories Canada Ltd.; 1:5000 in blocking solution), and then washed once more prior to antigen reactivity detection using an Enhanced Chemiluminescence Plus Western blotting system (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC, Canada).

After occludin detection, membranes were stripped and incubated with mouse monoclonal anti-actin antibody (JLA20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; 1:500 in blocking solution) and HRP-conjugated goat anti-mouse antibody (Bio-Rad Laboratories Canada Ltd.; 1:5000 in blocking solution), respectively as an internal loading control. The abundance of occludin and actin were quantified using a Molecular Imager Gel Doc XR+ System and Quantity One 1D analysis software (Bio-Rad Laboratories Canada Ltd.). Occludin is expressed as a normalized value relative to actin.

2.7. Statistical analysis

All data are expressed as mean values ± SEM (*n*), where *n* represents the number of filter inserts, except in Fig. 2, where *n* represents the number of fish sampled, and Fig. 5 where *n* represents the number of cell culture flasks. Significant differences ($P \leq 0.05$) between groups were determined using either a two-way or one-way analysis of variance as appropriate followed by a Student–Newman–Keuls test (Sigmastat Software; Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Cultured gill epithelia and effects of cortisol on TER, [^{3}H]PEG-4000 and net Na^+ flux

Under symmetrical culture conditions (L15 apical/L15 basolateral), untreated cultured trout gill epithelia preparations (i.e. 0 ng/ml cortisol added) exhibited a mean TER of ~2900 Ωcm^2 (Fig. 1a). The addition of cortisol to culture media (50 or 500 ng/ml) dose-dependently elevated TER (Fig. 1a). In accord with cortisol-induced elevations in TER, cortisol treatment of cultured gill preparations dose-dependently reduced the movement (efflux) of the paracellular permeability marker PEG-4000 (Fig. 1b).

When exposed to asymmetrical culture conditions (FW apical/L15 basolateral), dose-dependent effects of cortisol on TER and [^{3}H]PEG-4000 permeability were also observed (Fig. 1a and b). However, FW addition to the apical side of preparations significantly elevated TER in both the control and 50 ng/ml treated preparations relative to measurements under symmetrical conditions (Fig. 1a). In 500 ng/ml treated preparations, no additional significant increase in TER was observed (Fig. 1a). In association with changes in TER, [^{3}H]PEG-4000 flux significantly increased in control and 50 ng/ml cortisol-treated epithelia under asymmetrical

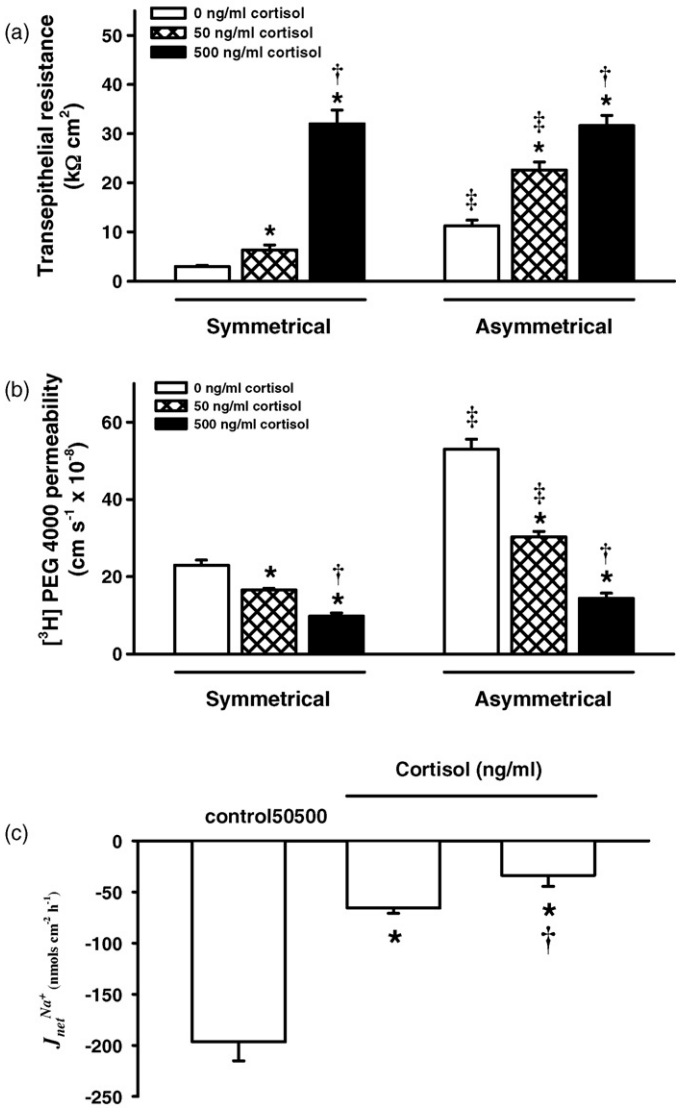

Fig. 1. Effect of cortisol on (a) transepithelial resistance (TER), (b) [^{3}H]PEG-4000 permeability and (c) net Na^+ flux rates across cultured rainbow trout gill epithelia. TER and [^{3}H]PEG-4000 permeability were measured under both symmetrical (L15 apical/L15 basolateral) and asymmetrical (FW apical/L15 basolateral) culture conditions while net Na^+ flux rates were measured under asymmetrical culture conditions only. Data are expressed as mean values ± SEM (n = 4–6). *Significant difference ($P \leq 0.05$) from control treatment (0 ng/ml cortisol), †Significant difference between cortisol doses (50 versus 500 ng/ml cortisol); ‡significant difference ($P \leq 0.05$) between symmetrical and asymmetrical culture conditions.

culture conditions (Fig. 1b) but no significant increase in [^{3}H]PEG-4000 flux was observed in epithelia treated with 500 ng/ml cortisol. Under asymmetrical culture conditions, cortisol dose-dependently reduced the efflux rates (basolateral to apical movement) of Na^+ (Fig. 1c).

3.2. Cloning of trout occludin cDNA, tissue expression profile and immunolocalization

Sequencing and analysis of the 1500 bp CDS of rainbow trout occludin revealed an open reading frame for a 500-amino acid protein that exhibited 46–48% amino acid sequence identity with mammalian occludin (i.e. human, mouse, rat, cow, dog, platypus and opossum), ~50% identity with frog occludin and ~63% identity with zebrafish occludin. Using qRT-PCR analysis, occludin tran-

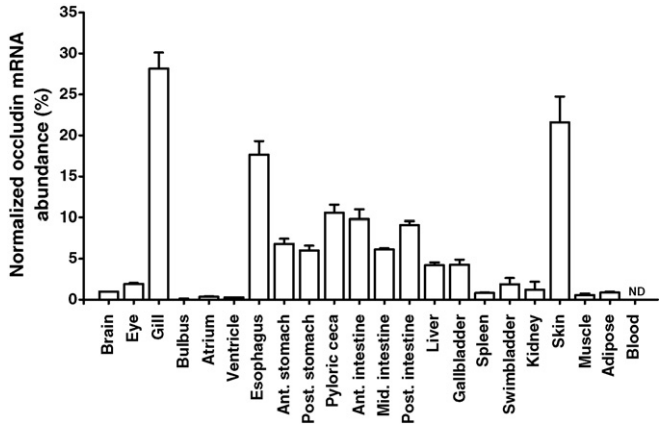

Fig. 2. qRT-PCR generated occludin expression profile for discrete rainbow trout tissues. Occludin mRNA abundance was normalized with β-actin and the abundance of occludin in each tissue was expressed relative to the brain assigned a value of 1.0. Data are expressed as mean values ± SEM ($n = 4$). Amplicon size was 340 bp and 354 bp for occludin and β-actin, respectively.

script was found to be broadly distributed in rainbow trout tissues (Fig. 2). Occludin mRNA was particularly abundant in gill tissue as well as some other tissues involved either directly or indirectly in the regulation of salt and water balance (e.g. skin and GI tract). Occludin was absent in red blood cells (a non-epithelial tissue). Immunocytochemical analysis of cultured preparations revealed occludin-ir along the cultured pavement cell periphery at the location of the TJ (Fig. 3a). A negative control exhibited no occludin-ir (Fig. 3b). Western blot analysis of cultured gill tissues using the custom-synthesized rainbow trout occludin antibody revealed a single immunoreactive band that resolved at ~70 kDa (Fig. 3c).

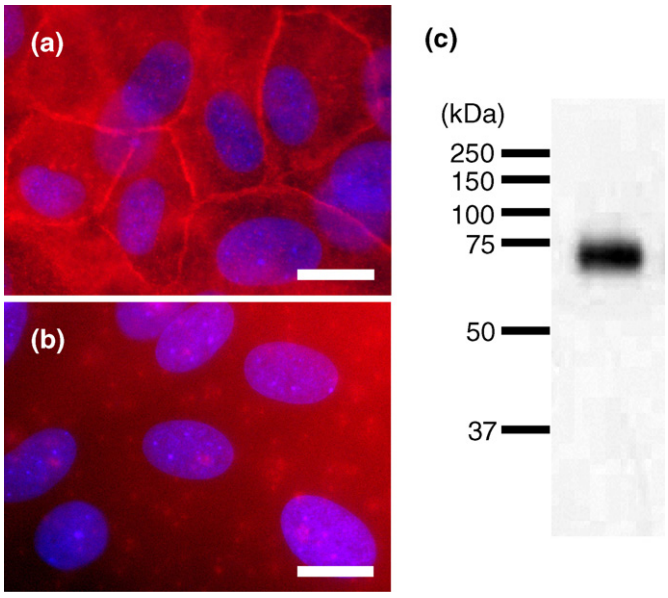

Fig. 3. (a) Immunolocalization of occludin (red) in cultured trout gill epithelia under symmetrical (L15 apical/L15 basolateral) culture conditions using a custom-synthesized rabbit polyclonal antibody raised against a synthetic region of trout occludin. Negative control (primary antibody omitted) is shown in (b). (c) Representative Western blot using the same custom-synthesized occludin antibody used for immunolocalization. A single occludin-immunoreactive band resolved at ~70 kDa. In panels (a) and (b), nuclei were stained with DAPI (blue) and each scale bar = 20 μm.

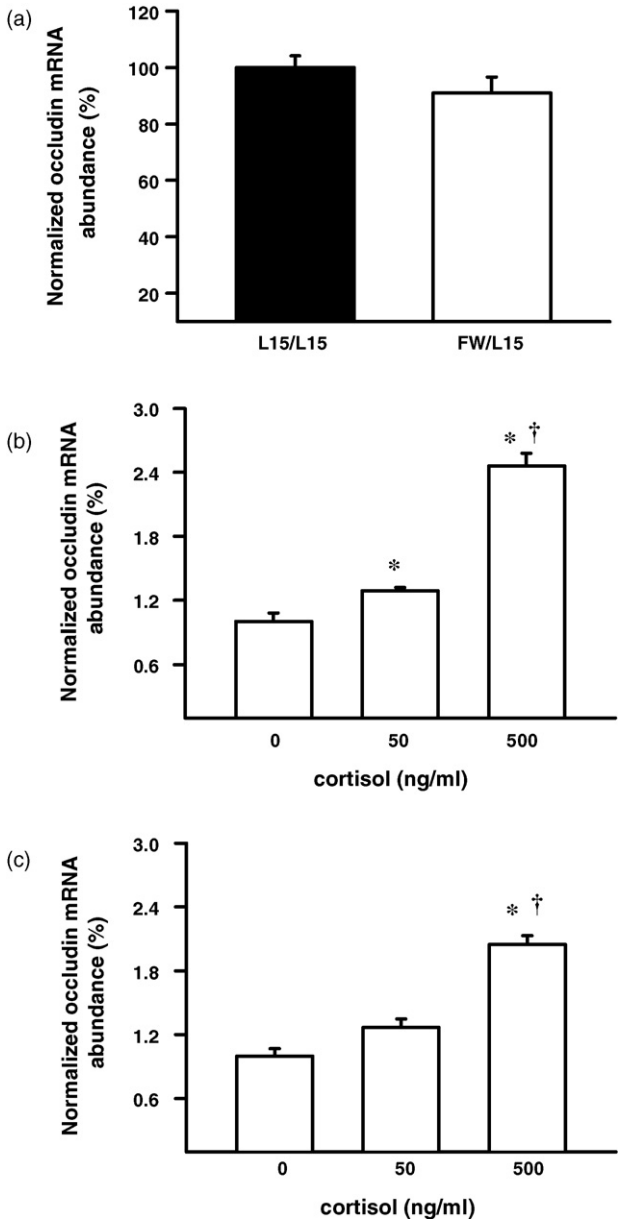

Fig. 4. Occludin mRNA abundance in response to (a) 12 h apical FW exposure and cortisol addition to media under (b) symmetrical (L15 apical/L15 basolateral) and (c) asymmetrical (FW apical/L15 basolateral) culture conditions. Data are expressed as mean values ± SEM ($n = 4$–6). In (a) β-actin normalized occludin mRNA abundance for epithelia exposed to apical FW (asymmetrical) is expressed relative to symmetrical culture conditions assigned a value of 1.0. In (b) and (c) β-actin normalized occludin mRNA abundance for cortisol-treated epithelia are expressed relative to untreated (0 ng/ml cortisol) epithelia assigned a value of 1.0. *Significant difference ($P \leq 0.05$) from control treatment (0 ng/ml cortisol); †significant difference between cortisol doses (50 versus 500 ng/ml cortisol).

3.3. *Effects of culture conditions and cortisol on occludin abundance*

In epithelia cultured on inserts, no significant differences in occludin mRNA abundance were observed between preparations held under symmetrical or asymmetrical culture conditions (Fig. 4a). In contrast, cortisol treatment significantly elevated occludin mRNA abundance in both 50 and 500 ng/ml treated epithelia in cell culture inserts under symmetrical conditions (Fig. 4b) and in 500 ng/ml treated preparations under asymmetrical culture conditions (Fig. 4c). No alterations in β-actin abundance were seen in any of these treatments (symmetrical versus asym-

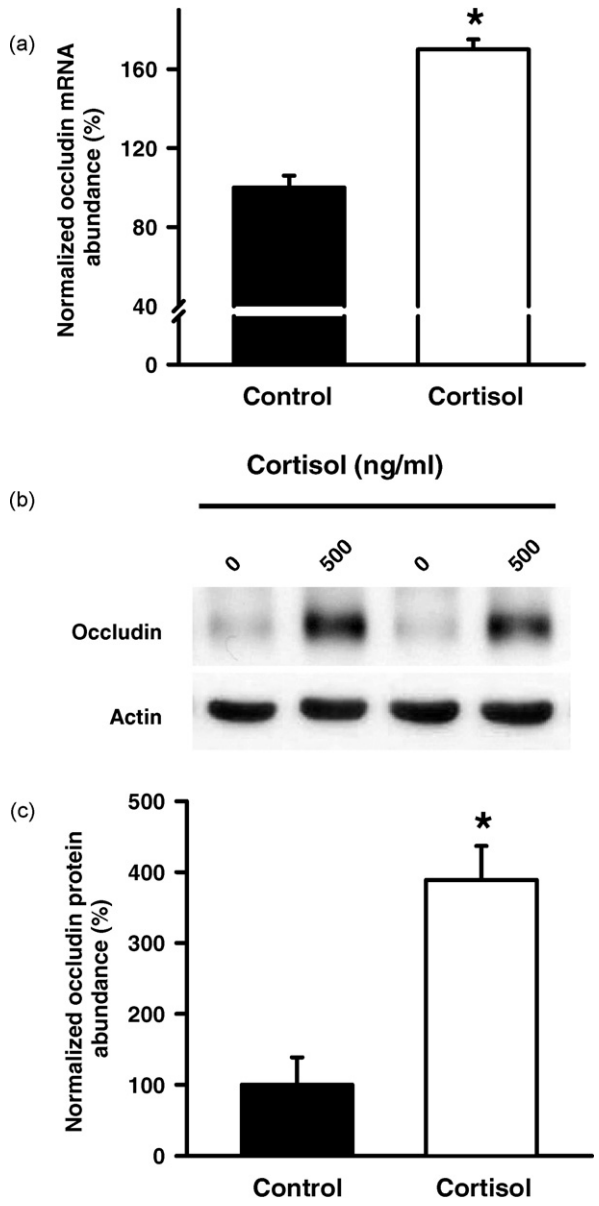

Fig. 5. Effect of cortisol (500 ng/ml) on occludin abundance in flask-cultured rainbow trout pavement cell epithelia. Alterations in occludin (a) mRNA and (b and c) protein abundance are shown. In (a) occludin mRNA abundance in cortisol-treated epithelia is normalized with β-actin and expressed relative to untreated (0 ng/ml cortisol) epithelia assigned a value of 1.0, (b) shows a representative Western blot where both occludin and actin immunoreactivity can be observed and (c) presents occludin protein abundance normalized to actin and expressed relative to untreated (0 ng/ml cortisol) epithelia assigned a value of 1.0. In (a) and (c), data are expressed as mean values ± SEM (n = 4–6). *Significant difference ($P \leq 0.05$) from untreated controls.

metrical, $P = 0.78$; control versus cortisol-treated inserts under symmetrical conditions, $P = 0.13$; control versus cortisol-treated inserts under asymmetrical conditions, $P = 0.18$).

In flask-cultured epithelia exposed to 500 ng/ml cortisol for 5 days and subsequently harvested for either mRNA or protein analysis, cortisol significantly elevated both transcript and protein abundance (Fig. 5). For these studies, actin was also used to normalize both mRNA and protein abundance. Again, no significant alterations in actin levels were observed as a result of cortisol treatment (mRNA, cortisol versus control $P = 0.19$; protein, cortisol versus control $P = 0.76$).

4. Discussion

4.1. Cultured gill epithelia and effects of cortisol on TER, [^{3}H]PEG-4000 and net Na^+ flux

The electrophysiological and permeability characteristics of untreated (0 ng/ml cortisol added) cultured trout pavement cell epithelia in symmetrical culture conditions were typical for these preparations (see Fig. 1 and Kelly et al., 2000), and cortisol treatment dose-dependently elevated TER in accord with previous reports (see Kelly and Wood, 2001). Since TER is a function of both transcellular and paracellular permeability, dose-dependent reductions in the efflux rates of the paracellular permeability marker PEG-4000 in cortisol-treated epithelia (see Fig. 1b) demonstrated that elevated TER measurements were driven at least in part by reductions in the permeability of the paracellular pathway. Under asymmetrical culture conditions, epithelia exhibited a qualitatively similar response to cortisol treatment (Fig. 1a and b). However, exposure of epithelia to apical FW caused a significant elevation in TER across control and 50 ng/ml treated preparations. In epithelia cultured in the presence of a high cortisol dose (i.e. 500 ng/ml), no further increase in TER was observed. In control epithelia, and epithelia treated with 50 ng/ml cortisol, elevated TER in response to apical FW addition occurs in conjunction with a paradoxical increase in paracellular permeability. This is usual for cultured trout gill epithelia and suggests that elevated TER under asymmetrical conditions in these preparations predominantly reflects decreased transcellular permeability, while paracellular permeability may actually increase upon acute FW exposure (Wood et al., 1998). Treatment with higher doses of cortisol appears to dampen this phenomenon, most likely by causing a reduction in both transcellular and paracellular conductance prior to FW exposure. When epithelia are exposed to apical FW, cortisol caused a dose-dependent reduction in the efflux rates (basolateral to apical movement) of Na^+. This has previously been hypothesized to reflect a beneficial reduction in passive ion loss across the gill surface of FW fishes under "stressed" conditions where cortisol would be naturally elevated in the circulatory system of a FW fish (see Kelly and Wood, 2001).

4.2. Cloning of trout occludin cDNA, tissue expression profile and immunolocalization

qRT-PCR analysis of occludin in discrete tissues of rainbow trout revealed a broad expression pattern. This is in accord with widespread distribution of occludin in other vertebrates (e.g. Furuse et al., 1993; Saitou et al., 1997; Feldman et al., 2005; Chasiotis and Kelly, 2009). In epithelia that are directly exposed to the external environment (such as the gill and skin), as well as other tissues involved in the regulation of salt and water balance in fishes, occludin was particularly abundant. Similar observations have been reported regarding occludin expression patterns in the goldfish (Chasiotis and Kelly, 2008). Occludin was also observed to immunolocalize to the periphery of cultured trout pavement cells, where TJs maintain epithelial integrity and contribute to the regulated separation of fluid compartments. This observation is in line with reports of occludin immunolocalization in numerous other vertebrate epithelia (e.g. Saitou et al., 1997; González-Mariscal et al., 2000; Ridyard et al., 2007; Chasiotis and Kelly, 2008; Chasiotis and Kelly, 2009). In goldfish gills, occludin has been reported to immunolocalize between epithelial cells in a similar manner. However, occludin has also been found to immunolocalize to the capillary endothelium (Chasiotis and Kelly, 2008). Since the cultured preparations are composed entirely of gill epithelial pavement cells, this model allows us to evaluate the effects of cortisol

on paracellular permeability properties and TJ protein mRNA abundance in gill epithelial cells only and negates any contribution from capillary endothelia.

4.3. Effects of culture conditions and cortisol on occludin abundance

In the absence of cortisol, exposure of cultured gill epithelia to asymmetrical culture conditions (i.e. apical FW) resulted in a significant increase in TER. However, occludin mRNA abundance did not significantly alter in response to asymmetrical culture conditions (see Fig. 4a). In this regard, elevated TER is not driven by a reduction in the permeability of the paracellular pathway as [^{3}H]PEG-4000 flux did not decrease in conjunction with an increase in TER (see Fig. 1 and discussion above). Therefore it is not unexpected that occludin mRNA levels did not increase. In contrast, it is noteworthy that occludin mRNA levels did not significantly decrease in association with an increase in PEG-4000 flux across cultured preparations. Despite these observations, cortisol treatment did significantly elevate occludin mRNA and protein abundance in association with both an increase in TER and decrease in PEG-4000 flux. This would suggest that the endocrine system, and more specifically corticosteroids, play an important role in regulating occludin abundance in the fish gill epithelium. In line with these observations, corticosteroid treatment of mouse mammary epithelia has been reported to result in both a decrease in paracellular permeability and increase in occludin abundance (Stelwagen et al., 1999). Furthermore, capillary endothelia of the blood–brain-barrier (BBB) as well as the blood–retinal barrier also exhibit a similar response (Antonetti et al., 2002; Förster et al., 2005). Correspondingly, it was recently shown that glucocorticoids directly up-regulate occludin expression via activated glucocorticoid receptor binding to a glucocorticoid response element within the human occludin gene promoter (Harke et al., 2008). Given that cortisol-induced alterations in the paracellular permeability of gill epithelial preparations can be almost entirely blocked using the glucocorticoid receptor antagonist RU486 (Kelly and Wood, 2002) it is plausible that a similar mechanism may also be in place in the gill epithelial tissue of fishes.

5. Conclusion

The response of occludin to corticosteroid treatment in gill epithelial tissue is qualitatively similar to reports of corticosteroid-induced occludin upregulation in the epithelia and endothelia of other vertebrates. Therefore this report highlights a conservation of fundamental alterations in the molecular machinery of the TJ complex in response to hormone treatment across vertebrate groups. While the current study strongly supports a role for occludin in regulating the barrier properties of osmoregulatory tissue such as the gill, within the confines of this study the endocrine regulation of this process appears to surpass any response to environmental change alone (i.e. no alteration in occludin mRNA abundance after switching apical culture conditions from L15 to FW). However, under natural conditions, a fish rapidly transitioning from saline conditions to FW would typically respond by elevating circulating cortisol levels (e.g. Scott et al., 2006). A key question to address in future studies with respect to the endocrine regulation of occludin and the role that this TJ protein plays in regulating epithelial permeability in tissues such as the gill will be to determine how alterations such as those described in the current study fit into the broader scheme of endocrine-mediated alterations in the molecular machinery of the TJ complex. For example, using a primary culture system similar to the cultured trout gill preparations used in this study, Bui et al. (2010) have recently demonstrated that mRNA encoding for 9 of 12 salinity-responsive claudin TJ proteins in puffer fish (*Tetraodon nigroviridis*) are found in cultured pavement cell epithelia and differentially respond to physiologically relevant doses of cortisol *in vitro*. Furthermore, Tipsmark et al. (2009) have also reported alterations in claudin mRNA abundance in cortisol-treated gill explants from salmon, albeit at doses higher than used here. These observations, together with the present findings, provide momentum for further study.

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