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RESPONSIVENESS OF GILL Na^+/K^+-ATPase TO CORTISOL IS RELATED TO GILL CORTICOSTEROID RECEPTOR CONCENTRATION IN JUVENILE RAINBOW TROUT

J. MARK SHRIMPTON* AND STEPHEN D. MCCORMICK

Conte Anadromous Fish Research Center, Biological Resources Division, USGS, Turners Falls, MA 01376, USA and *Department of Biology, University of Massachusetts, Amherst, MA 01002, USA*

*Present address: Biology Program, College of Science and Management, University of Northern British Columbia, 3333 University Way, Prince George, British Columbia, Canada V2N 4Z9 (e-mail: shrimptm@unbc.ca)

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Summary

A positive relationship between receptor concentration and tissue responsiveness is an often-assumed and rarely tested principle in endocrinology. In salmonids, seasonal changes in levels of plasma cortisol and gill corticosteroid receptors (CRs) during the spring indicate a potential role for this hormone in the parr–smolt transformation. It is not known whether these seasonal changes result in alterations in gill responsiveness to cortisol. The relationship between CR concentration and tissue responsiveness was, therefore, examined in the gills of juvenile rainbow trout (*Oncorhynchus mykiss*). Gill CR concentration (B_{max}) and affinity (K_d) were assessed using a radioligand binding assay with the synthetic glucocorticoid triamcinolone acetonide. Gill responsiveness to cortisol was quantified by measuring *in vitro* Na^+/K^+-ATPase activity. Gill CR concentration was manipulated by stress or hormonal treatments. Repeated handling stresses resulted in a significant reduction in CR numbers. The decrease in CR B_{max} corresponded to a reduction in gill responsiveness to cortisol. Triiodothyronine, but not growth hormone, treatment was found to increase CR B_{max} significantly. The increase in CR numbers was correlated with a marked increase in gill responsiveness to cortisol. A significant positive linear relationship exists between the *in vitro* gill Na^+/K^+-ATPase activity response to cortisol and CR B_{max} (r^2=0.614, P<0.001). We have demonstrated that binding sites for cortisol in the gills of rainbow trout have high affinity, high specificity and saturable binding and that the number of binding sites is correlated with the tissue response to cortisol.

Key words: cortisol, corticosteroid receptor, growth hormone, triiodothyronine, gill, Na^+/K^+-ATPase activity, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Many salmonids undergo a developmental process, the parr–smolt transformation, that is controlled by seasonal changes in both photoperiod and temperature. These environmental cues stimulate a series of physiological changes in juvenile salmon that culminate in the transformation of stream-dwelling parr into migratory smolts capable of surviving in the marine environment (Hoar, 1988). A variety of physiological and morphological changes have been shown to be associated with the parr–smolt transformation. Notable among these is an increase in gill Na^+/K^+-ATPase activity, which is correlated with the development of seawater tolerance (McCormick and Saunders, 1987). This enzyme has been shown to be regulated by cortisol *in vitro* (McCormick and Bern, 1989) and *in vivo* (for a review, see McCormick, 1995).

The action of cortisol in the gills is probably mediated by intracellular corticosteroid receptors (CRs). Hormone receptors are characterized by high affinity, high specificity and saturable binding, and by stimulating a response when bound to the appropriate hormone (Clark and Peck, 1977). Protein molecules that fit the first three criteria have been found in the gills of salmonids (Chakraborti et al., 1987; Maule and Schreck, 1990). A direct relationship, however, between tissue sensitivity to cortisol and corticosteroid receptor concentration or affinity has not been reported in the literature for teleosts. To understand the significance of changes in receptor concentration, it is important to establish a relationship between the tissue response and the receptor concentration. Plasma cortisol and gill CR concentration have been observed to change seasonally in conjunction with smolting in coho salmon *Oncorhynchus kisutch* (Shrimpton et al., 1994), steelhead trout *Oncorhynchus mykiss* (McLeese et al., 1994) and Atlantic salmon *Salmo salar* (Shrimpton and McCormick, 1998a). McCormick et al. (1991a) showed that seasonal changes in gill responsiveness to cortisol occurs in coho and Atlantic salmon. The seasonal changes in responsiveness to cortisol and receptor concentration associated with smolting suggest a functional relationship between these two variables; however, this relationship has not been established in a single study.

CR concentration has been shown to be altered by hormone

treatment. Shrimpton et al. (1995) found that growth hormone (GH) treatment increased the number of gill CRs in coho salmon. Shrimpton and McCormick (1998b) found that triiodothyronine (T_3) augmented the effect of growth hormone on increasing the number of CRs in Atlantic salmon. Cortisol treatment and stress led to a decrease in the number of gill CRs (Maule and Schreck, 1991; Shrimpton and Randall, 1994) and liver CRs (Pottinger et al., 1994). Methods exist, therefore, to manipulate the number of CRs in the gills of salmonids. Gill organ culture has been used to assess the responsiveness of the gill to hormones by measuring the increase in Na^+/K^+-ATPase activity *in vitro* (McCormick and Bern, 1989; McCormick et al., 1991a; Madsen and Bern, 1993). To assess the effect of differences in CR B_{max} on cortisol responsiveness, CR B_{max} was manipulated by stress and hormonal treatment, and responsiveness was measured from changes in Na^+/K^+-ATPase activity in organ culture.

Materials and methods

Experimental design

Experiment 1

Juvenile rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were transported from Sunderland State Trout Hatchery, Sunderland, MA, USA, to the Conte Anadromous Fish Research Center in Turner Falls, MA, USA, on 8 August 1995. Fish were reared in dechlorinated city water (19.4–19.8 °C) under natural photoperiod and fed to satiation twice daily. On 16 August 1995, juvenile rainbow trout (16.8±0.2 cm, 55.7± 2.3 g; means ± S.E.M., N=6) were placed into two tanks 1 m in diameter. Fish in the first tank were left undisturbed for 10 days. Fish in the second tank were acutely stressed twice daily for 10 days. One of three stressors was used to prevent accommodation of the fish to a specific stressor: confinement, water removal or chasing. For the confinement stress, fish were caught in a dip net and held for 15 min at a density such that all fish were in physical contact with one another. For the water removal stress, the water was drained from the tank until the fish were partially stranded and on their sides. After 60 s, the water was replaced. The third stress involved chasing the fish with a dip net for 15 min. At the end of the 10 day treatment, fish were left undisturbed for 3 days and then sampled as described below. This protocol has been shown to result in a significant reduction in CR concentration (Shrimpton and Randall, 1994). Fish were not fed throughout the experiment.

Experiment 2

Juvenile rainbow trout were transported from McLaughlin State Trout Hatchery, Belchertown, MA, USA, on 16 September 1996. We found no difference in CR B_{max} and responsivenes of gill Na^+/K^+-ATPase to cortisol between fish from the McLaughlin and Sunderland Hatcheries (J. M. Shrimpton and S. D. McCormick, unpublished results). Fish were held in filtered water drawn from the Connecticut River (13.5–14.1 °C) under natural photoperiod and fed to satiation twice daily. On 6 October 1996, rainbow trout (13.5±0.2 cm, 26.9±1.0 g; N=6) were anaesthetized with tricaine methane sulphonate (100 mg l^{-1}; neutralized and buffered with sodium bicarbonate, pH 7.0). Length and mass were measured, and the fish were injected with one of the following: vegetable oil as vehicle, 5.0 μg g^{-1} ovine growth hormone (GH; National Institute of Health, Bethesda, MD, USA), 1.6 μg g^{-1} T_3 (Sigma, St Louis, MO, USA) or 5.0 μg g^{-1} GH plus 1.6 μg g^{-1} T_3. Groups were identified by coloured acrylic paint injected between the fin rays of the anal fin. After recovery, the fish were placed in a circular tank 1 m in diameter. Four days later, fish were reinjected with the same treatment. On day 8, the fish were sampled as described below. Fish were not fed throughout the experiment.

Fish sampling

Fish were rapidly removed from their tanks and placed in 200 mg l^{-1} tricaine methane sulphonate (buffered with sodium bicarbonate, pH 7.0). Length and mass were measured within 5 min of first disturbing the fish to ensure that a stress-associated rise in cortisol level did not occur. The right first gill arch was removed and placed in minimal essential medium (MEM with Hanks' salts, Gibco) on ice. Approximately 6–8 primary gill filaments were removed and placed in 100 μl of SEI (150 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2EDTA, 50 mmol l^{-1} imidazole, pH 7.3) on ice for later determination of Na^+/K^+-ATPase activity. Samples were frozen at –80 °C within 30 min. The rest of the gill tissue was removed, placed in 2 ml of TEMS (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} Na_2EDTA, 12 mmol l^{-1} monothioglycerol, 20 mmol l^{-1} sodium molybdate, 10 % v/v glycerol, pH 7.4) and frozen immediately at –80 °C for later analysis of CR concentration and affinity.

Gill organ culture

The protocol of McCormick and Bern (1989) was followed for gill organ culture. Primary gill filaments were severed just above the septum and separated from one another. Filaments were moved using a positive displacement pipette to minimize tissue damage. Five to six primary gill filaments were placed in 0.5 ml of MEM with 25 mmol l^{-1} Hepes buffer, 4 mg ml^{-1} bovine serum albumin (Sigma radioimmunoassay grade), 292 μg ml^{-1} L-glutamine, 0.65 mg ml^{-1} $NaHCO_3$, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (adjusted to pH 7.8 with NaOH) in sterile 24-well culture plates. Gill filaments were preincubated in this medium on ice for up to 5 h. A stock solution of cortisol was prepared by dissolving 5 mg ml^{-1} in ethanol. Serial dilutions of cortisol in ethanol were made, and 60 μl was added to 100 ml of MEM to achieve final cortisol concentrations of 0.1, 1 and 10 μg ml^{-1}. The same volume of ethanol was added to the control MEM, but without cortisol. Ethanol at this concentration (0.06 %) does not affect Na^+/K^+-ATPase activity during *in vitro* exposure (McCormick and Bern, 1989). The preincubation medium was removed and replaced with 0.75 ml of MEM containing 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin and cortisol or vehicle that had been equilibrated with a 99 % oxygen and 1 % carbon dioxide gas mixture. Gill filaments were incubated at 15 °C for 48 h in a humidified chamber with 99 %:1 % O_2:CO_2 with gentle shaking. After culture, gill

filaments were removed with forceps and placed in 80 µl of SEI buffer on ice and then frozen at –80 °C until analysis of Na^+/K^+-ATPase activity.

Gill Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity was measured according to the microassay protocol of McCormick (1993). Gill filaments were homogenized in SEI buffer containing 0.1 % sodium deoxycholate. Following centrifugation (at 3000 ***g*** for 2 min) to remove insoluble particles, ouabain-sensitive ATPase activity was determined kinetically by following the hydrolysis of ATP linked to the oxidation of nicotinamide adenine dinucleotide (NADH), measured at 340 nm for 10 min at 25 °C in the presence and absence of 0.5 mmol l^{-1} ouabain. Protein content in the gill homogenate was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Specific activities were expressed as µmol ADP mg^{-1} protein h^{-1}.

Corticosteroid receptor assay

The method of Maule and Schreck (1990) as modified by Shrimpton and Randall (1994) was used for analysis of corticosteroid receptors. All procedures were carried out with samples on ice. Thawed gill tissue was scraped away from the cartilage and then homogenized in 2.0 ml of TEMS using a Tekmar TP 18/10S1 homogenizer for two 10 s bursts. Homogenates were centrifuged in a Beckman GPKR knee-well centrifuge at 3000 ***g*** for 15 min. The supernatant was removed and placed on ice. The pellet was resuspended with 0.5 ml of TEMS containing 50 µg ml^{-1} bacitracin, 20 µg ml^{-1} benzidine, 0.5 µg ml^{-1} aprotinin and 10 µg ml^{-1} *o*-phenanthroline, to wash more CRs from the pellet, and then recentrifuged at 3000 ***g*** for 15 min. The supernatants were combined and centrifuged at 48 000 ***g*** for 2 h in a Beckman J2-21M centrifuge with a JA-21 rotor. After this centrifugation, the supernatant was removed, mixed with 1.0 ml of TEMS containing 10 % (w/v) activated charcoal and 1.0 % (w/v) dextran and incubated for 10 min to remove endogenous steroids. To separate the charcoal from the liquid, the samples were centrifuged at 3000 ***g*** for 15 min. The final supernatant was used to quantify cortisol binding. Protein content was assayed with Bradford reagent (Bradford, 1976) using bovine serum albumin as a standard.

Cortisol binding receptor studies were conducted with [^{3}H]triamcinolone acetonide (TA; 1,4-pregnadien-9α-fluoro-11β,16α,-17β,21-tetrol-3,20-dione-16,17 acetonide) with a specific activity of 1620 GBq $mmol^{-1}$ (Dupont-NEN). In binding and competition studies on duplicate gill homogenates, TA and cortisol bound to the same number of receptors, but TA had a higher affinity. To determine the number of high-affinity cortisol receptors, 100 µl of the final supernatant was incubated in duplicate with 100 µl of buffer containing [^{3}H]TA with or without a 500-fold excess of cold TA. The final concentration of [^{3}H]TA ranged from 0.1 to 6 nmol l^{-1}. The tubes were vortexed and incubated for 2 h on ice. After the incubation period, 0.5 ml of TEMS containing 2.5 % (w/v) activated charcoal and 0.25 % (w/v) dextran was added and vortexed. After 10 min, the charcoal containing unbound ligand was separated from bound ligand by centrifugation at 3000 ***g*** for 15 min in a Beckman GPKR refrigerated centrifuge. The supernatant (0.5 ml) was added to 3 ml of aqueous counting scintillant (Scintisafe Econo 2 Fisher Scientific). Samples were counted on a Beckman LS 6000IC liquid scintillation counter. Specific binding was determined by subtracting the non-specific bound ligand from the total bound ligand.

Although the origin of CRs in the gills may be cytosolic or nuclear, they are referred to as cytosolic as they are found in the cytosol fraction following tissue processing. The CR concentration measured consists of the unbound receptor population. The equilibrium dissociation constant (K_d) and the concentration of corticosteroid receptor sites (B_{max}) were calculated according to Scatchard (1949). B_{max} was divided by the homogenate protein concentration, and CR concentration was expressed as fmol mg^{-1} protein. To estimate cooperativity between CR and ligand, the Hill coefficient was calculated according to Sandor et al. (1984).

Statistical analyses

For experiment 1, a *t*-test was conducted to determine differences in initial Na^+/K^+-ATPase activity, B_{max} and K_d between the control and stressed fish. A two-way analysis of variance (ANOVA) was used to determine the effect of hormone treatment and cortisol concentration on *in vitro* responsiveness of gill Na^+/K^+-ATPase activity. For experiment 2, a two-way ANOVA was used to determine the effects of GH and T_3 on *in vivo* Na^+/K^+-ATPase activity, B_{max} and K_d. A three-way ANOVA was used to determine the effects of GH treatment, T_3 treatment and cortisol concentration on *in vitro* responsiveness of gill Na^+/K^+- ATPase activity, followed by a Tukey's test to find significant differences among the means. Data from all the experiments were combined for regression analysis. Responsiveness of gill Na^+/K^+-ATPase activity to cortisol was regressed on B_{max}, K_d and B_{max}/K_d. Statistical significance was taken at a level of P=0.05. All values are expressed as means ± 1 S.E.M.

Results

Representative binding curves, Scatchard plots and Hill plots are shown in Fig. 1 for fish sampled in August 1995. The analysis indicated saturable binding. A single class of receptor was indicated by the linear Scatchard analysis. There was no indication of cooperative binding because the Hill plot was linear and the Hill coefficient was equivalent to 1.

Specificity for the receptor is shown in Fig. 2. The competition hierarchy of steroid competitors for gill CRs can be summarized as TA>cortisol=dexamethosone>11-deoxy-cortisol>corticosterone>17-hydroxyprogesterone>cortisone> progesterone.

Experiment 1

Following stress treatment, Na^+/K^+-ATPase activity was 1.22±0.09 and 1.40±0.14 µmol ADP mg^{-1} protein h^{-1} for the

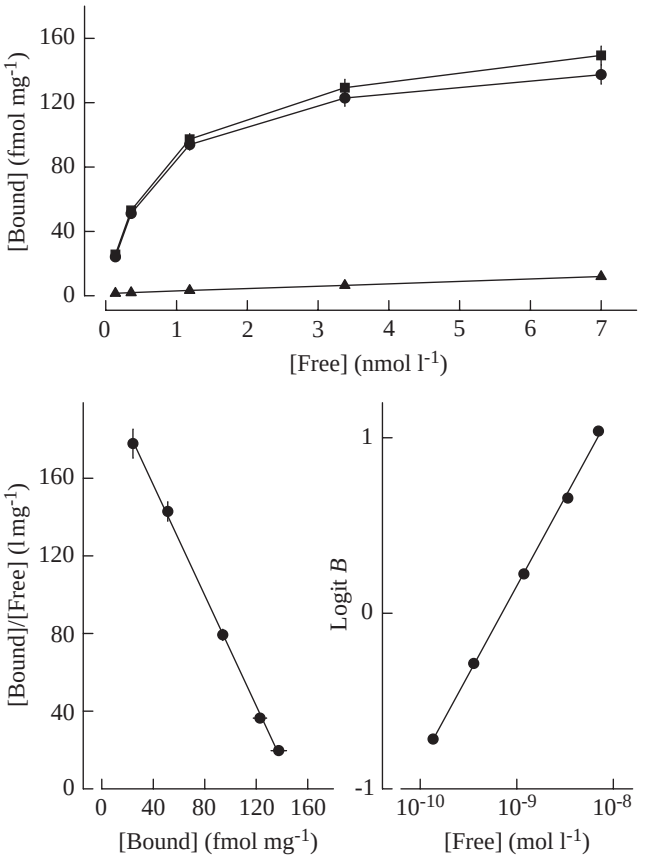

Fig. 1. Representative binding plot (A), Scatchard plot (B) and Hill plot (C) for gill corticosteroid receptors (CRs) in juvenile rainbow trout. ■, total binding; ▲, non-specific binding; ● specific (total minus non-specific) binding. Values are means ± 1 S.E.M. of six fish. Units are fmol mg^{-1} protein for bound CRs and nmol l^{-1} for free. $\text{Logit}B = \log\left(\frac{B}{B_{max} - B}\right)$, where B=bound ligand at different concentrations of free.

control and stress groups, respectively. These values did not differ significantly (P=0.33). K_d, also, did not differ significantly between the two groups (Fig. 3, P=0.12). There was, however, a significant 33 % reduction in B_{max} for the stressed group compared with the control (P<0.01).

The response of the gill to cortisol was significantly affected by stress treatment (P<0.001) and the cortisol concentration in the medium (P=0.011), but there was no interaction effect (P=0.77) (Fig. 4). At cortisol concentrations of 0.1, 1 and 10 μg ml^{-1}, gill tissue from stressed fish consistently exhibited a lower response than that from control fish.

Experiment 2

Hormone treatment had a significant effect on gill Na^+/K^+-ATPase activity. Enzyme activity in the vehicle-injected fish was 1.94±0.22 μmol ADP mg^{-1} protein h^{-1}. GH treatment increased this activity to 2.56±0.29 μmol ADP mg^{-1} protein h^{-1}. The gill Na^+/K^+-ATPase activity of T_3-treated fish was similar to that of the vehicle-injected group (1.93±0.20 μmol ADP mg^{-1} protein h^{-1}). GH+T_3 treatment induced the highest Na^+/K^+-ATPase activity of 2.94±0.14 μmol ADP mg^{-1} protein h^{-1}. Two-way ANOVA indicated that there was a significant effect of GH on *in vivo* gill Na^+/K^+-ATPase activity (P<0.01), but no effect of T_3 (P=0.45) and no interaction effect (P=0.41).

There was a twofold increase in CR B_{max} with T_3 and GH+T_3 treatment compared with the vehicle-injected controls (Fig. 5). Two-way ANOVA indicated a significant effect of T_3 treatment on CR B_{max} (P<0.01), but no effect of GH (P=0.76) and no interaction effect (P=0.84). K_d was not altered by any of the hormone treatments (Fig. 5). Two-way ANOVA results for GH, T_3 and their interaction were P=0.32, P=0.35 and P=0.54, respectively.

In vitro gill Na^+/K^+-ATPase activity was significantly affected by cortisol concentration in the incubation medium (P<0.001), T_3 treatment of the fish (P=0.02), but not GH treatment of the fish (P=0.87) (Fig. 6). There were no interaction effects between any of the factors. T_3- and GH+T_3-treated fish showed the greatest responsiveness to cortisol. The effect of GH treatment alone, however, was not significantly different from that for vehicle-injected fish. At 10 μg ml^{-1} *in vitro* cortisol, the percentage increase in gill Na^+/K^+-ATPase activity of the GH+T_3- and T_3-treated groups was twofold greater than for the vehicle- and GH-treated groups (Fig. 6; P<0.05).

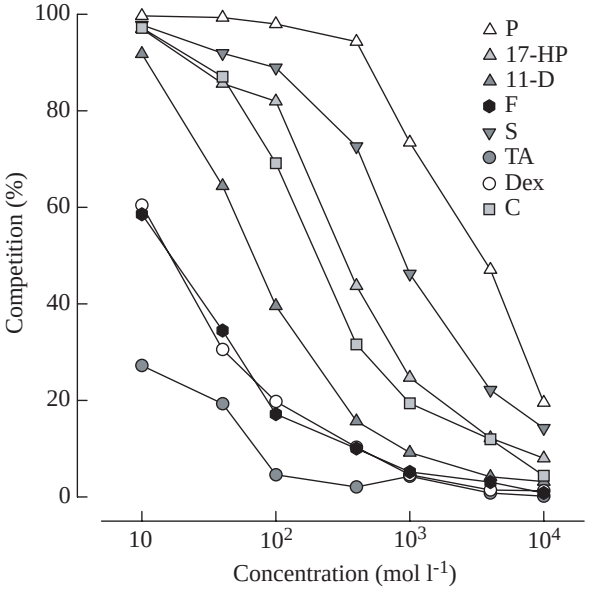

Fig. 2. Specificity of corticosteroid binding in rainbow trout gill homogenates. Pooled gill homogenates from four individuals were incubated in duplicate with 4 nmol l^{-1} [^{3}H]TA with or without 10, 40, 100, 400, 1000, 4000 and 10 000 nmol l^{-1} of unlabelled competitor. Specific binding was calculated as the difference between total binding and binding in the presence of 10 000 nmol l^{-1} unlabelled TA. Values are means of duplicate homogenates. P, progesterone; 17-HP, 17-hydroxyprogesterone; 11-D, 11-deoxycortisol; F, cortisol; S, cortisone; TA, triamcinolone acetonide; Dex, dexamethosone; C, corticosterone.

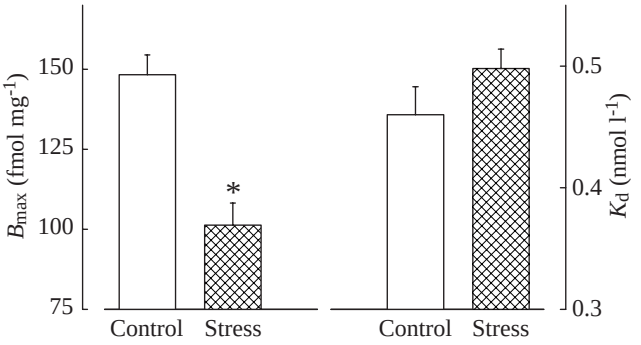

Fig. 3. Concentration (B_{max}, fmol mg^{-1} protein) and dissociation constant (K_d, nmol l^{-1}) of gill corticosteroid receptors from juvenile rainbow trout sampled 3 days after cessation of daily handling stress (N=6). The fish were stressed twice daily over a period of 10 days. An asterisk indicates that the value for the stressed group was significantly different from that for the control group (P<0.01). Values are means + 1 S.E.M.

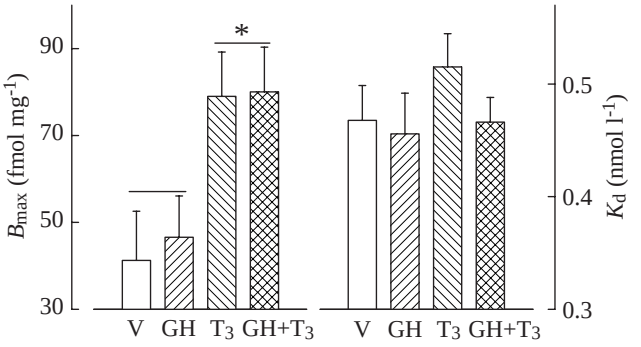

Fig. 5. Concentration (B_{max}, fmol mg^{-1} protein) and dissociation constant (K_d, nmol l^{-1}) of gill corticosteroid receptors from juvenile rainbow trout treated with vehicle (V, vegetable oil), 5.0 μg g^{-1} ovine growth hormone (GH), 1.6 μg g^{-1} triiodothyronine (T_3) or 5.0 μg g^{-1} GH + 1.6 μg g^{-1} T_3. An asterisk indicates that values for the T_3-treated groups are significantly different from those for groups not treated with T_3 (P<0.01). Values are means + 1 S.E.M. , N=6.

Regression analysis

There was a significant relationship between responsiveness of Na⁺/K⁺-ATPase activity and cortisol treatment at 10 μg ml^{-1} when regressed on B_{max} (P<0.001; r^2=0.614; Fig. 7). K_d was not significantly correlated with the responsiveness of Na⁺/K⁺-ATPase activity (P=0.612; r^2=0.004). The correlation between CR B_{max} and *in vitro* gill Na⁺/K⁺-ATPase activity, however, was slightly increased when B_{max} was divided by K_d (B_{max}/K_d) (P<0.001; r^2=0.657).

Discussion

In this study, we have demonstrated that the cortisol-binding sites in the gills of rainbow trout meet the four requirements of a receptor. The receptor has high affinity (Fig. 1), high specificity (Fig. 2) and shows saturable binding (Fig. 1), and a correlation exists between tissue response and receptor concentration (Fig. 7). Binding sites in the gills of rainbow trout showed affinity and B_{max} values comparable with values reported elsewhere for CRs in the gills of several species of salmonids (Sandor et al., 1984; Chakraborti et al., 1987; Maule and Schreck, 1990; Shrimpton and Randall, 1994). The specificity of branchial CRs is consistent with published results for several different salmonid tissues: gills (Chakraborti et al., 1987; Maule and Schreck, 1990), liver (Pottinger et al., 1994) and brain (Lee et al., 1992; Knoebl et al., 1996). The synthetic glucocorticoid TA competed most effectively. Of the natural steroids, cortisol was the most effective competitor and was similar in effect to dexamethasone, another synthetic

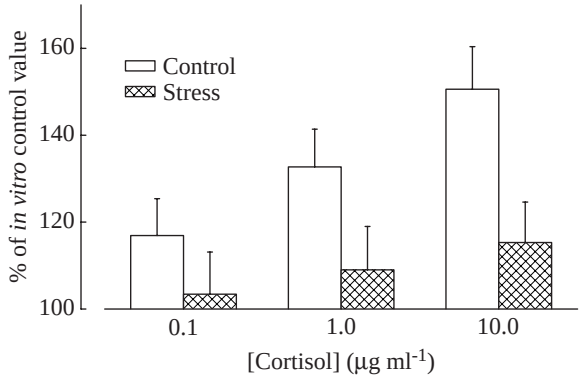

Fig. 4. *In vitro* gill Na⁺/K⁺-ATPase activity in response to cortisol for stressed and control fish. Gill filaments were incubated with 0, 0.1, 1 and 10 μg ml^{-1} cortisol for 2 days. Values are *in vitro* gill Na⁺/K⁺-ATPase as a percentage of *in vitro* gill Na⁺/K⁺-ATPase activity in control filaments not treated with cortisol. Values are means + 1 S.E.M., N=6.

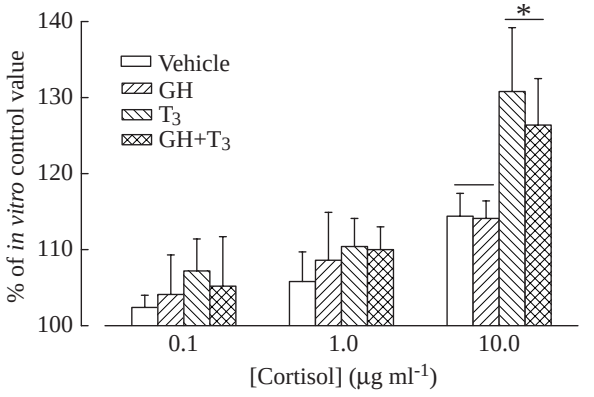

Fig. 6. *In vitro* gill Na⁺/K⁺-ATPase activity in response to cortisol for fish treated with vehicle (vegetable oil), 5.0 μg g^{-1} ovine growth hormone (GH), 1.6 μg g^{-1} triiodothyronine (T_3) or 5.0 μg g^{-1} GH + 1.6 μg g^{-1} T_3. Gill filaments were incubated with 0, 0.1, 1 and 10 μg ml^{-1} cortisol for 2 days. Values are *in vitro* gill Na⁺/K⁺-ATPase as a percentage of *in vitro* gill Na⁺/K⁺-ATPase activity in control filaments not treated with cortisol. An asterisk indicates that values for the T_3-treated groups are significantly different from those for groups not treated with T_3 (P<0.05). Values are means ± 1 S.E.M., N=6.

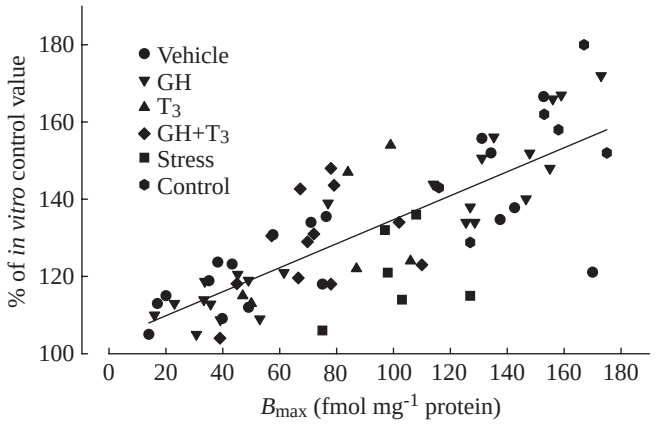

Fig. 7. *In vitro* Na^+/K^+-ATPase activity for gill filaments incubated with 10 μg ml^{-1} cortisol plotted against gill corticosteroid receptor concentration (B_{max}). Values are *in vitro* gill Na^+/K^+-ATPase as a percentage of *in vitro* gill Na^+/K^+-ATPase activity in control filaments not treated with cortisol. Values are for fish from experiment 2 injected with vehicle (vegetable oil), 5.0 μg g^{-1} ovine growth hormone (GH), 1.6 μg g^{-1} triiodothyronine (T_3) or 5.0 μg g^{-1} GH + 1.6 μg g^{-1} T_3 and for fish from experiment 1 subjected to daily handling stress. Each point corresponds to one individual. The equation for the regression line is y=0.311x+103.6 (r^2=0.614, P=0.001).

glucocorticoid. 11-Deoxycortisol, the precursor to cortisol, was less competitive than cortisol. The other precursors to cortisol (progesterone and 17-hydroxyprogesterone), the breakdown product of cortisol (cortisone) and corticosterone were less effective than cortisol in competing for CRs.

Most of the work examining the relationship between CR concentration and tissue responsiveness to cortisol has been conducted on mammalian cell lines, many of which are cancer cell lines. CR concentration has been correlated with a physiological response in mouse thymoma-derived cells (Danielsen and Stallcup, 1984), but not in human leukocytes from leukaemia patients (Homo et al., 1980). Although conflicting results do exist in the literature, most studies indicate that CR concentration is closely correlated with the magnitude of the response (Bamberger et al., 1996). Although cortisol receptors have been found in most tissues of fish, little is known of the relationship between cortisol receptor numbers and tissue responsiveness. In leukocytes from the anterior kidney of coho salmon, Maule et al. (1993) found a correlation between the *in vitro* immune response to cortisol and CR number. They did not find a similar relationship for splenic leukocytes, and speculated that other factors must be involved. In the present study, we have demonstrated a direct relationship between gill cytosolic CR B_{max} and *in vitro* responsiveness of the gill to cortisol.

The subcellular distribution of CRs in fish has not been investigated. In mammals, however, considerable controversy exists over the subcellular location of CRs. Recent studies have found the unoccupied CR to be located in the nucleus (Brink et al., 1992; Pekki et al., 1992) or the cytoplasm (Sackey et al., 1996). Using the protocol outlined above, gill CRs are found in the cytosolic fraction, possibly as a result of redistribution from the nucleus during tissue processing (Welshons and Jordan, 1987). We have been unable to quantify CRs in the nuclear fraction, as has been reported by Pottinger et al. (1994) and Knoebl et al. (1996). The CR concentration measured, therefore, consists of the unbound 'cytosolic' receptor population. Throughout this study, fish were anaesthetized and sampled in under 5 min to prevent an increase in plasma cortisol levels. By sampling rapidly and minimizing any potential increase in plasma cortisol levels, the number of CRs bound to cortisol remains low and the majority of CRs exist in the unbound 'cytosolic' receptor pool. The majority of the binding sites in liver and brain of rainbow trout have been reported to be cytosolic (Lee et al., 1992). Since the total population of CRs could not be measured, however, we do not know the extent to which the nuclear receptor population may have changed and affected the relationship between CR B_{max} and *in vitro* responsiveness to cortisol.

Responsiveness of gill Na^+/K^+-ATPase activity to cortisol *in vitro* changes seasonally and during development in salmonids. In coho salmon, the gills were unresponsive to cortisol in November, showed the highest responsiveness in January, and the response then declined until gill tissue was unresponsive in April, when *in vivo* gill Na^+/K^+-ATPase activity peaked (McCormick et al., 1991a). In separate studies on coho salmon, gill CR levels were low in November, highest in the early spring (Shrimpton, 1996) and then declined coincident with the peak in Na^+/K^+-ATPase activity (Shrimpton et al., 1994). In Atlantic salmon, McCormick et al. (1991a) reported that presmolts responded to cortisol *in vitro*, whereas smolts were unresponsive. Shrimpton and McCormick (1998a) found that CR B_{max} in Atlantic salmon was significantly greater in presmolts than in smolts, further supporting a relationship between CR B_{max} and response to cortisol. Another line of evidence within the literature that supports a relationship between gill CR concentration and gill responsiveness to cortisol was provided by Shrimpton et al. (1994). They found that wild coho salmon exhibited a higher gill CR B_{max} and a greater increase in plasma cortisol level, which corresponded to a significantly greater increase in gill Na^+/K^+-ATPase activity and the development of seawater tolerance, compared with their hatchery-reared counterparts.

Corticosteroid receptor B_{max} was three times greater in experiment 1 (August, Fig. 3) that in experiment 2 (October, Fig. 5). Seasonal changes in B_{max} of this magnitude have been observed in the gills of Atlantic salmon (Shrimpton and McCormick, 1998a) and coho salmon (Shrimpton et al., 1994; Shrimpton, 1996). Seasonal changes of smaller magnitude have also been found in the gills of hybrid rainbow/steelhead trout, but no significant seasonal differences were seen in steelhead trout (McLeese et al., 1994). It is not known whether changes of this magnitude are characteristic of this time of the year because most of the work examining seasonal changes in CRs has focused on the parr–smolt transformation during the spring. Shrimpton and McCormick (1998a) sampled Atlantic

salmon in early October and found little change in B_{max} until December. K_d changes seasonally in association with changes in B_{max} in most species examined (Shrimpton and McCormick, 1998a; Shrimpton, 1996). If B_{max} differences were associated with seasonal patterns of development, K_d might also be expected to differ. Values for K_d in the two experiments are very similar (0.484±0.015 and 0.476±0.015 nmol l^{-1} for experiment 1 and experiment 2, respectively), suggesting that seasonal differences may be small and that some other factor may account for the differences observed in B_{max}.

Temperature may have directly influenced CR B_{max} because it was approximately 19.6 °C for experiment 1 and 13.8 °C for experiment 2. The temperature of the water during experiment 1 was above the optimum temperature for growth in rainbow trout, but well below the thermal maximum for this species (Bidgood and Berst 1969), and the fish exhibited good feeding performance and growth. Since CR B_{max} was higher in fish from experiment 1 than from experiment 2, it is unlikely that the warmer temperature was stressful for the fish because CR B_{max} is downregulated by stress (Shrimpton and Randall, 1994; Pottinger et al., 1994; this study). In Atlantic salmon, we found that the seasonal decline in CR B_{max} occurred earlier in fish where the spring increase in water temperature was advanced. This response was independent of photoperiod (J. M. Shrimpton and S. D. McCormick, unpublished data). Further investigations are required to determine how temperature affects the seasonal cycles of CR B_{max} in rainbow trout.

Endocrine factors are known to alter the abundance of CRs. Cortisol and stress have been shown to decrease CR numbers in coho salmon gills (Maule and Schreck, 1991; Shrimpton and Randall, 1994) and rainbow trout liver (Pottinger et al., 1994). In the present study, we confirmed this finding for gill cytosolic CRs in rainbow trout. The reduction in CR number may be due to a decrease in the rate of production of CRs, because CR mRNA levels decline in response to dexamethasone treatment (Kalinyak et al., 1987). It is also possible that the rate of breakdown of CRs may change with stress or cortisol treatment because the degradation rate of CRs has been shown to increase following treatment with the steroid agonist TA (McIntyre and Samuels, 1985). Both these mechanisms may contribute to the downregulation of CRs in the gills of rainbow trout following repeated stress.

Increases in gill CR numbers have been found following GH treatment in coho salmon (Shrimpton et al., 1995) and Atlantic salmon (Shrimpton and McCormick, 1998b). The increase in CR B_{max} induced by GH has been proposed as a mechanism for the interaction between GH and cortisol that has been found in sea trout *Salmo trutta* (Madsen, 1990b), Atlantic salmon (McCormick, 1996) and rainbow trout (Madsen, 1990a). GH treatment in the present study on rainbow trout, however, was without effect on gill CR B_{max} and affinity. The absence of an effect of GH on CR in rainbow trout brings into question the proposed mechanism for interaction between GH and cortisol for this species. GH-induced increases in gill Na^+/K^+-ATPase activity may work directly through interaction with the GH receptor because GH has been shown to bind to membrane receptors in the liver, gill and kidney (Fryer and Bern, 1979; Sakamoto and Hirano, 1991). The increase in hypo-osmoregulatory ability due to GH may also be associated with the production of insulin-like growth factor-I (IGF-I). McCormick et al. (1991b) showed levels IGF-I to increase hypo-osmoregulatory ability significantly in rainbow trout. Whether the interaction between GH and cortisol may be mediated by IGF-I is not known, but McCormick (1996) showed that IGF-I and cortisol are additive in increasing gill Na^+/K^+-ATPase activity in Atlantic salmon.

The upregulation of gill CR B_{max} by T_3 has not been demonstrated previously. Shrimpton and McCormick (1998b) found that T_3 had an effect on B_{max} in Atlantic salmon, but that the increase was significant only when given in combination with GH. Endocrine factors modulating CR B_{max} thus appear to differ between species of salmonids. We do not know the mechanism by which T_3 increases gill CR B_{max}. In rat pituitary cells, T_3 treatment caused a significant increase in levels of CR mRNA (Williams et al., 1991).

The increase in CR B_{max} following T_3 treatment suggests that an interaction between T_3 and cortisol exists. Evidence for an interaction between thyroid hormones and cortisol in fish is limited. Thyroxine (T_4) has been shown to enhance the effect of cortisol on stimulating gill Na^+/K^+-ATPase activity in tilapia *Oreochromis mossambicus* (Dangé, 1986). In rainbow trout, Madsen (1990c) found that treatment with T_4 and cortisol for 1 week resulted in a significant increase in gill Na^+/K^+-ATPase activity compared with controls, whereas either hormone alone did not stimulate gill Na^+/K^+-ATPase activity significantly. T_4 treatment, however, was without effect after 14 and 28 days and did not alter the stimulatory effect of cortisol. The results of the present study indicate that upregulation of cortisol receptors by thyroid hormones is one mechanism for the interaction between these hormones.

The effectiveness of hormone receptors in the regulation of gene transcription is also correlated with hormone binding affinity (Bamberger et al., 1996). Mutations of the glucocorticoid receptor are associated with decreased hormone binding affinity of the receptor and are associated with clinical syndromes of glucocorticoid hyposensitivity (Hurley et al., 1991). Although changes in affinity due to mutations of the CR are much greater than differences in K_d that occur seasonally, they indicate that K_d changes can have an effect on tissue sensitivity. McCormick and Bern (1989) found a hierarchy of *in vitro* responsiveness of coho salmon gill filaments to dexamethasone, cortisol, 11-deoxycortisol and cortisone. The affinity of gill CRs for these four steroids was identical to that found for brook trout *Salvelinus fontinalis* (Chakraborti et al., 1987) and American eel *Anguilla rostrata* (Sandor et al., 1984). The relative affinity of the steroid for gill CRs, therefore, appears to play an important role in tissue sensitivity. The differences in K_d in the present study were not significant, and the potential effects on cortisol responsiveness are small in comparison with the changes in B_{max} observed. Regression of B_{max} on cortisol responsiveness accounted for 61 % of the

variance in the data. Division of B_{max} by K_d, however, strengthened the relationship slightly to account for 66 % of the variance in the data. CR affinity changes have been observed seasonally in gills of coho salmon (Shrimpton et al., 1994; Shrimpton, 1996) and Atlantic salmon (Shrimpton and McCormick, 1998a) and in coho salmon leukocytes (Maule et al., 1993), but not in steelhead trout gills (McLeese et al., 1994). Cortisol has also been shown to decrease CR affinity in coho salmon gills (Maule and Schreck, 1991; Shrimpton and Randall, 1994) and rainbow trout liver (Pottinger et al., 1994). GH also caused a decrease in CR affinity in gills of Atlantic salmon parr (Shrimpton and McCormick, 1998b).

Other regulatory factors may also affect the responsiveness of the gills to cortisol. Intracellular metabolism of cortisol by 11β-hydroxysteroid dehydrogenase (11β-HSD) (Bamberger et al., 1996) and removal of cortisol from the cell by specific membrane transporters (Thompson, 1995) are both mechanisms that prevent the interaction between glucocorticoids and CRs and alter the sensitivity of the tissue. Variation around the regression line exists for all treatment groups (Fig. 7). The stress-treated group, however, showed *in vitro* Na^+/K^+-ATPase activity in response to cortisol that was lower than that for other fish with a similar CR B_{max} because they consistently fell below the regression line (Fig. 7). One of these other regulatory factors may account for the observed difference. It is known that the half-life of cortisol in the plasma decreases with stress in juvenile coho salmon (Redding et al., 1984). We do not know whether the intracellular metabolism of cortisol in the gills was increased in the stress-treated group, but this could account for the difference seen in the stress-treated fish.

The present study demonstrates a strong correlation between CR concentration and *in vitro* responsiveness to cortisol in the gills of rainbow trout. Stress decreases, and T_3 treatment increases, CR abundance, resulting in significant differences in gill responsiveness to cortisol. The seasonal changes in CR abundance that have been observed during the parr–smolt transformation in several species of salmonids alter the response of the gill to cortisol. This finding supports the role of cortisol as an important endocrine factor in stimulating smolting and seawater tolerance in salmonids.

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