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MINERALOCORTICOID RECEPTORS AND IONIC REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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MINERALOCORTICOID RECEPTORS AND IONIC REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

The mineralocorticoid receptor (MR) was recently cloned from two fish species, *Haplochromis burtoni (H. burtoni*; *hb*MR) and rainbow trout (*Oncorhynchus mykiss*; rtMR). The objectives of the present study were to detect MR protein expression in rainbow trout ionoregulatory tissues, namely the gill and kidney, as well as to use the mammalian MR agonist aldosterone to selectively target and activate the rainbow trout MR to observe its effects on renal and branchial ionic regulation. Through immunohistochemistry and Western blot analysis using a heterologous antibody, MR protein expression was detected at the gill. Immunohistochemical analysis and flow cytometry data indicated that there was a high degree of colocalization of the rtMR to Na^+-K^+-ATPase rich cells at the gill, supporting the hypothesis that the rtMR is involved in branchial ionic regulation. Additionally, acute aldosterone treatment, but not cortisol treatment, stimulated a decrease in urine flow rate (UFR) and urinary sodium excretion at 1.5 h post-injection. The decrease in UFR occurred without any change in glomerular filtration rate (GFR), suggesting that the aldosterone-induced decrease in UFR was the result of increased tubular reabsorption of water, which in turn was presumably stimulated by enhanced Na^+ reabsorption . The results of this study support the hypothesis that the rtMR is involved in renal and branchial ionic regulation and help to clarify the mechanisms through which corticosteroid-mediated ionic regulation occurs in euryhaline teleosts.

Résumé

Le récepteur minéralocorticoïde (RM) a été recemment identifié chez deux espèces de poissons, *Haplochromis burtoni* et la truite arc-en-ciel (*Oncorhynchus mykiss*). Les objectifs de la présente étude ont été de détecter l'expression de protéines RM dans les tissus de régulation ionique de la truite arc-en ciel, particulièrement les branchies et les reins, et d'utiliser l'agoniste du RM des mammifères, l'aldostérone, pour cibler selectivement et activer le RM de la truite arc-en-ciel et observer ses effets sur les régulations ioniques rénale et branchiale. Employant les techniques d'immunohistochimie et d'analyse par immunobuvardage Western, l'expression de protéines RM a été détectée dans les branchies. Les résultats de l'analyse immunohistochimique et de la cytométrie en flux ont indiqué qu'il y avait un haut degré de colocalization du RM de poisson dans les cellules riches en Na^+-K^+-ATPase des branchies, supportant l'hypothèse que le RM est impliqué dans la régulation ionique des branchies. De plus, l'administration d'aldostérone, mais pas celle de cortisol, a stimulé une diminution dans le taux d'écoulement d'urine (TEU) et dans l'excrétion de sodium urinaire. La diminution du TEU s'est déroulée sans changer le taux de la filtration glomérulaire, suggérant que la réduction en TEU causée par l'aldostérone a été le résultat d'une augmentation de la récupération rénale d'eau, qui a été stimulée par l'augmentation de la récupération en Na^+. Les résultats de cette étude supportent l'hypothèse que les RM de la truite arc-en-ciel sont impliqués dans la régulation ionique rénale et branchiale et aident à clarifier les mécanismes de régulations ioniques contrôlées par les corticostéroïdes chez les poissons téléostéens.

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Abbreviations

ADP, adenosine diphosphate
Aldo, aldosterone
ATP, Adenosine triphosphate
BP, blocking peptide
BSA, bovine serum albumin
cAMP, cyclic adenosine monophosphate
CHIF, corticosteroid hormone-induced factor
CRE, corticosteroid response element
DA, dorsal aorta
DAPI, 4'6-diamidino-2-phenylindole
DBD, DNA binding domain
11-DOC, 11-deoxycorticosterone
EDTA, ethylenediaminetetra-acetic acid
ENaC, epithelial sodium channel
GFR, glomerular filtration rate
GR, glucocorticoid receptor
GTP, guanosine triphosphate
HRE, hormone response element
11β-HSD-II, 11β-hydroxysteroid dehydrogenase
hsp70, heat shock protein 70
i.p., intraperitoneal
$J_{net}Na^+$, net sodium flux
Ki-ras, Kirsten-Ras
MR, mineralocorticoid receptor
NADH, nicotinamide adenosine diphosphate (reduced)
NHE, Na^+/H^+ exchanger
NKA, Na^+-K^+-ATPase
OsO_4, osmium tetroxide
PBS, phosphate buffered saline
PE, polyethylene
PEG, polyethylene glycol
PFA, paraformaldehyde
PI3K, phosphotidylinositol 3-kinase
PNA, peanut lectin agglutinin
PVC, pavement cells
SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sgk, serum and glucocorticoid activated kinase
Spiron., spironolactone
UFR, urine flow rate
ZnI_2, zinc iodide

CHAPTER 1

GENERAL INTRODUCTION

Corticosteroids, Corticosteroid Receptors and Ionic Regulation in Fish

As a euryhaline species, rainbow trout have a remarkable ability to maintain ionic homeostasis despite large variations in freshwater ion levels ($[Ca^{2+}]$ = 10 μmol l^{-1} – 4 mmol l^{-1}; McDonald and Rogano, 1986). Owing to its large surface area, the gill is the principal organ involved in maintaining the ionic concentration of the body fluids. The freshwater gill epithelium is comprised of three main cell types: pavement cells (PVCs), mitochondria rich cells (MRCs) and mucous cells (Evans et al., 1999; Wilson and Laurent, 2002). With the exception of mucous cells, the relative abundance of these cell types can vary depending on physiological and environmental factors (Perry and Laurent, 1989; Laurent et al., 1994; Sakamoto et al., 2001). In general, PVCs occupy approximately 90% of the branchial epithelium while MRCs comprise the remaining 5-10% (Goss et al., 2001). However, changes in environmental salinity often induce proliferation and differentiation of MRCs in rainbow trout (Avella et al., 1987; Laurent and Hebibi, 1989; Perry and Laurent, 1989; Laurent et al., 1994).

Recently, Goss and colleagues (2001) determined that the branchial MRC population of freshwater rainbow trout is divisable into two cell subtypes. These cell types are distinguishable based on their ability to bind peanut lectin agglutinin (PNA), a protein previously used to differentiate between α- and β-intercalated cells of the mammalian kidney (Goss et al., 2001). Based on PNA binding the MRC cell population can be divided into PNA^+ MRCs (equivalent to the chloride cell) and PNA^- MRCs. PNA^- MRCs are thought to be a population of mitochondria rich PVCs that may be important in freshwater ionic regulation. To this end, Reid et al. (2003) have demonstrated that Na^+ uptake by PNA^- PVCs could be inhibited by Na^+ channel blockade and H^+-ATPase

inhibition, thus suggesting that this cell population may have a role in freshwater sodium uptake.

Despite the novel findings of Reid et al. (2003), most evidence suggests that the mitochondria rich freshwater chloride cell (CC) is the principal site of transbranchial ion uptake. In addition to a high concentration of mitochondria, the freshwater CC has a highly tabularized basolateral membrane housing a high concentration of Na^+-K^+-ATPase transporters (Perry, 1997; Wilson and Laurent, 2002; Hirose et al., 2003). The activity of the Na^+-K^+-ATPase establishes an electrochemical gradient required for Na^+ uptake across the basolateral membrane. Direct evidence describing the model for Na^+ uptake across the apical membrane is lacking, however, the current hypothesis is that apical Na^+ entry occurs through the activity of an apical H^+-ATPase that establishes an electrical gradient favourable for Na^+ entry (see review by Marshall, 2002).

Chloride cells are also important sites in ionic regulation in seawater adapted teleosts. However, there are several important differences between freshwater and seawater CCs. Firstly, freshwater CCs are characterized by the presence of apical microvilli and lack an apical crypt while seawater CCs have a smooth, cup-shaped apical membrane (Evans et al., 1999; Marshall, 2002). In addition to these ultrastructural differences freshwater and seawater CCs also have important differences in the type of ion transporters and channels in the apical and basolateral membrane (see reviews by Marshall, 2002 and Hirose et al., 2003 for current freshwater and seawater MRC models) that allow for NaCl extrusion and NaCl uptake in hyperosmotic and hypoosmotic environments, respectively. Another important difference between freshwater and seawater CCs is in their association with adjacent cells. In seawater, Na^+ is extruded at

the gill down its electrochemical gradient via a paracellular pathway formed by shallow apical junctions between CCs and accessory cells. Accessory cells are small MRCs that commonly associate with chloride cells in the seawater gill (Pisam et al., 1990; Marshall, 2002). Contrastingly, the association between CCs and PVCs, as is the case in the freshwater gill, occurs via deep, tight junctions that prevent passive ion loss and water gain (Marshall, 2002). Thus, mitochondria rich CCs and their association with adjacent cells are important regulators of transbranchial ion movement in both freshwater and seawater environments.

The importance of chloride cells in transbranchial ion movement became evident from studies that demonstrated a high correlation between chloride cell size and/or abundance, and transbranchial ion movement (Perry and Wood, 1985; Avella et al., 1987; Perry and Laurent, 1989; Perry et al., 1992). It has become well established that proliferation of the seawater type chloride cell occurs in many salmonid species transferred from freshwater to seawater (Laurent and Hebibi, 1989; Sakamoto et al., 2001) and is an important component of the smoltification process in which fish prepare for seaward migration (Langdon and Thorpe, 1984, 1985; Uchida et al., 1996). More recently, studies have found that chloride cell proliferation also occurs upon transfer of euryhaline fish from seawater to freshwater (Laurent and Perry, 1991) and freshwater to ion-poor water (Perry and Laurent, 1989; Uchida et al., 2002; Laurent et al., 1994; Greco et al., 1996).

Interestingly, chloride cell proliferation is induced not only by changes in environmental salinity, but can also be stimulated by cortisol treatment (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry et al., 1992; Bindon et al., 1994).

Moreover, cortisol treatment increases Na^+ uptake and restores plasma ion levels in interrenalectomized and hypophysectomized goldfish in fresh water (McCormick, 2001). For these reasons, cortisol is thought to be a key endocrine mediator of the changes in transbranchial ion movements that are critical for the maintenance of ionic homeostasis in euryhaline teleosts. In many salmonid species, the chloride cell proliferation that occurs during smoltification is thought to be stimulated, at least in part, by cortisol (Richman and Zaugg, 1987; Young et al., 1989; McLeese et al., 1994; Shrimpton et al., 1995; McCormick, 2001) Indeed, plasma cortisol becomes elevated in salmonid smolts prior to seaward migration (McLeese et al., 1994) and plasma cortisol levels have been found to be correlated with the hypoosmoregulatory ability of coho salmon (Young et al., 1989). Additionally, it has been observed that plasma cortisol levels may become transiently elevated in rainbow trout exposed to low calcium (Perry and Wood, 1985) or low NaCl (Perry and Laurent, 1989) environments. It should be noted however, that exposure to soft water does not always cause an increase in plasma cortisol (Sloman et al., 2001a), nor does an elevation in plasma cortisol always result in chloride cell proliferation. For example, Sloman et al. (2000) demonstrated that chronic elevation of plasma cortisol due to subordinate social status did not cause chloride cell proliferation. Similarly, elevation of plasma cortisol due to an acute stress (air exposure) also did not stimulate chloride cell proliferation in rainbow trout (Sloman et al., 2001). Together, this evidence points to a role for corticosteroid receptors in controlling the type of response that is elicited by cortisol.

Using radioligand binding studies, corticosteroid receptors have been identified in the gills of many teleost species including brook trout (Chakraborti et al., 1987),

American eel (DiBattista et al., 1984) rainbow trout (Sandor et al., 1984), coho salmon (Maule and Schreck, 1990) and Mozambique tilapia (Dean et al., 2003). These studies concluded that the fish gill contains a high-affinity, low-capacity corticosteroid receptor population. These receptors were characterized as being glucocorticoid-like receptors based on their high affinity for cortisol and triamcinalone acetonide (Sandor et al., 1984; Chakraborti et al., 1987; Maule and Schreck, 1990). Corroborating these results, Ducouret et al. (1995) have cloned two glucocorticoid-like receptors (rtGR1 and rtGR2) in various tissues of rainbow trout including the gill, intestine and kidney. Additionally, Uchida et al. (1998) used *in situ* hybridization and immunohistochemistry approaches to localize GR expression in the gills of chum salmon (*Oncorhynchus keta*) fry and found that GR mRNA and protein expression (using a polyclonal antibody raised against a synthetic peptide corresponding to a region of the hormone binding domain of tilapia and rainbow trout) was abundant in Na^+-K^+-ATPase rich chloride cells. Consistent with this, the GR is upregulated 24 h after exposure to seawater in killifish (*Fundulus heteroclitus*, Scott et al., 2004) and tilapia (*Oreochromis mossambicus*, Dean et al., 2003). Additionally, the GR-specific antagonist RU486 inhibits intestinal fluid absorption in Atlantic salmon smolts (Veillette et al., 1995) and impairs cAMP-stimulated increases in opercular membrane chloride secretion in killifish (Marshall, 2005). Together, these data demonstrate that the GR is involved in ionic homeostasis in several euryhaline teleost species.

In addition to the gill, corticosteroid receptor mRNA expression has also been identified in the kidney of cichlids (Greenwood et al., 2003) and rainbow trout (Sturm et al., 2005). The primary functions of the kidney in fish are in acid-base and ionic/osmotic

regulation (Hickman Jr. and Trump, 1969; Perry et al., 2003). In freshwater fish, the kidney acts to maintain ionic and osmotic homeostasis by reabsorbing the majority of sodium and chloride in the filtrate (Perry et al., 2003) and producing copious amounts of dilute urine (McDonald and Rogano, 1986). The kidney carries out these processes using an integrated set of transporters and channels not dissimilar to those of the gill (Perry et al. 2003). Although models for renal ion transport in fish remain poorly defined, there are several ion transporters that appear to be involved in ionic regulation. As in the gill, principal cells of the kidney have a high concentration of Na^+-K^+-ATPase transporters in the basolateral membrane (Epstein et al., 1969; Dantzler, 2003) as well as an apical H^+-ATPase that may be important for driving Na^+ reabsorption from the filtrate (Perry and Fryer, 1997; Perry et al., 2003). The general model for Na^+ and Cl^- reuptake in non-mammalian vertebrates also includes an apical Na^+-H^+-exchanger and a Na^+-$2Cl^-$-K^+-cotransporter (Dantzler, 2003). However, the primary mechanism for Na^+ and Cl^- uptake can vary from one segment of the nephron to another in mammals (Meneton et al., 2004) and remains largely undefined in fish. Thus, the mechanisms for Na^+ reuptake from the filtrate may vary depending on the segment of the kidney under consideration. Clearly, further investigation is required to elucidate the ion reabsorptive mechanisms of the various segments of the teleost kidney.

Studies describing the kidney as a target tissue for corticosteroid action in fish are sparse. However, Epstein et al. (1969) found that acclimation of seawater killifish (*fundulus heteroclitus*) to fresh water stimulated an increase in renal Na^+-K^+-ATPase activity. The involvement of cortisol in both hyperosmotic regulation (Evans, 2002) and increasing Na^+-K^+-ATPase activity (Evans, 2002; Richman and Zaugg, 1987; Yada and

Ito, 1999) evokes the possibility that activation of corticosteroid receptors by cortisol may have been involved in the ionoregulatory changes observed at the kidney by Epstein and colleagues (1969). In support of this hypothesis, it has been observed that treatment of salt-loaded rainbow trout with deoxycorticosterone decreases renal Na^+ excretion (Holmes, 1959). Furthermore, treatment of freshwater silver eels with cortisol prevents an increase in urine sodium concentration and a decrease in UFR observed in adrenalectomized eels (Butler, 1973). Thus, although there is some evidence for a role of corticosteroids in renal ionic regulation, further investigation is required to determine the type of corticosteroids and corticosteroid receptors that are involved in renal ionoregulatory responses in fish.

The one hormone – one receptor paradigm that was considered to adequately describe the function of cortisol in both ionic regulation and metabolic homeostasis as being mediated by a single, glucocorticoid-like receptor remained uncontested for many years (Wendelaar Bonga, 1997; Mommsen et al., 1999). However, the recent cloning of a mineralocorticoid-like receptor (MR) from rainbow trout testis by Colombe and colleagues (2000) followed by several similar studies (Greenwood et al., 2003; Bury et al., 2003; Sturm et al., 2004), clearly indicate that the model for corticosteroids and corticosteroid receptor systems in fish is much more complex than previously hypothesized. In fact, the use of molecular techniques has led to the cloning and characterization of several corticosteroid receptor subtypes in fish.

The teleost glucocorticoid receptor was first cloned and characterized in rainbow trout using a probe made from the DNA binding domain of the human GR (Ducouret et al., 1995). It has since been shown that there are two structurally similar but functionally

distinct GR isoforms (GR1 and GR2a) in rainbow trout (Bury et al., 2003) and cichlids (Greenwood et al., 2003). A third GR isotype (GR2b) was also identified in rainbow trout (Takeo et al., 1996) and cichlids (Greenwood et al., 2003). The GR2b isotype is characterized by a 9 amino acid insertion within the DNA binding domain of the receptor. Analysis of amino acid sequence identity of the three GR subtypes produced similar results, both within and between species. In general, a high degree of sequence similarity was found within the DNA and hormone binding domains (~83-97%) while the D domain (hinge region) and A/B domain, thought to be important for transcriptional activation, showed the lowest degree of homology (Ducouret et al., 1995; Bury et al., 2003; Greenwood et al., 2003). This sequence comparison is not only important for determining the evolutionary relationship of these receptors, but is also useful for trying to understand the physiological function of these receptors in fish.

In addition to the multitude of GRs, recent molecular work has led to the cloning of a MR in fish. The first partial sequence of the piscine MR was obtained by Colombe et al. (2000) from rainbow trout (rtMR) testis. Full-length sequences have since been obtained in rainbow trout (rtMRa and rtMRb, Sturm *et al.*, 2005) and in cichlids (*hb*MR, Greenwood *et al.* 2003). A phylogenetic analysis of the hormone binding domain revealed that the rtMR and *hb*MR cluster with the mammalian MRs (Sturm et al., 2005; Greenwood et al., 2003), while a comparison of amino acid identity among the three fish MRs indicated that the steroid and hormone binding domains are highly conserved, whereas the A/B and D domains are less conserved (Sturm et al., 2005).

Thus, the one hormone – one receptor paradigm has grown to become a system that involves at least one hormone and four corticosteroid receptor subtypes. The

challenge now is to determine the physiological role(s) of each of these receptor subtypes, as well as to determine how cortisol mediates several different processes, such as carbohydrate metabolism and ionic/osmotic balance. Although physiological data are lacking as yet, the available analyses of tissue localization, hormone sensitivity, and responses of receptor populations to different stressors have provided some interesting clues. Tissue localization can be an effective means of identifying tissues in which receptors play key roles. However, the tissue expression patterns of rtMR and *hb*MR mRNA suggest that the fish MR is expressed ubiquitously, although conclusive evidence in this respect requires data on MR protein expression. Using real-time PCR, Sturm et al. (2005) found that the rtMR is expressed at the highest level in the brain, while Greenwood et al. (2003) used the same technique to assess *hb*MR transcript levels in the liver, an organ largely involved in metabolic homeostasis, and found that transcript levels were approximately an order of magnitude higher than in the gill, an organ important for the maintenance of ionic homeostasis. Physiological approaches are now clearly imperative for understanding the processes mediated by corticosteroid receptors in fish.

The hormone sensitivity of the MR has been investigated as an initial step towards understanding the role of the MR in fish. *In vitro* transactivation studies, using a luciferase detection system, were used to characterize the binding kinetics of this receptor. In cichlids, the *hb*MR was most sensitive to cortisol (EC_{50}=0.02 nM) and aldosterone (EC_{50}=0.05 nM) treatment and was relatively unresponsive to treatment with estradiol, 11-ketotestosterone, testosterone and progesterone (Greenwood et al., 2003). Similarly, Sturm et al. (2005) found that aldosterone (EC_{50}=0.16 nM), cortisol (EC_{50}=1.1 nM) and 11-deoxycorticosterone (DOC; EC_{50}=0.11 nM) were the most effective

transactivators of the rtMR (Sturm et al., 2005). These *in vitro* transactivation studies are an important first step towards understanding the physiological function of the fish MR *in vivo*.

To date, most of the research conducted on the MR has been carried out on tetrapod systems. As a result, most of the nomenclature for corticosteroid receptors in fish is based on sequence similarity to tetrapod corticosteroid receptors, not on physiological function. Indeed, even in tetrapods such as marine reptiles and marine birds, the distinction between gluco- and mineralocorticoids is difficult to make because of phenomena such as glucomimetism by mineralocorticoids (Agarwal and Mirshahi, 1999). Thus, the involvement of the fish MR in metabolic homeostasis and the GR in ionic homeostasis cannot be discounted.

Several studies have demonstrated the involvement of GRs in ionic regulation by showing their responsiveness to salinity challenges. Indeed, GR abundance is increased in seawater exposed killifish (Scott et al., 2004), tilapia (Dean et al., 2003) and rainbow trout (McLeese et al., 1994). Additionally, GR mRNA and protein levels are significantly decreased 5 days after exposure of rainbow trout to ion-poor water (KM Gilmour, MJ Bell and MM Vijayan, unpublished observations). Collectively, this evidence suggests that GRs are important mediators of ionic homeostasis. In the future, the response of MRs to ionoregulatory challenges should also be investigated to determine the role, if any, of the MR in teleost ionic regulation. To this end, Metz and colleagues (2003) have observed that branchial Na^+-K^+-ATPase activity, in carp (*Cyprinus carpio* L.), was higher in fish acclimated to 15 °C water, where plasma cortisol levels were lowest, than in fish acclimated to 29 °C water in which plasma cortisol levels

were significantly elevated. Moreover, substantial elevations in plasma cortisol obtained by exogenous cortisol administration caused a significant increase in branchial Na^+-K^+-ATPase activity. In keeping with this, Sturm et al. (2005), found that the rtMR and the rtGR2 have a higher affinity (approximately 40-fold) for cortisol than does the rtGR1. Thus, as was eluded to by Metz et al. (2003), it is possible that rtMR and/or rtGR2 populations become activated and bind cortisol to initiate an ionoregulatory response without necessitating an increase in plasma cortisol.

The discovery of a fish MR strengthened similarities between the fish corticosteroid system and that of mammals. However, one of the main differences between fish and other vertebrates in this respect is that fish do not appear to produce aldosterone (Sandor et al., 1966; Idler and Sangalang, 1970; Butler, 1973; Butler and Youson, 1986; Gilmour, 2005). Analysis of the corticosteroidogenic activity of fish interrenal tissue (homologous to the mammalian adrenal cortex) *in vitro* indicated that cortisol is the primary corticosteroid synthesized and secreted from this gland (Idler and Sangalang, 1970; Butler and Youson, 1986; Balm et al., 1989). These results, coupled with the fact that plasma cortisol levels often increase during ionoregulatory challenges (Perry and Wood, 1985; Perry and Laurent, 1989; McLeese et al., 1994; McCormick, 2001), suggest that cortisol has a dual role as both a mineralocorticoid and a glucocorticoid hormone in fish. In accordance with this hypothesis, cortisol has a high binding affinity and transactivational potential for both GRs (K_d=1.43 nM, Sandor et al., 1984; EC_{50}=2.1-5.4 nM, Greenwood et al., 2003) and MRs (K_d=1.9 nM, Colombe et al., 2000; EC_{50}=0.02 nM, Greenwood et al., 2003) in rainbow trout. The high affinity of the MR for cortisol in fish presents a problem similar to that observed in mammals. That is,

if basal (unstressed or resting) cortisol levels (0-5 ng ml^{-1}; Sloman et al., 2001) are sufficient to activate the fish MR (EC_{50} ~0.2 ng ml^{-1}), then a mechanism to prevent continuous activation of the receptor would be required. In mammals, aldosterone is the specific MR ligand rather than cortisol. However, circulating cortisol levels are 100-1000 fold higher than circulating aldosterone levels and thus a mechanism must exist to prevent continuous occupation of the MR by cortisol (Agarwal and Mirshahi, 1999; Stockand et al., 2002). In some mammalian tissues, this protection is provided by the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD-II). This enzyme converts cortisol to its inactive metabolites, and is typically co-localized with the MR in aldosterone-sensitive epithelia, such as the kidney (Edwards *et al.*, 1988; Hirisawa *et al.*, 1997; Farman, 1999). Interestingly, Kusakabe *et al.* (2002) recently cloned rainbow trout 11β-HSD-II and found that several tissues, including gill and kidney, express 11β-HSD-II mRNA (Kusakabe *et al.*, 2003). This evidence suggests that the potential role of cortisol as a mineralocorticoid may be determined by the expression and activity of 11β-HSD-II within the cell, as well as that of the MR itself.

There is a paucity of evidence describing the physiological function of the fish MR. In fact, the only physiological evidence to date for the role of the rtMR in ionic regulation was obtained by Sloman and colleagues (2001), who reported that the chloride cell proliferation induced by softwater exposure could be inhibited by treatment with the high affinity MR antagonist spironolactone, but not the GR antagonist RU486 (Sloman et al., 2001). This evidence, in addition to the importance of the MR in ionic regulation in mammalian systems, provides the impetus for future studies investigating the role of the fish MR in ionic regulation.

The objectives of the experiments detailed in this thesis are, firstly, to describe MR protein expression in rainbow trout ionoregulatory tissues using techniques such as immunohistochemistry, Western blotting and flow cytometry. Secondly, the effects of acute and chronic aldosterone treatment on branchial and renal ionic regulation were investigated. The goal of these experiments was to identify a role for the rainbow trout MR in ionic regulation, as well as to differentiate between MR and GR-mediated ionoregulatory responses, as both of these receptor subtypes appear to be involved in ionic regulation in teleost fish. Specifically, the hypothesis tested was that activation of MRs at the gill and kidney would affect, respectively, branchial and renal Na^+ handling. On the basis of this hypothesis, it was predicted, firstly, that MR protein expression would occur largely in Na^+-K^+-ATPase-rich cells of the gill, given the importance of these cells in regulating transbranchial ion movements. Secondly, it was predicted that both acute and chronic aldosterone treatment would stimulate Na^+ recovery from the filtrate by increasing the activity of key ion transporters, namely the Na^+-K^+-ATPase and H^+-ATPase.

CHAPTER 2

MINERALOCORTICOID RECEPTORS AND IONIC REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

The mineralocorticoid receptor (MR) was recently cloned in two fish species, *Haplochromis burtoni (H. burtoni*; *hb*MR) and rainbow trout (*Oncorhynchus mykiss*; rtMR). The objectives of the present study were to detect MR protein expression in rainbow trout ionoregulatory tissues, namely the gill and kidney, as well as to use the mammalian MR agonist aldosterone to selectively target and activate the rainbow trout MR to observe its effects on renal and branchial ionic regulation. Through immunohistochemistry and Western blot analysis using a heterologous antibody, MR protein expression was detected at the gill. Immunohistochemical analysis and flow cytometry data indicated that there was a high degree of colocalization of the rtMR to Na^+-K^+-ATPase rich cells at the gill, supporting the hypothesis that the rtMR is involved in branchial ionic regulation. Additionally, acute aldosterone treatment, but not cortisol treatment, stimulated a decrease in urine flow rate (UFR) and urinary sodium excretion at 1.5 h post-injection. The decrease in UFR occurred without any change in glomerular filtration rate (GFR), suggesting that the aldosterone-induced decrease in UFR was the result of increased tubular reabsorption of water, which in turn was presumably stimulated by enhanced Na^+ reabsorption. The results of this study support the hypothesis that the rtMR is involved in renal and branchial ionic regulation and help to clarify the mechanisms through which corticosteroid-mediated ionic regulation occurs in euryhaline teleosts.

Introduction

The mineralocorticoid receptor belongs to a large family of steroid receptors that act as ligand-activated transcription factors in the cell (Agarwal and Mirshahi, 1999; Rogerson et al., 2003). In mammals, aldosterone is the endogenous mineralocorticoid produced and secreted from the adrenal cortex (Booth et al., 2002; Williams, 2005). A particularly well characterized response to aldosterone-induced MR activation in mammals is the stimulation of Na^+ reabsorption and potassium secretion in the kidney and colon (Booth et al., 2002; Verrey et al., 2003; Meneton et al., 2004; Williams, 2005) This endocrine response plays a key role in the maintenance of osmotic and ionic balance in the body fluids.

Recently, a mineralocorticoid-like receptor was cloned and characterized in two teleost species, rainbow trout (*Oncorhynchus mykiss*, Colombe et al., 2000; Sturm et al., 2005) and *Haplochromis burtoni* (Greenwood et al., 2003). Amino acid sequence analysis revealed that the fish MR clusters closely with the MR of human, rat and *Xenopus laevis* (Greenwood et al., 2003; Sturm et al., 2005). The highest degree of sequence similarity between the rtMR and the human MR (hMR) occurs in the DNA binding (98.5%) and hormone binding (76.5%) domains (Sturm et al., 2005). The highly homologous hormone binding domains might suggest that fish and human MR bind the same endogenous ligand. However, evidence supporting the presence of aldosterone in fish is mixed, and the current consensus is that aldosterone is not produced by fish interrenal tissue (Sandor et al., 1966; Idler and Sangalang, 1970; Butler and Yousen, 1986). Indeed, a detailed analysis of the adrenocortical synthetic capacity of the interrenal tissue of the European eel (*Anguilla anguilla*, Sandor *et al.*, 1966) and that of

tilapia (*Oreochromis mossambicus*, Balm *et al.*, 1989) revealed that aldosterone could not be synthesized. More recently, the key steroidogenic enzymes cytochrome P450(11β) (11β-hydroxylase) was cloned from the testis and head kidney of the eel (*Anguilla japonica,* Jiang et al., 1998). From this study it was concluded that P450(11β) is highly expressed in interrenal tissue but does not have significant aldosterone synthesizing ability *in vitro* (Jiang et al., 1998). Recently, Sturm et al. (2005) demonstrated that 11-deoxycorticosterone (DOC), which can be synthesized by teleost fish (Inoue et al., 1997) and appears to be present in the circulation at physiologically relevant levels (Campbell et al., 1980) is a more potent agonist of the rtMR than cortisol, thus proposing that DOC may be the preferred MR ligand *in vivo*. In addition, cortisol, the principal corticosteroid produced and secreted from fish interrenal tissue (Phillips et al., 1959; Balm et al., 1989), is known to be involved in several ionoregulatory processes in fish (see reviews by Mommsen et al., 1999; McCormick, 2001; Evans, 2002). Thus, further investigation is required to determine the endogenous ligand of the teleost MR.

In mammals, MR activation by aldosterone leads to activation of second messenger systems (Booth et al., 2002) as well as gene activation and repression (Agarwal and Mirshahi, 1999; Booth et al., 2002; Stockand, 2002). The function of the MR in mammalian systems becomes clear from experiments using transgenic MR-knockout mice which display impaired salt retention (Rogerson et al., 2003) as well as from human disorders involving aldosterone secretion and/or receptivity that are associated with salt/water imbalances (Stockand, 2002). Specifically, studies have shown that MR activation initiates signaling cascades that lead to an increase in the open probability of apical Na^+ channels (Rokaw et al., 1996; Chen et al., 1999; Booth et al.,

2002) and an increase in basolateral Na^+-K^+-ATPase activity (Stockand, 2002). The function of the MR in fish, however, is as yet unknown. Molecular approaches, in both trout and cichlids, indicate that MR mRNA is expressed in a wide range of tissues including the gill and kidney (Greenwood et al., 2003; Sturm et al., 2005); MR protein expression remains to be confirmed. Branchial and renal MR mRNA expression provides the potential for the teleost MR to be involved in ionic and osmotic regulation. Currently, the only physiological evidence supporting a role for the rtMR in ionic regulation was reported by Sloman et al. (2001), who demonstrated that softwater-induced proliferation of branchial mitochondria-rich (chloride) cells could be inhibited using the high affinity mammalian MR antagonist spironolactone, while the GR antagonist RU486 had no effect. Interestingly, however, Sturm et al (2005) reported that the commonly used MR antagonist, spironolactone, exhibited agonist activity in transactivation assays with the rtMR. Nevertheless, the observations of Sloman et al. (2001), coupled with the significant correlation between chloride cell surface area and the capacity for transbranchial ion movement (Perry and Wood, 1985; Avella et al., 1987; Laurent and Perry, 1990; Perry et al., 1992), suggest that the rtMR may mediate ionoregulatory responses at the gill. The role, if any, of the rtMR in ionoregulatory responses at the kidney remains unknown but clearly warrants investigation given the role of the MR in renal Na^+ handling in mammals (Agarwal and Mirshahi, 1999; Meneton et al., 2004), and the importance of the kidney in NaCl reabsorption in freshwater teleosts (Epstein et al., 1969; Hickman and Trump, 1969; Perry et al., 2003).

The objective of the present study was to examine the hypothesis that the rtMR in branchial and renal tissue in rainbow trout is involved in the maintenance of ionic and

osmotic homeostasis. Specifically, the role of the rtMR in mediating branchial and renal Na^+ handling was investigated using both acute and chronic aldosterone treatment. Selective MR activation was attempted using the high affinity mammalian MR ligand aldosterone because aldosterone has a high affinity (Colombe et al., 2000) and transactivational capacity (Sturm et al., 2005) for the rtMR *in vitro*, while having a low affinity for the rtGR (Sandor et al., 1984). Furthermore, fish do not appear to synthesize aldosterone (Sandor et al., 1966; Idler and Sangalang, 1970; Butler, 1973; Butler and Youson, 1986) and thus the effects of aldosterone administration would not be confounded by fluctuations in endogenous aldosterone within the blood. Together, these properties make aldosterone an ideal tool for selectively targeting the rtMR and studying its function *in vivo*. In addition, MR protein expression in the gills and kidneys of rainbow trout was assessed using a heterologous MR antibody in conjunction with Western analysis, immunohistochemistry and flow cytometry. The overall goal of these experiments was to better define mechanisms underlying corticosteroid-mediated maintenance of ionic homeostasis in fish.

Materials and Methods

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*; weight 178.5 ± 8.8 g [mean ± SEM], $N = 83$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Fish were held in 1150 l stock tanks containing flowing, dechloraminated and aerated city of Ottawa tap-water. The photoperiod was maintained at 12h L:12h D, and water temperature in the holding tanks was approximately 13 °C. Fish were fed to satiation twice daily and were allowed to acclimate to laboratory conditions for at least one week before any experiments were performed.

Experiment #1: Detection of MR protein expression in rainbow trout tissues

This experiment employed Western analysis and immunohistochemical, and flow cytometric approaches to attempt to detect and then localize MR protein expression in the gills and kidney of rainbow trout. Unfortunately, the MR antibody did not cross-react with kidney tissue sections. Antigen retrieval techniques (boiling in 1 mmol l^{-1} EDTA for 30 min., boiling in HCl, pH 2, for 30 min.) were employed to enhance the immunoreactivity of kidney tissue sections to try to overcome this limitation. Epitopes masked during fixation may be exposed by antigen retrieval, or new epitopes may be revealed by refolding of proteins. Although kidney tissue section immunoreactivity was enhanced by antigen retrieval, MR-specific immunostaining was not obtained. Thus, only gill tissue sections were examined by immunohistochemistry.

Sampling, fixing and sectioning of tissue

Fish were killed by a blow to the head and gill filaments were severed from the first or second gill arch. Filaments were placed into 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) overnight, followed by 15% sucrose for 2 h, and 30% sucrose for 2 h. Filaments were mounted in Thermo Shandon Cryomatrix at –25 °C. Cryosections were cut (10 μm), transferred to electrostatically charged slides (SuperFrost Plus, Fisher Scientific) and left to dry for 10 minutes at room temperature. Slides were stored at -20 °C.

Antibodies

Currently, a homologous fish MR antibody is not commercially available. For this reason, a polyclonal goat-anti-human primary antibody raised against the N-terminal region of the human MR (diluted 1:500 in PBS; Santa Cruz Biotech.) was used. Alexa-Fluor 488 anti-goat (diluted 1:200; Molecular Probes) was used as a secondary antibody. For immunohistochemical localization of Na^+-K^+-ATPase, a monoclonal antibody raised against the α-subunit of the chicken Na^+-K^+-ATPase was used (diluted 1:200). This antibody was developed by Douglas M. Fambrough and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Alexa-Fluor 546 anti-mouse antibody (diluted 1:200; Molecular Probes) was used as a secondary antibody.

Immunohistochemistry

Two sections on each slide were circled with an Aqua Hold Pap Pen (Fisher). Sections were washed in phosphate buffered saline (PBS; 2 x 10 min) and incubated in blocking solution (10% donkey serum, 2% BSA, 0.01% NaN_3, 0.3% triton-X) for 1 h at room temperature. Slides were aspirated dry, and sections were then incubated in anti-human MR primary antibody (described above) diluted 1:500 in PBS at room temperature overnight in a humid chamber. For double labeling experiments, sections were incubated in anti-MR and anti-α5 primary antibodies simultaneously at room temperature overnight in a humid chamber. Sections were washed in PBS (3 x 15 min) and incubated in the appropriate secondary antibodies (detailed above) for 2 h at room temperature in a humid chamber. Sections were washed (3 x 15 min) in PBS, a drop of VectaShield mounting medium with 4'6-diamidino-2-phenylindole (DAPI) was applied to each section, and a coverslip was applied. Slides were viewed using a Zeiss AxioPhot microscope with the appropriate filters in place.

To demonstrate that MR immunoreactivity was specific, two control experiments were carried out. First, primary antibody (diluted to 0.02 μg ml^{-1}) was incubated for 1 h at room temperature with the peptide against which the antibody was raised (diluted to 0.2 μg ml^{-1}; Santa Cruz Biotech.). Following this pre-absorption, the standard immunolabeling protocol (described above) was followed. To account for the possibility of non-specific binding of the Alexa Fluor 488 secondary antibody, the primary antibody was replaced with PBS and the standard immunolocalization protocol was followed.

Western blotting

Western blot analysis was used to examine MR protein expression in rainbow trout gill and kidney as well as to confirm the specificity of the heterologous anti-MR antibody for the fish MR. Gill or kidney tissue was homogenized under liquid nitrogen using a mortar and pestle and resuspended in 1.5 ml of extraction buffer (200 µl of 25x Complete Mini Protease Inhibitor Cocktail (Fisher Scientific), 50 mmol l^{-1} Tris-Cl, pH 8, 150 mmol l^{-1} NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate). The homogenate was centrifuged at 14 000 x *g* for 10 minutes, the supernatants were separated from the pellets, and all samples were frozen in liquid nitrogen and stored at –20 °C until analysis.

The protein content of each sample was quantified using the Bradford method. Samples were then diluted appropriately in loading buffer (40% glycerol, 450 mmol l^{-1} Tris-HCl, pH 8.45, 4% sodium dodecyl sulphate (SDS), 0.002% Phenol Red, 0.04% Coomassie Brilliant Blue G-250, 720 $mmol^{-1}$ β-Mercaptoethanol) to give equal protein loading, and heated for 5 minutes at 95 °C. The proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% acrylamide gel. PageRuler Prestained Protein Ladder (10-180 kilodaltons (kDa); Fermentas) was used for determination of molecular length. Following electrophoresis, the proteins were transferred at constant voltage (25V), for 14 h through a cooled transfer apparatus (Hoefer) onto a 0.45 µm Pure Nitrocellulose Membrane (BioRad).

Prior to immunoblotting, membranes were incubated in 5% skim-milk at room temperature for 1 h. After blocking, membranes were incubated in primary antibody (diluted 1:100 in 0.1% Tween-20 in PBS, pH 7.4) for 1 h. For pre-absorption, a duplicate

membrane was incubated in primary antibody that was previously incubated with an excess (10x) of blocking peptide. The membranes were rinsed 5 times for 5 minutes each time in 0.1% Tween-20 in PBS, and incubated in secondary antibody (1:1000 in 0.1% Tween-20 in PBS; Molecular Probes) for 1.5 h at 37 °C. To remove any unbound secondary antibody, the membrane was washed (5 x 5 min) in 0.1% Tween-20 in PBS. Immunoreactive bands were visualized using a horseradish-peroxidase conjugated secondary antibody and Western Lightning™ Chemiluminescence reagents according to the manufacturers' instructions (Perkin Elmer).

Flow Cytometry

The technique used for the isolation of gill epithelial cells was adapted from Goss et al. (2001). Briefly, trout were removed from the holding tank and killed by a blow to the head. All gill arches were excised from two fish and placed into 30 ml ice-cold phosphate buffer (PB, pH 7.4). Small pieces of filaments cut from the gill arch were placed in trypsin-EDTA (0.5% trypsin, 5.3 $mmol^{-1}$ EDTA, Invitrogen) and shaken vigorously (8 min at ~300 rpm; Gyratory Shaker-G2). The resulting cell suspension was passed through a 100 μm nylon filter (Fisher) into PB containing 10% bovine serum. The material that did not pass through the filter was resuspended in 5 ml trypsin-EDTA and again shaken vigorously (8 minutes at ~300 rpm). This procedure was repeated three times or until the filaments appeared white. The resultant cell suspension was centrifuged at 5 °C for 8 min at 250 x *g*. The supernatant was removed and added to 5-10 ml of double-distilled water (to lyse red blood cells). A few minutes later, 30 ml of PB was added, and the suspension was centrifuged as before. This step was repeated (on

average 3 times) until all red blood cells had been removed from the pellet. At this point, the pellet was resuspended in 5 ml of 4% paraformaldehyde (PFA) in PB and incubated at 4 °C for 4 hours. The fixed cells were then resuspended in 30 ml PB, centrifuged (5 °C for 8 minutes at 250 x *g*), and the resultant cell pellet was washed twice, and resuspended in PB. The final cell pellet was resuspended in 3 ml of PB and stored at 4 °C until immunostaining and flow cytometry were performed.

For analysis by flow cytometry, approximately 5-10 x 10^6 cells were transferred to a 2 ml tube and centrifuged (5 min at 420 x *g*). The pellet was resuspended in 0.2% Tween-20 in PB and shaken vigorously for 30 minutes at room temperature to permeabilize the cells. This reaction was stopped by centrifuging (5 min at 420 x *g*) the cell suspension and the pellet was then resuspended in 200 µl of primary antibody mixture (diluted 1:500 in 0.2% Tween-20 in PBS and α5 primary antibody diluted 1:100 in 0.2% Tween-20 in PBS). The cells were incubated in primary antibody at 4 °C overnight, then 1.5 ml of PB was added to the mixture and the cells were centrifuged (5 min at 420 x *g*) and resuspended in PBS twice. Cells were then resuspended in 200 µl of secondary antibody mixture (both α5 and MR secondary antibodies were diluted 1:100 in PBS) and incubated for 1 hour at room temperature. Finally, the cells were washed and resuspended in PB twice, and diluted in a final volume of 0.8 ml of PB.

Flow cytometry was performed on a FCS-500 Flow Cytometer. Flow cytometer was calibrated using Flow-check Fluorospheres/Flow-check™ (Beckman Coulter; bead size was 3.5 and 10 µm for FL1-FL3 calibration). Based on analysis of control samples, regions were drawn to exclude cellular debris (particles less than approximately 5 µm) from subsequent analysis. The software (CXP Data Acquisition Software Version 2.0,

Beckman Coulter Inc.) was programmed to count 8,000-10,000 events from each sample. The flow cytometer had a standard optical filter configuration with band pass of 525 ± 25 nm (argon laser) for FL1 and 675 ± 25 nm (helium neon laser) for FL4.

Experiment #2: The role of the rtMR in renal and branchial Na^+ handling

The objective of this experiment was to test the hypothesis that MRs in rainbow trout play a role in Na^+ handling analogous to that observed in mammalian systems. Aldosterone was used as a tool to selectively stimulate MRs, and its effects were compared with those of cortisol injection, a treatment that would be expected to activate both GRs and MRs. Renal and branchial Na^+ fluxes were assessed individually owing to the roles of the kidneys and gills in, respectively, reabsorption of Na^+ from the filtrate and active uptake of Na^+ from the environment. In addition, glomerular filtration rate (GFR) was assessed in a separate group of fish to determine whether selective activation of MRs had any effect on this parameter. Because in mammalian systems aldosterone-induced effects may be observed within 1-4 h of aldosterone elevation (Booth et al., 2002 and Verrey et al., 2003), the approach adopted in the present experiment was to monitor renal or branchial Na^+ fluxes acutely following steroid or saline injection via the DA cannula.

The desired plasma cortisol concentration for these experiments was 400 ng ml^{-1}. This dose was chosen because it is in the upper physiological range for this species (Zhou et al., 2003). The target plasma aldosterone concentration was 4,000 ng ml^{-1} based on evidence by Colombe et al. (2000) that demonstrated the ability of cortisol to outcompete aldosterone for binding to the rtMR *in vitro*. Thus, equal effective concentrations of the

corticosteroids in the plasma could be obtained by administering a higher dose of aldosterone.

Fish preparation

Fish were anaesthetized in an oxygenated solution of benzocaine (0.05 g l^{-1} ethyl-p-aminobenzoate). Once loss of equilibrium occurred, the fish was placed on a surgical table that allowed the gills to be continuously irrigated with the same anesthetic solution (0.05 g l^{-1} ethyl-p-aminobenzoate). A guide wire technique was employed for the insertion of an indwelling cannula of flexible polyethylene tubing (PE 50, Clay Adams) into the dorsal aorta (DA), as described by Axelsson and Fritsche (1994).

Fish were in addition fitted with an external catheter for the collection of naturally discharged urine from the urogenital papilla (Curtis and Wood, 1991; Wood and Patrick, 1994). As outlined by Curtis and Wood (1991), the advantage of this method is that the urinary sphincters function normally, and thus any modifications to the urine made by the bladder can be detected. The catheter was made from a 46 cm Bard All Purpose Urethral Catheter with the dilated end used as a funnel. The catheter was stitched around the urinary papilla and anus by 16-18 evenly spaced purse-string ligatures (2-0 silk) and any leaks were sealed by applying a layer of Vetbond tissue cement between the fish body wall and the catheter. The catheter was then tested for leaks by filling it with water and visually inspecting the suture for escaping fluid. Additionally, urine ammonia levels were monitored for several fish and were at the expected level of approximately 1 mmol l^{-1} (Wood et al., 1999; data not shown). Prior to surgery, fish were starved for 3 days to empty the gastrointestinal tract. To prevent any remaining faecal matter from blocking

the catheter, a small incision was made in the ventral body wall and the posterior intestine was ligated with 2-0 surgical silk.

Fish were recovered by ventilating the gills with fresh, aerated water, and were then transferred to individual 4 l black boxes supplied with flowing, aerated water. Fish were allowed 18-24 h for recovery prior to initiating experiments. Urine was allowed to flow by gravity into scintillation vials held outside the experimental chamber, approximately 4 cm below the water level. The DA cannulae were flushed daily with heparinized (100 i.u. sodium heparin ml^{-1}) modified (4.5 mmol l^{-1} $NaHCO_3$) Cortland saline.

Experimental protocol

To examine the effect of MR activation on renal or branchial sodium handling, renal sodium excretion or branchial net sodium flux ($J_{net}Na^+$) was estimated prior to and following treatment of the fish with saline (control), aldosterone, or cortisol. For measurement of renal Na^+ excretion, the experiment consisted of one 2 h and two 3 h periods of urine collection, with blood samples being withdrawn at the midpoint of each urine collection period. Following an initial 2 h 'pre' period, fish were injected with one of saline (2 ml kg^{-1} fish, 6% ethanol in saline, $N = 5$), aldosterone (0.6 mg kg^{-1} fish, injected in 6% ethanol in saline 2 ml kg^{-1} fish, $N = 5$) or cortisol (60 μg kg^{-1} fish, injected in 6% ethanol in saline 2 ml kg^{-1} fish, $N = 5$), and were then monitored for an additional 6 h (2 x 3-h urine collection periods). For each urine collection period, urine volume was measured and urine samples were then frozen for subsequent analysis of Na^+ concentration. Blood samples (0.3-0.4 ml) were separated into plasma and red blood

cells by centrifugation. Red blood cells were re-suspended in an equivalent volume of saline and returned to the fish, while plasma samples were frozen in liquid nitrogen and stored at -80°C for subsequent analysis of Na^+, cortisol and aldosterone concentrations.

The determination of $J_{net}Na^+$ was carried out on separate groups of fish (saline, N = 5, aldosterone, N = 5, cortisol, N = 5) to determine if effects of corticosteroid treatment on Na^+ handling at the gill reflected those that were observed at the kidney. Using an experimental protocol similar to that described above, except that a 2-h pre period was followed by three 3 h post-injection periods. In addition, water samples were analysed rather than urine samples. In each period, the supply of water to the experimental chamber was removed, and the water level was adjusted to a known volume; note that each chamber was supplied with independent aeration. Water samples (10 ml) were withdrawn at the beginning and end of each flux period and stored at –80 °C for subsequent analysis of Na^+ concentrations. Between flux periods, the chambers were flushed with flowing water.

Measurement of GFR

Glomerular filtration rate was measured as previously described (Pane et al., 2005). Fish (control, N = 5; aldosterone, N = 6) were injected with 0.6 ml of Cortland saline containing 17 μCi of [^{3}H] polyethyleneglycol-4000 (PEG-4000; American Radiolabeled Chemicals; specific activity 2.7 mCi g^{-1}). The radiotracer was allowed to equilibrate in the blood and tissues of the fish for 20 h prior to initiation of the experiment. PEG-4000 was chosen as a radiolabeled marker over inulin because it more accurately reflects the actual GFR in fish (Pane et al., 2005).

Urine was collected gravimetrically for 2-h periods, one prior to injection of saline (2 ml kg^{-1} fish) or aldosterone (0.6 mg kg^{-1} fish, injection volume 2 ml kg^{-1} fish), and two post-injection, to accurately determine UFR. Blood samples (0.3 ml) were withdrawn at the midway point of each urine collection period, and centrifuged at 5,000 x *g* for 30 s. Red blood cells were re-suspended in saline and re-injected in the fish, while 25 µL aliquots of plasma were counted (Beckman Coulter LS-6500 Multipurpose Scintillation Counter) in 4.975 ml double-distilled water and 10 ml of scintillant (Ready Safe Liquid Scintillation Cocktail, Beckman Coulter). Urine aliquots (100 µl) were counted in 4.9 ml of double-distilled water and 10 ml of scintillant. Samples were mixed and incubated at 4 °C for 24 h prior to counting. GFR was calculated as follows:

$$\text{UFR} = \text{urine volume (ml)} / \text{mass (kg)} \times \text{time (h)}$$

$$\text{GFR} = (\text{UFR})\ (\text{cpm})_u / (\text{cpm})_p$$

Experiment #3: Effects of chronic aldosterone treatment on branchial and renal ion transport activity

Previous work (Sloman et al., 2001) suggested that in fish, MRs may play a role in processes, such as chloride cell proliferation, that take place over a longer time frame than the acute effects investigated above. Hence, the objective of this experiment was to investigate the influences of 48 – 72 h of MR activation on branchial and renal H^+- and Na^+-K^+-ATPase activity. Longer-term activation of MRs was achieved by treatment of the fish with slow-release aldosterone-containing implants. Na+-K+-ATPase activity was analyzed because of the importance of this ion transporter in generating the

electrochemical gradient necessary for basolateral Na^+ movement. H^+-ATPase activity was analyzed because of the functional link thought to exist between H^+-ATPase activity and apical Na^+ entry through a putative epithelial Na^+ channel.

Experimental protocol

Fish were lightly (i.e. to the point of losing equilibrium) anaesthetized in an oxygenated solution of benzocaine (0.05 g l^{-1} ethyl-*p*-aminobenzoate) and randomly allocated to one of two experimental groups. Fish in the control group ($N = 10$) were given an intraperitoneal (i.p.) injection of warm cocoa butter (4 ml kg^{-1} fish), whereas aldosterone-treated fish ($N = 10$) were given an i.p. injection of warm cocoa butter containing aldosterone (36 mg aldosterone kg^{-1}fish, volume of injection 4 ml kg^{-1} fish. When warmed slightly, cocoa butter exists in the liquid state but it rapidly forms a solid pellet at the holding temperature (13 °C) of the trout. Fish were then placed (in groups of 10) into 115 l holding tanks for 48 h. This period was chosen based on previously conducted pilot studies of the time-course of elevation of circulating aldosterone concentrations (see Appendix 1).

After 2 days, fish were terminally anaesthetized in a solution of benzocaine (0.5 g l^{-1} ethyl-*p*-aminobenzoate). Blood samples (~0.5 ml) were withdrawn by caudal puncture and centrifuged at 5,000 *g* for 30 s. Plasma was drawn off, flash frozen in liquid nitrogen and stored at –20 °C for later analysis of aldosterone concentrations. Samples of gill (from second arch) and kidney tissue were dissected out and placed into SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2 EDTA, 50 mmol l^{-1} imidizole, pH 7.3), flash frozen in

liquid nitrogen, and stored at -80 °C for later analysis of Na^+-K^+-ATPase and H^+-ATPase activities.

Analytical techniques

Water and plasma Na^+ concentrations were measured using a flame emission spectraphotometer (Varian, model spectra AA 250 Plus). Plasma cortisol and aldosterone concentrations were measured using commercial ^{125}I radioimmunoassay kits for cortisol (ICN pharmaceutical) and aldosterone (Diagnostic Systems Laboratories Inc.), respectively. Na^+-K^+-ATPase and H^+-ATPase activities were measured in gill and kidney tissue using the basic method of McCormick (1993), with modifications for H^+-ATPase activity outlined by Lin and Randall (1993). Briefly, tissue samples were homogenized in 25 µl of SEID (1 g sodium deoxycholate added to 20 ml SEI buffer) by 10 strokes of a dounce homogenizer, and the homogenate was centrifuged at 5000 x *g* for 30 s to remove cartilage. The resultant supernatant was used for determination of protein concentration and ATPase activities. ATPase activity was measured for 10-15 minutes by adding 10 µl of tissue homogenate to 200 µl of assay reagent (50 mmol l^{-1} imidazole buffer, 2 mmol l^{-1} phosphoenolpyruvate, 0.2 mmol l^{-1} NADH, 0.7 mmol l^{-1} ATP, 4.6 i.u. lactic dehydrogenase, 5.1 i.u. pyruvate kinase, pH 7.5) in a 96 well microplate. For determination of Na^+-K^+-ATPase activity, 13 mmol l^{-1} of ouabain was added to the assay reagent. For determination of H^+-ATPase activity, 17 mmol l^{-1} of N-ethylmaleimide was added to the assay reagent. Final Na^+-K^+- or H^+-ATPase activity is calculated by subtracting the rate of NADH hydrolysis in the presence of the inhibitor from that of the control. All samples were assayed in duplicate, and absorbance were measured at 340

nm using a Spectramax 340PC microplate reader (Molecular Devices). Protein concentrations were measured in triplicate using the BioRad protein assay, and ATPase activity was expressed as μmol ADP^{-1} mg $protein^{-1}$ h^{-1}.

Statistical Analyses

Data are presented as mean values ± 1 SEM. Whether differences among control, cortisol and/or aldosterone treated groups were statistically significant for acute exposure experiments was determined by two-way repeated measures (RM) analysis of variance (ANOVA) followed by the Bonferroni *post hoc* multiple comparisons test, as appropriate. The statistical significance of differences between control and aldosterone-treated groups in chronic exposure experiments was determined using Student's *t*-tests. The fiducial limit of significance in all analyses was 0.05, and all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc).

Results

Branchial rtMR protein expression

Western blot analysis was used to demonstrate the specificity of the heterologous (human) MR antibody for the fish MR. Two bands were detected in the gill corresponding to a size of approximately 180-200 kDa (Fig.2-1). Equal protein loading produced a single band in the kidney (arrowhead) of substantially lower intensity than that obtained in the gill (arrows). These bands were significantly reduced in intensity after pre-incubation of the primary antibody with excess (10x) blocking peptide (Fig. 2-1).

Immunohistochemical analysis of MR protein expression in rainbow trout gill tissue using the same heterologous MR antibody revealed a positive signal in cells located both on the lamellae, and on the filament in the interlamellar region (Fig. 2-2A,B). The intracellular signal distribution was diffuse (Fig. 2-2B), suggesting that the protein was largely present in the cytoplasm. Sections incubated with only secondary antibody (Fig. 2-2C) or with an excess (100x) of MR blocking peptide (Fig. 2-2D) lacked immunoreactivity, indicating that the immunostaining observed was specific for the MR.

Colocalization of the rtMR to Na^+-K^+-ATPase-rich cells was investigated by double-labeling of gill tissue sections with the MR antibody and α5, an antibody that is widely used to label Na^+-K^+-ATPase (Piermarini and Evans, 2000; Wilson et al., 2000; Witters et al., 1996). In double-labeled sections, cells on both the lamellae and filament fluoresced for both the MR and Na^+-K^+-ATPase (Fig. 2-3C,D; yellow fluorescence). Double-labeled sections incubated with an excess of blocking peptide showed staining

only for Na^+-K^+-ATPase (Fig. 2-3E), demonstrating the specificity of the MR signal. Interestingly, cells rarely showed only MR or Na^+-K^+-ATPase immunoreactivity alone.

Flow cytometry was used to analyse the relative percentages of isolated branchial cells that expressed only Na^+-K^+-ATPase, only MRs or both Na^+-K^+-ATPase and MRs (Fig. 2-4). Cells were identified as belonging to a particular group on the basis of immunofluorescence for α5, the MR antibody, or both, and results would therefore be expected to be comparable to those obtained by immunohistochemistry, in which the same antibodies were employed. The flow cytometry results indicated that approximately 6% of cells expressed Na^+-K^+-ATPase in abundance. Interestingly, a small population of cells expressed only MRs, but most of the cells that expressed Na^+-K^+-ATPase also expressed the MR.

Effects of acute aldosterone treatment on renal and branchial Na^+ handling

To examine the effectiveness of the steroid injection protocol, plasma cortisol and aldosterone concentrations were measured as a function of time in control, cortisol- and aldosterone-treated fish (Fig.2-5A,B). In fish injected with cortisol, plasma cortisol concentrations were significantly elevated at 1.5h post-injection (Fig.2-5A; two-way RM ANOVA with sample time and treatment group as factors, $P < 0.001$ for sample time, treatment group and for interactions between these two factors). There were no significant differences in plasma cortisol concentrations within or between control and aldosterone treated fish (Fig. 2-5A). Analysis of plasma aldosterone concentrations in aldosterone-treated fish revealed significant elevation at 1.5 h post-injection (Fig.2-5B; two-way RM ANOVA with sample time and treatment group as factors, $P < 0.001$ for

sample time, treatment group and for the interaction term). Plasma aldosterone levels in control fish were close to the detection limit of the assay. Several fish were excluded from further analysis, because cortisol levels were significantly elevated (>100 ng ml^{-1}) during the pre-injection time period on the premise that these fish did not adequately recover from surgery. Also, fish with an average post-injection plasma cortisol concentration of greater than 100 ng ml^{-1} were also excluded from further analysis to avoid potentially confounding results from fish that were highly stressed during the experiment.

An analysis of UFR showed that there was no significant difference among control, cortisol and aldosterone treated groups prior to injection (Fig. 2-6A). However, UFR was significantly reduced in aldosterone treated fish 1-3 h following injection (Fig.2-6A). There was no significant difference in UFR within or between control and cortisol treated fish throughout the experiment (Fig.2-6A). Additionally, there was no significant effect of cortisol or aldosterone treatment on plasma (Table 2-1) or urine Na^+ levels (Fig. 2-6B). However, urinary Na^+ excretion was found to be significantly lower in the aldosterone treated group than in the control or cortisol treated groups (Fig. 2-6C). Although urinary Na^+ excretion values were low (relative to controls) initially in the aldosterone treated group, the decrease in urinary Na^+ excretion at 1.5 h post-injection (relative to pre-injection values) was significant in aldosterone treated fish while this effect was not apparent in control and cortisol treated fish (Fig.2-6C, see inset), thus suggesting that aldosterone treatment stimulated a decrease in urinary Na^+ excretion.

To determine if the aldosterone-induced decrease in UFR was due to a decrease in GFR, GFR was measured prior to and following acute aldosterone and saline treatment.

GFR values were within the range of previous values obtained for euryhaline teleost fish (Hofmann and Butler, 1979; Pane et al., 2005). There were no effects of treatment group (aldosterone mean change in GFR = 2.56 ± 1.21 ml kg^{-1} h^{-1} at 1.5 h post-injection and 0.02 ± 1.44 ml kg^{-1} h^{-1} at 4.5 h post-injection; saline mean change in GFR = 2.38 ± 3.94 ml kg^{-1} h^{-1} at 1.5 h post-injection and –5.06 ± 1.01 ml kg^{-1} h^{-1} at 4.5 h post injection) or sampling time on GFR (two-way RM ANOVA with sample time and treatment group as factors, P = < 0.349 for sample time, P = 0.084 for treatment group and P = 0.326 for the interaction term).

The effect of cortisol and aldosterone treatment on net Na^+ movement across the gill was investigated. There were no significant effects of either sampling time or treatment group (2-way RM ANOVA, P = 0.874 for treatment group, P = 0.173 for sampling time, P = 0.471 for the interaction term) on branchial net Na^+ flux (Fig.2-7). Consistent with these results, there was no effect of either acute cortisol or aldosterone exposure on plasma Na^+ levels (Table 2-1; 2-way RM ANOVA, P = 0.523 for treatment group, P = 0.102 for sampling time, P = 0.622 for the interaction term).

Effects of chronic aldosterone treatment on ion transport activity

Plasma aldosterone levels were significantly (rank sum test, P = < 0.001) elevated to 6071 ± 795 pg ml^{-1} (N = 10) at 48 h after fish were given an intraperitoneal injection of cocoa butter containing aldosterone (36 mg aldosterone kg^{-1}) compared to sham injected controls (30 ± 5 pg ml^{-1}, N = 9). Although plasma aldosterone levels were significantly elevated there were no significant effects of aldosterone treatment on gill or kidney Na^+-K^+-ATPase or H^+-ATPase activities (Table 2-2).

Figure 2-1. A representative Western blot showing mineralocorticoid receptor (MR) protein expression in the gill and kidney of rainbow trout (*Oncorhynchus mykiss*). Two bands were observed in the gill at approximately 180 kDa (arrows). Only a single band of lower intensity was obtained in the kidney (arrowhead). Immunoreactivity was significantly reduced in both gill and kidney when the membrane was blotted with primary antibody and excess (10x by weight) of blocking peptide (MR + BP).

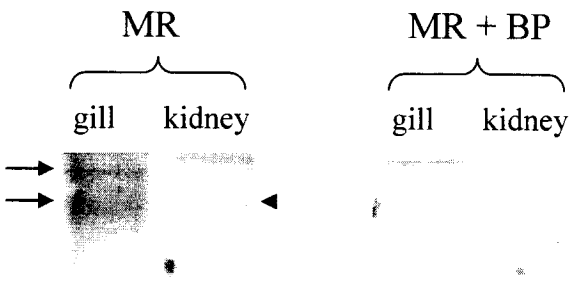

MR
MR + BP
gill
kidney
gill
kidney

Figure 2-2. Mineralocorticoid receptor (MR) immunoreactivity (green staining) in the gills of rainbow trout (*Oncorhynchus mykiss*) at low (A) and high (B) magnification. Nuclei were visualized using 4'6-diamidino-2-phenylindole (DAPI) and are shown in overlay. Various controls were performed including sections incubated in secondary antibody only (C), and sections incubated in primary antibody and an excess (100x) of MR blocking peptide (D). Control sections in no case exhibited immunoreactivity, indicating that the MR signal was specific.

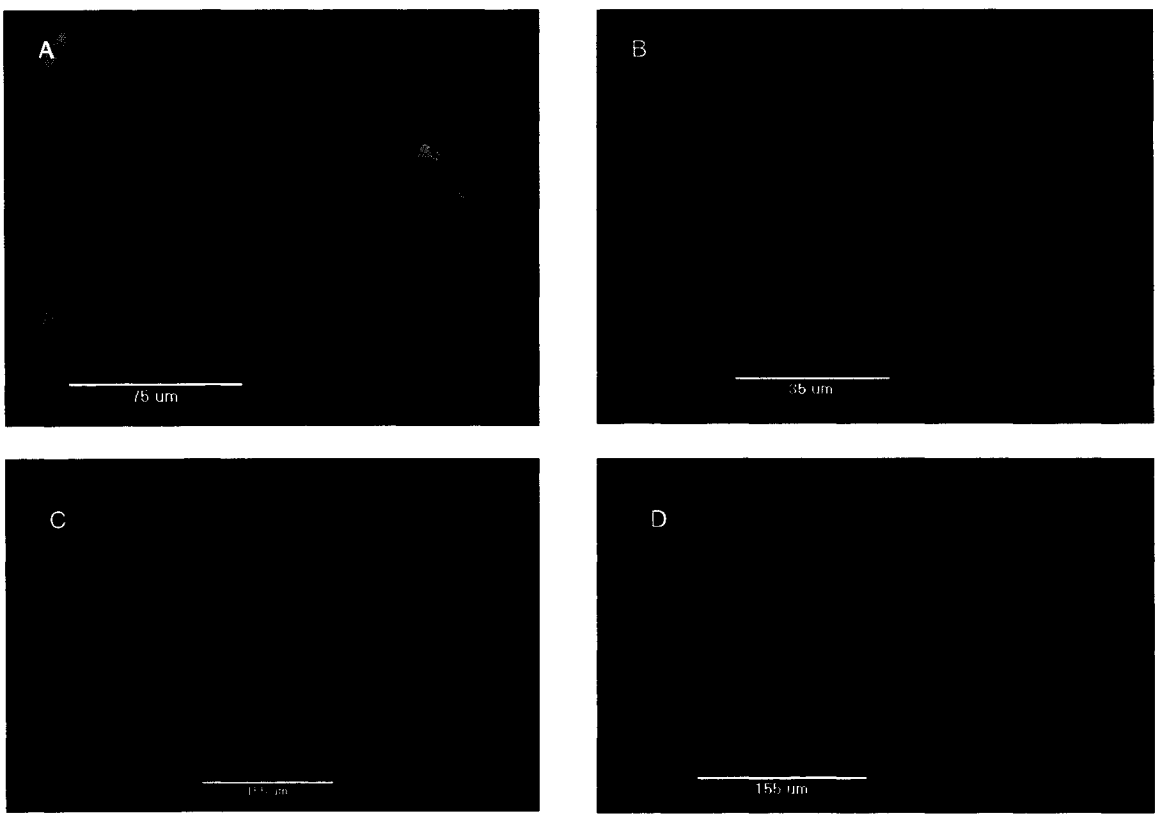

A
75 um
B
35 um
C
D
155 um

Figure 2-3. Immunoreactivity of Na^+-K^+-ATPase (red) and mineralocorticoid receptor (MR; green) co-localized with the Na^+-K^+-ATPase (yellow) in gill tissue sections from rainbow trout. Sections were labeled with (A) α5 (for Na^+-K^+-ATPase) only, (B) MR only, or (C, D) both MR and α5 antibodies. In addition, (E) presents a representative image of a section that was double-labeled with MR and α5 in the presence of excess (100x) of MR blocking peptide. All sections are shown in overlay, with nuclei visualized with DAPI.

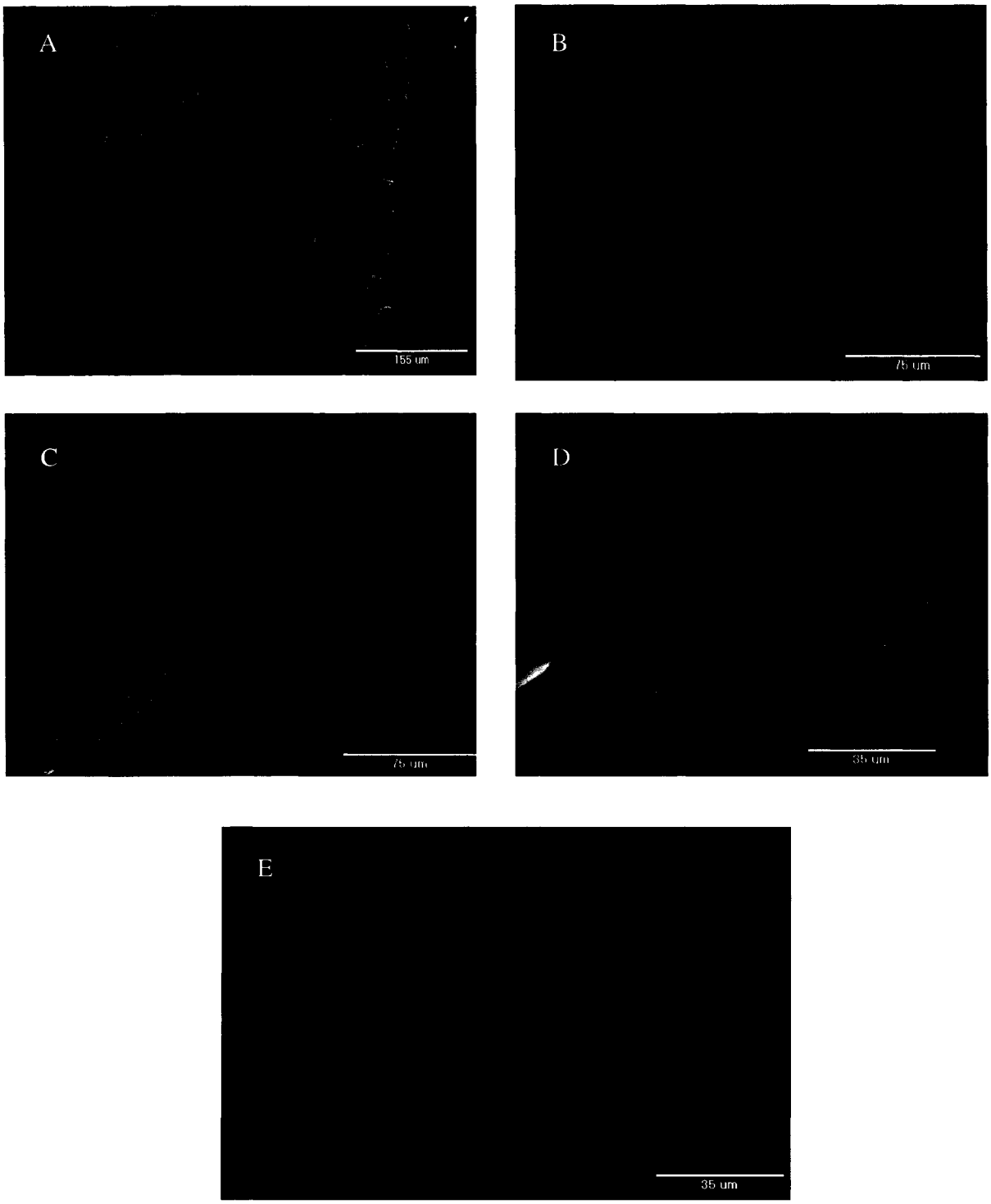
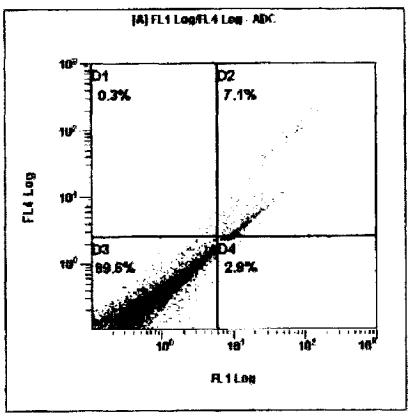

A
155 um
B
75 um
C
75 um
D
35 um
E
35 um

Figure 2-4. Representative scatter plot of flow cytometry data (A) and the mean relative abundance of cells expressing Na^+-K^+-ATPase, MRs, both proteins or neither protein within populations ($N = 5$) of gill cells (B). Branchial cells were isolated and incubated with anti-Na^+-K^+-ATPase and anti-MR antibodies. Data presented are the mean percentages of cells that were unlabeled, double-labeled for Na^+-K^+-ATPase (NKA) and the mineralocorticoid receptor (MR), singly labeled for the MR, or singly labeled for NKA.

A)


[A] FL1 Log/FL4 Log - ADC
D1
0.3%
D2
7.1%
D3
89.6%
D4
2.9%
FL4 Log
FL1 Log

B)

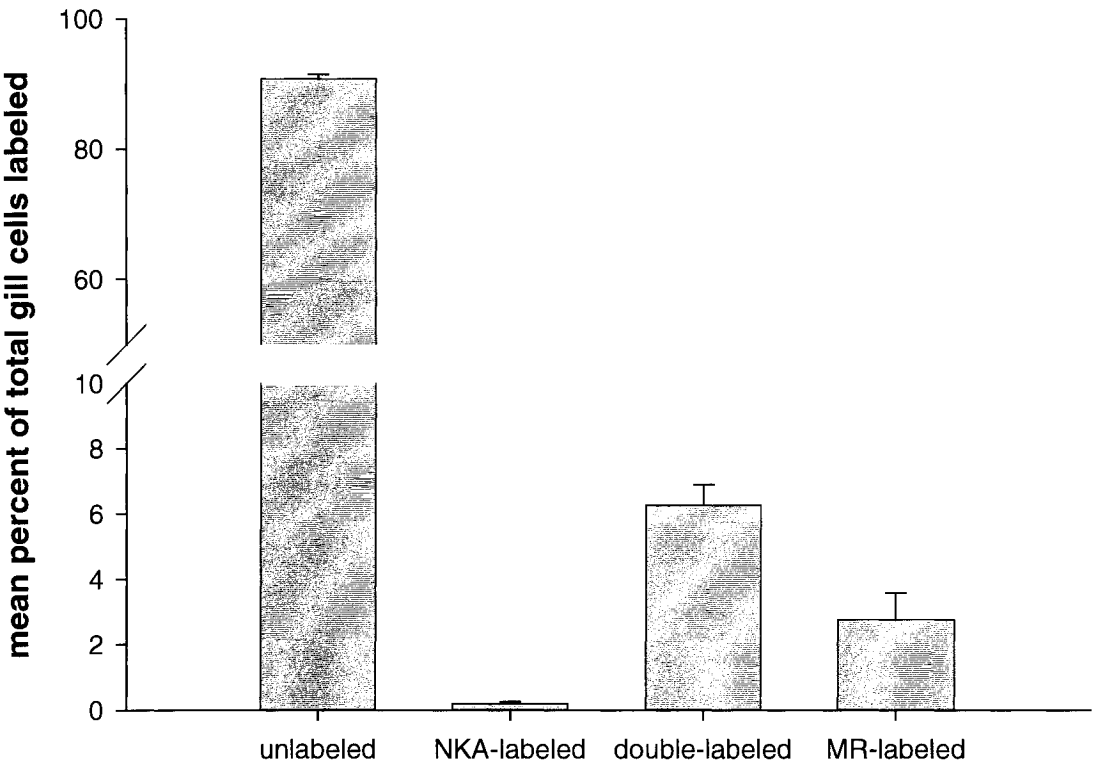

mean percent of total gill cells labeled
100
80
60
10
8
6
4
2
0
unlabeled
NKA-labeled
double-labeled
MR-labeled

Figure 2-5. Plasma (A) cortisol and (B) aldosterone concentrations in rainbow trout (*Oncorhynchus mykiss*) prior to and following an injection of saline (2 ml kg^{-1} fish), cortisol (60 μg mg^{-1} fish), or aldosterone (0.6 mg kg^{-1} fish). Data are presented as mean values ± 1 SEM; N = 10-11 for cortisol treated fish 1.5 h pre-injection – 7.5 h post injection and N = 5 for 10.5 h post-injected fish. N = 8-10 for aldosterone-treated fish 1.5 h pre-injection – 7.5 h post injection and N = 4-5 for 10.5 h post-injected fish. Only those fish for which branchial Na^+ movement was measured were monitored until 10.5 h post-injection. Asterisk (*) indicates a value that is significantly different from the pre-injection value within a treatment group; † indicates a value that is significantly different from other treatment groups within a sampling time (two-way RM ANOVA with sampling time and treatment group as factors, $P < 0.001$ for sampling time, for treatment group and for the interaction of these two terms for both plasma cortisol and aldosterone concentrations).

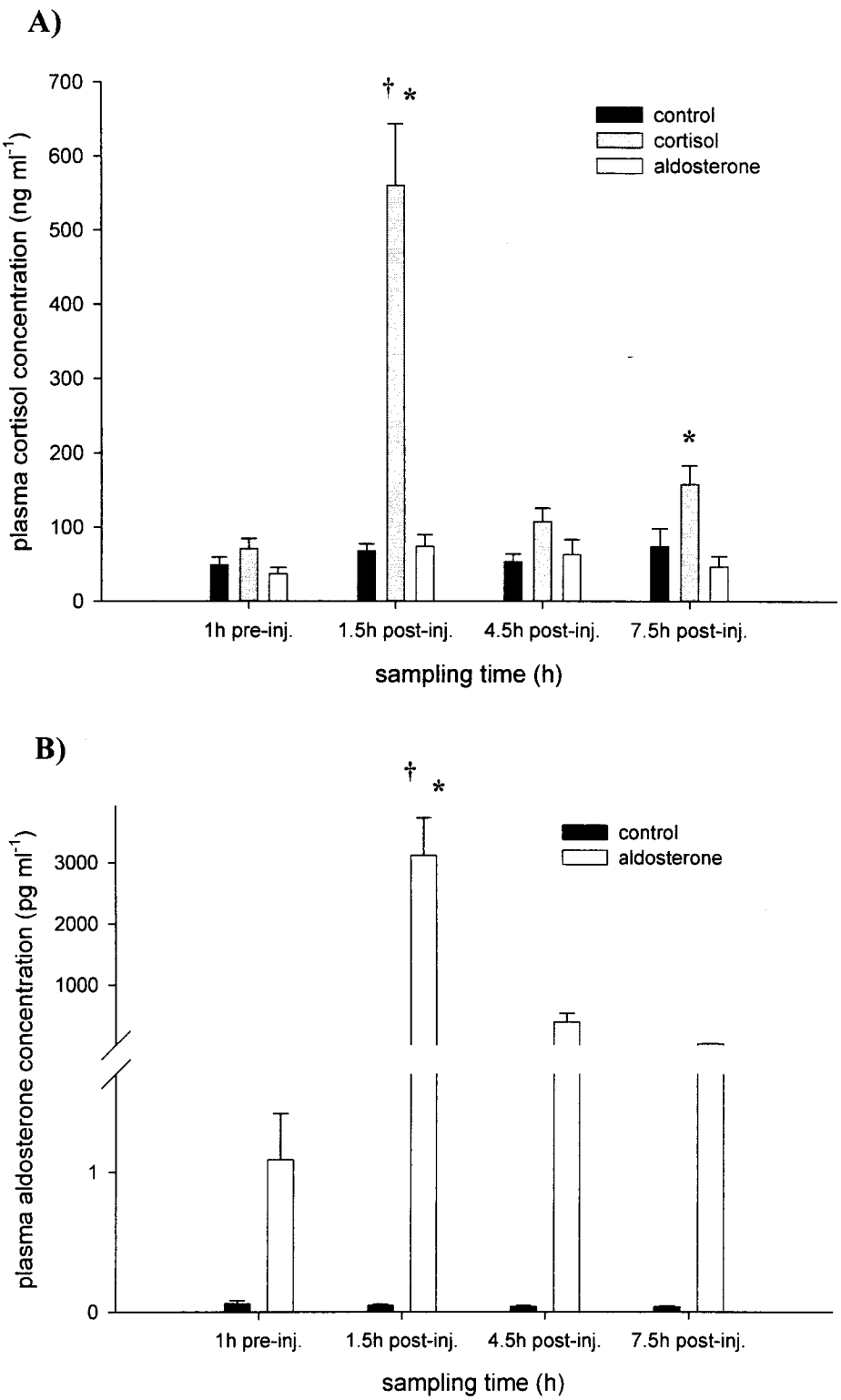
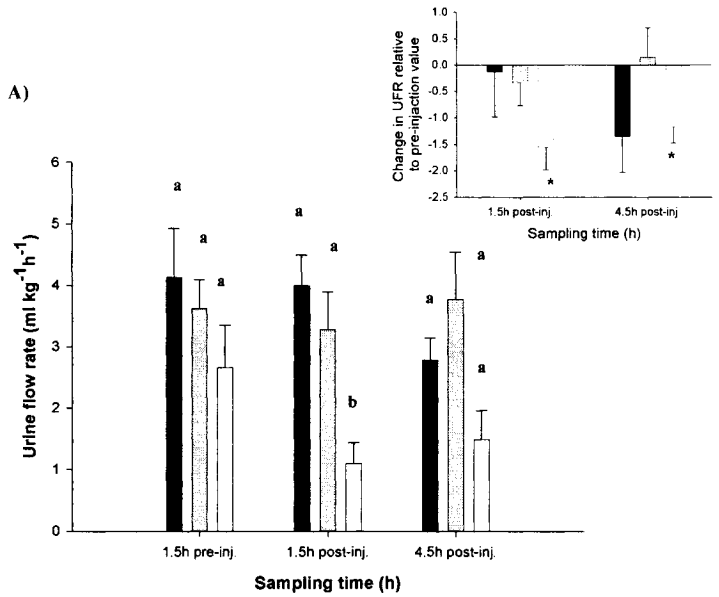
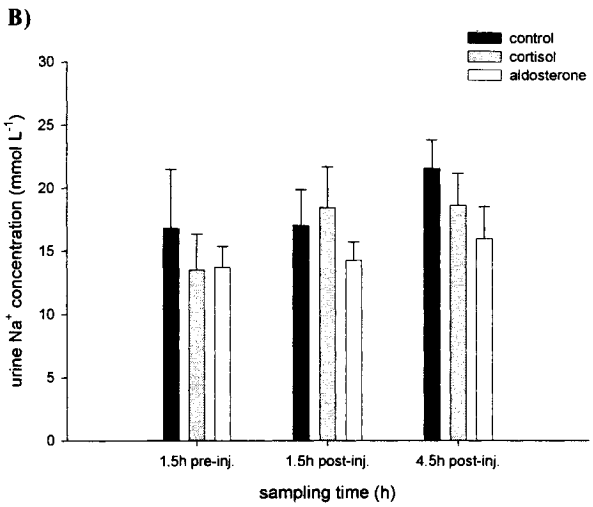

A)
700
600
500
400
300
200
100
0
plasma cortisol concentration (ng ml-1)
† *
*
control
cortisol
aldosterone
1h pre-inj.
1.5h post-inj.
4.5h post-inj.
7.5h post-inj.
sampling time (h)
B)
3000
2000
1000
1
0
plasma aldosterone concentration (pg ml-1)
† *
control
aldosterone
1h pre-inj.
1.5h post-inj.
4.5h post-inj.
7.5h post-inj.
sampling time (h)

Figure 2-6. Urine flow rate (A), urine Na^+ concentration (B) and urinary Na^+ excretion (C) in rainbow trout (*Oncorhynchus mykiss*) prior to and following treatment with saline (2 ml kg^{-1} fish), cortisol (60 μg kg^{-1} fish) or aldosterone (0.6 mg kg^{-1} fish). Inset figures show the change in urine flow rate (A inset) and urinary Na^+ excretion (C inset) relative to pre-injection (control) values. Data are presented as mean values ± 1 SEM; $N = 5$ for all treatment groups. Treatment groups that do not share a letter are significantly different from one another (two-way RM ANOVA with sampling time and treatment group as factors for UFR, $P = 0.031$ for sampling time, $P = 0.034$ for treatment group and $P = 0.063$ for the interaction of these two terms; for urine sodium concentration, $P = 0.13$ for sampling time, $P = 0.449$ for treatment group and $P = 0.784$ for the interaction of these two terms; for inset figures, asterisk (*) indicates a value that is significantly different from the pre-injection value (zero), one-sample *t*-test, $P < 0.01$).


A)
Urine flow rate (ml kg-1h-1)
Sampling time (h)
1.5h pre-inj.
1.5h post-inj.
4.5h post-inj.
Change in UFR relative to pre-injection value
1.5h post-inj.
4.5h post-inj
Sampling time (h)


B)
control
cortisol
aldosterone
urine Na+ concentration (mmol L-1)
1.5h pre-inj.
1.5h post-inj.
4.5h post-inj.
sampling time (h)

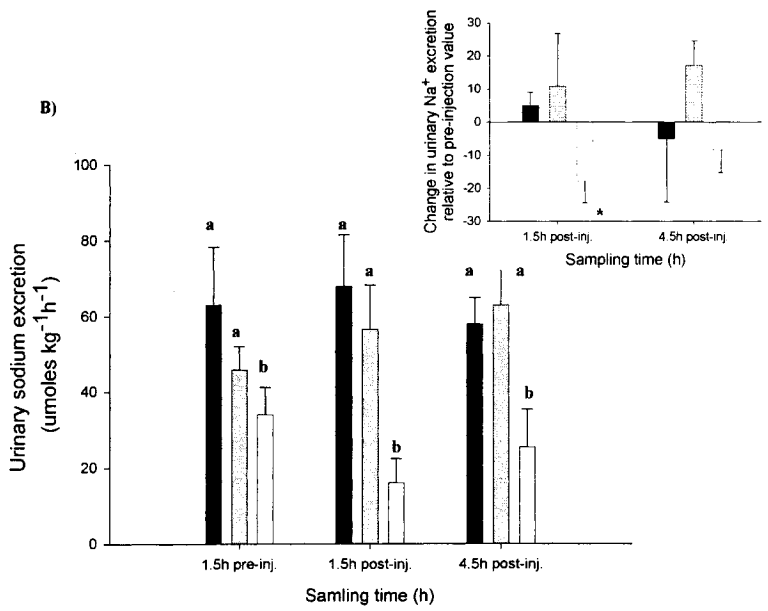

B)
Urinary sodium excretion (umoles kg-1h-1)
Change in urinary Na+ excretion relative to pre-injection value
1.5h post-inj.
4.5h post-inj
Sampling time (h)
1.5h pre-inj.
1.5h post-inj.
4.5h post-inj.
Samling time (h)

Figure 2-7. Branchial net Na^+ flux as a function of sampling time and treatment group in rainbow trout (*Oncorhynchus mykiss*) injected with saline (2 ml kg^{-1} fish), cortisol (60 μg kg^{-1} fish) or aldosterone (0.6 mg kg^{-1} fish). Values are means ±1 SEM, N=5 for all treatment groups. There were no statistically significant effects of sampling time or treatment group (two-way RM ANOVA, $P = 0.874$ for sampling time, $P = 0.173$ for treatment group, $P = 0.471$ for the interaction term).

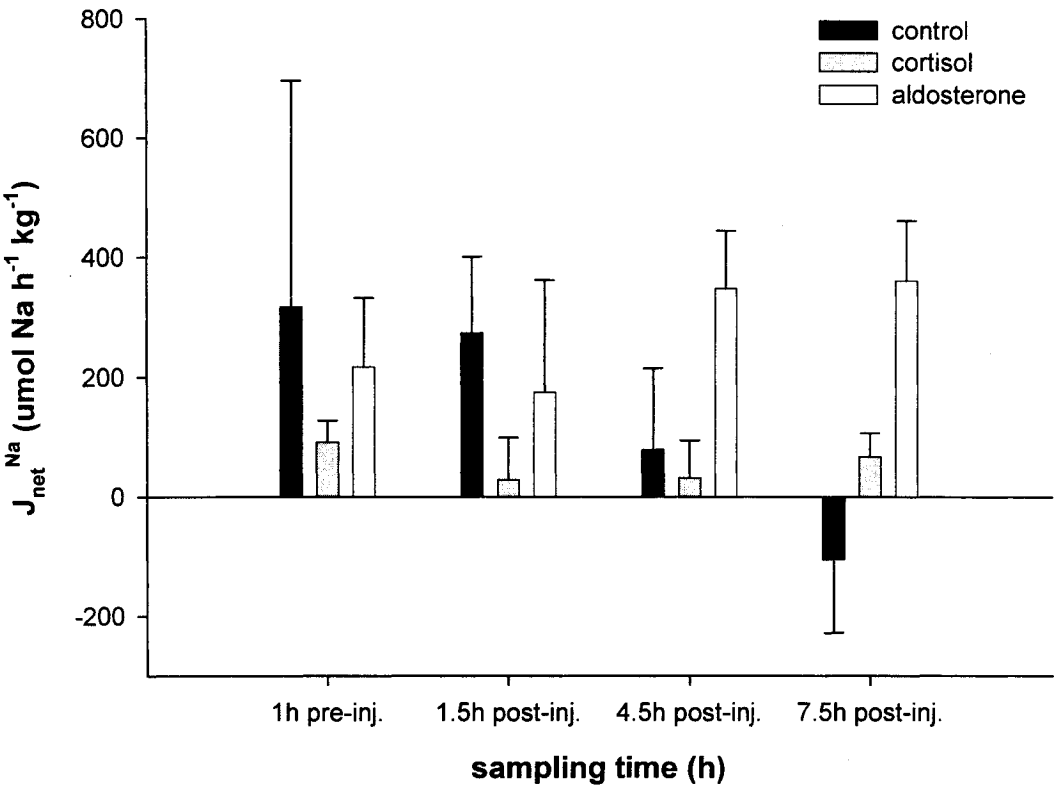

800
600
400
200
0
-200
control
cortisol
aldosterone
J_{net}^{Na} (umol Na h^{-1} kg^{-1})
1h pre-inj.
1.5h post-inj.
4.5h post-inj.
7.5h post-inj.
sampling time (h)

Table 2-1. Plasma Na^+ concentration (mmol l^{-1}) in rainbow trout (*Oncorhynchus mykiss*) prior to and following an injection of saline (2 ml kg^{-1} fish), cortisol (60 μg kg^{-1} fish) in saline or aldosterone (0.6 mg kg^{-1} fish) in saline.

	Plasma sodium concentration (mmol l^{-1})			
Treatment group	0-2 h pre-injection	3-5 h post-injection	6-8 h post-injection	9-11 h post-injection
Control	112.5 ± 3.1 (11)	111. ± 3.0 (11)	107.3 ± 2.1 (11)	113.6 ± 3.1 (6)
Cortisol	125.2 ± 4.9 (5)	114.5 ± 6.7 (5)	117.2 ± 7.3 (5)	Not measured
Aldosterone	111.7 ± 2.9 (9)	111.7 ± 2.5 (9)	112.1 ± 3.7 (9)	103.3 ± 6.9 (4)

Numbers in parentheses are *N* values. Data are presented as means ± 1 S.E.M. There were no statistically significant effects of sampling time or treatment group (two-way RM ANOVA, $P = 0.102$ for sampling time, $P = 0.523$ for treatment group, $P = 0.622$ for the interaction term).

Table 2-2. Branchial and renal Na^+-K^+-ATPase and H^+-ATPase activities following 48 h sham (control) or aldosterone treatment (68 mg kg^{-1} fish).

	Na^+-K^+-ATPase activity (μmoles ADP mg protein $^{-1}$ h^{-1})		H^+-ATPase (μmoles ADP mg protein $^{-1}$ h^{-1})	
	Gill	Kidney	Gill	Kidney
Control	0.25 ± 0.03 (10)	1.31 ± 0.087 (10)	0.48 ± 0.04 (10)	1.00 ± 0.18 (10)
Aldosterone	0.22 ± 0.02 (10)	1.40 ± 0.078 (10)	0.48 ± 0.03 (10)	1.12 ± 0.25 (10)

Numbers in parentheses are *N* values. Data are presented as means ± 1 S.E.M. Two-tailed Student's *t*-test were performed to compare values for control and aldosterone treatment groups, but in no case were the differences significant ($P > 0.05$ in all cases).

Discussion

Identification of branchial MR protein expression

Mineralocorticoid receptor (MR) gene expression was analysed at the tissue level in both *H. burtoni* (Greenwood et al., 2003) and rainbow trout (Sturm et al., 2005). These studies revealed that MR mRNA is expressed in fish ionoregulatory tissues such as the gill, kidney and intestine (Greenwood et al., 2003; Sturm et al., 2005). Sturm et al. (2005) observed that branchial MR mRNA expression levels were low relative to tissues such as the brain and eye, and suggested that localized expression of the MR within a particular cell type could account for this observation. In the present study, the distribution of MR protein within the gill was examined using a heterologous antibody in conjunction with immunohistochemistry and flow cytometry. Although heterologous antibodies must be used with caution, a number of factors suggest that the human MR antibody used in the present study reacted specifically to the trout MR. First, the heterologous antibody used in this experiment is specific for the N-terminal region of the receptor. This N-terminal region exhibits very low levels of sequence similarity with other corticosteroid receptors (Greenwood et al., 2003; Strum et al., 2005) making it unlikely that cross-reactivity with the GR or any other corticosteroid receptor is occurring. To confirm this, a multiple sequence alignment was performed for the human (h)MR, the rtMR and the rtGR1 and rtGR2. The results showed that the N-terminal region of hMR and the rtMR shared greater identity than did the rtMR and either of the rtGRs, supporting the idea that the antibody is specific for the rtMR and is not interacting with either of the rtGRs. In addition, Western analysis of trout gill tissue using the human MR antibody revealed a prominent band corresponding to an approximate

molecular weight of 180 kDa. Although this band corresponds to a protein larger than that of the mammalian MR (approximately 110 kDa, Kalman and Spencer, 2002), the intensity of the bands were greatly diminished when the antibody was pre-absorbed using the peptide against which it was raised. Similarly, pre-absorption of the MR antibody selectively attenuated or abolished apparent MR immunoreactivity in gill tissue sections. Finally, it is unlikely that the heterologous, anti-human MR antibody is displaying cross-reactivity with the GR because Uchida et al. (1998) have shown, using *in situ* hybridization and immunocytochemistry, that branchial GR expression occurs at low but detectable levels in PVCs as well as in Na^+-K^+-ATPase-rich cells. Thus, cross-reactivity of the MR antibody with the GR would have resulted in a broader distribution of MR immunoreactivity at the gill than was observed in the present study.

Examination of rainbow trout gill tissue in the present study using immunohistochemistry and flow cytometry revealed that only a small proportion of cells at the branchial epithelium (~9%) exhibited MR expression (Figs. 2-2 and 2-4), in agreement with the hypothesis of Sturm et al. (2005). Furthermore, essentially all Na^+-K^+-ATPase-positive cells also expressed the MR (Figs. 2-3C,D and 2-4). A characteristic feature of mitochondria rich or chloride cells is the abundance of Na^+-K^+-ATPase located in the basolateral membrane (Perry, 1997; Wilson et al., 2000; Wilson and Laurent, 2002). The localization of MR-immunoreactivity to Na^+-K^+-ATPase-rich cells at the gill supports the hypothesis that the rtMR may play a role in mediating branchial ionic regulation. A small population of cells (~3%) expressed only the MR (Fig. 2-4). It is unlikely that these "MR-only" cells are mitochondria-poor pavement cells (PVCs) given that PVCs occupy approximately 90-95% of the branchial epithelium (Goss et al., 2001).

However, Goss et al. (2001) recently identified a small population (~6%) of branchial mitochondria-rich PVCs (peanut lectin agglutinin (PNA) negative mitochondria rich cells, as opposed to the PNA positive mitochondria rich chloride cells) that lack the highly folded basolateral membrane characteristic of chloride cells. It is tempting to speculate that the "MR-only" cells identified in the present study by flow cytometry represent a population of mitochondria rich PVCs. Interestingly, mitochondria rich PVCs are hypothesized to be the site of Na^+ channel/H^+-ATPase driven Na^+ uptake at the gill (Galvez et al., 2001), and MR expression in these cells would provide a framework for MR involvement in transbranchial Na^+ uptake. Alternatively, the "MR-only" cells may be undifferentiated cells. Uchida et al. (1998) identified cortisol receptor protein and mRNA expression in a small population of undifferentiated gill cells in the interlamellar region. These undifferentiated cells did not display immunoreactivity for Na^+-K^+-ATPase and were thought to be precursors of filamental chloride cells (Uchida et al., 1998). Thus, the small population of Na^+-K^+-ATPase-poor MR cells identified in the present study may reflect an involvement of the MR in chloride cell proliferation induced by ionoregulatory challenges (Laurent and Perry, 1991; Jurss and Bastrop, 1995; Perry et al., 1996).

Effects of acute corticosteroid administration on renal and branchial Na^+ handling

Although MR mRNA (Sturm et al., 2005) and protein (Fig. 2-2A,B) expression were detected in the gills and kidneys of trout (although MR protein expression could not be localized in the kidney by immunohistochemistry in the present study), the role(s) of the MR *in vivo* remain unclear. To investigate the physiological function of the receptor

in vivo, acute corticosteroid administration was employed. In tetrapods, acute aldosterone treatment has rapid effects on epithelial sodium handling (i.e. 1-3 h post-aldosterone treatment, Booth et al., 2002; Verrey et al., 2003). In the tetrapod kidney, aldosterone acts primarily at the distal tubule and collecting duct where it stimulates Na^+ reabsorption and K^+ secretion by activating apical Na^+ channels (ENaCs, generally the rate limiting step) and increasing basolateral Na^+-K^+-ATPase activity (Verrey et al., 1989; Stockand, 2002; Verrey et al., 2003; Williams, 2005). Based on these well characterized effects of aldosterone in tetrapods, it was predicted that aldosterone treatment would stimulate Na^+ recovery from the filtrate in trout kidney. In agreement with prediction, aldosterone injection in rainbow trout elicited a significant decrease in renal Na^+ excretion that was the result of a significant fall in UFR in the face of constant urine Na^+ concentrations (Fig. 2-6B,C). Contrasting results were obtained for cortisol injection, which had no significant impact on UFR or urinary sodium excretion up to 4.5 h post-injection (Fig. 2-6A,C). These observations are consistent with those of previous studies in which cortisol treatment of freshwater silver eels or lamprey had no effect on UFR, whereas aldosterone administration caused UFR to decrease (Butler, 1973). UFR and urinary sodium excretion are the product of glomerular filtration followed by tubular reabsorption and/or secretion. In the present experiment, as in the work of Holmes and McBean (1963), neither aldosterone injection nor vehicle (saline) alone had any significant effect on GFR, a finding that suggests that aldosterone-induced increases in renal water reabsorption must account for the reduction in UFR. Water reabsorption by the nephron is an important ionoregulatory process in both freshwater- and seawater-acclimated rainbow trout, in which 60 and 79% of tubular water is

reabsorbed, respectively (Hofmann and Butler, 1979). The results of the present study suggest that tubular water reabsorption is, at least in part, regulated by the MR in rainbow trout.

The effect of MR activation in enhancing tubular water reabsorption may occur through enhanced tubular Na^+ reabsorption. In support of this, renal fluid reabsorption in most vertebrates is approximately isosmotic and in reptiles, Na^+ and water recovery from the filtrate occur at equivalent rates and may in fact be linked (Dantzler, 2003). This link between Na^+ and water recovery from the filtrate, in turn, could account for the lack of effect of aldosterone administration on urine Na^+ concentrations (Fig. 2-6B). Similarly, aldosterone treatment was without effect on urine Na^+ levels despite inducing a decrease in UFR in freshwater lamprey (Butler, 1973). Thus, the teleost MR may mediate both Na^+ and water recovery from the filtrate in freshwater environments.

The rtMR is highly responsive to both cortisol (EC_{50}=1.1 nM) and aldosterone (EC_{50}=0.16 nM) *in vitro* (Sturm et al., 2005), yet cortisol and aldosterone affected UFR and urinary Na^+ excretion quite differently *in vivo* (Fig. 2-6A,C). Unlike aldosterone, cortisol binds both GRs and MRs with a high affinity. Additionally, GR mRNA expression is known to occur in the kidney of rainbow trout (Ducouret et al., 1995; Bury et al., 2003) and *H. burtoni* (Greenwood et al., 2003) Thus, the absence of effect of cortisol treatment on UFR and urinary Na^+ excretion might reflect counteracting influences of MR and GR activation. However, preliminary trials using the GR-selective agonist dexamethasone did not reveal GR-mediated increases in UFR or urinary Na^+ excretion (data not shown), refuting this explanation. An alternative and more likely possibility given the situation in mammals is that cortisol is prevented from binding to

MRs. In mammals, the enzyme 11ß-HSD-II is co-localized with the MR in aldosterone-sensitive epithelia and acts to convert cortisol to its inactive metabolites to prevent MR activation (Edwards et al., 1988; Kusakabe et al., 2003). The trout enzyme was cloned recently (Kusakabe *et al.*, 2002), and mRNA tissue distribution analysis suggests that 11β-HSD-II is expressed in several tissues including the gill and kidney (Kusakabe et al., 2003). Although the function of 11β-HSD-II has not yet been characterized in trout gill or kidney, it is possible that the presence of such an enzyme-based sentinel system prevents cortisol-induced decreases in UFR equivalent to those observed with aldosterone treatment.

Although the aldosterone-induced increase in renal Na^+ reabsorption ultimately might be expected to elevate plasma Na^+ levels, no change in plasma Na^+ concentration was measured in aldosterone treated fish over the course of the present experiment (Table 2-1). This result is not unexpected, given that at 1.5 h post-aldosterone injection urinary Na^+ excretion decreased by 18 μmoles kg^{-1} fish h^{-1} (Fig. 2-6C). In a 179 g fish (the average mass of fish used in this experiment) this corresponds to an addition of 0.09 mmol l^{-1} of Na^+ to the extracellular fluid. Given that the average plasma Na^+ concentration for aldosterone treated fish was 110 mmol l^{-1}, it is unlikely that any change in plasma would be detected. In terms of overall Na^+ regulation in freshwater fish, however, the gill rather than the kidney is the key player owing to its role in active Na^+ uptake from the environment, and aldosterone injection was without effect, at least acutely, on branchial net Na^+ flux (Fig. 2-7). Interestingly, Holmes and Butler (1963) observed a significant decrease in plasma Na^+ concentrations in aldosterone-treated rainbow trout, a result that was attributed to the storage of Na^+ in the tissues as muscle

Na^+ levels underwent an increase 5 h after an aldosterone injection. Another explanation for the lack of effect of aldosterone on branchial net Na^+ flux and plasma Na^+ levels is that the ENaC that is present in tetrapod epithelia may not be present in fish. The ENaC is the limiting factor in transepithelial Na^+ uptake in tetrapods (Booth et al., 2002) and is upregulated by aldosterone (Stockand, 2002; Rogerson et al., 2003). However, the ENaC has not been cloned from fish tissue (Perry et al., 2003) and evidence supporting the presence of ENaC in fish is sparse (Wilson et al., 2000; Perry et al., 2003). Thus, the evidence presented here supports the hypothesis that the vertebrate ENaC is not present in fish and suggests that apical Na^+ transport may occur through other transport mechanisms that are insensitive to aldosterone treatment.

Somewhat unexpectedly, cortisol treatment was also without effect on branchial net Na^+ flux. Treatment of gill filaments with cortisol, *in vitro*, stimulates an increase in Na^+-K^+-ATPase activity (McCormick and Bern, 1989; Shrimpton and McCormick, 1999). Consistent with this, Zhou et al. (2003) found that exposure of cultured branchial epithelia from rainbow trout to cortisol promotes Na^+ uptake. Additionally, cortisol treatment, *in vivo*, is linked to changes in plasma Na^+ (Redding et al., 1991) and branchial Na^+-K^+-ATPase activity (Madsen, 1990). However, in each of these experiments, changes in plasma Na^+ and Na^+-K^+-ATPase activity were only observed 1-7 days after cortisol exposure. Thus, the lack of effect of acute cortisol treatment (1.5 h – 7.5 h post injection) on $J_{net}Na^+$ may be due to an insufficient amount of time allowed for cortisol to induce measurable effects on transbranchial Na^+ movement.

The effects of chronic aldosterone treatment on renal and branchial ion transport activity

The high degree of colocalization of MRs to Na^+-K^+-ATPase-rich cells (Fig. 2-4) suggests that MR activation by aldosterone may induce changes in branchial Na^+-K^+-ATPase activity. In addition, Sloman et al. (2001) provided evidence for a role for MRs in inducing chloride cell proliferation during acclimation to ion-poor water, a process that requires 24 – 48 h (Laurent et al. 1994). For these reasons, Na^+-K^+-ATPase activity was assessed at 48 h of aldosterone treatment in the present study. The results indicated that neither branchial Na^+-K^+-ATPase activity, nor H^+-ATPase activity, a transport protein that is thought to be critical for Na^+ uptake in freshwater fish (Lin and Randall, 1995; Perry and Fryer, 1997), was altered (Table 2-2) in fish given an aldosterone implant. Whether chloride cell numbers were affected, however, remains to be determined; unfortunately, the OsO_4/ZnI_2 technique commonly used to identify chloride cells by light microscopy (Garcia-Romeu and Masoni, 1970; Shreiber and Specker, 1999) failed to stain gill tissue samples from aldosterone-treated fish in the present study.

The apparent lack of effect of aldosterone treatment on branchial and renal Na^+-K^+-ATPase and H^+-ATPase activities may reflect a requirement for corticosteroid receptor up-regulation to occur prior to aldosterone treatment. Several lines of evidence suggest that the status of the corticosteroid receptors, rather than the levels of corticosteroids in the blood, may be the most important determinant of the physiological response of the tissue. For example, Sloman et al. (2001) found that chloride cell proliferation after 7 days of softwater exposure was not accompanied by elevated plasma cortisol levels but could be blocked by treatment with a MR antagonist. Furthermore, elevations in plasma cortisol due to stress from air emersion (Sloman et al., 2001) or

subordinate social status (Sloman et al., 2000) did not stimulate chloride cell proliferation. Together, these studies imply that the effective size of the corticosteroid receptor pool may be the key determinant of the ability of a tissue to respond to corticosteroid exposure. Indeed, Shrimpton and McCormick (1999) demonstrated that cortisol-induced increases in Na^+-K^+-ATPase activity were dependent on the abundance of corticosteroid receptors in the gill. Glucocorticoid receptor populations in fish are highly labile (Weisbart et al., 1987; Pottinger, 1990; Maule and Schreck, 1991; McLeese et al., 1994; Mommsen et al., 1999; Dean et al., 2003) and external cues, such as changes in environmental salinity alter GR number at the gill (Weisbart et al., 1987; Dean et al., 2003), as does cortisol treatment (Shrimpton and Randall, 1994; Mommsen et al., 1999). It is likely that a degree of plasticity also exists in MR populations. Thus, detection of aldosterone-induced changes in branchial and renal ion transport activity may require that fish be pre-exposed to an ionoregulatory challenge prior to aldosterone treatment to elicit appropriate corticosteroid receptor expression.

In conclusion, the present study provides two significant advances. First, MR protein expression has been localized to the gills of rainbow trout. While the MR expression is largely confined to Na^+-K^+-ATPase-rich cells, a small population of branchial cells that expresses only the MR also appears to exist. Second, evidence has been provided to support a physiological role for the MR in renal Na^+ handling. The use of aldosterone to selectively target MRs revealed that UFR and urinary Na^+ excretion can be modified through MR activation. The impact of MR activation on UFR and urinary Na^+ excretion appear to reflect a specific impact on tubular Na^+ reabsorption, thus suggesting that the teleost renal MR has function similar to that of the mammalian renal

MR. Future studies should examine the effects of ionoregulatory disturbances on rtMR populations in various tissues, particularly gill and kidney, to help clarify the role of the rtMR in ionic regulation.

CHAPTER 3

GENERAL DISCUSSION

Discussion

Despite the wealth of information describing the strong link between cortisol and ionic regulation (McCormick and Bern, 1989; Bern and Madsen, 1992; McLeese et al., 1994; Mommsen et al., 1999; McCormick, 2001; Evans, 2002), considerably less is known about the mechanism by which cortisol elicits a response at its target tissue. The use of sensitive molecular techniques has lead to the identification of three GRs and two MRs in rainbow trout (*Oncorhynchus mykiss*). These receptors have a broad tissue distribution and display highly varied binding kinetics for cortisol (Colombe et al., 2000; Bury et al., 2003; Sturm et al., 2005). However, there is a paucity of evidence linking any of these corticosteroid receptors to a particular physiological process. Thus, there is clearly a great deal of uncertainty regarding corticosteroids and corticosteroid receptor-mediated processes in fish.

The general objective of this thesis was to gain a better understanding of the physiological function of the fish MR, using a working hypothesis that the rtMR is involved in renal and branchial ionic (specifically Na^+) regulation. In support of this hypothesis, immunohistochemical analysis and flow-cytometry data indicated that the rtMR is expressed mainly in Na^+-K^+-ATPase-rich cells (see Figs. 2-2 and 2-4). This cellular colocalization of the rtMR and Na^+-K^+-ATPase led to an investigation of whether the gill was responsive to aldosterone treatment *in vitro*. In amphibian epithelia, there is strong evidence for an aldosterone-induced increase in Na^+-K^+-ATPase activity during the late phase (i.e. > 6 h post-aldosterone exposure) of the response (Palmer et al., 1993; Booth et al., 2002). However, in the present study there was no change in Na^+-K^+-ATPase activity after 24 h or 5-day of aldosterone treatment (see Figs. A1 and A2). This

apparent lack of effect may reflect the complexity of an MR-mediated endocrine response. Indeed, there are several endocrine and non-endocrine factors known to affect branchial ionic regulation, including prolactin (Eckert et al., 2001), growth hormone, insulin-like growth factor-I (McCormick et al., 1991; Seidelin and Madsen, 1999), epinephrine, cAMP, vasoactive intestinal peptide and urotensin I (McCormick, 2001). Some of these factors, such as prolactin and growth hormone, have been shown to act synergistically with cortisol (McCormick, 2001; Eckert et al., 2001). Shrimpton et al. (1995) demonstrated that juvenile coho salmon treated with exogenous prolactin or growth hormone displayed an increase in corticosteroid receptor abundance thus, presumably, allowing cortisol to exert its effect at the gill more efficiently. An absence of one or more of these endogenous factors in our *in vitro* system may explain the lack of effect of both cortisol and aldosterone on branchial Na^+-K^+-ATPase activity.

In addition to investigating the effects of aldosterone treatment on branchial ion transport *in vitro*, the hormone was also administered *in vivo*, both acutely and chronically. Flow cytometric analyses indicated that chronic (48 h) aldosterone treatment caused a substantial reduction in the relative proportion of double-labeled, Na^+-K^+-ATPase rich cells (see Fig. A7). This result implies that branchial MR activation leads to chloride cell loss and/or inhibits proliferation of stem cells into chloride cells. Consistent with this observation, a decrease in MR mRNA expression has been observed in gills of rainbow trout exposed to soft water for 5 days (unpublished data, KM Gilmour, M Bell and MM Vijayan). In apparent opposition to these results, however, Sloman et al. (2001) reported that MR blockade using spironolactone prevented chloride cell proliferation during softwater acclimation, a finding which suggests that the MR is involved in

promoting chloride cell proliferation. The discrepancies between the studies are difficult to resolve and may partly be explained by the fact that one experiment examines the relationship between the rtMR and chloride cell proliferation using softwater exposure while the other attempts to induce MR activation by pharmacological means. As an alternative explanation, Sturm et al. (2005) observed that spironolactone acted as an agonist of the rtMR, not an antagonist. If spironolactone does indeed activate the rtMR *in vivo*, the lack of chloride cell proliferation observed in spironolactone treated fish by Sloman et al. (2001) may be explained by the activation of the rtMR rather than by the presumed blockade of the receptor from cortisol-induced activation. Several studies have demonstrated the dynamic nature of corticosteroid receptor populations in the gill (Weisbart et al., 1987; Pottinger, 1990; McLeese et al., 1994; Shrimpton and Randall, 1994; Shrimpton et al., 1995; Dean et al., 2003). This plasticity of receptor populations, combined with the observation that plasma cortisol elevations are not always correlated with an ionoregulatory response (Sloman et al., 2001a, 2001b; Metz et al., 2003), suggests that it may be the state of the corticosteroid receptor population, rather than the level of corticosteroid in the plasma, that is most important in determining whether cortisol elicits a response at the target tissue. If this hypothesis is correct, it may explain why there was no effect of chronic aldosterone treatment, *in vivo*, on renal or branchial Na^+-K^+-ATPase and H^+-ATPase activity in the present study. Future studies should focus on the effects of aldosterone treatment in fish exposed to an ionoregulatory challenge. Such experiments will help determine whether an external ionoregulatory disturbance is required as a cue to change the status of the corticosteroid receptor pool so as to respond more effectively to aldosterone treatment.

In addition to investigating the effects of chronic aldosterone treatment on fish ionic regulation, acute effects (1–7.5 h post exposure) were also examined. Interestingly, acute exposure to a high dose of aldosterone promoted a decrease in UFR that appeared to be the result of increased tubular water reabsorption (see Fig. 2-6). In tetrapods, aldosterone-induced increases in tubular water reabsorption can be accomplished by at least two means; an increase in basolateral Na^+-K^+-ATPase activity and/or an increase in luminal Na^+ entry (Verrey et al., 1989; Stockand, 2002; Verrey et al., 2003). The activity of the epithelial Na^+ channel (ENaC) is the limiting step in transepithelial sodium uptake and can be increased by enhancing sodium channel activity or by recruiting vesicular ENaC stores to the apical membrane (Chen et al., 1999). Although a piscine ENaC remains to be identified (Hirose et al., 2003; Perry et al., 2003; Reid et al., 2003), it is possible that acute aldosterone exposure stimulated an MR-mediated, early phase (within 1-3 h of aldosterone exposure) response that increased the open probability of putative sodium channels, or perhaps increase the expression and/or activity of other Na^+ transporters such as the Na^+/H^+-exchanger (NHE) or $Na^+/Cl^-(HCO_3^-)$ cotransporter. Such effects are known to occur in tetrapods (Stockand, 2002) and would stimulate Na^+ (and presumably water) reabsorption without producing a measurable change in Na^+-K^+-ATPase activity.

Regardless of the mechanism by which UFR was reduced in aldosterone-treated fish, it is interesting that acute cortisol treatment did not produce a similar response. Sturm et al. (2005) demonstrated that cortisol is also a potent agonist of the rtMR (EC_{50}=1.1 nM), but in cortisol-treated fish, UFR and urinary Na^+ excretion paralleled the corresponding values for saline-injected (control) fish (see Figs. 2-6). This effect may be

the result of the enzymatic catabolism of cortisol by 11β-HSD-II. In trout, 11β-HSD-II mRNA is expressed in the posterior and head kidney as well as in the gill (Kusakabe et al., 2004) and although the function of this enzyme has not been characterized in fish, in tetrapods it metabolizes cortisol to prevent activation of MRs (Edwards et al., 1988; Stauffer et al., 2002). In support of the hypothesis that the function is equivalent in trout, the cortisol breakdown product cortisone, has been detected in fish (Greenwood et al., 2003), providing indirect evidence for the possible presence of 11β-HSD-II. Interestingly, the evidence presented here, combined with previous observations (Holmes and Butler, 1963; Butler, 1973; Sturm et al., 2005), suggests that cortisol may not be the endogenous MR ligand. Sturm et al. (2005) found that the naturally occurring fish corticosteroid, 11-deoxycorticosterone (DOC) is a more potent transactivator of the rtMR than cortisol. However, direct evidence for DOC as a fish mineralocorticoid is lacking. Future experiments should focus on establishing links between DOC, MRs and ionic regulation. Additionally, specific 11β-HSD-II inhibitors such as dithiocarbamates (Atanasov et al., 2003) could be co-administered with cortisol in experiments similar to those of the present study to determine if 11β-HSD-II is preventing cortisol from acting as a mineralocorticoid in fish.

Additional mechanisms that may exist to prevent continuous activation of the rtMR by cortisol include the binding of cofactors and/or posttranslational modification of the rtMR. In mammals, corticosteroid receptors are a target of protein kinase A, and phosphorylation occurs at the N-terminus (Masaad et al., 1999). In fish, as in mammals, the N-terminus A/B domain is highly variable (Funder, 1997; Greenwood et al., 2003; Sturm et al., 2005). In fact, the *hb*MR bears less than 20% amino acid identity with

*hb*GR1 and *hb*GR2 at the N-terminus (Greenwood et al., 2003). Thus, posttranslational modifications in the A/B domain may be one way in which the effective sizes of the MR and GR pools are altered, so as to control the type of response that corticosteroids elicit within a cell.

Although little information on posttranslational modifications of fish corticosteroid receptors exists, there is evidence that corticosteroid receptors bind different protein cofactors in the cytoplasm. In fish, heat shock proteins are important for maintaining an active GR population in the cell (Sathiyaa and Vijayen, 2003). Recently, treatment of trout hepatocytes with galdanamycin, a hsp90 inhibitor, was found to cause proteosomal degradation of the GR and decreased GR mRNA levels (Sathiyaa and Vijayan, 2003). Additionally, Basu et al. (2003) demonstrated that stress induced by heat and cortisol treatment caused an increase in the amount of hsp70 associated with GRs in trout hepatocytes. Chaperone protein–MR interactions have not yet been described in fish, but heat shock proteins are known to associate with the ligand binding domain of both fish (Basu et al., 2003) and mammalian corticosteroid receptors (Bledsoe et al., 2002; Ramirez et al., 2004), and this region is conserved between GRs and MRs in fish (Greenwood et al., 2003). Thus, it is possible that chaperone proteins have an important role in regulating ligand binding to MRs as well as GRs in fish. Future examination of the interaction of chaperone proteins with the rtMR may help explain how MR- and GR-mediated pathways are differentiated in the cell.

Posttranslational modifications and chaperone proteins provide interesting examples of how external factors can affect hormone binding and corticosteroid receptor activation in the cell. However, there are also intrinsic properties of corticosteroid

receptors that may influence hormone binding and receptor activation. For example, Rogerson et al. (2003) demonstrated that the N- and C-terminal regions of the mammalian MR interact with each other during aldosterone binding, but not during cortisol binding, to induce transactivation of the receptor *in vitro*. Mechanisms of this nature could also account for the differences between cortisol and aldosterone with respect to stimulating decreases in UFR and urinary Na^+ excretion. Examination of N- and C-terminal interactions during cortisol, aldosterone and DOC binding is clearly warranted for fish MRs.

Regardless of the type of ligand that the rtMR binds and the mechanism by which it becomes activated, all activated receptors must translocate to the nucleus and dimerize to exert a physiological effect (Farman, 1999). Recent studies indicated that both homodimers (MR-MR and GR-GR) and heterodimers (MR-GR) form *in vivo* and produce transcription complexes with a broad range of transactivational capacities (Farman, 1999). Although very little is known about these interactions in fish, it is possible that regulation does occur at the level of receptor dimerization, particularly since cortisol is a relatively potent agonist of both MRs and GRs. Interestingly, Sturm et al. (2005) found that the rtMR (EC_{50}=1.1nM) and rtGR2 (EC50=0.72nM) exhibited similar EC_{50} values for cortisol, whereas cortisol was approximately a 50-fold less potent agonist of rtGR1 (EC_{50}=46 nM). These differences imply that plasma cortisol levels will influence the relative sizes of the MR-MR, GR-GR and MR-GR pools within the cell. Fluctuations in the relative sizes of the dimerized pools in the cell, in turn, will most likely give rise to downstream genomic consequences, since these transcription complexes have very specific interactions with hormone response elements (HREs) in

promoter regions (Green et al., 1988; Umesono and Evans, 1989; Lethimonier et al., 2002; Nagaich et al., 2004).

Members of the steroid/thyroid receptor family bind similar HREs and are sensitive to changes in the amino acid sequence of the DNA binding domain (DBD, Umesono and Evans, 1989). For example, mutation of a glycine residue within the DBD of the mammalian GR to a glutamate residue will cause the GR to bind to both the glucocorticoid response element and the estrogen response element (Umesono and Evans, 1989). Thus, even though both the teleost GRs and MRs are highly homologous in the DBD (Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005), they may recognize and bind different HREs, as slight sequence variations can have major effects on DNA binding specificity. The spacing of the two zinc finger regions of the DBD differs between rtGR1 and rtGR2 (Bury et al., 2003), and this difference will govern the type of HRE that the receptor binds (Lethimonier et al., 2002). Interestingly, Sturm et al. (2005) identified two rtMR subtypes of high sequence similarity but differing in 10 amino acids in the A/B domain, and 3 amino acids between the zinc finger regions. Thus, the two MRs expressed in rainbow trout could potentially activate the transcription of different genes, despite binding the same ligand. Such differences in target gene activation would likely also occur between GRs and MRs, and may be an important mechanism allowing for differentiation between glucocorticoid and mineralocorticoid responses in the cell.

Current evidence indicates that the role of MRs and GRs in fish is overlapping. There is ample evidence supporting the role of the fish GR in ionic regulation (Weisbart et al., 1987; McLeese et al., 1994; Shrimpton et al., 1995; Dean et al., 2003). There is also limited evidence for the role of the rtMR in ionic regulation (see Fig. 2-6; Sloman et

al., 2001). It is important to note that fish corticosteroid receptor nomenclature is based only on sequence similarity to tetrapod corticosteroid receptors (Colombe et al., 2000; Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005), not physiological function. Even in tetrapods, where GR and MR mediated pathways are more distinct than in fish (due to the presence of aldosterone and 11β-HSD-II), phenomena such as glucomimetism by mineralocorticoids (Agarwal and Mishahi, 1999) and vice versa (Farman, 1999; Rogerson et al., 2003) have been observed. It is probable that this type of cross-talk also occurs in fish in which there are multiple corticosteroid receptor subtypes, all with similar affinities for cortisol (Greenwood et al., 2003; Sturm et al., 2005). Thus, the tetrapod corticosteroid receptor nomenclature may not apply to fish and only through future investigation, involving the integration of molecular and physiological experiments, will the true identity corticosteroid receptors in fish be revealed.

Conclusions

Our knowledge of the complexity of corticosteroid receptor pathways in fish has grown recently from a one hormone/one receptor paradigm to a system that involves one-or-more hormones and at least three receptors. The challenge now is to determine the function of each corticosteroid receptor subtype in fish, and how different receptor pathways are integrated into the overall physiological response of the fish to a particular ionic, osmotic or metabolic challenge. The overall goal of this thesis was to gain a better understanding of the physiological function of the MR in rainbow trout. The present study demonstrates that MR protein is expressed in Na^+-K^+-ATPase rich cells at the gill and presents evidence in support of the hypothesis that the rtMR is involved in the

regulation of renal Na^+ handling. Future investigation should focus on the external stimuli that may lead to activation of the rtMR, as well as the downstream targets of this receptor. Such experiments will help uncover the mechanisms through which euryhaline fish, such as rainbow trout, survive the ionoregulatory challenges presented by their aquatic environments.

APPENDIX:

The effect of aldosterone treatment *in vitro* and *in vivo* on branchial ionic regulation

Introduction

The mineralocorticoid receptor (MR), like all corticosteroid receptors, acts as ligand-activated transcription factor in the cell. Once activated, the receptor translocates to the nucleus, binds to corticosteroid response elements (CREs) and initiates a genomic response. The nature of the genomic response to MR activation has not yet been examined in fish. In tetrapods, the classical model describing the aldosterone-induced effect on Na^+ transport is divided into three phases: the latent, early, and late phases (Booth et al., 2002). The early phase is described by an aldosterone-induced activation of signaling proteins that posttranslationally modify ion channels and transporters to increase the Na^+ transporting capacity of the epithelium (Booth et al., 2002). Some of the transcription factors that are activated are serum- and glucocorticoid-inducible kinase (sgk), a GTP binding protein called Kirsten-Ras (Ki-ras), phosphotidylinositol 3-kinase (P13K) and corticosteroid hormone-induced factor (CHIF; Booth et al., 2002). The late phase of the response is characterized by an activation of the epithelial Na^+ channel (ENaC) and the Na^+-K^+-ATPase by the aforementioned signaling molecules (Booth et al., 2002). In fish, the evidence for the presence of ENaC is limited (Wilson et al., 2000). However, the current model for active Na^+ uptake from the environment by the freshwater fish gill (Wilson et al., 2000; Marshall 2002; Perry et al., 2003) describes Na^+ uptake as occurring, at the apical membrane, through a Na^+ channel. This Na^+ entry is thought to be powered by an electrochemical gradient established by a V-type H^+-ATPase (Wilson et al., 2000). Na^+ ions that enter the branchial epithelial cells then exit basolaterally into the blood via Na^+-K^+-ATPase (Evans et al., 1999; Marshall, 2002). The tight mechanistic linkage between the putative Na^+ channel and the H^+-ATPase in

Na^+ uptake provides a rationale for investigating the effects of *in vitro* and *in vivo* aldosterone treatment on renal and branchial H^+-ATPase activity. Similarly, the importance of the renal and branchial Na^+-K^+-ATPase in mediating transepithelial ion transport prompted an investigation of the effects of the same aldosterone treatments on the activity of this ion transporter.

The experiments presented and discussed in this appendix either did not produce conclusive results, or produced results that could not be further investigated due to time constraints. The results, however, are discussed to a limited extent and explanations are offered for those findings that were inconclusive.

Materials and Methods

Experimental animals

Fish acquisition and holding conditions were as described in Chapter 2.

The effect of in vitro aldosterone treatment on gill explant ion transporter activities

The objective of this experiment was to determine if the rtMR is involved in the regulation of branchial Na^+-K^+- and H^+-ATPase activity. The methods used in this experiment were based upon those of McCormick and Bern (1989) and Mazon et al. (2004). In brief, fish were terminally anaesthetized in a solution of benzocaine (0.5 g l^{-1} ethyl-*p*-aminobenzoate). The first three gill arches were excised and rinsed in ice-cold Cortland saline. The primary filaments were severed from the arch above the septum, placed in saline and then transferred into sterile 6-well cell culture plates using sterile forceps. All filaments were handled at the severed end only, to minimize damage to the tissue. Each well contained 5 filaments in 3 ml of L-15 medium containing 25 mmol l^{-1} HEPES, 4 mg ml^{-1} BSA, 0.65 mg ml-1 $NaHCO_3$, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The gill filaments were preincubated in this medium at 13 °C for 3-5 h. The preincubation medium was then removed from the wells by aspiration and replaced with fresh L-15 medium (modified as above) containing in addition the appropriate hormone or vehicle.

The culture treatment groups used were: control (ethanol), cortisol (0.1 μg ml^{-1}), cortisol (1.0 μg ml^{-1}), cortisol (10 μg ml^{-1}, for 5-day treatment only), aldosterone (0.1 μg ml^{-1}), aldosterone (1.0 μg ml^{-1}), aldosterone (10 μg ml^{-1}), aldosterone (100 μg ml^{-1}, for 5-day treatment only). Hormones were dissolved in ethanol and the highest concentration

of ethanol used was added to the control medium (~2% ethanol). Gill filaments were incubated for either 24 h or 5 days at 13 °C. Following incubation, filaments were placed into 100 µl of SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2 EDTA, 50 mmol l^{-1} imidizole, pH 7.3), flash frozen in liquid nitrogen, and stored at -80 °C until analysis.

The effect of aldosterone treatment in vivo on ion transporter activities

This experiment was conducted to determine if chronic aldosterone treatment affects Na^+-K^+- and/or H^+-ATPase activity and to determine if these effects are mediated through a glucocorticoid- or a mineralocorticoid-like receptor. Fish were lightly anaesthetized (*i.e.* until the loss of equilibrium) in a benzocaine solution (0.05 g l^{-1} ethyl-*p*-aminobenzoate) and were then randomly allocated to one of four treatment groups (N = 7-8 for each group); sham-injected controls (cocoa-butter only, 2 ml kg^{-1} fish), aldosterone treated (36 mg kg^{-1} fish; Steraloids) fish, aldosterone (36 mg kg^{-1} fish) plus the GR antagonist RU486 (0.5 µg kg^{-1} fish; mifepristone, Sigma), and aldosterone (36 mg kg^{-1} fish) plus the mammalian MR antagonist spironolactone (0.1 µg kg^{-1} fish, Sigma). Fish were treated with aldosterone and/or receptor antagonists via a single, intraperitoneal (i.p.) injection of warm cocoa butter containing the appropriate chemicals. Following injection, fish within a treatment group were placed into separate 115 l holding tanks. After 5 days, fish were killed by a blow to the head, and gill and kidney tissue were dissected out, placed into SEI buffer, flash frozen in liquid nitrogen, and stored at - 80 °C for later analysis of Na^+-K^+-ATPase and H^+-ATPase activities.

The effect of aldosterone treatment in vivo on MR protein expression

The objective of this study was to determine if chronic aldosterone treatment, *in vivo*, affects branchial ion transporter activity and/or the abundance of Na^+-K^+-ATPase-rich cells at the gill. The results from the control group in this experiment are presented in Chapter 2 (see Fig. 2-7). Briefly, fish were lightly anaesthetized in benzocaine (0.05 g l^{-1} ethyl-*p*-aminobenzoate) until equilibrium was lost and then randomly allocated to one of two treatment groups. Control fish (N=10) received an i.p. injection of warm cocoa butter, while aldosterone-treated fish (N=8) received an i.p. injection of warm cocoa butter containing aldosterone (36 mg aldosterone kg^{-1}). Fish were then placed in their respective treatment groups into 115 l tanks for 48 h, a time period chosen on the basis of the previous experiment.

After 2 days fish were terminally anaesthetized in benzocaine (0.5 g l^{-1} ethyl-*p*-aminobenzoate). Blood samples (0.5 ml) were withdrawn by caudal puncture and centrifuged at 5,000 *g* for 30 s. Plasma was drawn off, flash frozen in liquid nitrogen, and stored at -20° for later analysis of plasma aldosterone concentrations. Gill and kidney tissue were dissected and placed into SEI buffer, flash frozen in liquid nitrogen and stored at -80 °C for later analysis of Na^+-K^+-ATPase and H^+-ATPase activities. The remaining gill tissue was subjected to a protocol for the isolation of epithelial cells developed by Goss et al. (2001) and described in detail in Chapter 2 (see materials and methods section entitled Flow cytometry). The isolated cells were then labeled with a polyclonal goat IgG raised against the N-terminal region of the human MR (diluted 1:500 in 0.2% Tween-20 in PBS) and α5 primary antibody (described in materials and methods section entitled Antibodies; diluted 1:100 in 0.2% Tween-20 in PBS) and analysed by

flow cytometry as detailed in Chapter 2 (see materials and methods section entitled Flow cytometry).

Analytical techniques

Plasma aldosterone was measured using a commercial (^{125}I) radioimmunoassay kit (Diagnostic Systems Laboratories Inc.). On the day of the assay, filaments were thawed, 25 ul of SEID buffer (1 g sodium deoxycholate added to 20 ml SEI buffer) was added to the SEI buffer, and filaments were homogenized using a Wheaton Overhead Stirrer. All homogenates were kept on ice until protein concentration, and Na^+-K^+-ATPase and H^+-ATPase activity could be measured. Na^+-K^+-ATPase and H^+-ATPase activity was measured in gill and kidney tissue using the method of McCormick (1993; see materials and methods section entitled Analytical techniques).

Statistical Analysis

Data are presented as mean values ± 1 SEM. The significance of statistical differences among control, cortisol- and aldosterone-treated groups for *in vitro* experiments was determined by one-way ANOVA. Statistically significant differences between control and aldosterone-treated groups in *in vivo* and in flow cytometry experiments was determined using the Student's *t*-test. All flow cytometry data were arcsin transformed prior to statistical analysis. The fiducial limit of significance in all analyses was 0.05, and all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc).

Results

A gill explant culture system was used to assess the effects of cortisol or aldosterone on branchial Na^+-K^+-ATPase and H^+-ATPase activities. The 24 h experiment consisted of two cortisol doses, one within the physiological range (~100 ng ml^{-1}) and one above this range (~1000 ng ml^{-1}). Corresponding doses of aldosterone were employed together with a dose that was 10-fold higher (~10 000 ng ml^{-1}). The higher dose was included because the competition studies carried out by Colombe et al. (2000) suggested that cortisol out-competed aldosterone for binding to the rtMR *in vitro* and thus, higher aldosterone levels would be required to attain an equal effective concentration of hormone in the plasma. No statistically significant effect of any hormone treatment on either Na^+-K^+-ATPase or H^+-ATPase activity was detected (Fig. A1).

This experiment was repeated over a longer time course and with a wider range of cortisol and aldosterone doses to investigate the possibility that the lack of effect of hormone treatment on Na^+-K^+-ATPase activity at 24 h was because changes were gradual or long-term change. Again, however, no significant effect of cortisol or aldosterone treatment on Na^+-K^+-ATPase activity was apparent (Fig. A1).

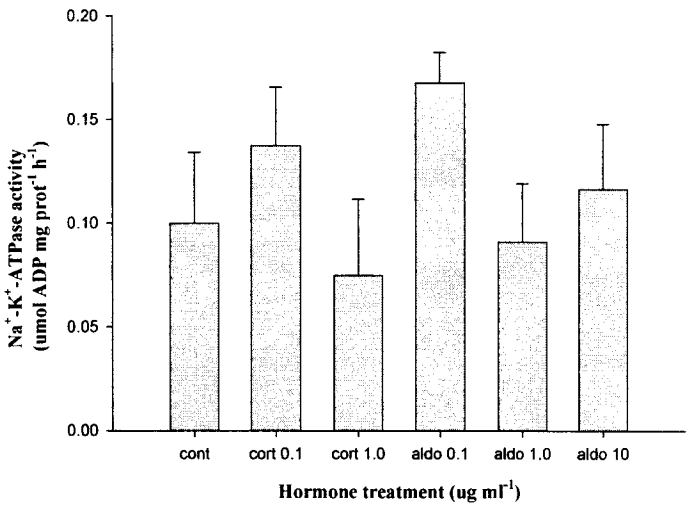
The potential effects of aldosterone treatment on branchial and renal Na^+-K^+-ATPase and H^+-ATPase activities were also examined *in vivo*. The effects of 48 h (Fig. A4a,b) or 5 days (Fig. A5a,b and Fig A6a,b) of aldosterone treatment using slow-release i.p. implants were investigated. No significant effect of aldosterone treatment on renal or branchial Na^+-K^+-ATPase or H^+-ATPase activity relative to controls was detected for either treatment period. Moreover, in the 5-day experiment, treatment groups in which

aldosterone was administered together with either the GR-antagonist RU486 or the putative MR-antagonist spironolactone were assessed. No significant differences among treatment groups were detected. However, plasma aldosterone levels after 5 days of aldosterone treatment were only 26.9 ± 7.6 pg ml^{-1} ($N = 7$), compared to a control value of 4.87 ± 0.84 pg ml^{-1} ($N = 7$; Student's t-test, $P = 0.013$). By contrast, plasma aldosterone levels after 48 h of aldosterone treatment were 4519 ± 586 pg ml^{-1} ($N = 4$) as compared to a control value of 67 ± 14 ($N = 2$; Student's t-test, $P = 0.007$).

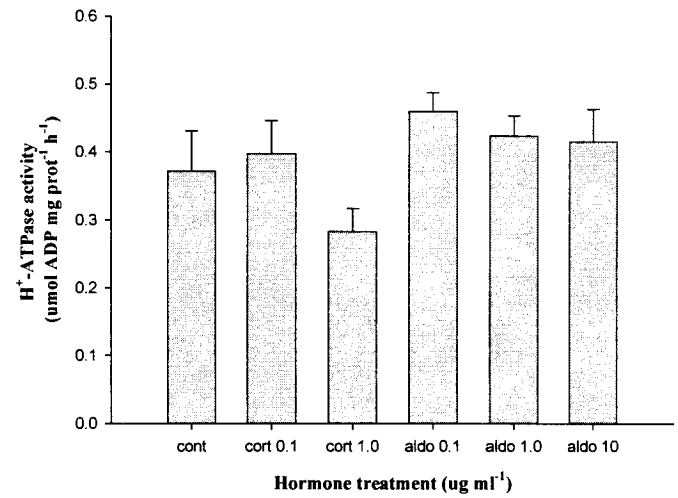
Analysis of gill cell populations by flow cytometry revealed a clear, significant (Student's t-test, $P = 0.002$) reduction in the relative proportion of cells that were double-labeled for Na^+-K^+-ATPase ($\alpha 5$) and the MR (Fig. A7). Moreover, there was a slight reduction in the relative proportion of cells staining for the MR only, although this difference was not statistically significant. The reductions in double-labeled and MR-labeled cells in the aldosterone-treated group appeared to be counterbalanced by a significant increase (Student's t-test, $P < 0.001$) in the relative proportion of unlabeled cells.

Figure A1. The effect of cortisol (cort) or aldosterone (aldo) treatments *in vitro* for 24 h (A, B) or 5 days (C) on Na^+-K^+-ATPase (A, C) and H^+-ATPase (B) activities of gill tissue explants prepared from rainbow trout (*Oncorhynchus mykiss*). Data are presented as mean values ± 1 SEM; $N = 5$ for all treatment groups. No significant differences among treatment groups were detected in any case (one-way ANOVA, $P > 0.05$).

A)


0.20
0.15
0.10
0.05
0.00
Na+-K+-ATPase activity (umol ADP mg prot-1 h-1)
cont
cort 0.1
cort 1.0
aldo 0.1
aldo 1.0
aldo 10
Hormone treatment (ug ml-1)

B)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
H+-ATPase activity (umol ADP mg prot-1 h-1)
cont
cort 0.1
cort 1.0
aldo 0.1
aldo 1.0
aldo 10
Hormone treatment (ug ml-1)

C)

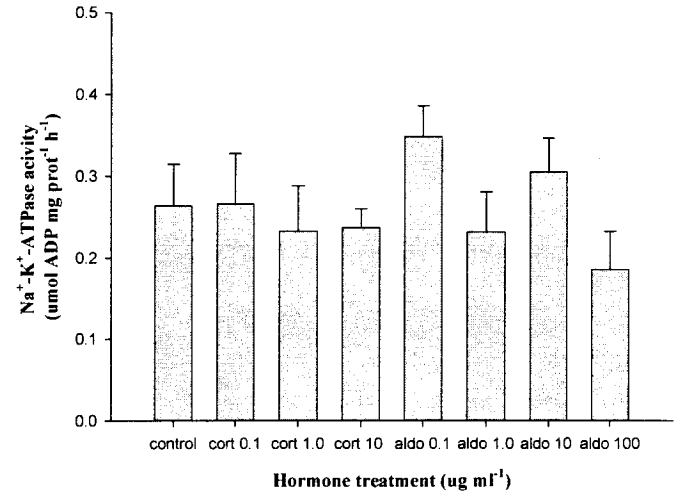
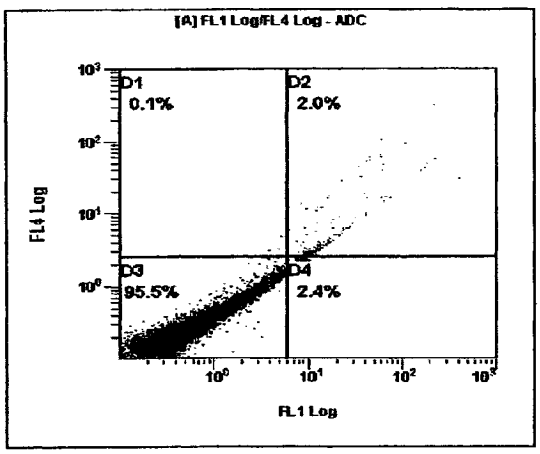
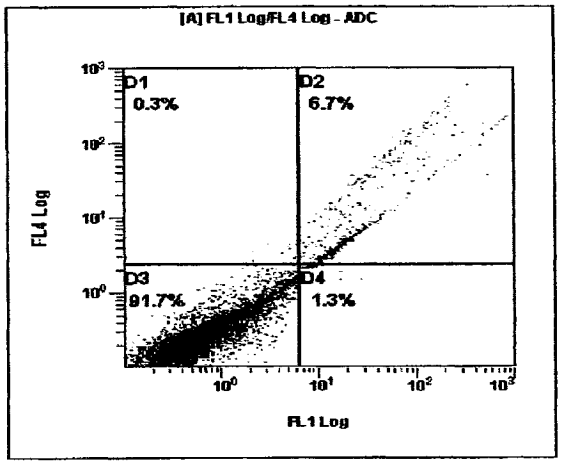

0.5
0.4
0.3
0.2
0.1
0.0
Na+-K+-ATPase acivity (umol ADP mg prot-1 h-1)
control
cort 0.1
cort 1.0
cort 10
aldo 0.1
aldo 1.0
aldo 10
aldo 100
Hormone treatment (ug ml-1)

Figure A2. Representative scatter plot of flow cytometry data for control (A) and aldosterone (B) treated fish indicating and the mean relative proportions of cells from a whole-gill cell mixture that show no fluorescence (unlabeled), positive immunostaining for Na^+-K^+-ATPase (NKA), positive immunostaining for both NKA and the mineralocorticoid receptor (MR), or positive staining for the MR only (C). Fish gill cells were separated following 48 h sham (control, $N = 5$) or aldosterone treatment ($N = 3$). Data are presented as mean values ± 1 SEM. An asterisk (*) indicates a significant difference between treatment groups within a particular cell population (Student's *t*-tests, $P < 0.001$ for unlabelled and $P = 0.002$ for NKA+MR, data were arcsin transformed prior to statistical analysis).

A)


[A] FL1 Log/FL4 Log - ADC
D1
0.1%
D2
2.0%
D3
95.5%
D4
2.4%
FL4 Log
FL1 Log

B)


[A] FL1 Log/FL4 Log - ADC
D1
0.3%
D2
6.7%
D3
91.7%
D4
1.3%
FL4 Log
FL1 Log

C)

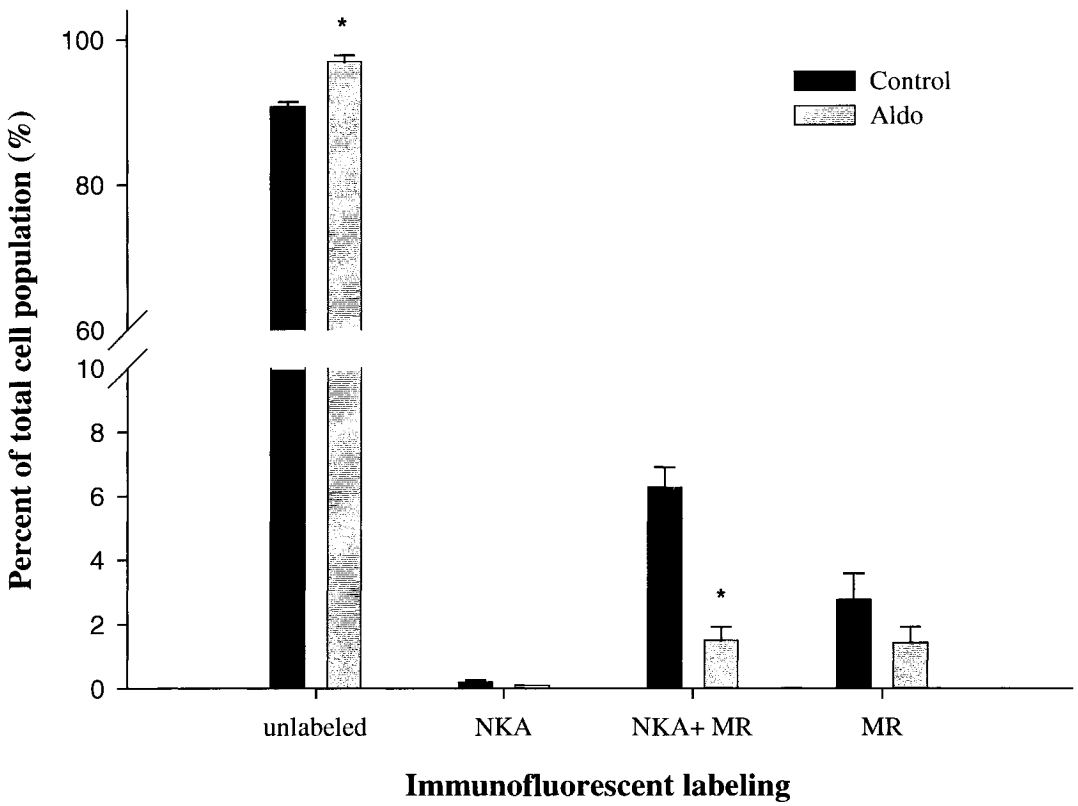

Percent of total cell population (%)
100
80
60
10
8
6
4
2
0
*
*
Control
Aldo
unlabeled
NKA
NKA+ MR
MR
Immunofluorescent labeling

Table A1. Branchial and renal Na^+-K^+-ATPase activity in rainbow trout (*Oncorhynchus mykiss*) 48 h after an injection of cocoa butter (control) and cocoa butter containing aldosterone (36 mg kg^{-1} fish).

Ion transport activity (μmol ADP mg $prot^{-1}$ h^{-1})	Control	Aldosterone
Branchial Na^+-K^+-ATPase	0.27 0.03 (5)	0.29 0.06 (4)
Renal Na^+-K^+-ATPase	1.38 0.06 (4)	1.44 0.18 (4)

Numbers in parentheses are *N* values. Data are presented as means ± 1 S.E.M. No statistical differences were measured between control and aldosterone treated groups (statistical analysis performed was Student's *t*-test, $P > 0.05$).

Table A2. Na^+-K^+-ATPase and H^+-ATPase activity in gill and kidney tissue of rainbow trout (*Oncorhynchus mykiss*) 5 days following an injection of a cocoa butter implant (control), an implant containing aldosterone, an implant containing aldosterone and RU486 (aldo+RU486) or an implant containing aldosterone and spironolactone (aldo+spiron.).

	Control	Aldosterone	Aldosterone + RU486	Aldosterone + spironolactone	*P* value for one-way ANOVA
Branchial Na^+-K^+-ATPase	0.38 0.09 (8)	0.64 0.22 (8)	0.67 0.21 (7)	0.80 0.23 (8)	0.25
Renal Na^+-K^+-ATPase	1.45 0.11 (8)	1.43 0.21 (8)	1.63 0.15 (7)	1.60 0.21 (8)	0.84
Branchial H^+-ATPase	0.43 0.15 (8)	0.36 0.05 (7)	0.55 0.14 (5)	0.44 0.15 (6)	0.37
Renal H^+-ATPase	1.41 0.25 (7)	1.38 0.11 (8)	1.17 0.18 (6)	1.05 0.17 (8)	0.41

Numbers in parentheses are *N* values. Data are presented as means ± 1 S.E.M.

Discussion

A role for the rtMR in ionic regulation was investigated, *in vitro*, using the mammalian MR agonist aldosterone. In tetrapods, aldosterone stimulates an increase in Na^+-K^+-ATPase activity and an increase in sodium reabsorption across epithelia (Booth et al., 2002) as a part of the late genomic response phase. Sturm et al. (2005) reported that aldosterone is a potent agonist of the rtMR in transactivation assays, identifying it as a useful tool for studying rtMR function *in vitro* and *in vivo* since fish lack endogenous aldosterone production (Sandor et al., 1966; Idler and Sangalang, 1970; Butler and Yousen, 1986). In the present study, however, exposure of gill explants to aldosterone failed in all cases to elicit a change in Na^+-K^+-ATPase activity (Fig A1). Similarly, cortisol treatment was without effect on gill explant Na^+-K^+-ATPase activity even when administered at supraphysiological levels (Fig.A1). The latter result was unexpected given that McCormick and Bern (1989) demonstrated that cortisol treatment *in vitro* resulted in a slight but statistically significant increase in Na^+-K^+-ATPase activity in gill tissue explants from coho salmon. Similar results were obtained by Shrimpton and McCormick (1999) for rainbow trout. However, branchial Na^+-K^+-ATPase activity was found to be unresponsive to short-term cortisol exposure in sea bream (Deane and Woo, 2005) and 24 h exposure of rainbow trout gill filaments to cortisol, *in vitro*, had no effect on the number of Na^+-K^+-ATPase-rich cells (Mazon et al., 2004). Thus, although previous studies have found cortisol-induced increases in branchial Na^+-K^+-ATPase activity in vitro, these effects were relatively small and required supraphysiological concentrations of cortisol (McCormick and Bern, 1989). Collectively, the evidence presented here, combined with results from previous studies indicate that an *in vitro*

system, used to detect hormone-induced changes in Na^+-K^+-ATPase activity, may not be the most effective and sensitive method for investigating the function of corticosteroids and corticosteroid receptors in fish. The lack of effect of hormone treatment on branchial Na^+-K^+-ATPase activity in gill explants could also be explained by the absence of essential response components *in vitro*, such as the continuous delivery of hormone through the bloodstream as well as additional factors that act synergistically with hormones to stimulate a response (Bern and Madsen, 1992; Eckert et al., 2001; McCormick, 2001). With such possibilities in mind, aldosterone was administered *in vivo* for 48 h and 5 days. Again, however, aldosterone treatment failed to elicit changes in renal or branchial Na^+-K^+-ATPase or H^+-ATPase activities. A number of possible explanations for these results exist. Perhaps the most attractive of these explanations is that MR activation targets a step other than Na^+-K^+-ATPase or H^+-ATPase activity in ionic regulation, as these transport proteins are unlikely to represent the limiting steps in ion uptake pathways. Alternatively, the timing and/or dose of aldosterone may have been inappropriate. The plasma aldosterone concentration in fish 5 days following an i.p. injection of aldosterone was only slightly higher than in sham injected controls, indicating that the peak in plasma aldosterone was missed and may explain why no effect on ion transport activity was detected.

In salmonid fish, changes in the salinity of the environment can elicit morphological changes in the branchial epithelium (Perry and Wood, 1985; Avella et al., 1987; Perry and Laurent, 1989; Uchida et al., 1996). These changes are largely due to changes in the size and abundance of chloride cells in the epithelium. For example transfer from freshwater to seawater stimulated an increase in filamental chloride cell size

in chum salmon fry (Uchida et al., 1996) and an increase in lamellar surface area in rainbow trout (Laurent and Hebibi, 1989). Furthermore, transfer of rainbow trout to ion-poor water stimulates an increase in chloride cell size (Perry and Laurent, 1989; Perry et al., 1996) and abundance (Laurent and Hebibi, 1989; Laurent and Perry, 1991). Sloman et al. (2001) recently observed that the chloride cell proliferation induced by exposure to ion-poor water was inhibited by the MR antagonist spironolactone, but not by the GR antagonist RU486. This observation suggests that the rtMR mediates chloride cell proliferation during acclimation to soft water. To further investigate the role of the rtMR in regulating chloride cell proliferation, we used flow cytometry to analyse aldosterone-induced changes in the relative proportions of cells expressing Na^+-K^+-ATPase and MR within the branchial cell population as a whole. Interestingly, a significant decrease in the percentage of.double-labeled cells was observed 48 h after the administration of an aldosterone implant (Fig.A7). This decrease, in what is presumed to be a population of Na^+-K^+-ATPase-rich chloride cells, was unexpected given the evidence suggesting the involvement of the rtMR in softwater-induced chloride cell proliferation (Sloman et al., 2001). It is in keeping, however, with results that suggest a down-regulation of MR mRNA occurs in response to softwater acclimation in rainbow trout (unpublished data, K.M. Gilmour, M. Bell and M.M. Vijayan). Interestingly, Laurent et al. (1994) found evidence supporting the role of cortisol in promoting the differentiation of immature chloride cells at the gill and found that cortisol was not localized to undifferentiated stem cells nor did it affect the rate of branchial cell division. Together, this evidence coupled with the results of the flow cytometry experiments presented here, suggest that undifferentiated, immature and mature branchial cells may express different

corticosteroid receptor subtypes at different times so as to respond appropriately to corticosteroids in the blood. Clearly, the role of corticosteroid receptors in both the proliferation and degradation of chloride cells warrants further investigation. Additional experiments are required to confirm the preliminary flow cytometry results presented here, and to examine the relative abundance of Na^+-K^+-ATPase-rich, MR-expressing cells in the gills of fish exposed to an ionoregulatory challenge or treated with cortisol (a potent MR and GR agonist), or dexamethasone (a potent GR agonist). Such experiments will help to clarify the role of corticosteroid receptors in altering the cellular composition of the branchial epithelium.

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