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Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout

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Summary

Fish acclimated to ion-deficient water exhibit proliferation of branchial chloride cells. The objective of the present study was to investigate the role of cortisol in this response using the corticosteroid receptor antagonists RU486 and spironolactone. RU486 is a potent antagonist of the glucocorticoid actions of cortisol, whereas spironolactone exhibits high-affinity binding to mineralocorticoid receptors, with a resulting blockade of mineralocorticoid properties in mammals. Untreated rainbow trout, as well as rainbow trout given a single intraperitoneal implant of coconut oil alone, coconut oil containing RU486 (0.5 mg g^{-1}) or coconut oil containing spironolactone (0.1 mg g^{-1}), were exposed to either dechlorinated city-of-Ottawa tapwater or artificial softwater for 7 days. Neither corticosteroid antagonist nor acclimation condition affected circulating plasma cortisol levels, plasma ion concentrations or gill Na^+-K^+-ATPase activity. Kidney Na^+-K^+-ATPase activity was significantly higher in softwater-acclimated fish than in fish held in dechlorinated tapwater. In addition, whereas RU486 treatment was found to be without effect on gill morphometrics, treatment with spironolactone inhibited the proliferation of chloride cells normally associated with acclimation to ion-deficient water. The results of the present study provide further evidence for the mineralocorticoid actions of cortisol in freshwater fish, specifically in eliciting chloride cell proliferation. Furthermore, these results support the hypothesis that distinct glucocorticoid and mineralocorticoid receptor populations are present in teleost fish, despite the apparent absence of the classic mineralocorticoid hormone, aldosterone.

Key words: rainbow trout, *Oncorhynchus mykiss*, chloride cell, softwater, cortisol, spironolactone, RU486, gill.

Introduction

In mammals and tetrapod lower vertebrates, aldosterone is the main mineralocorticoid hormone, playing a key role in the regulation of sodium transport across epithelia, and glucocorticoid functions are regulated by the corticosteroid hormones cortisol, cortisone and/or corticosterone (Agarwal and Mirshahi, 1999). The mineralocorticoid and glucocorticoid hormones exert their effects through separate, well-characterised receptors that exhibit high sequence homology; both the aldosterone receptor (mineralocorticoid receptor, MR) and glucocorticoid receptor (GR) are members of the steroid receptor superfamily (Arriza et al., 1987). By contrast, cortisol, the main corticosteroid in teleost fish, plays a dual role in contributing to the regulation of both carbohydrate metabolism and salt/water balance (Bern and Madsen, 1992; Wendelaar Bonga, 1997; Mommsen et al., 1999). Until recently, in the absence of identification of a distinct mineralocorticoid receptor in fish, both the mineralocorticoid and glucocorticoid actions of cortisol have been considered to be mediated by a single corticoid receptor of the GR subtype; see review by Mommsen et al. (1999). GRs have been characterised in a number of fish species and tissues, see for example table 3 in Mommsen et al. (1999). Recently, however, a novel steroid receptor was cloned from rainbow trout testis (Colombe et al., 2000). This receptor exhibited high homology to mammalian and amphibian MR cDNA sequences at both nucleotide and amino acid levels, as well as steroid binding characteristics consistent with those of other MRs, leading to the conclusion that rainbow trout possess a putative mineralocorticoid-like receptor (rtMR) (Colombe et al., 2000). The tissue distribution and physiological function of the new receptor, however, remain unclear.

In the absence of aldosterone in fish, cortisol plays an important role in the maintenance of salt and water balance (Bern and Madsen, 1992; Wendelaar Bonga, 1997; Mommsen et al., 1999). The mineralocorticoid actions of cortisol have received particular attention with respect to the osmoregulatory processes required for adaptation to sea water. The transfer of fish from fresh water to sea water is accompanied by a prolonged elevation of plasma cortisol that is widely accepted

to contribute to seawater acclimation by stimulating the proliferation and differentiation of ion-transporting chloride cells within the gill epithelium, as well as by stimulating Na^+-K^+-ATPase activity within osmoregulatory organs including the gill, intestine and kidney (Madsen et al., 1995; Marshall et al., 1999; Seidelin et al., 2000) (for reviews, see Bern and Madsen, 1992; McCormick, 1995).

Cortisol also appears to contribute to the ionic/osmotic regulation of freshwater fish. Cortisol treatment induces chloride cell proliferation (Laurent and Perry, 1990; Laurent et al., 1994; Bindon et al., 1994; Dang et al., 2000) and increases whole body and/or branchial ion (Na^+, Ca^{2+}, Cl^-) influx in freshwater rainbow trout (Perry and Laurent, 1989; Flik and Perry, 1989; Laurent and Perry, 1990; Bindon et al., 1994). In addition, transient elevations of plasma cortisol concentrations have been found to be associated with exposure of rainbow trout to water deficient in NaCl, Ca^{2+} or both (Perry and Wood, 1985; Perry and Laurent, 1989; Flik and Perry, 1989). Softwater-acclimated fish experience a chloride cell proliferation (for reviews, see Laurent and Perry, 1991; Perry and Laurent, 1993; Perry, 1997) that is believed to contribute to the maintenance of ionic homeostasis by enhancing branchial ion uptake, e.g. Perry and others (Perry and Wood, 1985; Perry and Laurent, 1989; Flik and Perry, 1989; Greco et al., 1996), and cortisol is hypothesised to play a role in this process by stimulating the chloride cell proliferation (Perry and Laurent, 1989; Flik and Perry, 1989). However, despite several studies that have highlighted an apparent association between the elevation of plasma cortisol concentrations in freshwater fish and chloride cell proliferation, the proximate stimulus for chloride cell proliferation during softwater acclimation remains poorly understood.

The present study was thus conceived with two aims. Firstly, the hypothesis that cortisol contributes to chloride cell proliferation in rainbow trout during softwater acclimation was tested by blocking cortisol receptors in fish exposed to ion-deficient water and examining gill morphology. Secondly, by using both the GR antagonist RU486, which has been shown to have antiglucocorticoid effects in fish (for a review, see Mommsen et al., 1999), and spironolactone, a known MR antagonist in amphibians and higher vertebrates (Delyani, 2000), the hypothesis that rainbow trout possess distinct populations of glucocorticoid and mineralocorticoid receptors was tested.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 38.0±0.6 g; experimental N=144) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Fish were maintained on a 12 h:12 h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated city-of-Ottawa tapwater at 13 °C. Trout were fed to satiation on alternate days on a diet of commercial trout pellets and were allowed at least 2 weeks to acclimate to the holding conditions before any experiments were performed.

Acclimation conditions and experimental protocol

Fish were randomly allocated to one of four treatment groups (N=24 in each group); untreated, sham, RU486-treated or spironolactone-treated. In each case, the trout were anaesthetised in a solution of benzocaine (0.05 mg ml^{-1}), fork length and mass were recorded, and a fin was either clipped or marked with Alcian Blue dye, according to the treatment group to which the fish was allocated. All fish except those in the untreated group were also given a single intraperitoneal injection of warm hydrogenated coconut oil (0.005 ml g^{-1} body mass) alone (sham group), or containing RU486 (0.5 mg g^{-1}; mifepristone, Sigma) or spironolactone (0.1 mg g^{-1}; Sigma).

Half of the fish in each treatment group were maintained in dechlorinated city-of-Ottawa tapwater (control groups, N=12 in each group), while the remainder (N=12 in each group) were provided with dechlorinated tapwater diluted with reverse osmosis (RO) water (artificial softwater; see Table 1 for water ion concentrations). Fish were exposed to the softwater condition by a gradual increase in the proportion of RO water over a 24 h period until the final conditions were met; day 1 of softwater exposure was taken to be the first day in full-strength artificial soft water. Softwater-acclimated fish were fed to satiation daily, the quantity of food consumed was recorded, and the control fish were fed accordingly (average=2.6 % of body mass per day).

After 7 days, all fish were killed by anaesthetic overdose, fork lengths and mass were recorded, and a blood sample (0.5 ml) was withdrawn by caudal puncture. The 7-day acclimation period was chosen to balance the contrasting requirements of sufficient time for chloride cell proliferation to occur (Greco et al., 1996), and the expected duration of effectiveness of the cortisol receptor blocker implants. Blood samples were centrifuged and separated plasma was frozen in liquid N_2 and stored at −80 °C for later analysis of plasma cortisol (N=6 for each group) or ion (N=6 for each group) concentrations. Tissue samples were then collected for microscopy (N=6 for each group) or analysis of Na^+-K^+-

Table 1. *Water ion concentrations for the control (city-of-Ottawa dechlorinated tapwater) and softwater (artificial softwater) conditions to which rainbow trout were acclimated*

[Ion] (mmol l^{-1})	City-of-Ottawa dechlorinated tapwater	Artificial softwater
Na^+	0.163±0.003 (39)	0.032±0.002 (63)*
K^+	0.021±0.002 (39)	0.007±0.0004 (63)*
Ca^{2+}	0.397±0.009 (39)	0.096±0.005 (63)*
Cl^-	0.173±0.008 (28)	0.050±0.004 (58)*

Values are means ± 1 S.E.M. (N).

*Significant difference from the corresponding control value (Student's t-test or Mann–Whitney rank sum test, P<0.05).

ATPase activity (N=6 for each group). Gill and kidney tissue samples were collected for analysis of Na^+-K^+-ATPase activity. The heart was exposed *via* a ventral mid-line incision and a cannula (PE160) was inserted through the ventricle into the ventral aorta. Heparinised saline (10 i.u. ml^{-1} sodium heparin in 0.9 % NaCl; 20 ml) containing isoproterenol (as a vasorelaxant; 10^{-6} mol l^{-1}) was then infused with a 60 ml syringe. All gill arches were removed from the fish, and the filaments were cut from the arches and frozen in liquid N_2. The trunk kidney was stripped out and immediately frozen in liquid N_2. Tissue samples were stored at –80 °C until analysis (up to 6 months).

For microscopy, the left second gill arch was excised and rinsed in 0.9 % saline. Small samples of filaments were removed and fixed in buffered glutaraldehyde (5 % glutaraldehyde in phosphate buffer; 1 h; 4 °C). The gill tissue was stained with an osmium-zinc iodide preparation (1:4 2 % OsO_4:3 % ZnI_2; 18 h; 20 °C), which causes a reduction of osmic acid to osmium, blackening the phospholipids. Following staining, the tissues were dehydrated in an ethanol series (30 %, 50 %, 75 % and 95 %, followed by 100 % ethanol (twice), 20 min in each; 20 °C), rinsed in Histochoice® (2× 20 min; 20 °C) and Paraplast® (2× 20 min; 55 °C), embedded in Paraplast®, and cut into 7 μm sections.

In a separate experiment, the time course of any changes in plasma cortisol concentrations resulting from acclimation to ion-poor water was assessed. A group (N=60) of (untreated) trout was exposed to softwater as described above. After exposure to softwater at 24, 48, 72, 96, 120, 144 and 168 h, six fish were removed from the tank and killed by anaesthetic overdose. A blood sample (0.5 ml) was withdrawn by caudal puncture and centrifuged, and the separated plasma was frozen in liquid N_2 and stored at –80 °C for later analysis of plasma cortisol concentrations.

Gill morphology

Eight slides, with eight sections per slide, were prepared for each fish, producing a total of 64 sections per fish. Slides were viewed using a light microscope (25× objective; Leitz Dialux® microscope) and digital images were acquired (JVC TK1381 1/2”CCD digital video colour camera, 470 lines resolution PCI Image Capture card). Six images each for four fish from each treatment group were captured from randomly selected sections; the slide was positioned so that the field of view contained approximately seven lamellae and a small portion of the filament at the bases of the lamellae. For quantification, the gill tissue area in a picture was recorded using Scion Image software and the number of chloride cells per unit tissue area was calculated by visually counting the stained cells (Fig. 1). The thickness of the lamellae was measured (two measurements per image) and also the interlamellar distance (two measurements per image) (Fig. 1). The value for each fish was taken as the mean of the values for the six images.

Analytical techniques

Water and plasma Na^+, Ca^{2+} and K^+ concentrations were determined by flame emission spectrophotometry (Varian, model SpectraAA 250 Plus). Cl^- concentrations in water and plasma were determined by a mercuric thiocyanate spectrophotometric assay method. A commercial radioimmunoassay (ICN Pharmaceuticals) was used for the measurement of plasma cortisol concentrations. Na^+-K^+-ATPase activities in gill and kidney tissue homogenates were measured according to the method of McCormick (1993). In brief, samples were thawed on ice and homogenised for 30 s in SEID buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA and 50 mmol l^{-1} imidazole at pH 7.3 with 0.1 % w/v sodium deoxycholate), then centrifuged for 1 min at 5000 ***g***. Samples (10 μl) of the resultant supernatant were run in two sets of duplicates in a 96-well microplate, one set containing only assay mixture (300 i.u. lactate dehydrogenase, 375 i.u. pyruvate kinase, 2.1 mmol l^{-1} phosphoenolpyruvate, 2.6 mmol l^{-1} ATP, 0.17 mmol l^{-1} NADH, 50 mmol l^{-1} imidazole, 47 mmol l^{-1} NaCl, 2.6 mmol l^{-1} $MgCl_2$, and 10.5 mmol l^{-1} KCl, pH 7.5), and the other containing assay mixture plus 0.4 mmol l^{-1} ouabain. Na^+-K^+-ATPase activity was determined by subtracting the oxidation rate of NADH to NAD in the presence of ouabain from that in the absence of ouabain. Absorbance was measured at 340 nm using a SpectraMAX 340PC microplate reader (Molecular Devices). Supernatant total protein levels were measured using the Bradford method allowing Na^+-K^+-ATPase activities to be expressed as μmol ADP mg^{-1} protein h^{-1}.

Statistical analyses

Data are presented as mean values ± 1 S.E.M. Statistical differences among the two acclimation conditions (control or softwater) and four treatment groups (untreated, sham, RU486-treated and spironolactone-treated) in plasma cortisol concentrations, plasma ion levels, gill morphometric variables and tissue Na^+-K^+-ATPase activities were assessed using two-way analysis of variance (ANOVA) followed by Bonferroni’s *post hoc* multiple-comparisons test, as appropriate. Bonferroni’s test was used to make comparisons against controls, which were designated as untreated fish (treatment) and dechlorinated tapwater (acclimation condition). The effect of acclimation time on plasma cortisol concentrations was assessed by means of a one-way ANOVA. Comparisons in water ion levels between tapwater and softwater were carried out using Student’s t-tests. Where assumptions of normality or equal variances were violated, equivalent non-parametric statistical analyses were employed. The fiducial limit of significance in all analyses was 5 %.

Results

Neither acclimation condition (control *versus* softwater) nor treatment (untreated, sham, RU486-treated or spironolactone-treated) had any significant effect on plasma cortisol concentrations at 7 days (Table 2). Moreover, in a separate group of (untreated) fish in which plasma cortisol levels were measured at 24 h intervals during a 7-day acclimation to soft

Table 2. *Plasma cortisol concentrations in control and softwater-acclimated rainbow trout in untreated, sham, RU486-treated or spironolactone-treated groups*

Treatment	City-of-Ottawa dechlorinated tapwater	Artificial softwater
Untreated	1.32±0.17	1.14±0.07
Sham	3.48±1.13	1.93±0.25
RU486	7.82±6.46	3.46±1.11
Spironolactone	1.64±0.23	3.47±1.13

Cortisol levels were determined after 7 days of treatment (see Materials and methods for details).

Values are means ± 1 S.E.M. (N=6 for each treatment group).

Table 3. *Plasma ion concentrations and gill and kidney Na^+-K^+-ATPase activities measured in control and softwater-acclimated rainbow trout*

	City-of-Ottawa dechlorinated tapwater	Artificial softwater
$[Na^+]$ (mmol l^{-1})	159.9±1.5	159.8±3.2
$[K^+]$ (mmol l^{-1})	3.09±0.09	3.33±0.06*
[Total calcium] (mmol l^{-1})	3.26±0.04	3.17±0.06
$[Cl^-]$ (mmol l^{-1})	141.3±2.0	144.5±2.2
Gill Na^+-K^+-ATPase activity (μmol ADP mg^{-1} protein h^{-1})	0.179±0.020	0.191±0.020
Kidney Na^+-K^+-ATPase activity (μmol ADP mg^{-1} protein h^{-1})	2.93±0.20	3.56±0.25*

Values are means ± 1 S.E.M. (N=22 and 24 for fish acclimated to control and softwater, respectively).

As there were no significant effects of treatment on plasma ion concentrations or tissue Na^+-K^+-ATPase activities, data for treatment groups within an acclimation condition have been combined.

*A significant difference from fish acclimated to dechlorinated tapwater (two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons test, P<0.05).

water, no significant differences were detected (data not shown). In both cases, all groups exhibited plasma cortisol concentrations characteristic of unstressed fish [overall means were 3.00±0.84 ng ml^{-1} (N=48) and 2.03±0.20 ng ml^{-1} (N=48), respectively, for the two experiments described above; mean ± 1 S.E.M.].

Plasma ion concentrations at 7 days as well as gill and kidney Na^+-K^+-ATPase activities were similarly unaffected by treatment group. However, acclimation condition had a significant effect on plasma K^+ concentrations and kidney Na^+-K^+-ATPase activities, both of which were higher in fish acclimated to ion-deficient water than in control fish (Table 3). Acclimation condition had no effect on gill Na^+-K^+-ATPase activity or plasma Na^+, Ca^{2+} or Cl^- levels (Table 3).

A different picture emerged from the analysis of gill morphology. Fig. 1 presents representative light micrographs of trout gills sampled from untreated (Fig. 1A,B), RU486-treated (Fig. 1C,D) and spironolactone-treated fish (Fig. 1E,F) acclimated to control (Fig. 1A,C,E) or softwater (Fig. 1B,D,F) conditions to illustrate the general morphological appearance of the filaments and lamellae. In general, the densely stained chloride cells were more numerous and larger in the gills of fish acclimated to softwater, and the higher chloride cell densities were associated with increased lamellae thickness and decreased interlamellar distances. Gills from the spironolactone-treated fish acclimated to softwater (Fig. 1F), in resembling those from fish acclimated to control water, provided the exception to these general trends.

Statistical analysis of the morphometric data revealed that acclimation to softwater resulted in significant increases in chloride cell density (Fig. 2A) and lamellar thickness (Fig. 2B) together with a significant reduction in interlamellar distance (Fig. 2C) in all treatment groups except that treated with spironolactone. No significant effect of acclimation condition was detected for any gill morphometric parameter in spironolactone-treated fish (Fig. 2). Among the fish acclimated to dechlorinated city-of-Ottawa tapwater, small but significant sham effects on chloride cell density (Fig. 2A) and lamellar thickness (Fig. 2B) were detected, while interlamellar distance was significantly greater in spironolactone-treated fish than in untreated fish (Fig. 2C); gill morphology was otherwise unaffected by treatment group among fish acclimated to control conditions. Among the fish acclimated to artificial softwater, no significant differences were detected between untreated and sham- or RU486-treated fish with the exception of a slightly, but significantly lower lamellar thickness in RU486-treated fish in comparison to the untreated group (Fig. 2B).

Discussion

By demonstrating that cortisol receptor blockade prevents chloride cell proliferation in rainbow trout exposed to ion-poor water, the results of the present study support the hypothesis that cortisol plays a pivotal role in softwater acclimation in freshwater fish. Moreover, the observation that chloride cell proliferation was blocked by the MR antagonist spironolactone but not by the GR antagonist RU486 is further evidence for the presence of a mineralocorticoid-like receptor in rainbow trout, as proposed by Colombe et al. (2000). These two key findings will be discussed separately.

Cortisol receptors in teleost fish

Until the recent cloning of a mineralocorticoid-like receptor (rtMR) from rainbow trout testis and characterisation of its steroid binding domain using the recombinant protein (Colombe et al., 2000), all effects of cortisol in teleost fish were considered to be exerted through a glucocorticoid receptor (Ducouret et al., 1995). In the present study, specific GR and MR antagonists were used to test the hypothesis that rainbow trout possess distinct populations of glucocorticoid and mineralocorticoid receptors. The steroid analogue RU486 has been found to exhibit high-affinity binding to cortisol receptors

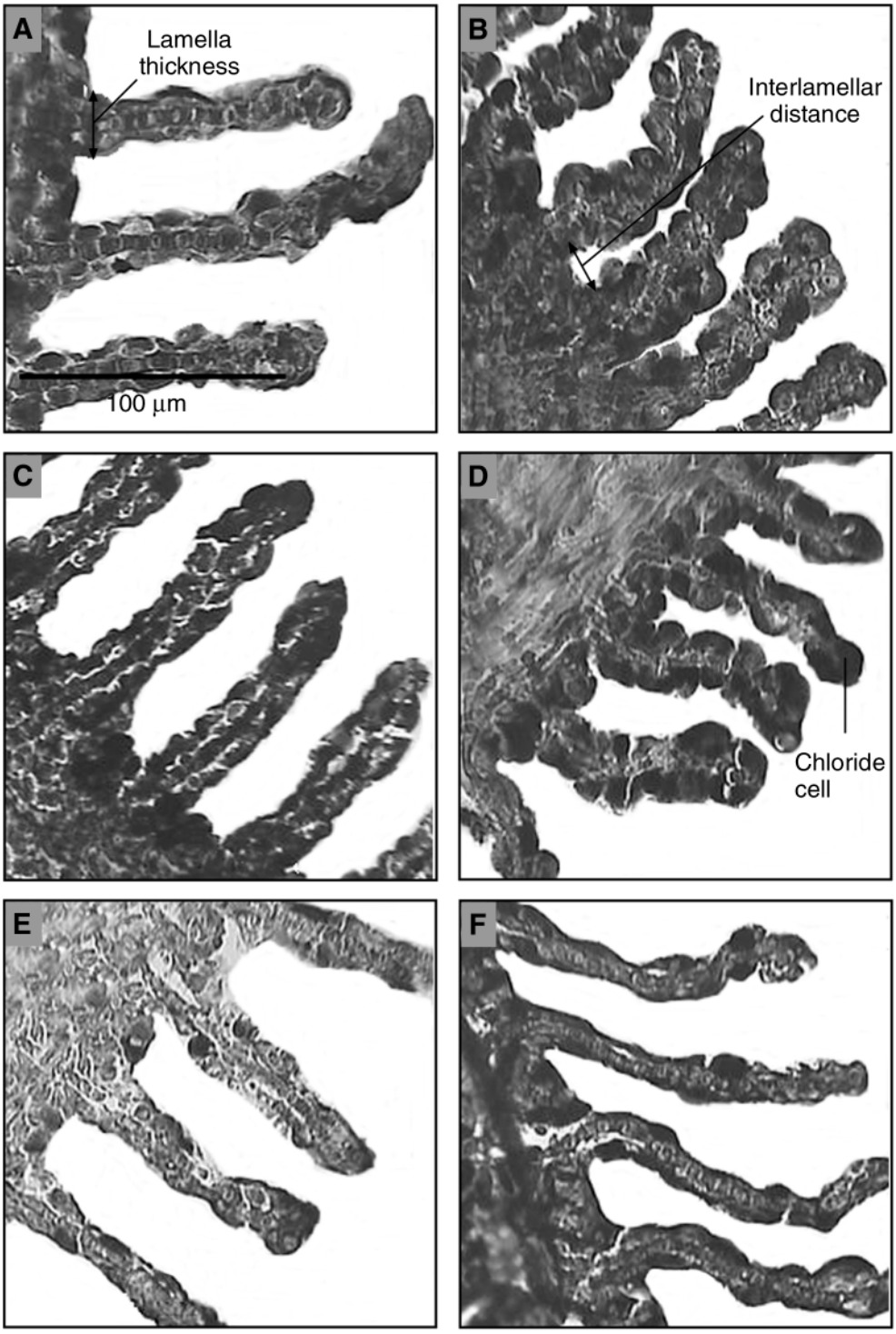

Fig. 1. Representative light micrographs of trout gills showing filament and lamellae from untreated (A,B), RU486-treated (C,D) and spironolactone-treated (E,F) fish acclimated to dechlorinated city-of-Ottawa tapwater (control; A,C,E) or artificial softwater (B,D,F) for 7 days. Chloride cells, which are densely stained, have proliferated over the entire surface of the gill epithelium of the softwater-acclimated fish, with the exception of those fish treated with spironolactone (F). Accompanying the chloride cell proliferation is an increase in lamellar thickness and a reduction in interlamellar distances. Chloride cells of fish acclimated to control conditions (A,C,E) and of spironolactone-treated fish acclimated to softwater (F) are smaller and far less numerous.

in several fish tissues (Lee and Bols, 1989; Pottinger, 1990), with a resultant blockade of glucocorticoid properties both *in vitro* (Lee and Bols, 1989; Dasmahapatra and Lee, 1993; Weyts et al., 1998) and *in vivo* (Vijayan and Leatherland, 1992; Vijayan et al., 1994; Reddy et al., 1995). In reviewing the usefulness of RU486 as an experimental tool, Mommsen et al. (1999) concluded that it was an excellent means of blocking GR-induced activities in fish systems. Interestingly, RU486 was also found to inhibit intestinal fluid uptake in Atlantic salmon smolts (Veillette et al., 1995), an example of a mineralocorticoid action of cortisol mediated by a GR. Spironolactone, by contrast, has been relatively little used in fish systems. A known MR antagonist in mammalian and amphibian systems (Delyani, 2000), spironolactone was found to be without effect on renal function in dogfish (Churchill et al., 1985) and to be ineffective in blocking dexamethasone-

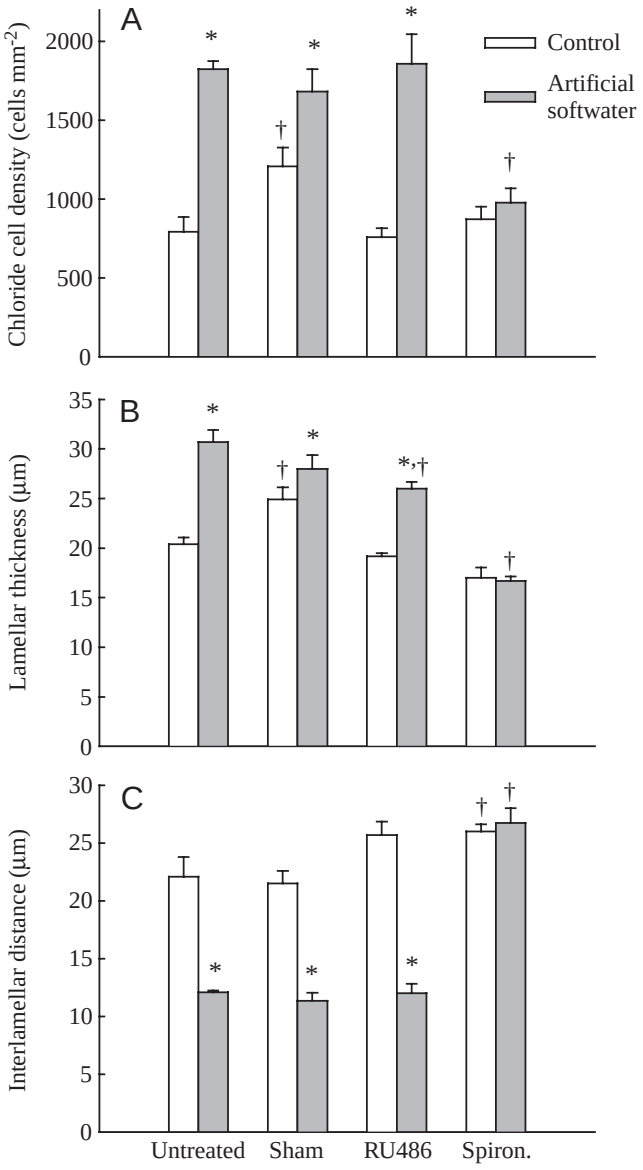

Fig. 2. The effect of acclimation condition (control *versus* softwater) and treatment (Untreated, Sham, RU486-treated and spironolactone-treated, Spiron.) on morphometric measurements for rainbow trout gills. (A) Chloride cell density, (B) lamellar thickness, (C) interlamellar distance. Values are means ± 1 S.E.M. (N=4 for each group). *A significant difference between control and softwater-acclimated fish within the same treatment group; † a significant difference within an acclimation condition (control or softwater) from the untreated group (two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons test, P<0.05).

induced effects in trout hepatocytes (Dasmahapatra and Lee, 1993). In the present study, however, the chloride cell proliferation induced by acclimation to ion-deficient water (see below) was blocked by treatment with spironolactone but not RU486 (Figs 1, 2). This result therefore provides the first physiological evidence in a teleost fish of a mineralocorticoid action of cortisol mediated by a mineralocorticoid-like receptor.

Assuming a branchial location for the mineralocorticoid-like receptor involved in mediating chloride cell proliferation, it is likely that the rtMR is co-expressed with a GR. Receptor assays have revealed the presence of GR activity in the gills of several fish species, including rainbow trout (Sandor et al., 1984; Weisbart et al., 1987; Maule and Schreck, 1990; Shrimpton and McCormick, 1998; Shrimpton and McCormick, 1999; Marsigliante et al., 2000). The corticosteroid receptor activity in these studies has been identified as a GR on the basis of its steroid-binding characteristics, including the inability of aldosterone to compete significantly for binding sites (Sandor et al., 1984; Maule and Schreck, 1990). The fish GR has also been cloned (Ducouret et al., 1995), and GR gene expression has been detected in rainbow trout gills (Ducouret et al., 1995). *In situ* hybridisation and immunocytochemical studies were used to localise GR gene expression more specifically to chloride cells and, to a lesser extent, to pavement cells and undifferentiated cells in the interlamellar regions in the gills of chum salmon (Uchida et al., 1998).

Evidence that this branchial GR activity is involved in the mediation of the mineralocorticoid actions of cortisol has been obtained from studies of cortisol-induced branchial cellular changes *in vitro* (Bury et al., 1998). In addition, studies of the osmoregulatory changes associated with the transition from fresh water to sea water have been important in this regard. For example, changes in branchial GR activity have been detected during seawater adaptation (Weisbart et al., 1987; Marsigliante et al., 2000), as well as following treatment with hormones (such as growth hormone, cortisol) that tend to enhance seawater tolerance (Weisbart et al., 1987; Shrimpton and McCormick, 1998). Moreover, Shrimpton and McCormick (1999) have demonstrated that a strong correlation exists between GR concentration and the responsiveness of gill tissue to cortisol *in vitro*, where branchial Na^+-K^+-ATPase activity was used as the index of gill responsiveness to cortisol. While the evidence supporting the involvement of GRs in mediating the mineralocorticoid actions of cortisol is mounting, the impact of exposure to ion-poor water on branchial GR activity or GR gene expression does not yet appear to have been investigated.

The apparent involvement of a mineralocorticoid receptor in chloride cell proliferation indicated by the results of the present study, in conjunction with the evidence for branchial GRs and branchial GR involvement in salt/water balance, suggest that both MRs and GRs are present in teleost fish gills and that both are involved in mediating the mineralocorticoid effects of cortisol. While GRs have been localised to chloride cells, pavement cells and undifferentiated cells (Uchida et al., 1998), the cellular distribution of MRs remains to be determined. In addition, the different roles envisaged for chloride and pavement cells in ionic regulation (for a review, see Perry, 1997) raises the intriguing possibility of differential expression and/or regulation of GRs and MRs in different cell types. Clearly, the roles of the corticosteroid receptors in teleost ionic and osmotic regulation warrant further investigation.

Cortisol and chloride cell proliferation in ion-poor water

Exposure of rainbow trout to ion-deficient water for 1 week elicited chloride cell proliferation (Fig. 2A), the response that was expected on the basis of previous studies (e.g. Perry and Wood, 1985; Avella et al., 1987; Perry and Laurent, 1989; Laurent et al., 1994; Greco et al., 1996). The increased density of the voluminous chloride cells, in turn, caused a marked increase in the lamellar thickness (Fig. 2B) and an associated narrowing of the interlamellar water channels (Fig. 2C); these consequences of chloride cell proliferation are similar to those reported by others (Bindon et al., 1994; Greco et al., 1996). In the present study, this well-characterised response was used to test the hypothesis that cortisol plays a role in stimulating chloride cell proliferation during exposure to ion-deficient water. Several previous studies have reported either transient (Perry and Wood, 1985; Perry and Laurent, 1989) or maintained (Flik and Perry, 1989) increases in plasma cortisol levels during exposure to ion-deficient water. Indeed, this mobilisation of cortisol is one of the factors on which the hypothesis that cortisol triggers chloride cell proliferation is based. However, no significant changes in plasma cortisol concentrations were detected in the present study. Both at the end of the acclimation period (Table 2) and at 24 h intervals throughout the exposure to ion-poor water, cortisol concentrations were at a level indicative of unstressed fish (typically $<5\,ng\,ml^{-1}$) (Gamperl et al., 1994; Pickering and Pottinger, 1995). It is possible that a transient increase occurred over the initial 24 h period during which water ion levels were adjusted, and that cortisol concentrations had returned to basal levels by the first sampling time. A rapid recovery of cortisol to basal levels is not unusual in response to an acute stressor, for example, see Pickering et al. (1982). Transient increases in plasma cortisol levels in response to treatment with the cortisol receptor antagonists may also have occurred owing to reduced stimulation of regulatory negative feedback pathways. Bernier et al. (1999) demonstrated that RU486 administration elicits an acute increase in plasma cortisol levels, but that cortisol concentrations return to resting values by 3 days after treatment, a time course that would account for the apparent lack of any effect of antagonist treatment on plasma cortisol concentrations found in the present (Table 2) and other studies (Vijayan et al., 1994; Reddy et al., 1995), but see Veillette et al. (1995). The overall lower levels of cortisol in the present study, compared to those reported previously for fish exposed to ion-deficient water (Perry and Wood, 1985; Perry and Laurent, 1989; Flik and Perry, 1989) may also reflect the generally less severe ionic challenge to which the fish were exposed, as well as the very gentle transition from normal to artificial softwater that was employed.

Although cortisol mobilisation was not detected in the present study, cortisol receptor blockade nevertheless prevented chloride cell proliferation (Figs 1, 2), clearly indicating the important role played by cortisol in this response to ionoregulatory challenge. Exposure of rainbow trout to water deficient in NaCl, Ca^{2+}, or both, results initially in pronounced ion losses to the environment caused by reductions in ion influx rates (McDonald and Rogano, 1986; Perry and Laurent, 1989; Flik and Perry, 1989). After hours to days of exposure, however, net ion fluxes gradually return to normal values, largely through a restoration of ion influx rates that is correlated with chloride cell proliferation (Perry and Wood, 1985; Perry and Laurent, 1989). Na^+-K^+-ATPase has been localised to chloride cells (McCormick, 1995; Perry, 1997), in accordance with their role in ion uptake, and therefore it might be expected that chloride cell proliferation would be accompanied by increases in branchial Na^+-K^+-ATPase activity. Additionally, increases in both Na^+-K^+-ATPase activity and chloride cell number have been detected in fish treated with cortisol (Dang et al., 2000). However, while Flik and Perry (1989) reported an increase in branchial Na^+-K^+-ATPase activity in rainbow trout after 8 days exposure to Ca^{2+}-deficient water, no impact of ion-poor water on gill Na^+-K^+-ATPase activity was detected in the present study (Table 3) or by McCormick (1995). By contrast, kidney Na^+-K^+-ATPase activity in the present study was significantly increased by exposure to ion-deficient water (Table 3). McDonald and Rogano (1986) reported a significant decrease in renal NaCl excretion in rainbow trout after 9 days of softwater acclimation and suggested that it might be attributable to an increase in tubular NaCl reabsorption rates. Elevation of renal Na^+-K^+-ATPase activity could provide one mechanism by which such an enhancement of tubular NaCl reabsorption might be accomplished.

Depending on the ion, the severity of the ionoregulatory challenge and the extent to which acclimation has occurred, plasma ion levels may remain depressed during exposure to ion-deficient water (e.g. McDonald and Rogano, 1986; Avella et al., 1987; Perry and Laurent, 1989; Flik and Perry, 1989; Greco et al., 1995), or may be maintained at or restored to normal values by the adaptational responses (e.g. Perry and Wood, 1985; Avella et al., 1987; Greco et al., 1995), as was the case in the present study for Na^+, Ca^{2+} and Cl^- levels (Table 3). The increased plasma K^+ concentration of softwater-acclimated fish, while statistically significant (Table 3), was unlikely to have been of physiological significance. Perhaps surprisingly, even the softwater-acclimated spironolactone-treated fish, which did not exhibit chloride cell proliferation, maintained plasma ion levels that were not significantly different from control values. It is possible that, in the absence of chloride cell proliferation, other mechanisms may allow ionic homeostasis to be maintained. For example, a substantial decrease in branchial ion efflux rates in trout exposed to softwater was reported by McDonald and Rogano (1986), together with reductions in urine flow rate and renal NaCl excretion; the net effect of these adjustments would be to minimise ion losses. Dietary ion intake may also provide a means of compensating for ionoregulatory disturbances, e.g. D'Cruz et al. (1998).

In conclusion, the results of the present study clearly indicate that softwater-induced chloride cell proliferation is mediated by activation of a mineralocorticoid-like receptor. What is perhaps less clear, given the failure to detect cortisol mobilization in the present study, is the agent that stimulates

these receptors. The simplest explanation is that MRs are activated by cortisol acting in a mineralocorticoid capacity and that a transient (within 24 h of lowering ion levels) elevation of plasma cortisol levels triggers chloride cell proliferation. In this context it is notable that Laurent et al. (1994) detected significant effects of a single injection of cortisol 12–24 h after administration. A second possibility is that exposure to ion-deficient water elicits MR-upregulation *via* an as-yet-unidentified signal transduction pathway and that the resultant MRs then respond to (existing) circulating levels of cortisol. The documented labile nature of GRs in fish gills (e.g. Shrimpton and McCormick, 1998, 1999) and the high affinity for cortisol of the mineralocorticoid-like receptor from trout testis (K_d=1.9 nmol l^{-1} or 0.9 ng ml^{-1}; Colombe et al., 2000) lend credence to this possibility. Thirdly, other hormones may be involved. Both growth hormone and insulin-like growth factor-I are known to have effects on the branchial epithelium (e.g. Seidelin and Madsen, 1999). Disentangling the potential role that these and/or other hormones play in eliciting chloride cell proliferation in fish exposed to ion-deficient water will require a better understanding of the linkages among the different hormone systems. Clearly, the hypothesis that two populations of cortisol receptors exist in fish gills, and that the abundance and type of cortisol receptors available contribute to regulating the physiological effects of cortisol, warrants further investigation. The branchial localisation of MRs is a critical first step in this process.

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