Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway

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Abstract

Based on real-time RT-PCR, analysis of transcripts of selected ion-regulatory proteins (Na^+, K^+-ATPase α1a and α1b subunit, Na^+, K^+, $2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator (CFTR), and H^+-ATPase B-subunit), the regulatory role of cortisol and the associated receptor signaling pathway (glucocorticoid (GR) versus mineralocorticoid (MR)) of cortisol was investigated in the salmon gill. Using a gill organ culture technique, the effect of cortisol with and without addition of specific hormone receptor antagonists (RU486 and spironolactone) was analyzed in gills from freshwater (FW) and seawater (SW) acclimated fish. The effect of cortisol was highly dependent on acclimation to salinity. In FW, cortisol stimulated the transcript levels of CFTR-I and Na^+, K^+-ATPase α1a and α1b. Addition of RU486 totally abolished the cortisol effects on CFTR-I and Na^+, K^+-ATPase α1b, suggesting that signaling was mediated via GR. Interestingly, both spironolactone and RU486 were able to inhibit the cortisol effect on Na^+, K^+-ATPase α1a indicating a role for both *MR* and *GR* in regulation of this target gene. In SW, cortisol increased the transcript levels of CFTR-I, CFTR-II, Na^+, K^+-ATPase α1a and α1b, and NKCC. Interestingly, the effect of cortisol on CFTR-I and Na^+, K^+-ATPase α1a was mediated through GR and MR respectively, while both GR and MR signaling were required in the regulation of CFTR-II and Na^+, K^+-ATPase α1b. In FW gills, GR1 and MR transcript levels were not significantly affected by cortisol. In SW gills, GR1 and MR transcripts were downregulated by cortisol; GR1 was regulated via the MR and MR regulation was mediated via GR.

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Introduction

Cortisol, the major corticosteroid in teleost fish, is considered a dual regulator of chloride cell differentiation and function. During seawater (SW) acclimation, cortisol stimulates chloride cell numbers and Na^+, K^+-ATPase enzymatic activity in the gill and increases overall salinity tolerance in synergy with insulin-like growth factor-I (IGF-I) and growth hormone (GH; reviewed by McCormick 2001). In freshwater (FW) environment, cortisol presumably cooperates with prolactin (PRL) to ensure ion-osmotic homeostasis (reviewed by McCormick 2001). Cortisol alone stimulates overall Na^+, K^+-ATPase enzyme activity and mRNA levels of certain ion transport proteins in the gill *in vitro*: Na^+, K^+, $2Cl^-$ cotransporter (NKCC), α1a and α1b subunits of the Na^+, K^+-ATPase (McCormick & Bern 1989 (FW coho salmon, *Oncorhynchus kisutch*); Tipsmark *et al.* 2002 (FW brown trout, *Salmo trutta*, and Atlantic salmon, *Salmo salar*); Shrimpton & McCormick 1999 (FW rainbow trout, *Oncorhynchus mykiss*); and Deane & Woo 2005 (SW silver sea bream, *Sparus sarba*)).

The mineralocorticoid-like role of cortisol is unique to fish, since in vertebrates ranging from amphibians to mammals aldosterone serves this function by acting through the mineralocorticoid receptor (MR; Agarwal & Mirshahi 1999). The absence of aldosterone in fish (see Prunet *et al.* 2006) may be compensated for by the ability of cortisol to bind both glucocorticoid receptors (GR) and MR in fish (Bury *et al.* 2003, Sturm *et al.* 2005), and that these receptors bind identical response elements in target gene promoters (Farman & Rafestin-Oblin 2001). The possibility that differential signaling by cortisol may occur through GR and MR in fish has yet to be elucidated. Recently, 11-deoxycorticosterone (DOC), an intermediate in aldosterone synthesis in mammals, was identified in fish as a potential mineralocorticoid hormone. DOC has high affinity for MR (Sturm *et al.* 2005) and seems to be the ancestral mineralocorticoid (Bridgham *et al.* 2006). The information concerning the function and significance of DOC is very limited, but the emergence of this potential hormone further emphasizes the requirement to investigate the signaling pathways of GR and MR in fish.

The knowledge of osmoregulatory gene regulation by cortisol in fish gill is scarce and little is known about exact cortisol signaling mechanisms through GR and MR. This is the first study that specifically addresses these questions. In

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order to investigate the specific cortisol effect on gene expression in Atlantic salmon gill, an *in vitro* gill organ culture system was used. Using this technique, isolated effects of cortisol on gene expression were investigated in intact gill tissue, where cells remain in their natural cellular and junctional environment. QPCR analysis was applied to examine differential regulation of ion transport genes representing SW (Na^+, K^+-ATPase α1b, NKCC, the cystic fibrosis transmembrane conductance regulator isoform I and II (CFTR-I and CFTR-II)) and FW gills (Na^+, K^+-ATPase α1a and the V-type H^+-ATPase B-subunit). Specific antagonists for GR and MR (RU486 and spironolactone respectively) were applied to further examine the signaling pathway of cortisol.

Materials and Methods

Fish and sampling

In September, 1·5-year-old post-smolt *S. salar* (Burishoole stock) from Danmarks Center for Vildlaks (Randers, Denmark) were acclimated at the Odense Campus (University of Southern Denmark) at 14° C, 12 h light:12 h darkness cycle in 500 l fiberglass tanks with either flow through tap water or 30 ppt artificial SW for at least 30 days prior to sampling. The fish were fed *ad libitum* once daily with pelleted trout feed. When sampled, fish were stunned with a blow to the head and killed by cutting the spinal cord and pithing the brain.

Gill block incubation

To examine the effect of cortisol on gene expression, a gill block incubation experiment modified from McCormick & Bern (1989) was conducted using a repeated measures design. Excised gill arches from each fish were placed in separate glass Petri dishes placed on ice with 25 ml cold preincubation medium (Salmon Ringer's containing 400 000 IU/l streptomycin/penicillin and 2 mg/l amphotericin B. Salmon Ringer: 140 mM NaCl, 15 mM $NaHCO_3$, 2·5 mM KCl, 1·5 mM $CaCl_2$, 1·0 mM KH_2PO_4, 0·8 mM $MgSO_4$, 10 mM D-glucose, and 5·0 mM EPPS (pH 7·8); equilibrated with 99% O_2/1% CO_2 for 10 min) for minimum half-an-hour. The gill filaments were dissected away from the cartilage arch and blocks of three to four filament pairs were cut out. Upon dissection, five to six gill blocks were frozen in liquid N_2 for RNA isolation to serve as preincubation controls. Groups of 10–12 gill blocks were carefully transferred to 6-well plates containing 2 ml incubation medium (Salmon Ringer's with 100 000 IU/l streptomycin/penicillin, and 2 mg/l amphotericin B) with or without the following agonist/antagonist combination: control, DMSO only (0·003 v/v); cortisol hemisuccinate + DMSO (F), 10μg/ml (28μM); RU486, 20μg/ml (47μM); Spironolactone (Spi), 20μg/ml (48μM); F + RU486, F + Spi. Cortisol hemisuccinate sodium salt (Sigma–Aldrich) was dissolved in Salmon Ringer, whereas RU486 and spironolactone (Sigma–Aldrich) were dissolved in DMSO and mixed with Ringer to obtain the above concentrations. The 6-well plates were incubated at 14° C in a 99% O_2/1% CO_2 atmosphere with shaking for 3 days. The gas in the incubation chamber was changed every day; the incubation medium was changed on day 2. Mucus was carefully removed by wiping the gill blocks on a paper towel and the gill blocks were stored at −80° C until analysis.

RNA purification, cDNA synthesis, and real-time semi-quantitative PCR

Total RNA was purified by the TRIzol procedure (Invitrogen) using 1 ml TRIzol/100 mg gill tissue according to manufacturer's recommendation. RNA concentration and purity was determined by measuring A_{260}/A_{280}. One microgram RNA was treated with 1 U RQ1 DNase (Promega) for 30 min at 37° C in a total volume of 20μl followed by 5 min at 75° C to inactivate RQ1 DNase. Reverse transcription was carried out on 1μg DNase-treated RNA with 2μg random hexamers (GE Healthcare Bio-Sciences, Little Chalfont, UK) and 200 U. Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) for 1 h at 37° C in the presence of 40 U RNAguard (GE Healthcare) in a total volume of 25μl. At the end the cDNA was diluted with 50μl milliQ H_2O. Semi-quantitative real-time PCR analysis using SYBR Green detection was carried out on a *Mx*3000P instrument (Stratagene, La Jolla, CA, USA) using standard software settings including adaptive baseline for background detection, moving average, and amplification-based threshold settings with the built-in FAM/SYBR filter (excitation wavelength: 492 nm and emission wavelength: 516 nm). Reactions were carried out with 1μl cDNA, 7·5 pmol glucocorticoid receptor (GR1) and mineralocorticoid receptor (MR) or 5 pmol (Na^+, K^+-ATPase α1a, Na^+, K^+-ATPase α1b, CFTR-I, CFTR-II, H^+-ATPase, and NKCC) forward and reverse primer (DNA technology A/S, Denmark), 12·5μl 2× Brilliant SYBR green master mix (Stratagene) in a total volume of 25μl. Cycling conditions: 95° C for 30 s, 60° C for 60 s, and 72° C for 5 s in 50 cycles (GR1 and MR) or 40 cycles (Na^+, K^+-ATPase α1a, Na^+, K^+-ATPase α1b, CFTR-I, CFTR-II, H^+-ATPase, and NKCC). Melting curve analysis was carried out routinely with 30 s for each 1° C interval from 55 to 95° C.

Amplification efficiency, normalization, and calculations

For each primer set, cDNA was diluted 4, 16, 64, 256, and 1024 times in duplicates and analyzed by QPCR to determine amplification efficiency calculated as the slope of the resultant linear graph of threshold cycle (*C*t) versus log cDNA concentration. The amplification efficiency (E_a) for each primer set was used for calculation of relative copy numbers of the respective target gene. EF1α was used as normalization gene according to Olsvik *et al.* (2005). To verify the

normalization, 18S rRNA was used as a second normalization gene. No appreciable differences were observed between the results obtained with the two normalization genes (not shown). Relative copy number of the target gene cDNAs was calculated as $2^{(-Ct/Ea)}$, where *Ct* is the threshold cycle number and E_a is the amplification efficiency. Normalized units were obtained by dividing the relative copy number of the target gene cDNA with the relative copy number of the normalization gene cDNA.

Primers

Primer sequences are listed in Table 1. CFTR-I, CFTR-II, Na^+, K^+-ATPase α1a, NKCC, H^+-ATPase B-subunit, GR1, MR, elongation factor (EF)1α, and 18S rRNA primers were designed using the NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA) with standard settings and double checked using the Primer3 software (Rozen & Skaletsky 2000) and BLASTed against the GenBank no. and EST sequences. Na^+, K^+-ATPase α1b primers are identical to the Na^+, K^+-ATPase subunit specific primers based on *O. mykiss* mRNA sequences described by Richards *et al.* (2003). These primers have previously been successfully used in gill tissue from *S. salar* (Bystriansky *et al.* 2006). All primers were tested for nonspecific product amplification and primer–dimer formation using both melting curve analysis and agarose gel verification. In case of lack of *S. salar* mRNA sequence information, PCR primers based on conserved regions of *O. mykiss*, mRNA sequences were used when necessary (Na^+, K^+-ATPase α1a and α1b, GR1, and MR). The high degree of conservation of nucleotide sequence between salmon and trout permit the use of primers between these two species. Moreover, the functionality and significance of the NKCC and H^+-ATPase genes have yet to be validated since only partial sequences of these target genes is available so far.

All primers were synthesized by DNA Technology A/S (Århus, Denmark).

Statistical analysis

Data were analyzed using a repeated measures one-way ANOVA followed by a Bonferroni adjusted Fisher's least significant difference (LSD) test where appropriate, taking into account the total number of pairwise comparisons. When necessary, data were transformed to obtain normality and homogeneity of variances (Zar 1999). In all cases a significance level of $\alpha = 0{\cdot}05$ was used. All tests were performed using SAS (Version 9.1 for Windows, by SAS Institute Inc., Cary, NC, USA).

Results

Cortisol increased CFTR-I mRNA levels in FW- and SW-adapted salmon gill (Fig. 1A and B). The GR antagonist, RU486, decreased CFTR-I mRNA levels and totally abolished the effect of cortisol. The MR antagonist spironolactone had no effect on CFTR-I transcript levels and did not interfere with the effect of cortisol. While there was no effect of cortisol on CFTR-II mRNA levels in FW gills, an increase was observed in SW gills (Fig. 2A and B). RU486 reduced CFTR-II mRNA levels when compared with control and abolished the stimulatory effect of cortisol observed in SW gills. Spironolactone had no effect in FW gills (Fig. 2A) but reduced CFTR-II levels and abolished the effect of cortisol in SW gills (Fig. 2B). In FW and SW gills, Na^+, K^+-ATPase α1a transcript level increased after cortisol treatment (Fig. 3A and B). Addition of both RU486 and spironolactone abolished the cortisol effect in FW gill, whereas only spironolactone antagonized the cortisol effect in SW gill. Cortisol increased Na^+, K^+-ATPase α1b subunit transcript levels in both FW and SW gills (Fig. 4A and B), an effect which was fully antagonized by RU486 at both salinities. Spironolactone had no effect in FW gills but abolished the cortisol effect in SW gills (Fig. 4B).

In FW gills no effect of cortisol on NKCC mRNA level was observed (Fig. 5A). GR and MR antagonists both increased NKCC transcript levels in FW gills. Cortisol

Table 1 Sequences and target genes of primers used to investigate mRNA levels after *in vitro* incubation of gills from freshwater (FW) and seawater (SW) adapted Atlantic salmon

Target sequence	Forward primer 5′–3′	Reverse primer 5′–3′	Gene bank acc. number
Salmo salar CFTR-I	CCTTCTCCAATATGGTTGAAGAGGCAAG	GCACTTGGATGAGTCAGCAG	AF155237
Salmo salar CFTR-II	GCCTTATTTCTTCTATTTGTATGCACTT	GCCACCATGAAAAACTAAA GAGTACCTCAG	AF161070
O. mykiss Na^+, K^+-ATPase α1a	CCCAGGATCACTCAATGTCAC	CCAAAGGCAAATGGGTTTAAT	AY319391
O. mykiss Na^+, K^+-ATPase α1b	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	AY319390
Salmo salar NKCC	ACGTGTCCCACATCTCAG	GAGGGCTTGGATGAGTCT	AJ417890
Salmo salar H^+-ATPase B-subunit	GAACATGGAAACTGCTCG	TGGTAGGCCAGGTACTGA	AJ250811
O. mykiss GR1	ACGACGATGGAGCCGAAC	ATGGCTTTGAGCAGGGATAG	Z54210
O. mykiss MR	AGACTCGACCCCCACCAAG	CGTTAGTGGGACTGGTGCTC	AF209873
Salmo salar EF1α	GAGAACCATTGAGAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	AF321836
Salmo salar 18S rRNA	TATTGTGCCGCTAGAGGTGA	CCTCCGACTTTCGTTCTTGA	AJ427629

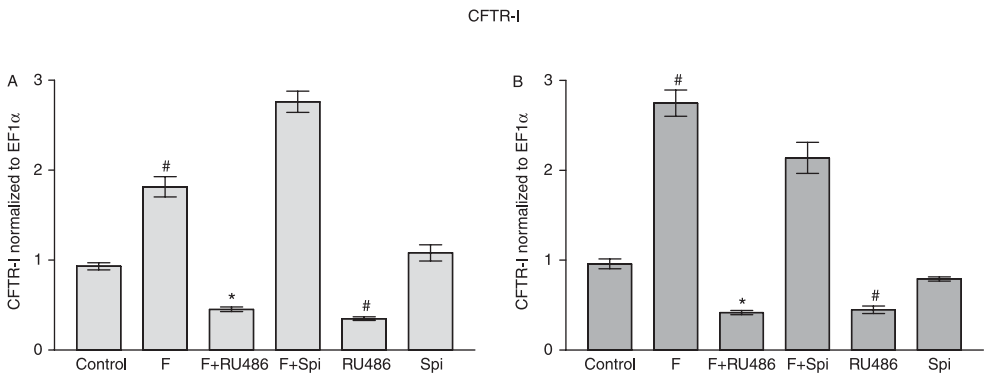

Figure 1 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on CFTR-I mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. CTFR-I mRNA levels were normalized to mRNA levels of EF1α. Values (means ± S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. An overall effect of treatment was detected in the FW and the SW experiments ($P<0{\cdot}0001$). #Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

stimulated NKCC mRNA levels in SW gills (Fig. 5B). This was not antagonized by RU486 but tended to be diminished by the addition of spironolactone ($P=0{\cdot}2082$; Fig. 5B).

No significant effect of cortisol or antagonists on H^+-ATPase B-subunit transcript levels were found either in FW or in SW gills (Fig. 6A and B).

In FW gills there was no effect of cortisol on GR1 mRNA levels (Fig. 7A). In SW gills, cortisol decreased GR1 levels when compared with the control level (Fig. 7B). RU486 had no effect but spironolactone antagonized this effect completely. No significant effect of cortisol and/or antagonists was found on the MR mRNA level in FW gills (Fig. 8A). However, cortisol tended to lower the MR transcript level ($P=0{\cdot}1374$). Also in SW gills, cortisol tended to lower the MR transcript level ($P=0{\cdot}1356$), a tendency which was abolished by RU486 but not spironolactone (Fig. 8B). Yet, only an overall effect of treatment and no single significant differences was detected.

Stability of the investigated mRNA species during culture was investigated by comparing normalized mRNA levels in preincubation control gills and after 3-day incubation (Table 2). The level of CFTR-I, CFTR-II, Na^+, K^+-ATPase α1b, and NKCC transcripts decreased during incubation of FW and SW gills, whereas that of H^+-ATPase mRNA increased. Na^+, K^+-ATPase α1a transcript level decreased significantly during incubation of FW gills but increased in SW gills. The GR1 transcript level decreased during incubation of FW gills and was unchanged in SW gills. MR transcript levels were unchanged during incubation of FW gills but increased during incubation of SW gills.

A summary of all effects of cortisol is shown in Table 3.

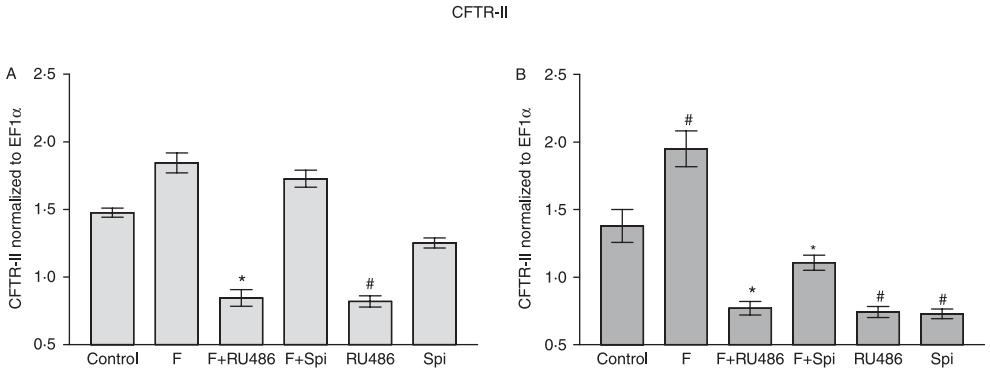

Figure 2 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on CFTR-II mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. CTFR-II mRNA levels were normalized to mRNA levels of EF1α. Values (means ± S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD. An overall effect of treatment was detected in the FW and the SW experiment ($P<0{\cdot}0001$). #Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

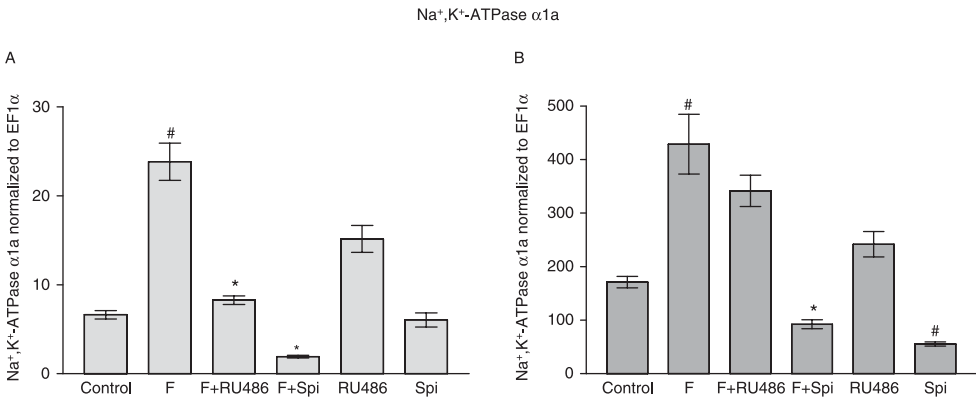

Figure 3 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on Na^+, K^+-ATPase α1a mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. Na^+, K^+-ATPase α1a mRNA levels were normalized to mRNA levels of EF1α. Values (means±S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. An overall effect of treatment was detected in the FW and SW experiments ($P<0{\cdot}0001$). #Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

Discussion

Cortisol is considered as a fundamental regulator of chloride cell differentiation and proliferation, and numerous experiments confirm the pivotal role of cortisol in salinity acclimation of fish (reviewed by McCormick 2001). However, in terms of the basic signaling pathways and molecular mechanisms involved, cortisol regulation of gene expression in fish is still poorly understood. GR elements (GREs) have so far only been identified in very few piscine promoters (lactate hydrogenase-B in killifish, *Fundulus heteroclitus*: Schulte *et al.* 2000; α-amylase in seabass, *Lates calcarifer*: Ma *et al.* 2004; growth hormone 2 in rainbow trout: Yang *et al.* 1997 and PRL in rainbow trout: Argenton *et al.* 1996). No functional GRE has been identified in the osmoregulatory target genes investigated here although GREs have been identified in the human GR and Na^+, K^+-ATPase α1 and β1promoters (Derfoul *et al.* 1998, Kolla *et al.* 1999, Yudt & Cidlowski 2002).

To determine the cortisol signaling pathway, the specific antagonists RU486 (GR) and spironolactone (MR) were used. These antagonists have previously been employed in several fish studies (Sloman *et al.* 2001, Bury *et al.* 2003, Scott *et al.* 2005, Shaw *et al.* 2007) without appreciably unspecific effects. However, the limited use and the insufficient validation of specificity of the antagonists in fish systems might impute some uncertainty to the conclusions. In fact, agonistic effects of spironolactone (Sturm *et al.* 2005) and RU486 (Mazon *et al.* 2004) have been reported, but there was no consistent evidence for such effects in this study when

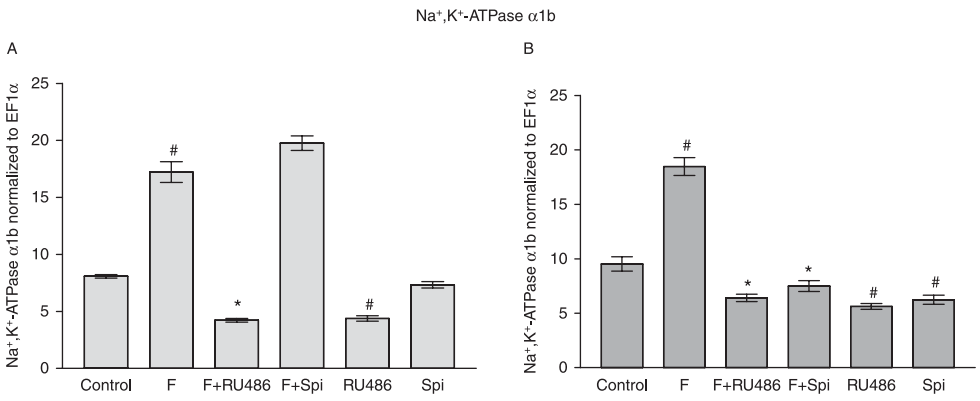

Figure 4 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on Na^+, K^+-ATPase α1b mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. Na^+, K^+-ATPase α1b mRNA levels were normalized to mRNA levels of EF1α. Values (means±S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. An overall effect of treatment was detected in the FW and the SW experiment ($P<0{\cdot}0001$). #Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

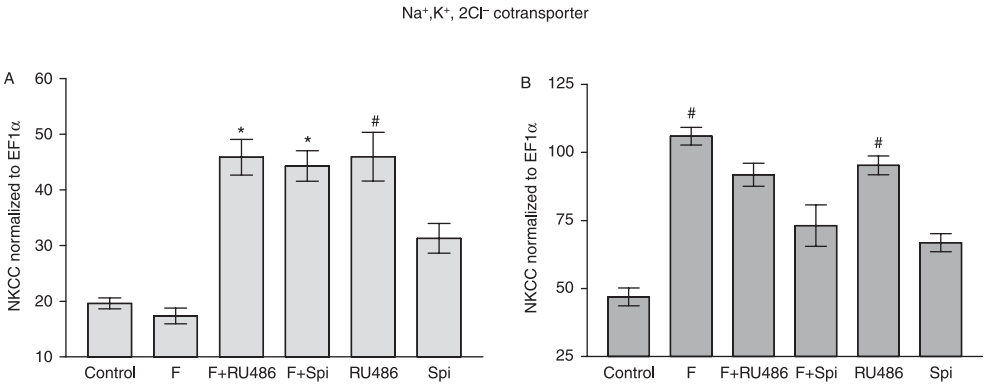

Figure 5 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on NKCC mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. NKCC mRNA levels were normalized to mRNA levels of EF1α. Values (means ± S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. An overall effect of treatment was detected in the FW and the SW experiments ($P<0{\cdot}005$). # Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

using similar antagonist concentrations. Despite lowering of the basal transcript level of some of the targets by antagonists, no overall pharmacological effect was observed.

The gill tissue organ culture

The main advantage of using the present organ culture approach is that isolated effects of cortisol may be studied in gill tissue, in which cells remain in their natural structural and junctional organization. Despite the fact that isolation of gill tissue from the organism removes all endogenous input (neural and hormonal), which may be vital for the integrity and functionality of the tissue, several studies have validated the application of this technique, and demonstrated good viability of the tissue even after 4-day incubation (McCormick & Bern 1989, Bury *et al.* 1998, Shrimpton & McCormick 1999, Mazon *et al.* 2004, Deane & Woo 2005). Our study confirms that the tissue is indeed responsive to experimental manipulations. Three-day incubation *per se* had an effect on the majority of the investigated transcripts, but apparently there is no conformity in the direction of the changes in mRNA level. Among the transcripts investigated in the present paper, several transcripts (CFTR-I, CFTR-II, Na^+, K^+-ATPase α1b, and NKCC) showed decreased levels during 3-day control incubation. However, others were unchanged (MR) or even increased (V-type H^+-ATPase) during incubation. This demonstrates the differential influence of the endogenous input to the gill.

Regulation of CFTR-I and CFTR-II

Two isoforms of the chloride transporter CFTR are present in Atlantic salmon (Chen *et al.* 2001), but their expression regulation and separate functions are largely unknown. Here

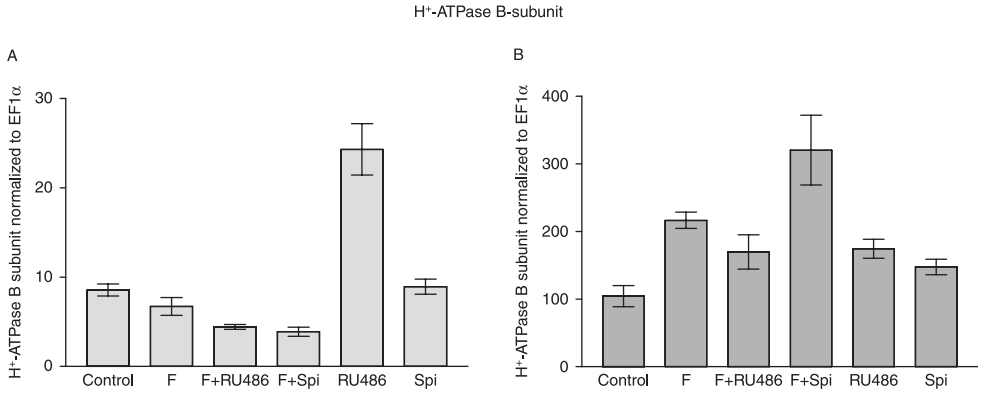

Figure 6 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on H^+-ATPase B subunit mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. H^+-ATPase mRNA levels were normalized to mRNA levels of EF1α. Values (means ± S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. No overall effect of treatment was detected in the FW or the SW experiments ($P<0{\cdot}05$).

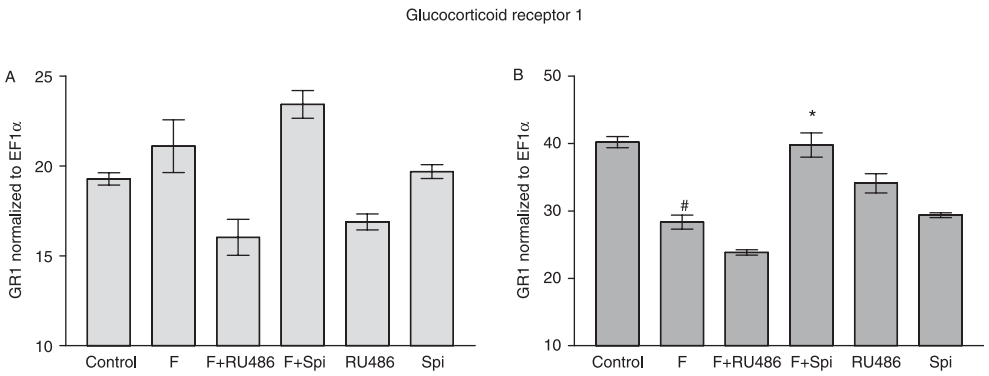

Figure 7 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on GR1 mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. GR1 mRNA levels were normalized to mRNA levels of EF1α. Values (means±S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. No overall effect of treatment was detected in the FW experiment ($P>0{\cdot}05$). An overall effect of treatment was detected in the SW experiment ($P<0{\cdot}001$). #Indicates significant difference from the control group and * indicates a significant difference from the cortisol-treated group ($P<0{\cdot}05$).

we show that the two isoforms are differentially regulated by cortisol. Cortisol increased the CFTR-I transcript level in both FW and SW, whereas CFTR-II levels were only affected in the SW gill. This agrees with a report by Singer *et al.* (2003) where cortisol treatment of FW Atlantic salmon stimulates CFTR-I but not CFTR-II mRNA levels. The CFTR-I isoform is also upregulated during smoltification and after SW-transfer in salmon, while CFTR-II is only transiently affected under these conditions (reviewed by Marshall & Singer 2002; T. Nilsen, Univ. of Bergen, pers. comm.). Altogether, the two CFTR isoforms seem to be differentially regulated and expressed in the gill. The effect of cortisol on CFTR-I transcript levels in salmon is opposite to that seen in FW-adapted striped bass gill (*Morone saxatilis*), where cortisol caused a downregulation of the CFTR mRNA level *in vitro* (Madsen *et al.* 2007). The effect of cortisol on CFTR-I and CFTR-II levels in FW and SW gills was consistently abolished by RU486, indicating that cortisol signals through the GR in both hyper- and hypo-osmoregulating gill tissue. An inhibitory effect of RU486 was also shown for CFTR in the gill of SW-acclimating (Shaw *et al.* 2007), but not FW-acclimating (Scott *et al.* 2005) killifish. The present reduction of CFTR levels by RU486 alone when compared with control levels may be due to blocking of endogenous cortisol left in the tissue or due to a nonspecific effect of the antagonist. Putative GR-binding sites have been identified in the promoter region of the *F. heteroclitus CFTR* gene (Schulte & Keir 2003); however, the authors reported that these binding sites were nonfunctional when tested in cell culture and transgenic fish.

There was no evidence for cortisol signaling through MR in CFTR-I and CFTR-II regulation in FW-acclimated salmon in this study. However, in the SW gill cortisol stimulation of CFTR-II transcript levels was blocked by

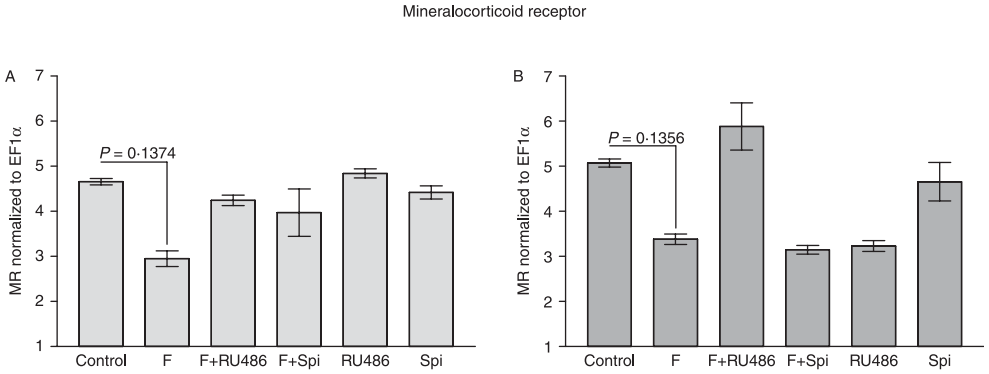

Figure 8 Effect of cortisol (F) and GR (RU486) and MR (Spi) antagonists on MR mRNA levels in gill blocks from (A) FW- and (B) SW-acclimated Atlantic salmon. MR mRNA levels were normalized to mRNA levels of EF1α. Values (means±S.E.M., $n=6$) were analyzed with a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test. No overall effect of treatment was detected in the FW experiment ($P>0{\cdot}05$). An overall effect of treatment was detected in the SW experiment ($P<0{\cdot}05$) but no single significant effects was detected ($P>0{\cdot}05$).

Table 2 Comparison of mRNA levels before (preincubation controls) and after (3-day controls) incubation of freshwater (FW)- and seawater (SW)-acclimated Atlantic salmon gill

	FW		SW	
	Preincubation	3-Day control	Preincubation	3-Day control
Target gene				
CFTR-I	2·8±0·6	0·9±0·1[a]	5·3±0·5	1·0±0·1[a]
CFTR-II	5·2±0·6	1·5±0·1[a]	4·0±0·2	1·4±0·3[a]
Na^+, K^+-ATPase α1a	19·7±8·0	6·6±1·2[a]	76·5±13·9	170·9±25·9[a]
Na^+, K^+-ATPase α1b	34·4±2·3	8·1±0·5[a]	129·3±18·9	9·5±1·6[a]
NKCC	36·0±8·7	19·6±3·4[a]	233·0±25·5	46·9±8·0[a]
H^+-ATPase B	0·7±0·3	8·6±2·3[a]	13·2±3·3	104·4±38·5[a]
GR	46·5±3·0	1·9±1·2[a]	46·1±2·6	40·2±2·1
MR	5·6±0·2	4·7±0·2	3·6±0·3	5·1±0·2[a]

[a]Denotes significant difference between preincubation and control values as determined by a repeated measures one-way ANOVA followed by Bonferroni adjusted Fisher's LSD test conducted on the datasets from Figs 1 to 8. Values are means±S.E.M. of six fish. mRNA levels are depicted as relative copy number of the target gene normalized to the relative copy number of EF1α.

spironolactone suggesting additional regulation through MR. Thus far the role of MR in CFTR regulation has only been investigated in killifish where no effect of spironolactone was reported after BW to FW transfer (Scott *et al.* 2005).

Regulation of Na^+, K^+-ATPase α1 isoforms

Recently, two Na^+ -ATPase α1 isoforms were identified in the rainbow trout gill displaying reverse responses to salinity changes (Richards *et al.* 2003): the α1a isoform, characteristic of the FW-gill and the α1b isoform which increases during SW acclimation. This pattern has been characterized in other salmonids as well (*S. salar* and Arctic char, *Salvelinus alpinus*; Bystriansky *et al.* 2006). Cortisol stimulated both transcripts in gills from both salinities thus agreeing with its dual role in osmoregulation (reviewed by McCormick 2001). Cortisol has previously been shown to increase overall Na^+, K^+-ATPase protein (Seidelin *et al.* 1999) and α-subunit mRNA (Tipsmark *et al.* 2002) levels in the salmonid gill, but this is the first study to distinguish cortisol effects on the distinct Na^+, K^+-ATPase α1 isoforms and to give evidence for the involvement of both GR and MR. Curiously, the signaling pathway is opposite at the two salinities. The α1a isoform is consistently regulated by the MR (in FW and SW gills) and by additional GR signaling in the FW gill. The α1b isoform is consistently regulated via GR signaling (in FW and SW) and by additional MR signaling in the SW gill. This pattern of regulation complies with the hypothesis that GR signaling has a primary role in developing the gill for ion excretion, while MR signaling may be more important in stimulating ion retention (Sloman *et al.* 2001, Scott *et al.* 2005, Kiilerich *et al.* 2007). Future experiments should investigate to what degree the present cortisol effects may be modulated *in vivo* by interaction with other hormones in salinity acclimation, such as PRL, growth hormone, and IGF-I.

Table 3 Summary of cortisol action and the deduced signaling pathway. Arrows indicate upregulation (↑), downregulation (↓) and no regulation (→) of the target mRNA.

	FW		SW	
	Cortisol	Receptor	Cortisol	Receptor
Target cDNA				
CFTR-I	↑	GR	↑	GR
CFTR-II	→	–	↑	GR and MR
Na^+, K^+-ATPase α1a	↑	GR and MR	↑	MR
Na^+, K^+-ATPase α1b	↑	GR	↑	GR and MR
NKCC	→	–	↑	(MR)
H^+-ATPase	→	–	→	–
GR	→	–	↓	MR
MR	(↓)	(GR and MR)	(↓)	(GR)

Brackets denote that the effect was statistically nonsignificant. See text for details

Regulation of Na^+, K^+, $2Cl^-$ cotransporter

Consistent with the SW-adaptive role of NKCC (reviewed by McCormick 2001, Tipsmark *et al.* 2002), cortisol increased NKCC mRNA levels but only in gills from SW-acclimated salmon. Previously, cortisol stimulated NKCC mRNA in FW gill *in vitro* (Tipsmark *et al.* 2002) and NKCC protein level *in vivo* (Pelis & McCormick 2001). A reason for the disparity in response to cortisol in the FW gill may be found in seasonal variation of sensitivity of the gill, since post-smolt salmon were used in this study whereas Tipsmark *et al.* (2002) used smolting salmon. Thus, stimulation of this particular SW-type ion transporter may depend on the developmental stage of chloride cells. No antagonist was able to efficiently inhibit the effect of cortisol in SW gill, but blocking of MR slightly lowered the NKCC transcript level when compared with cortisol alone. On the other hand, both RU486 and spironolactone increased the NKCC transcript level significantly in the FW gill. The reason for this is unknown, and further conclusions concerning the signaling pathway should await further research. Nonetheless, the lack of antagonist effect on NKCC mRNA expression in SW gill is in good agreement with Scott *et al.* (2005), where neither RU486 nor spironolactone implants affected the suppression of NKCC mRNA levels in BW- to FW-transferred killifish (Scott *et al.* 2005).

Regulation of H^+-ATPase B-subunit

In addition to participating in acid–base regulation, vacuolar-type H^+-ATPase is suspected to facilitate Na^+ uptake across the gill from hypo-osmotic environments (Marshall 2002, Hirose *et al.* 2003) Accordingly, protein levels have been shown to increase upon FW acclimation (Wilson *et al.* 2002). There is only limited information on the endocrine regulation on H^+-ATPase expression, and only at the protein level. Based on *N*-ethylmaleimide-sensitive inhibition of V-type H^+-ATPase activity, cortisol injection was shown to stimulate enzyme activity in the FW rainbow trout gill (Lin & Randall 1993). Our data, however, did not confirm this effect of cortisol at the mRNA level, when applied *in vitro*.

Regulation of GR and MR

Downregulation of SW gill GR1 transcript level by cortisol reported here is in good agreement with the downregulation of GR protein levels after corticosteroid treatment of gill, brain and liver of coho salmon, and rainbow trout (Pottinger 1990, Maule & Schreck 1991, Lee *et al.* 1992, Shrimpton & Randall 1994). However, an increase in GR mRNA levels upon cortisol treatment of rainbow trout hepatocytes (Sathiyaa & Vijayan 2003) was also reported. Interestingly, a dual mode of GR feedback regulation was recently reported by Takahashi *et al.* (2006) where chronic cortisol treatment *in vivo* increased GR levels, whereas a single cortisol injection caused a decrease in GR transcript levels (Takahashi *et al.* 2006). Curiously, the cortisol regulation of GR1 transcript levels seen in SW gill is signaled through the MR. The GR has been linked to SW acclimation since the GR1 mRNA levels and the corticosteroid-binding ability increase in the gill during smoltification and after SW transfer in salmonids (reviewed by Prunet *et al.* 2006, Kiilerich *et al.* 2007). Consistent with this is the fact that cortisol only affects the gill GR1 mRNA levels in SW-acclimated salmon.

The MR was recently discovered in fish (Colombe *et al.* 2000, Sloman *et al.* 2001, Greenwood *et al.* 2003, Sturm *et al.* 2005) and its function is still puzzling. There is evidence that MR plays a role in chloride cell proliferation and differentiation in FW rainbow trout and killifish (Sloman *et al.* 2001, Scott *et al.* 2004, 2005) and a FW-adaptive role for MR has been proposed in salmon (Kiilerich *et al.* 2007). The present data show no convincing effects of cortisol on the transcript level of MR, but only a trend of a downregulation by cortisol is to occur via the GR, at least in the SW gill. Future experiments should include the possible role of DOC in modulating MR level.

Conclusion and perspectives

The present study demonstrated the important role of cortisol in regulating osmoregulatory gene expression in the salmon gill. Most transcripts investigated were stimulated by cortisol in both FW and SW (Na^+, K^+-ATPase α1b, CFTR-I) or in SW only (CFTR-II, NKCC). The difference in responsiveness may be due to developmental and functional differences of the target cells involved. This may come down to a matter of functional equipment with corticosteroid receptors. The involvement of GR and MR varies with respect to gene of interest and acclimation salinity, thus strongly supporting a role for both signaling pathways in salinity acclimation as well as interaction between them. All effects of cortisol and antagonists in FW and SW are listed in Table 3.

The differences in target gene regulation may be due to several molecular differences along the hormone signaling pathway, e.g., differences in GRE composition of individual promoters as has been described in killifish (Schulte *et al.* 2000). Colocalization of the 11βhydroxysteroid dehydrogenase 2 enzyme, which converts cortisol to the receptor inactive cortisone (Funder *et al.* 1988) with the MR, may add another level of control of cortisol signaling through the MR. This is particularly interesting because of the recent identification of DOC – an intermediate in cortisol synthesis in fish – as a potent and specific ligand for MR in fish (Sturm *et al.* 2005, Prunet *et al.* 2006). Future studies should emphasize structural analyses of promoter and GRE sequences in osmoregulatory genes in fish in conjunction with functional studies of GR/MR effects of cortisol and DOC.

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