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RESEARCH ARTICLE

Glucocorticoid and mineralocorticoid receptors regulate paracellular permeability in a primary cultured gill epithelium

Scott P. Kelly* and Helen Chasiotis

Department of Biology, York University, Toronto, ON, Canada, M3J 1P3

*Author for correspondence (spk@yorku.ca)

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SUMMARY

The role of corticosteroid receptors (CRs) in the regulation of gill permeability was examined using a primary cultured trout gill epithelium. The epithelium expressed both glucocorticoid receptors (GR1 and GR2) and a mineralocorticoid receptor (MR), and cortisol treatment significantly increased transepithelial resistance (TER) and decreased paracellular [^{3}H]PEG-4000 flux. Epithelial permeability was unaffected by deoxycorticosterone or aldosterone. The GR antagonist RU486 as well as MR antagonists spironolactone and RU26752 significantly reduced, but did not completely block, the effects of cortisol. The MR antagonist eplerenone was without effect. Only RU486 + spironolactone or RU486 + RU26752 treatment completely suppressed the effects of cortisol. On its own, RU486 had cortisol-like effects which could be blocked by spironolactone, suggesting that although RU486 is a GR antagonist, in this system it may also have agonistic properties that are mediated through the MR. The GR agonist dexamethasone increased TER and reduced [^{3}H]PEG-4000 flux across cultured epithelia and was unaffected by MR antagonists. Therefore, alterations in transcript abundance of select tight junction (TJ) proteins were examined in response to cortisol, dexamethasone (a GR agonist) and RU486 (as a MR agonist). Occludin and claudin-7, -8d, -12 and -31 mRNA were significantly elevated in response to cortisol, dexamethasone or RU486 treatment. Claudin-3a mRNA was significantly elevated in response to cortisol or dexamethasone only, and claudin-28b and -30 mRNA were significantly altered following cortisol or RU486 treatment only. The data support a role for the GRs and MR in regulating gill permeability and suggest that TJ proteins are responsive to cortisol through both or individual CR types.

Key words: claudin, occludin, ZO-1, RU486, spironolactone, RU26752, aldosterone, deoxycorticosterone.

INTRODUCTION

The role of corticosteroids in the regulation of vertebrate salt and water balance is well documented (for a review, see McCormick and Bradshaw, 2006). In aquatic vertebrates such as fish, this phenomenon has been the subject of most attention in the Teleostei (McCormick, 2001). A long-held dogma in this area was that cortisol, which is the principal corticosteroid in teleosts, exerts both glucocorticoid and mineralocorticoid effects solely through a glucocorticoid receptor (GR) (for a review, see Mommsen et al., 1999). However, recent developments indicate that teleost fish possess both a GR and a mineralocorticoid receptor (MR), and in the case of the GR, genome duplication has resulted in the appearance of two distinct isoforms (i.e. GR1 and GR2) (for a review, see Bury and Sturm, 2007). In light of these insights, a new area to address is a potential role for the MR in the maintenance of salt and water balance in fish. In this regard, two views could be considered and these need not be mutually exclusive. The first is that teleosts possess both a glucocorticoid and a mineralocorticoid hormone, the latter of which would operate through the MR. The second view is that a single ligand (i.e. cortisol) differentially utilizes each corticosteroid receptor (CR) type. In the current literature, there is evidence to support and contest both of these positions. For example, the mineralocorticoid hormone aldosterone is an agonist of the MR in fish but is either absent or is measured at circulating levels too low to be considered physiologically relevant in teleosts (for a review, see Prunet et al., 2006). However, deoxycorticosterone is a potent agonist of the MR (Sturm et al., 2005) and is measured at circulating levels of ~0.5–10 nmol l^{-1} in trout (Campbell et al., 1976), leading to the idea that this corticosteroid could contribute to the endocrine control of osmoregulation (see Prunet et al., 2006). Despite these observations, the administration of either aldosterone or deoxycorticosterone in Atlantic salmon parr did not significantly alter the response of fish to a 24 h seawater challenge (McCormick et al., 2008), and this lack of response is in agreement with a small number of additional studies that have similarly reported an absence of effect when considering a role for either aldosterone or deoxycorticosterone in salt and water balance in fish (Chan et al., 1967; Takahashi et al., 2006; Umminger and Gist, 1973). Nevertheless, studies have reported that the MR antagonist spironolactone can alter endpoints of osmoregulation in fish and that the effects of spironolactone can differ from the effects of the GR antagonist RU486 (Kiilerich et al., 2007; Scott et al., 2005; Shaw et al., 2007; Sloman et al., 2001).

Using primary cultured ‘reconstructed’ gill preparations, a role for cortisol in the regulation of gill epithelial permeability has been established (see Kelly and Wood, 2001; Kelly and Wood, 2002; Wood et al., 2002). Measurements of epithelial permeability, such as transepithelial resistance (TER), paracellular radiotracer movement (i.e. [^{3}H]PEG-4000 flux) and passive (‘outwardly directed’) ionic flux, exhibit tremendous sensitivity to cortisol treatment. For example, cultured trout gill epithelia have been shown to respond to levels of cortisol as low as 10 ng ml^{-1} (Kelly and Wood,

2001). Recent studies indicate that cortisol-induced alterations in the paracellular permeability characteristics of cultured gill preparations relate to changes in the abundance of tight junction (TJ) proteins and transcript encoding these proteins (Bui et al., 2010; Chasiotis et al., 2010). This makes cultured gill preparations well suited for studying: (1) the effects of corticosteroids on the regulation of gill permeability, (2) the potential role that CRs play in mediating corticosteroid-induced alterations in gill permeability, and (3) alterations in the molecular components of the TJ complex that may be responsible for changing the permeability properties of the paracellular pathway in gill epithelia. Indeed, regarding the role that CRs may play in mediating the effects of cortisol, previous studies using epithelia generated from the gills of freshwater tilapia (*Oreochromis niloticus*) indicated that GRs may occupy a dominant role in the regulation of gill permeability in tilapia at least, as the effects of cortisol in this species could be ablated by the GR antagonist RU486 (Kelly and Wood, 2002). In light of the above, the objective of this suite of studies was to use GR and MR agonists/antagonists to experimentally dissect the potential role that the GRs and the MR play in regulating corticosteroid-induced alterations in the permeability properties of a cultured gill preparation derived from freshwater rainbow trout.

MATERIALS AND METHODS

Cultured rainbow trout gill epithelia

Cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) gill epithelia were prepared according to methodology originally developed by Wood and Pärt (Wood and Pärt, 1997), as outlined in detail previously (Kelly et al., 2000). Cultured preparations were composed exclusively of gill pavement cells (i.e. SSI preparations) and derived from stock trout (150–750 g) held in flow-through dechlorinated tap water (composition in $\mu mol\,l^{-1}$: Na^+ 590, Cl^- 920, Ca^{2+} 900, K^+ 50, pH 7.4). Water temperature ranged from 8 to 10°C and photoperiod was 12 h:12 h L:D. Cells were initially cultured in 25 cm^2 surface area flasks for ~5 days in Leibovitz's L-15 media containing L-glutamine and supplemented with 6% fetal bovine serum. Following flask culture, pavement 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) which were housed in companion cell culture plates (Falcon BD). Twenty-four hours later, non-adherent material was removed by aspiration and preparations were cultured with L-15 media (as described above) bathing both the apical and basolateral sides of epithelia over a period of 4–5 days until a plateau in TER was measured.

Treatment of cultured gill epithelia with cortisol, dexamethasone, deoxycorticosterone, aldosterone, RU486, spironolactone, RU26752, eplerenone or various combinations of these factors (for details, see below) commenced after seeding cells into cell culture inserts. Therefore, in each case epithelia were treated with hormone or pharmacological agents for approximately 5 days. In all experiments, the above factors were added to media bathing the basolateral side of the cultured epithelia only.

Hormones and pharmacological agents

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Hormones were dissolved in appropriate solutions and stored as single use stock aliquots at –30°C until use. Cortisol was used at a single dose of 500 $ng\,ml^{-1}$, which was selected based on the response of cultured trout gill epithelia to cortisol treatment as observed in previous studies (Chasiotis et al., 2010; Kelly and Wood, 2001) as well as levels of cortisol seen in stressed salmonids (see Pottinger, 2010). Deoxycorticosterone and aldosterone were used at doses of 5, 50 and 500 $ng\,ml^{-1}$ and dexamethasone was used at a final concentration of 1.4 $\mu mol\,l^{-1}$ (i.e. ~500 $ng\,ml^{-1}$). Receptor antagonists RU486, spironolactone, RU26752 and eplerenone were used at concentrations approximately 10-fold higher than cortisol or dexamethasone (i.e. 14 $\mu mol\,l^{-1}$) unless otherwise stated. Dexamethasone, RU486 and spironolactone were dissolved in 100% ethanol while eplerenone and RU26752 were dissolved in DMSO. The final concentration of each vehicle in culture media was ≤0.1%. Ethanol or DMSO at ≤0.1% has no effect on cultured gill preparations.

TER measurements and [^{3}H]PEG-4000 permeability

TER was measured using chopstick electrodes (STX-2) fitted to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL, USA). After seeding cells into inserts, TER was recorded every 24 h to monitor epithelial development. TER is reported as a background-corrected value, taking into account the resistance measured across a 'vacant' culture insert bathed with culture media on both sides.

The paracellular permeability marker [^{3}H]polyethylene glycol (molecular mass 4000 Da, 'PEG-4000'; PerkinElmer, Woodbridge, ON, Canada) was used to examine alterations in paracellular permeability across cultured gill epithelia. The methodology and calculations have previously been outlined in detail (see Kelly and Wood, 2001). Briefly, [^{3}H]PEG-4000 (0.5 or 0.75 $\mu Ci\,ml^{-1}$) was added to the basolateral compartment of culture preparations and its appearance in the apical compartment was monitored as a function of time and epithelial surface area. [^{3}H]PEG-4000 flux experiments were run for either 12 or 18 h. TER was recorded at the start and end of each [^{3}H]PEG-4000 flux experiment. A mean of the two values recorded was used to represent TER during the flux period.

RT-PCR and qRT-PCR of rainbow trout CRs

Total RNA was isolated from rainbow trout gill tissue and cultured rainbow trout gill epithelia using TRIzol® Reagent (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's instructions. Extracted 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.). Gene-specific primers for rainbow trout corticosteroids were designed (see Table 1) and the expression of rainbow trout GR1, GR2 and MR in gill tissue and cultured gill epithelia was examined by reverse transcriptase PCR (RT-PCR) under the following reaction conditions: 1 cycle of denaturation (95°C, 4 min); 40 cycles of denaturation (95°C, 30 s), annealing (see Table 1, 30 s) and extension (72°C, 30 s); and a final single extension cycle (72°C, 5 min). Rainbow trout β-actin mRNA abundance was used as a loading control and was amplified using the primers shown in Table 1. RT-PCR amplicons were resolved by agarose gel electrophoresis and images of gels stained with ethidium bromide were captured using a Molecular Imager Gel Doc XR+ System and Quantity One 1D analysis software (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada).

qRT-PCR analysis of CR mRNA abundance was conducted using the aforementioned gene-specific CR primers, SYBR Green I Supermix (Bio-Rad Laboratories Canada Ltd) and a Chromo4™ Detection System (CFB-3240; Bio-Rad Laboratories Canada Ltd) under the following reaction conditions: 1 cycle of denaturation (95°C, 4 min), followed by 40 cycles of denaturation (95°C, 30 s),

Table 1. Primer sets and accession numbers for genes encoding rainbow trout corticosteroid receptors, tight junction proteins and β-actin

Gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	Annealing temperature (°C)	Accession no.
GR1	F: GGACTGAAACACAGCAAGGAC R: GCAATACTCGCCTCCAACAG	335	59	NM_001124730
GR2	F: AGAACACGTCTGCCATGC R: CTGGAGAAAGCGGAGGTAG	346	57	NM_001124482
MR	F: TGTGTCTGGGTAATGGTAGC R: CGTTGTTGTTGTTCTCTTGG	369	56	AY495584
Occludin*	F: CAGCCCAGTTCCTCCAGTAG R: GCTCATCCAGCTCTCTGTCC	341	58	GQ476574
Claudin-3a	F: TGGATCATTGCCATCGTGTC R: GCCTCGTCCTCAATACAGTTGG	285	60	BK007964
Claudin-7	F: CGTCCTGCTGATTGGATCTC R: CAAACGTACTCCTTGCTGCTG	261	61	BK007965
Claudin-8d	F: GCAGTGTAAAGTGTACGACTCTCTG R: CACGAGGAACAGGCATCC	200	60	BK007966
Claudin-12	F: CTTCATCATCGCCTTCATCTC R: GAGCCAAACAGTAGCCCAGTAG	255	60	BK007967
Claudin-28b	F: CTTTCATCGGAGCCAACATC R: CAGACAGGGACCAGAACCAG	310	60	EU921670
Claudin-30	F: CGGCGAGAACATAATCACAG R: GGGATGAGACACAGGATGC	297	59	BK007968
Claudin-31	F: TCGGCAACAACATCGTGAC R: CGTCCAGCAGATAGGAACCAG	311	61	BK007969
Claudin-32a	F: ATTGTGTGCTGTGCCATCC R: AGACACCAACAGAGCGATCC	321	60	BK007970
ZO-1	F: AAGGAAGGTCTGGAGGAAGG R: CAGCTTGCCGTTGTAGAGG	291	60	HQ656020
β-Actin*	F: GGACTTTGAGCAGGAGATGG R: GACGGAGTATTTACGCTCTGG	355	58	AF157514

*Primer sets from Chasiotis et al. (Chasiotis et al., 2010).
GR, glucocorticoid receptor; MR, mineralocorticoid receptor. F, forward; R, reverse.

annealing (see Table 1, 30 s) and extension (72°C, 30 s). To ensure a single product was synthesized during reactions, a melting curve was carried out after each qRT-PCR run. For all qRT-PCR analyses, CR mRNA abundance was normalized to β-actin transcript abundance.

Identification, cloning and qRT-PCR analysis of mRNA encoding trout TJ proteins

Putative rainbow trout claudin sequences were identified using a BLAST search (Altschul et al., 1997) to compare known *Fugu* and/or Atlantic salmon claudin coding sequences against a rainbow trout EST database (available at the NCBI, www.ncbi.nlm.nih.gov). ClustalX multiple sequence alignment (Larkin et al., 2007) was then used to assemble overlapping regions of EST sequences into full-length or partial coding sequences for rainbow trout claudins. The following *Fugu* sequences (and respective accession numbers) were used in the BLAST search: claudin-3a (AY554377), claudin-7b (AY554347), claudin-8d (AY554390), claudin-12 (AY554346), claudin-31 (AY554351) and claudin-32a (AY554360). The following Atlantic salmon sequences were used in the BLAST search: claudin-3a (BK006381), claudin-7 (BK006387), claudin-12 (NM_001140081), claudin-30 (BK006405) and claudin-31 (BK006406). Amino acid sequence alignment of translated rainbow trout claudins with orthologous *Fugu* and Atlantic salmon proteins revealed greater sequence similarity with salmon than with *Fugu* claudin orthologs. Rainbow trout claudins were therefore named according to Atlantic salmon claudin nomenclature (see Tipsmark et al., 2008), except for claudin-8d and claudin-32a, which were named according to *Fugu* terminology originally described by Loh et al. (Loh et al., 2004) as salmon orthologs for these claudins have yet to been identified. Full-length and partial coding sequences for rainbow trout claudin-3a, -7, -8d, -12, -30, -31 and -32a were submitted to the Third Party Annotation (TPA) database and accession numbers are shown in Table 1.

Based on highly conserved regions within coding sequences for mouse (NM_009386), rat (NM_001106266), human (NM_003257), dog (NM_001003140), chicken (XM_413773) and zebrafish (XM_001922655) ZO-1 (tight junction protein-1, TJP1) orthologs (as determined by a ClustalX multiple sequence alignment), degenerate primers were designed and used to clone a partial rainbow

trout ZO-1 cDNA fragment by RT-PCR under the reaction conditions described above (see 'RT-PCR and qRT-PCR of rainbow trout CRs'). A putative ZO-1 fragment of the predicted amplicon size was verified using agarose gel electrophoresis, purified using a QIAquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada) and then sequenced at the York University Core Molecular Biology and DNA Sequencing Facility (Department of Biology, York University, ON, Canada). A partial coding sequence for rainbow trout ZO-1 was confirmed using a BLAST search and submitted to GenBank (see Table 1).

For qRT-PCR, gene-specific primer sets for rainbow trout claudins and ZO-1 were designed based on the full-length and partial coding sequences determined above (see Table 1). To verify that gene-specific primer sets were targeting the correct genes, RT-PCR amplicons were confirmed by sequence analysis (York University Core Molecular Biology and DNA Sequencing Facility). Rainbow trout occludin mRNA was amplified using primers previously described (Chasiotis et al., 2010) and primers for rainbow trout claudin-28b were designed based on GenBank accession number EU921670 (see Table 1). qRT-PCR analysis of TJ mRNA abundance was conducted as described previously under the following reaction conditions: 1 cycle of denaturation (95°C, 4 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (see Table 1, 30 s) and extension (72°C, 30 s). TJ mRNA abundance was normalized to β-actin transcript abundance.

Statistical analysis

All data are expressed as mean values ± s.e.m., and N represents the number of cell culture inserts in each treatment group. Significant differences ($P \leq 0.05$) between groups were determined using a one-way analysis of variance (ANOVA) followed by a Holm–Sidak multiple comparison procedure (SigmaPlot Build 11.0.0.77, Systat Software Inc., www.systat.com). When data compared control *versus* cortisol-treated preparations only, a *t*-test comparison was conducted using the software cited above.

RESULTS

CRs in trout gill epithelia and the effects of cortisol on cultured gill permeability

Trout gills and cultured trout gill epithelia expressed transcripts encoding a MR as well as two GRs (see Fig. 1). These observations confirm a previous study that reported CRs in a primary cultured trout gill epithelium (see Leguen et al., 2007). Throughout these studies, baseline permeability properties of cultured gill epithelia exhibited a natural variation that is typical for these preparations (for details, see Wood et al., 1998; Kelly et al., 2000; Wood et al., 2002). However, all are within the expected range for SSI preparations and epithelia treated with cortisol always exhibited a significant increase ($P \leq 0.05$) in TER and a decrease ($P \leq 0.05$) in [^{3}H]PEG-4000 permeability (see Figs 2–4, 6 and 8). Generally, TER increased ~5-fold (i.e. between 4- and 6-fold) when treated with cortisol.

Effects of aldosterone and deoxycorticosterone on cultured gill permeability

When added at doses ranging from 5 to 500 ng ml^{-1}, aldosterone had no significant effect on TER across cultured gill preparations (Fig. 2). At the same doses, deoxycorticosterone also had no effect on TER (Fig. 2). The same preparations used for this series exhibited a significant increase in TER in response to cortisol treatment. Because aldosterone and deoxycorticosterone had no significant effect on TER, [^{3}H]PEG-4000 permeability was not examined in these preparations.

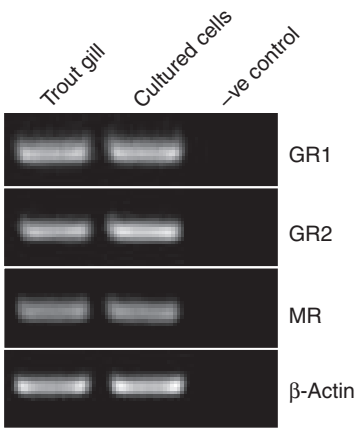

Fig. 1. An RT-PCR and gel electrophoresis profile of transcripts encoding glucocorticoid receptors 1 (GR1) and 2 (GR2), and a mineralocorticoid receptor (MR) in rainbow trout gills and a primary cultured trout gill epithelium composed exclusively of gill pavement cells (SSI preparation). β-Actin mRNA was used as a loading control.

Effects of receptor antagonists RU486, RU26752, spironolactone and eplerenone on cultured gill permeability

The addition of the GR antagonist RU486 to cortisol-treated preparations significantly reduced TER (Fig. 3A), and significantly increased [^{3}H]PEG-4000 permeability (Fig. 3B). However, blocking the effects of cortisol with RU486 did not result in TER or [^{3}H]PEG-4000 flux values comparable to those observed in untreated control epithelia. In the absence of cortisol, RU486 significantly increased TER and reduced [^{3}H]PEG-4000 permeability in cultured epithelia (Fig. 3). The MR antagonists RU26752 and spironolactone also significantly reduced TER and increased [^{3}H]PEG-4000 flux in cortisol-treated epithelia. In both cases, the effects of these MR antagonists did not result in TER or [^{3}H]PEG-4000 flux reaching values comparable with those observed in untreated control epithelia. RU26752 and spironolactone were without effect when added to epithelia alone (see Fig. 3).

The effects of eplerenone were evaluated in a separate series of epithelia from those used to evaluate the effects of RU486, RU26752 and spironolactone (see above). Eplerenone did not alter TER or [^{3}H]PEG-4000 permeability in cortisol-treated preparations and had no significant effect when added to epithelia in the absence of cortisol. In the epithelial preparations used to evaluate the effects of eplerenone, TER values (kΩ cm^2) were as follows: control 2.66±0.18 (N=6), cortisol 17.41±1.19 (N=8), eplerenone 2.31±0.12 (N=6), cortisol + eplerenone 17.49±1.88 (N=6). [^{3}H]PEG-4000 flux values ($\times 10^{-7}$ cm s^{-1}) were as follows: control 3.03±0.10 (N=6), cortisol 1.75±0.05 (N=8), eplerenone 3.26±0.07 (N=6), cortisol + eplerenone 1.69±0.06 (N=6).

Dose-dependent effects of RU486 on cultured gill permeability

When cultured preparations were treated with a range of RU486 doses (0.14, 1.4 and 14 μmol l^{-1}), a stepwise increase in TER was observed across epithelia that corresponded with increasing RU486 dose (see Fig. 4A). An increasing dose of RU486 also resulted in a downward trend in [^{3}H]PEG-4000 permeability (Fig. 4B). RU486, at all doses used, significantly reduced the cortisol-induced elevation in TER across cultured epithelia (Fig. 4A) and significantly increased [^{3}H]PEG-4000 permeability (Fig. 4B). However, in contrast to the effects of RU486 alone, varying the dose of this GR antagonist in

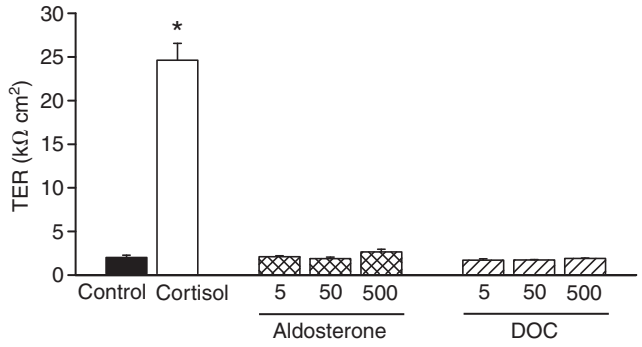

Fig. 2. The effects of cortisol and varying doses (ng ml^{-1}) of aldosterone and deoxycorticosterone (DOC) on transepithelial resistance (TER) across cultured rainbow trout gill epithelia. Data are expressed as mean values + s.e.m. (N=6 inserts per group). A one-way ANOVA was used to compare groups. *Significant difference (P≤0.05) from control (no hormone) treatment.

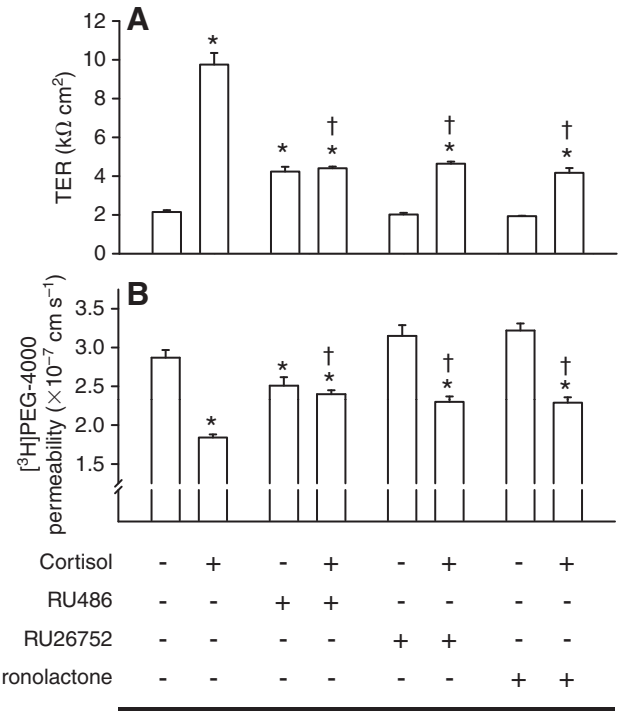

Fig. 3. The effects of cortisol, RU486, RU26752 and spironolactone on (A) TER and (B) [^{3}H]PEG-4000 permeability across cultured rainbow trout gill epithelia. Data are expressed as mean values + s.e.m. (N=4–8 inserts per group). A one-way ANOVA was used to compare groups. *Significant difference (P≤0.05) from control (no cortisol and/or corticosteroid agonist/antagonist) treatment; †significant difference between cortisol treatment (500 ng ml^{-1} cortisol) and cortisol + antagonist treatment (i.e. 500 ng ml^{-1} cortisol + GR or MR antagonist).

the presence of cortisol had no significant dose-dependent effect on TER or [^{3}H]PEG-4000 across cultured gill epithelia (Fig. 4). At all doses examined, RU486-treated epithelia exhibited significantly altered TER and [^{3}H]PEG-4000 permeability when compared with untreated controls.

Effects of dexamethasone and MR antagonists on cultured gill permeability

Dexamethasone significantly increased TER and significantly reduced [^{3}H]PEG-4000 permeability in cultured gill epithelia (see Fig. 5). The addition of eplerenone, spironolactone or RU26752 to epithelial preparations treated with dexamethasone did not significantly alter TER (Fig. 5A) or [^{3}H]PEG-4000 permeability (Fig. 5B).

Combined actions of GR and MR antagonists on cultured gill permeability

Cortisol-treated cultured gill epithelia exposed to RU486, RU26752 and spironolactone responded in a manner identical to that observed above (see 'Effects of receptor antagonists RU486, RU26752, spironolactone and eplerenone on cultured gill permeability' and Fig. 3). That is, TER significantly declined in response to each of these antagonists (Fig. 6A) and [^{3}H]PEG-4000 permeability significantly increased (Fig. 6B). In addition (and again consistent with the above observations, Fig. 3), the changes brought about by exposing cortisol-treated epithelia to these antagonists did not result in TER or [^{3}H]PEG-4000 values that were equal to those exhibited by control epithelia (see Fig. 6). In contrast, when cortisol-treated epithelia were exposed to either RU486 + spironolactone or RU486 + RU26752, TER declined to levels not significantly different from those of control epithelia (Fig. 6A). Similarly, these same combination treatments significantly increased [^{3}H]PEG-4000 permeability to levels that were not significantly different from those of control preparations (Fig. 6B). When cortisol-treated epithelia were exposed to a combination of MR antagonists (i.e. spironolactone + RU26752), TER remained significantly elevated relative to that of control epithelia (see Fig. 6A) and [^{3}H]PEG-4000 permeability was significantly reduced relative to that of control preparations (Fig. 6B).

To confirm that the cortisol-like effects of RU486 could be blocked by a MR antagonist, epithelia were treated with RU486 or RU486 + spironolactone in the absence of cortisol (see Fig. 7). In these preparations, RU486 increased TER (Fig. 7A) and reduced [^{3}H]PEG-4000 permeability (Fig. 7B) relative to control epithelia. The addition of spironolactone abrogated the effects of RU486 (Fig. 7).

Alterations in CR and TJ protein mRNA abundance following cortisol treatment

In epithelial preparations harvested to examine cortisol-induced alterations in TJ protein mRNA abundance, TER of the control group was 8.95±0.27 kΩ cm^2 (N=10) *versus* 39.10±1.96 kΩ cm^2 (N=10) for the cortisol-treated group (statistically different at P<0.05, see Fig. 8A). The dose of cortisol and duration of exposure (i.e. 500 ng ml^{-1} for 4–5 days) used throughout these studies did not alter transcript abundance of CRs in the cultured gill epithelium (see Fig. 8B). In response to cortisol treatment, mRNA encoding occludin and claudin-3a, -7, -8d, -12, -28b, -30 and -31 was significantly elevated (see Fig. 8C). In contrast, transcript encoding claudin-32a and ZO-1 did not exhibit any significant alteration in abundance following cortisol treatment (Fig. 8C). A comparison of control *versus* cortisol-treated gill epithelium β-actin mRNA abundance revealed no significant difference between groups (P=0.34).

Alterations in TJ protein mRNA abundance following dexamethasone or RU486 treatment

Alterations in TJ protein mRNA abundance were also examined following treatment with dexamethasone (a GR agonist) or RU486 (as a novel MR agonist in this system). For these studies, control

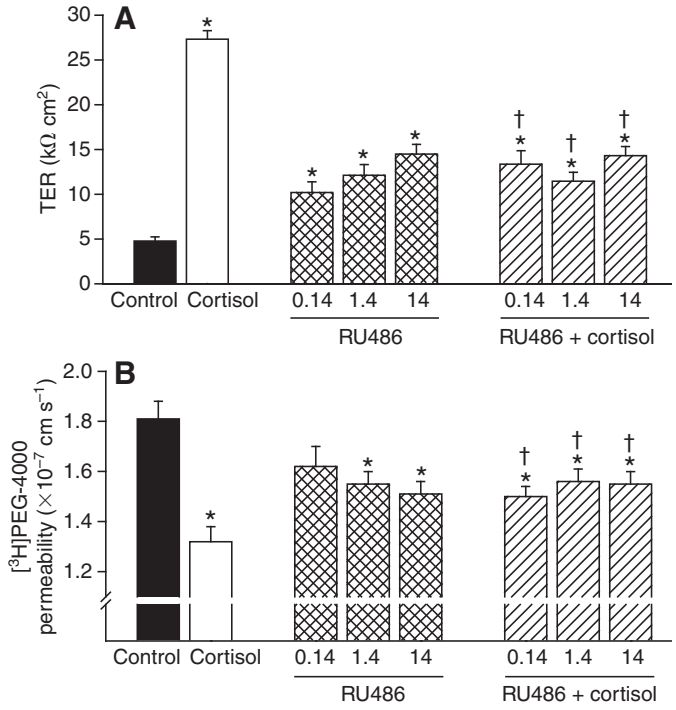

Fig. 4. The effects of varying doses (μmol l^{-1}) of RU486 on (A) TER and (B) [^{3}H]PEG-4000 permeability across cultured rainbow trout gill epithelia in the presence and absence of cortisol (500 ng ml^{-1}). Data are expressed as mean values + s.e.m. (N=6 inserts per group). A one-way ANOVA was used to compare groups. *Significant difference (P≤0.05) from control (no cortisol and/or RU486) treatment; †significant difference between cortisol treatment (500 ng ml^{-1} cortisol) and cortisol + RU486 treatment.

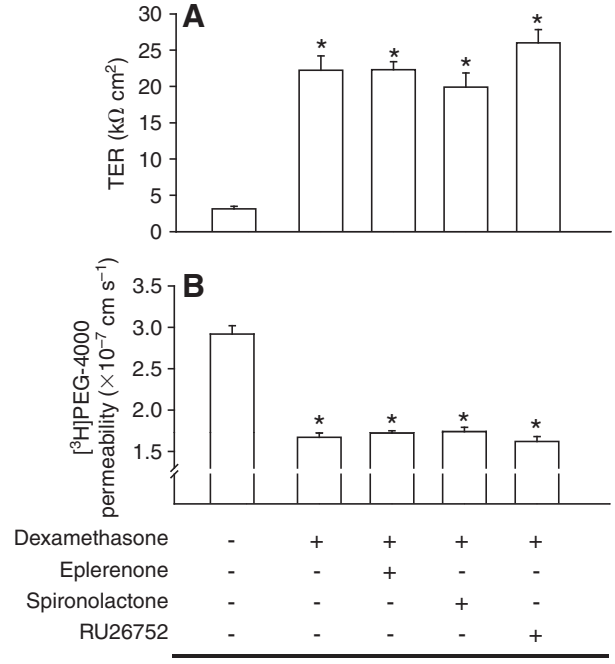

Fig. 5. The effects of dexamethasone or dexamethasone + MR antagonist (eplerenone, spironolactone or RU26752) on (A) TER and (B) [^{3}H]PEG-4000 permeability across cultured rainbow trout gill epithelia. Data are expressed as mean values + s.e.m. (N=8 inserts per group). A one-way ANOVA was used to compare treatments. *Significant difference (P≤0.05) from control (no dexamethasone or MR antagonist) treatment.

preparations exhibited a TER of 1.77±0.24 kΩ cm^2 (N=5). Dexamethasone-treated preparations exhibited a TER of 28.51±1.86 kΩ cm^2 (N=5) and RU486-treated preparations exhibited a TER of 7.27±1.50 kΩ cm^2 (N=5). Statistical analysis indicated that the TER of dexamethasone-treated preparations was significantly greater than that of both control and RU486-treated epithelia. The TER of RU486-treated epithelia was significantly greater than the TER of control epithelia.

In response to dexamethasone treatment, transcript encoding occludin and claudin-3a, -7, -8d, -12 and -31, and ZO-1 significantly increased in cultured gill preparations (see Fig. 9). Dexamethasone had no significant effect on mRNA encoding claudin-28b, -30 (see Fig. 9) or -32a (data not shown). When compared with untreated control epithelia, RU486 treatment of cultured gill preparations resulted in a significant increase in transcript abundance of all TJ proteins examined except for claudin-3a (Fig. 9) and -32a (data not shown). A comparison of β-actin mRNA abundance between control epithelia and those treated with dexamethasone or RU486 revealed no significant alteration in transcript abundance (P=0.20).

DISCUSSION

The effect of corticosteroids on the permeability characteristics of select vertebrate epithelia and endothelia is well established. Cultured epithelial and endothelial preparations derived from tissues as diverse as the rodent mammary gland (Zettl et al., 1992), mouse brain [cerebral endothelial cells (Förster et al., 2005; Weidenfeller et al., 2005)] and bovine retina [bovine retinal endothelial cells (Antonetti et al., 2002; Felinski et al., 2008)] have been reported to exhibit corticosteroid-induced alterations in permeability endpoints that reflect a 'tightening' response (i.e. increased TER and/or reduced paracellular radiotracer flux). These changes occur following treatment with GR agonists such as dexamethasone or hydrocortisone (cortisol) (see Felinski et al., 2008; Förster et al., 2005; Weidenfeller et al., 2005; Zettl et al., 1992). However, additional evidence also supports a role for MR agonists as endocrine factors capable of reducing the permeability of vertebrate epithelia. For example, deoxycorticosterone has been reported to reduce TJ conductance in the cortical collecting duct of the rabbit nephron (Sansom and O'Neil, 1985) and aldosterone has been reported to increase TER across epithelia generated from A6 kidney cells (Paccolat et al., 1987). Despite these observations, the response of epithelia to MR agonists appears to be less consistent than that observed for GR agonists. More specifically, evidence also suggests that aldosterone can decrease TER across cell lines derived from the cortical collecting duct of the rodent nephron (Bens et al., 1999; Djelidi et al., 2001; Le Moellic et al., 2005) as well as the toad urinary bladder (Handler et al., 1979).

Dexamethasone or cortisol treatment of primary cultured gill epithelia derived from freshwater fish gill tissue (see Chasiotis et al., 2010; Kelly and Wood, 2001; Kelly and Wood, 2002; Wood et al., 2002) (this study) causes alterations in permeability characteristics that are consistent with glucocorticoid-induced changes observed in mammalian preparations (Felinski et al., 2008; Förster et al., 2005; Weidenfeller et al., 2005; Zettl et al., 1992). However, to the best of our knowledge there is no functional evidence to suggest a role for either MR ligands or MRs in the regulation of gill permeability. As such, the current study provides

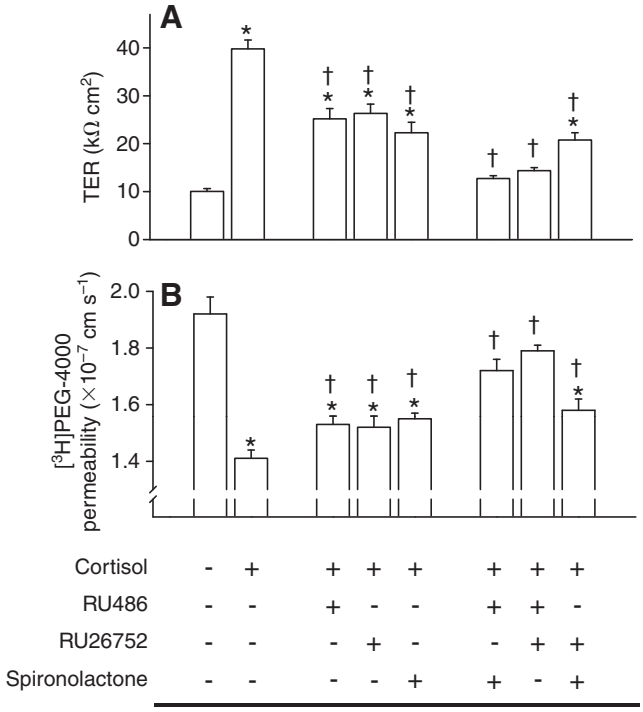

Fig. 6. The effect of the combined use of MR or GR antagonists on cortisol-induced alterations in (A) TER and (B) [^{3}H]PEG-4000 permeability across cultured rainbow trout gill epithelia. Data are expressed as mean values + s.e.m. (N=5–7 inserts per group). A one-way ANOVA was used to compare treatments. *Significant difference (P≤0.05) from control (no cortisol and/or corticosteroid agonist/antagonist) treatment; †significant difference between cortisol treatment (500 ng ml^{-1} cortisol) and cortisol + antagonist treatment (i.e. 500 ng ml^{-1} cortisol + GR and/or MR antagonist).

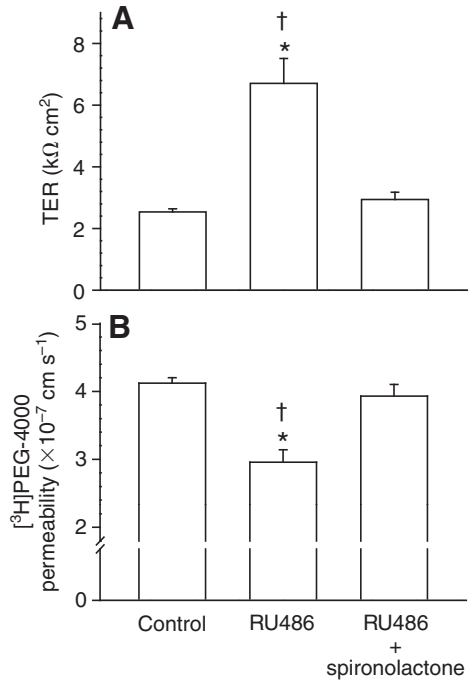

Fig. 7. The effect of RU486 and RU486 + spironolactone on (A) TER and (B) [^{3}H]PEG-4000 permeability across cultured rainbow trout gill epithelia. Data are expressed as mean values + s.e.m. (N=10–11 inserts per group). A one-way ANOVA was used to compare treatments. *Significant difference (P≤0.05) from control (no agonist/antagonist) treatment; †significant difference between RU486 and RU486 + spironolactone treatment.

the first data to suggest that the mineralocorticoid hormones aldosterone and deoxycorticosterone have no apparent effect on the epithelial permeability of fish gills. In the case of aldosterone, this is not surprising because all evidence to date suggests that aldosterone is either absent or present only at very low levels in teleost fish (Prunet et al., 2006). However, the presence of deoxycorticosterone at levels biologically relevant enough to impact salt and water balance in teleost fish has been considered plausible (Prunet et al., 2006; Sturm et al., 2005). Despite these reports, recent *in vivo* studies do not provide evidence to support a role for aldosterone or deoxycorticosterone in the regulation of salt and water balance in Atlantic salmon parr at least (McCormick et al., 2008), and data from the current study are in accord with these observations.

In contrast, cortisol appears to be capable of altering properties of the paracellular pathway in the gill epithelium by working through both GRs and the MR. This is supported by the antagonistic effects of RU486, spironolactone and RU26752. However, none of these antagonists alone was capable of completely blocking the effects of cortisol. With regard to RU486, this drug has previously been reported to block the epithelial 'tightening' effect of cortisol in cultured tilapia gill epithelia (Kelly and Wood, 2002), but in tilapia preparations RU486 was not reported to have an agonistic effect. This differs from the current suite of studies where cortisol-like agonistic effects of RU486 were observed. The response of trout gill epithelia to RU486 does not appear to be related to the use of a high dose as changes in TER and [^{3}H]PEG-4000 permeability following a 10- or 100-fold reduction in RU486 dose were modest. Therefore, although RU486 can act as a GR antagonist in cultured gill epithelia, there appear to be species-specific differences in the response of primary cultured gill preparations to the drug itself. The agonistic properties of RU486 make it difficult to utilize this drug as a specific tool for GR blockade in this model. In light of these observations, the effects of the GR agonist dexamethasone were evaluated in cultured trout gill epithelia in order to bring into play a tool that could be used to alter permeability characteristics of the epithelium specifically through GRs. Like cortisol, dexamethasone increased TER and reduced [^{3}H]PEG-4000 flux across cultured trout gill epithelia. This response was consistent with the effects of dexamethasone on cultured tilapia gill epithelia (Kelly and Wood, 2002) as well as other vertebrate epithelia (e.g. Zettl et al., 1992). As this drug is a GR agonist, these effects should be mediated through the GRs only. But because of the unexpected results generated by RU486, a precautionary measure of evaluating the effects of MR antagonists (spironolactone, RU26752 and eplerenone) in conjunction with dexamethasone treatment was taken. These MR antagonists did not alter the response of the gill epithelium to dexamethasone, confirming that this drug can be used as an exclusive GR agonist in this model.

The antagonistic effects of spironolactone on cortisol-mediated physiological change in primary cultured trout gill epithelia are consistent with the ability of this drug to alter transcellular transport properties of the gill epithelium, as reported in previous studies (see Kiilerich et al., 2007; Scott et al., 2005). In addition, spironolactone did not alter the permeability of the primary cultured gill epithelium

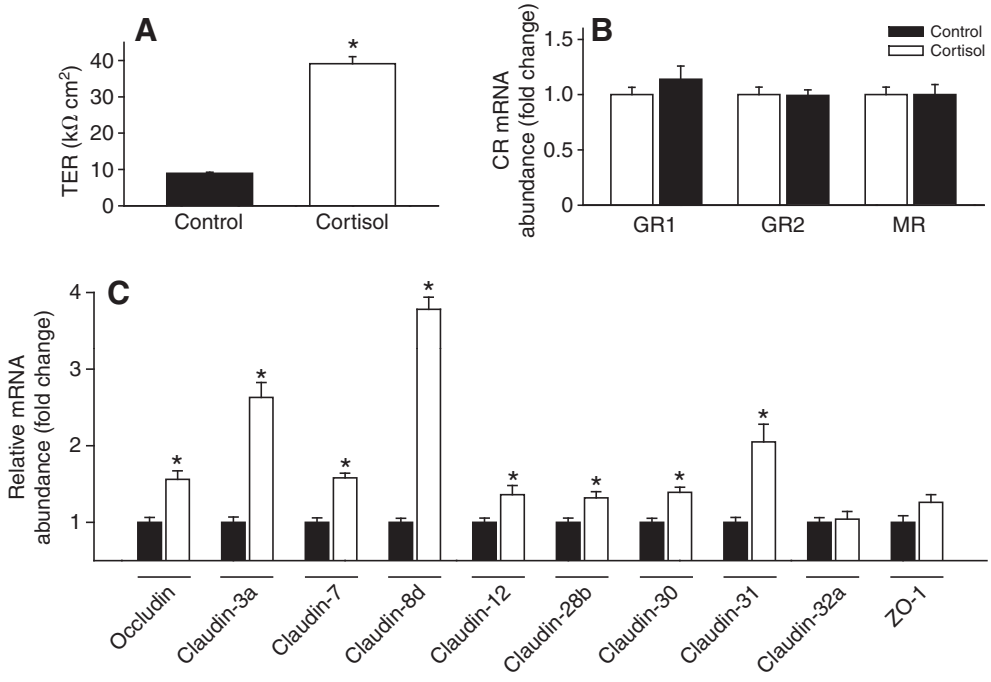

Fig. 8. The effect of cortisol (500 ng ml^{-1}) on (A) TER, (B) GR1, GR2 and MR transcript abundance and (C) occludin, claudin and ZO-1 transcript abundance in cultured trout gill epithelia. CR and TJ protein mRNA abundance was normalized with β-actin and is expressed relative to that of untreated control epithelia, which was assigned a value of 1.0. Data are expressed as mean values + s.e.m. (N=10 inserts per group). For each gene, control and cortisol-treated preparations were compared using a t-test. *Significant difference (P≤0.05) from control epithelia.

when present alone. This contrasts with recent reports that suggest spironolactone can act as an agonist when it is used to treat COS-7 cells transiently transfected with trout MR (Sturm et al., 2005) or CV-1 cells transiently transfected with zebrafish MR (Pippal et al., 2011). Furthermore, in mammalian models, the *in vivo* off target effects of spironolactone (e.g. gynocomastia, male impotence, menstrual abnormalities) resulting from antiandrogenic and progestomimetic actions of the drug are well documented (for a review, see Agarwal and Mirshahi, 1999). This last observation led to the development of MR antagonists such as RU26752 and eplerenone, which do not cause the same side effects (Agarwal and Mirshahi, 1999; Garthwaite and McMahon, 2004). Therefore, it is encouraging to note that RU26752 elicited a physiological response in cultured gill epithelia that was very similar to that produced by spironolactone. To the best of our knowledge, there are no other studies that have used RU26752 as a MR blocker in fish. Therefore, it remains to be investigated how this MR blocker will alter other aspects of gill epithelium physiology either *in vivo* or *in vitro*. In contrast to spironolactone and RU26752, eplerenone did not have any effect in the cultured trout gill epithelium. This is surprising as eplerenone is a highly selective MR antagonist in mammalian models (Garthwaite and McMahon, 2004). However, it has been reported that eplerenone is 40-fold less potent than spironolactone at blocking aldosterone activation of MRs (Garthwaite and McMahon, 2004); therefore, the dosage used in the current study may be an issue. However, a dose of 10 μmol l^{-1} eplerenone (which is equivalent to the dose used in the current study) has recently been reported to inhibit aldosterone-induced activation of zebrafish MR transiently transfected into CV-1 cells (Pippal et al., 2011). This again suggests potential species differences in the response of fish to CR antagonists.

Given that GR and MR antagonists were individually capable of partially, but not completely, blocking the effects of cortisol in cultured trout gill epithelia, it was rationalized that the combined use of these antagonists may result in a more pronounced, and perhaps complete, blockade of cortisol. To explore this possibility, cortisol-treated cultured gill epithelia were exposed to GR + MR antagonist treatment (i.e. RU486 + spironolactone or RU486 + RU26752). GR + MR antagonist treatment reduced TER and increased [^{3}H]PEG-4000 permeability in cortisol-treated preparations to values that were not significantly different from those of control epithelia. In contrast, doubling the presence of MR antagonists (i.e. spironolactone + RU26752) did not significantly alter the permeability characteristics of gill epithelia when compared with treatment with either spironolactone or RU26752 alone. These observations further support the idea that cortisol elicits its effect on the permeability properties of this model through both the MR and GRs. It is important to note, however, that combined antagonist (GR + MR) treatments included RU486 as the anti-glucocorticoid agent, and because RU486 possesses agonistic properties in this model, it would seem logical to speculate that the presence of a MR antagonist in combined treatments also blocks the agonistic effects of RU486. To directly address this possibility, preparations were exposed to RU486 or RU486 + spironolactone in the absence of cortisol. Spironolactone completely blocked the effects of RU486 on TER and [^{3}H]PEG-4000 permeability, providing support for the idea that although RU486 possesses GR antagonist properties in this model, it can also be used as a novel MR agonist. This concept may seem peculiar at first, but it should be noted that the mineralotropic properties of glucocorticoids have been documented in amphibians as well as mammals, both of which are generally accepted to possess corticosteroid hormones and systems with a clearer separation of mineralocorticoid and glucocorticoid function than what may be expected in teleost fishes (for a review, see Agarwal and Mirshahi, 1999).

Recent studies have reported that cortisol treatment of primary cultured gill epithelia (Bui et al., 2010; Chasiotis et al., 2010) or gill explants (Tipsmark et al., 2009) can alter the protein and/or mRNA abundance of select TJ proteins. However, alterations in the permeability of the gill epithelium have only been examined in conjunction with measured changes in occludin abundance (Chasiotis et al., 2010). To date, there is no information relating measured changes in gill epithelium permeability to alterations in TJ proteins such as ZO-1 or members of the claudin superfamily.

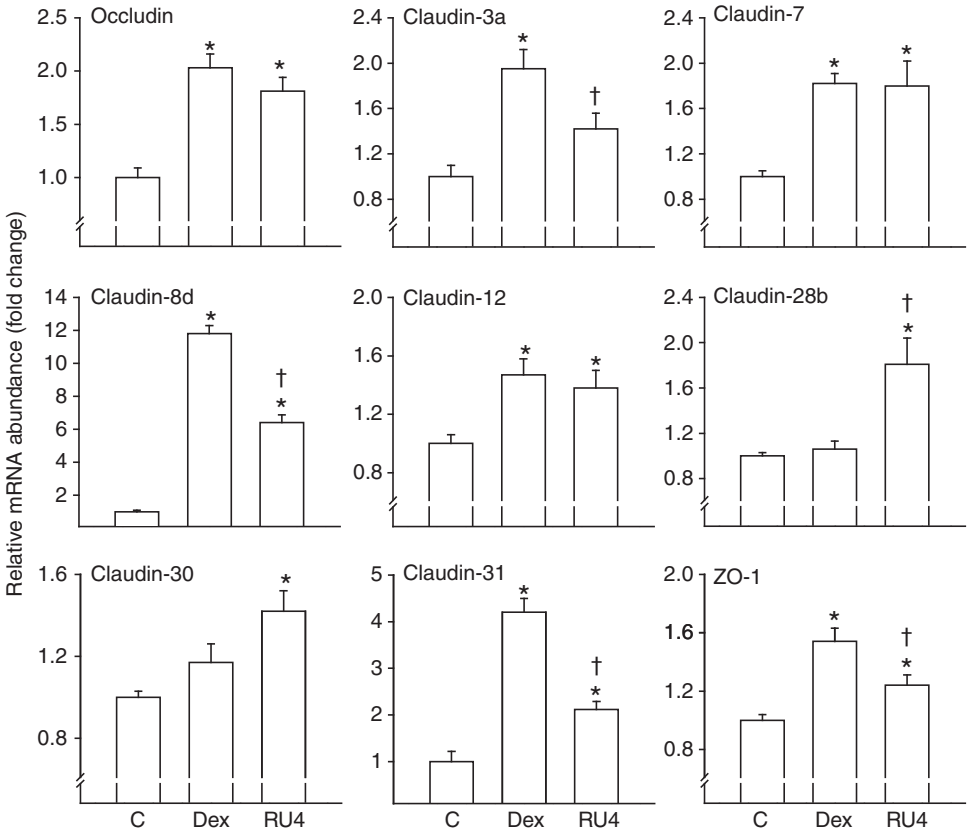

Fig. 9. The effect of dexamethasone (Dex) and RU486 (RU4) on occludin, claudin and ZO-1 mRNA abundance in cultured trout gill epithelia. Tight junction protein mRNA abundance was normalized with β-actin and expressed relative to that of untreated control (C) epithelia, which was assigned a value of 1.0. Data are expressed as mean values + s.e.m. (N=5 inserts per group). A one-way ANOVA was used to determine significant differences between treatments. *Significant difference ($P \leq 0.05$) from untreated control epithelia; †significant difference between Dex and RU4 groups.

Furthermore, the experimentally dissected role that CRs play in the regulation of epithelial permeability can be examined at the molecular level by using dexamethasone and RU486 (as GR and MR agonists, respectively) to evaluate what effects, if any, these factors have on TJ transcript abundance.

Cortisol treatment of cultured gill epithelia significantly increased the transcript abundance of occludin and claudin-3a, -7, -8d, -12, -28b, -30 and -31, but did not significantly alter the mRNA abundance of claudin-32a and ZO-1. The effects of cortisol on occludin confirm recent studies that reported an increase in gill epithelium occludin mRNA and protein in response to this hormone (Chasiotis et al., 2010). In addition, the effects of cortisol on claudin-30 mRNA abundance in this study are in accord with recent observations that claudin-30 mRNA abundance is significantly elevated in whole gill tissue in response to cortisol treatment (Tipsmark et al., 2009). However, claudin-28b transcript abundance was not previously reported to alter in whole gill tissue in response to cortisol treatment *in vitro* (Tipsmark et al., 2009). It is possible that this dissimilarity may reflect differences in the cortisol sensitivity of the two models, as alterations in TJ mRNA abundance of the whole gill preparation required higher doses of cortisol than those used in the current study. Cortisol had a particularly significant effect on claudin-3a and -8d in trout gill epithelia. Transcript encoding these proteins markedly increased in response to hormone treatment, suggesting that increased abundance of claudin-3a and -8d may be linked to a reduction in epithelial permeability. In line with this contention, both claudin-3 and -8 are considered to be barrier-promoting (or 'tightening') tight junction components in mammalian systems (Milatz et al., 2010; Yu et al., 2003). Furthermore, both claudins have previously been linked to epithelial tightening in the gills and kidney of fish (Bagherie-Lachidan et al., 2008; Bagherie-Lachidan et al., 2009; Duffy et al., 2011). However, in contrast to these observations, a primary cultured epithelium generated using puffer fish gill cells did not exhibit any alteration in claudin-8d mRNA abundance in response to cortisol treatment and, in the same preparation, claudin-3a transcript abundance decreased in response to cortisol (Bui et al., 2010). It will be of interest to further examine the reason for this variation (e.g. species-specific differences, differences in culture conditions). In fish gills, the presence of transcript encoding claudin-7, -12 and -31 has been reported in *Fugu rubripes* (Loh et al., 2004) and that encoding claudin-12 noted in zebrafish (Clelland and Kelly, 2010). However, to the best of our knowledge, no study has examined whether any of these TJ factors are involved in the maintenance of salt and water balance in fish. In mammals, the role of claudin-7 in maintaining salt and water balance has recently been consolidated (Tatum et al., 2010). That is, *cldn7*$^{-/-}$ mice, which are viable at birth, succumb to salt loss and dehydration after a number of days. Transcript encoding claudin-7 increased in cortisol-treated trout gill epithelia and as cortisol has previously been reported to cause a reduction in passive salt loss across these preparations (Kelly and Wood, 2001), it seems reasonable to propose that increased claudin-7 presence may be

involved. In contrast, little is known about the physiological role of claudin-12 in vertebrates and nothing is known about claudin-31, which so far has been described only in fish (Loh et al., 2004). By virtue of its association with the high-resistance mammalian uroepithelium, claudin-12 seems likely to be a barrier-forming protein of some description (Acharya et al., 2004; Khandelwal et al., 2009). In cortisol-treated trout gill epithelia, claudin-12 transcript abundance increased, also suggesting barrier-forming properties. Similarly, transcript encoding claudin-31 increased in preparations treated with cortisol, which is suggestive of barrier-forming properties. However, in both cases, these ideas will require further investigation using techniques that impart greater resolution.

Of the 10 TJ proteins examined in this study, mRNA abundance for all but claudin-28b, -30 and -32a was significantly elevated in response to dexamethasone and in association with decreased permeability. Claudin-32a transcript abundance also did not alter in response to cortisol or RU486. Therefore, it seems likely that claudin-32 is not responsive to corticosteroids in this system. However, a significant increase in claudin-28b and -30 mRNA was observed following cortisol and RU486 treatment. Assuming that cortisol mediates its effects on TJ permeability through both the MR and the GRs and that the response to RU486 occurs through the MR, this would suggest that claudin-28b and -30 are not responsive to the actions of corticosteroids through GRs but instead solely through the MR. Conversely, claudin-3a mRNA significantly increased in response to cortisol and dexamethasone, but was not significantly changed by RU486 alone. Again, this indicates that claudin-3a mRNA may only be responsive to cortisol through a specific CR type, in this case the GR. In line with these observations, select TJ proteins have previously been reported to respond to corticosteroid treatment through specific CR types (Felinski et al., 2008; Le Moellic et al., 2005). For example, occludin and claudin-5 gene expression in retinal endothelial cells has been suggested to increase exclusively in response to glucocorticoids through the GR (Felinski et al., 2008), and a rapid and transient phosphorylation of claudin-4 can be observed in RCCD2 cells in response to the actions of aldosterone through the MR and not in response to dexamethasone through the GR (Le Moellic et al., 2005). However, little else is known about the specific and/or separate actions of MR and GRs on epithelial permeability in vertebrates and this is clearly an area worthy of continued attention.

Primary cultured gill epithelia provide us with an opportunity to gain considerable insight into how transepithelial permeability in the architecturally and physiologically complex fish gill is controlled. When generated using trout gills, the permeability properties of the primary cultured gill epithelium are exquisitely sensitive to cortisol treatment. Therefore, this preparation is well suited to investigations of how corticosteroids and CRs contribute to alterations in salmonid gill permeability. The current study suggests that the MR agonists aldosterone and deoxycorticosterone do not impact the permeability properties of this gill model, but that cortisol does by working through both GRs and the MR. Furthermore, the current suite of experiments encourages the view that even in a simplified model system composed exclusively of pavement cells, the role of corticosteroids and CRs in the regulation of gill permeability is complex. Taking into consideration the fact that the molecular components of the salmonid gill TJ complex still require full enumeration, and that there is evidence for the expression of cell-specific TJ proteins in the gills of fish (see Bui et al., 2010), it seems likely that further studies will not only be revealing but also significantly add to the complexity of the system.

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