General and Comparative Endocrinology 124, 134–143 (2001)
doi:10.1006/gcen.2001.7703, available online at http://www.idealibrary.com on IDEAL®

Effects of Growth Hormone and Cortisol on Na^+–K^+–$2Cl^-$ Cotransporter Localization and Abundance in the Gills of Atlantic Salmon

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Accepted July 25, 2001

The hormones responsible for the regulation of the teleostean gill Na^+–K^+–$2Cl^-$ cotransporter have not been elucidated. With Western blotting and immunocytochemistry, Na^+–K^+–$2Cl^-$ cotransporter abundance and localization were examined in the gills of Atlantic salmon (*Salmo salar*) following 2-week treatment with growth hormone (GH; 5.0 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$), and both hormones in combination (GH+cortisol). GH and cortisol treatments increased gill Na^+–K^+–$2Cl^-$ cotransporter abundance over levels seen in controls, and both hormones together (GH+cortisol) produced a greater effect than either hormone alone. Gill Na^+,K^+–ATPase activity was also elevated by all three hormone treatments. Compared to controls, Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament were greater in number and size following all three treatments. Although the number of immunoreactive chloride cells on the secondary lamellae did not differ among the treatment groups, GH+cortisol increased their size. These data indicate that GH and cortisol increase gill Na^+–K^+–$2Cl^-$ cotransporter abundance through chloride cell proliferation and differentiation in the gills of Atlantic salmon and are likely the hormones responsible for Na^+–K^+–$2Cl^-$ cotransporter regulation during smolting and seawater acclimation.

Key Words: Salmo salar; growth hormone; cortisol; gill; chloride cell; Na^+,K^+–ATPase; osmoregulation.

INTRODUCTION

The osmoregulatory organs (gill, gut, and kidney) of euryhaline teleosts maintain ionic homeostasis and have different functions depending on the environmental salinity. The gill actively absorbs sodium and chloride in freshwater and secretes sodium and chloride in seawater. In anadromous salmonids, smolting occurs in freshwater and is a developmental period that is a preparatory adaptation for seawater entry. Numerous physiological changes occur in the gill during smolting and are responsible for greater seawater tolerance of smolts (McCormick and Saunders, 1987). Among these physiological changes is an increase in the enzyme Na^+,K^+–ATPase, which is required for ion secretion by the gill. Gill chloride cells, ion transporting cells that are rich in Na^+,K^+–ATPase, also increase in number and size during smolting (Boeuf, 1993).

The endocrine system modulates the osmoregulatory ability of teleost fish (Bern and Madsen, 1992). Whereas prolactin has been established as an important hormone for the maintenance of ionic homeostasis in freshwater, growth hormone (GH) and cortisol are recognized as the major seawater-adapting hormones (McCormick, 1995). Although many hormones are elevated in smolts, increased levels of plasma GH and cortisol occur at a time of heightened seawater tolerance (Young *et al.*, 1989). Treatment with exogenous (*in vivo*) GH and cortisol increase gill Na^+,K^+–ATPase activity and enhance osmoregulatory ability

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0016-6480/01 $35.00

following seawater transfer (see reviews by Barron, 1986; Bern and Madsen, 1992; Sakamoto *et al.,* 1993; McCormick, 1995). *In vivo* treatment with GH and cortisol has also been shown to stimulate the number and size of gill chloride cells (Madsen, 1990a).

In the current model of chloride cells from seawater-acclimated teleosts Na^+,K^+–ATPase and the Na^+–K^+–$2Cl^-$ cotransporter are located at the basolateral surface of the cell. Na^+,K^+–ATPase creates a sodium gradient that drives the transport of sodium, potassium, and two chloride ions into the cell via the Na^+–K^+–$2Cl^-$ cotransporter. Chloride ions are secreted into the external medium through apical Cl^--channels and sodium ions are transported back into the basal lamina via Na^+,K^+–ATPase. Sodium is transported down an electrochemical gradient and into the external medium via leaky paracellular junctions between adjacent cells. We have previously shown that the Na^+–K^+–$2Cl^-$ cotransporter is abundant in gill chloride cells of Atlantic salmon and is upregulated during seawater acclimation and smolting (Pelis *et al.,* 2001). Furthermore, changes in Na^+–K^+–$2Cl^-$ cotransporter abundance parallel changes in gill Na^+,K^+–ATPase activity, indicating coordinated roles for these proteins in salt secretion by the gill. However, the hormonal regulation of the Na^+–K^+–$2Cl^-$ cotransporter has not been studied in teleosts. We hypothesize that the Atlantic salmon gill Na^+–K^+–$2Cl^-$ cotransporter should exhibit similarities to Na^+,K^+–ATPase in its regulation by GH and cortisol. The objectives of this study were to monitor changes in the abundance and localization of the Na^+–K^+–$2Cl^-$ cotransporter within the gill epithelium of Atlantic salmon following *in vivo* treatment with GH alone, cortisol alone, and GH and cortisol in combination (GH+cortisol).

MATERIALS AND METHODS

Animals and Hormones

Juvenile Atlantic salmon (*Salmo salar*) parr were maintained under simulated natural photoperiod in a 1-m-diameter tank with flow-through freshwater held at 10 ± 0.5° (Connecticut River, MA) and fed to satiation daily with commercial feed (Zeigler Bros., Gardners, PA). Fish were acclimated to the aforementioned conditions 1 week prior to receiving a single injection. Treatment groups included GH (5 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$; hydrocortisone; Sigma), and GH and cortisol in combination (GH, 5 $\mu g \cdot g^{-1}$ + cortisol, 50 $\mu g \cdot g^{-1}$), with eight fish allocated to each treatment group. Ovine GH (oGH-15) was a generous gift from NIDDK's National Hormone and Pituitary Program and A. F. Parlow. Hormones were suspended in a 1:1 mixture of vegetable shortening (Crisco) and peanut oil, and injections were administered intraperitoneally at 5 $\mu l \cdot g^{-1}$ of body weight (McCormick, 1996). The control group ($n = 8$) received the vehicle only (1:1 mixture). Fish in each treatment were given an identifying paint mark on the anal fin. Fish from all treatments were kept in the same tank, and rearing conditions were kept the same following injections, except for the withholding of food. All fish were sampled on November 26, 1999, 2 weeks following injection.

Sampling

At the time of sampling all fish were anesthetized with 100 mg/L MS-222 (pH 7.0, 12 mM $NaHCO_3$) and weighed to the nearest 0.1 g, and fork lengths were recorded. To measure gill Na^+,K^+–ATPase activity, gill biopsies (five to six gill filaments) were placed in 100 μl of ice-cold SEI (250 mM sucrose, 10 mM Na_2EDTA, and 50 mM imidazole) and stored at −80°. A single gill arch from each fish was removed, plunged into fixative (80% absolute methanol:20% dimethyl sulfoxide at −20°), and stored at −20° for later use in immunocytochemistry. The remaining gill tissue was removed, snap-frozen on dry ice, and stored at −80°.

Gill Na^+,K^+–ATPase Activity Measurements

Gill Na^+,K^+–ATPase activity was measured by the method of McCormick (1993). Gill tissue taken from biopsies was thawed immediately prior to assay and homogenized in 125 μl of 0.1% sodium deoxycholate in SEI buffer for 10–15 s. The resulting homogenate was centrifuged at 5000*g* for 30 s and the supernatant retained and assayed for Na^+,K^+–ATPase activity. Each sample of gill homogenate was plated in quadruplicates of 10 μl; two contained 0.5 mM ouabain and two did not. Fifty microliters of salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM $MgCl_2 \times 6H_20$

and 42 mM KCl) and 150 μl of assay mixture (50 mM imidazole, 2 mM phosphoenolpyruvate, 0.16 mM nicotinamide adenine dinucleotide, 0.5 mM adenosine triphosphate, 3.3 U/ml lactic dehydrogenase, and 3.6 U/ml pyruvate kinase) were added to each well. The kinetic assay was read at a wavelength of 340 nm at 25° with a run time of 10 min and intervals of 10 s. Na^+,K^+–ATPase in this assay system is linear between 5 and 25°, and 25° was chosen because it is within the thermal limit of Atlantic salmon and most teleosts and provides higher sensitivity than use of lower temperatures (McCormick and Bern, 1989). Protein concentration of the gill homogenate was determined with the bicinchoninic acid method (BCA Protein Kit; Pierce, Rockford, IL). The difference between the kinetic reading with and without ouabain is the Na^+,K^+–ATPase activity and is expressed as micromoles ADP per milligram protein per hour.

SDS–PAGE and Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were used to quantify the amounts of Na^+–K^+–$2Cl^-$ cotransporter present in the gill. Frozen gill tissue was thawed, rinsed in ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 adjusted to pH 7.3), and blotted on a paper towel. Gill epithelia were cut away from the arches and placed in 10 volumes of ice-cold homogenization buffer (2 mM EDTA and 30% sucrose w/v in PBS) along with the following protease inhibitors: 0.2 mM [4-(2-aminoethyl)benzenesulfonylfluoride, HCl] (AEBSF), 100 μM *N*-tosyl phenylalanine chloromethyl ketone, 1 μM pepstatin A, 10 μM chymostatin, 10 μM leupeptin, and 50 μM *o*-phenanthroline. Gill tissue was homogenized at low speed with a tissue homogenizer (Tekmar; SDT-182EN fitted with a saw tooth generator) and centrifuged at 5000*g* for 10 min at 4°. The resulting supernatant was centrifuged at 20,000*g* for 10 min, and the pellet was removed to discard mitochondria and cellular debris. The supernatant was centrifuged at 48,000*g* for 2 h at 4°. The final pellet was resuspended in homogenization buffer and total protein was determined with the BCA protein assay.

Membranes were placed in Laemmli sample buffer (250 mM Tris base, 10% glycerol w/v, 2% SDS w/v, 1% beta mercaptoethanol w/v, 0.05% bromophenyl blue w/v in deionized water adjusted to pH 6.8) and heated to 60° for 15 min. Membranes were loaded on 7% SDS–PAGE gels at 50 μg of protein per lane. Gels were run overnight followed by transfer to immobilon P (PVDF) transfer membranes (Millipore, Bedford, MA). PVDF membranes were immersed for 1.5 h in blocking buffer (7.5% nonfat dry milk and 0.1% Tween 20 w/v in PBS) at room temperature and incubated overnight at 4° in T4–Na^+–K^+–$2Cl^-$ cotransporter primary antibody diluted 600 μg/ml in blocking buffer. The T4–Na^+–K^+–$2Cl^-$ cotransporter antibody, developed by Dr. Christian Lytle and Dr. Bliss Forbush III, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa. The T4–Na^+–K^+–$2Cl^-$ cotransporter antibody was produced against 310 amino acids within a highly conserved region in the carboxy terminus of the human colonic secretory Na^+–K^+–$2Cl^-$ cotransporter. This antibody has been used to identify the Na^+–K^+–$2Cl^-$ cotransporter within a wide variety of species including mammals and fish (Lytle *et al.,* 1995; Pelis *et al.,* 2001). PVDF membranes were washed five times in blocking buffer followed by a 2-h incubation at room temperature in peroxidase-labeled goat anti-mouse IgG, H + L (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 2 μg/ml in blocking buffer. PVDF membranes were washed four times in blocking buffer, once in 0.1% Tween 20 (w/v) in PBS, and once in deionized water. Immunoreactivity was visualized with DAB buffer (1.38 mM 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.0084 mM $CuCl_2$, and 0.001% H_2O_2, w/v in PBS).

Digital photographs were taken immediately following incubation with DAB. Band staining intensity was measured from the digital photographs with Image calc (C. H. A. van de Lest, Dutch Asthma Foundation). To obtain staining intensity, an entire lane on a Western blot is selected, and Image calc scans the selected part of the image from top to bottom, averaging the 8-bit gray scale values that are located on each horizontal line. The average 8-bit gray scale values on each horizontal line are then summed to obtain cumulative 8-bit gray scale values for each particular band. To standardize for differences in background intensity between Western blots, the background 8-bit gray scale value was subtracted from each horizontal

line average 8-bit gray scale value. Na^+–K^+–$2Cl^-$ cotransporter abundance, as measured by staining intensity, is recorded as cumulative 8-bit gray scale.

Immunocytochemistry

Immunocytochemical procedures were modified from Ginns *et al.* (1996). Following fixation (80% absolute methanol:20% dimethyl sulfoxide) at −20°, tissue was placed on ice and allowed to warm before being placed in Ringer's at 4°. The tissue was then equilibrated in cryoprotectant (30% sucrose w/v in Ringer's) before being embedded in Histo Prep Embedding Media (Fisher Scientific, PA). Tissue was frozen (−25°), cryosectioned at 10 μm, placed on warm poly-l-lysine subbed slides, and washed twice with high-salt Ringer's (360 mM NaCl and 1% BSA w/v in Ringer's). Sections were washed three times in glycine wash (50 mM glycine and 1% BSA w/v in Ringer's) and incubated overnight in T4 primary antibody at 4° diluted 300 μg/ml in antibody dilution (0.1% NaN_2 and 1% BSA w/v in Ringer's). After incubation with primary antibody, tissue was washed five times in high-salt Ringer's and incubated for 2 h at 4° in Cy 3-labeled goat anti-mouse IgG, H + L (Kirkegaard & Perry Laboratories) diluted 2 μg/ml in antibody dilution. Tissue was washed twice in Ringer's prior to the viewing. All washes were 10 min in length. Controls omitting theT4 primary antibody were performed and yielded no immunoreactivity.

Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament and secondary lamellae were tallied separately. From each fish, immunoreactive chloride cells were counted from at least seven sagittal sections of gill filament and expressed per millimeter of primary filament. Mean numbers of immunoreactive chloride cells on the primary filament and secondary lamellae for each group were obtained with the means calculated from each fish. Due to the uneven distribution of chloride cells in the gill, an entire piece of gill arch was sectioned and images were taken from randomly selected sagittal sections. Cell area (μm^2/cell) and shape factor were also obtained from immunoreactive chloride cells. Shape factor is defined as $4\pi A/P^2$ (with A and P being area and perimeter, respectively), with values close to one indicating a round shape and less than one a more elongate shape. The use of different filters in the acquisition of digital images of immunoreactive chloride cells prevented staining intensities from being analyzed. Fifty primary and 50 secondary immunoreactive chloride cells were analyzed from a minimum of five sagittal sections of gill filament from each fish. For vehicles, fewer immunoreactive cells (≥30 immunoreactive cells) were used for analysis due to fewer immunoreactive cells. Means for each group were calculated from the means from individual fish. Cell number, size, and shape factor were obtained with MetaMorph 4.1.2 (Universal Imaging Corp. 1992–2000).

Data Analysis

Two-way analysis of variance on ranks was used to test the significance of hormones and their interaction. Tukey's HSD test on ranks was used for pairwise comparisons of individual treatments. All statistical analyses were deemed significant at the $\alpha = 0.05$ level and conducted with Sigma Stat 2.0 (Jandel Corp.).

RESULTS

Membrane preparations of Atlantic salmon gill tissue produced three broadly stained bands centered at 285, 160, and 120 kDa on Western blots probed with the T4–Na^+–K^+–$2Cl^-$ cotransporter antibody (Fig. 1). Compared to the control group, GH and cortisol treatments alone increased gill Na^+–K^+–$2Cl^-$ cotransporter abundance 6- and 13-fold, respectively ($P < 0.05$; Tukey's HSD; Fig. 2). Fish treated with cortisol had significantly greater amounts of gill Na^+–K^+–$2Cl^-$ cotransporter than fish treated with GH ($P < 0.05$; Tukey's HSD). GH+ cortisol had a greater effect than either hormone alone ($P < 0.05$; Tukey's HSD) and resulted in Na^+–K^+–$2Cl^-$ cotransporter levels that were 18 times higher than those in the control group. There was no significant interaction of GH and cortisol on Na^+–K^+–$2Cl^-$ cotransporter abundance within the gill ($P > 0.05$; two-way ANOVA).

Mean gill Na^+,K^+–ATPase activity of the control group was 2.5 μmol ADP · mg protein^{-1} · h^{-1}, and GH and cortisol treatments alone significantly increased Na^+,K^+–ATPase activity within the gill ($P < 0.05$; Tukey's HSD; Fig. 2). GH treatment increased gill

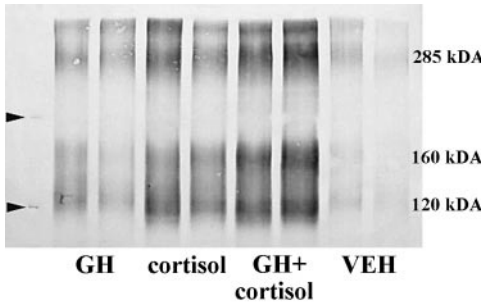

FIG. 1. Western blot of the gill Na^+–K^+–$2Cl^-$ cotransporter from Atlantic salmon parr treated with growth hormone (GH; 5 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$), and both hormones together (GH+cortisol) for 2 weeks. The control group (VEH) was injected with vehicle only. Each treatment has two representative lanes. Immunoreactive bands occur at 285, 160, and 120 kDA. Standards are marked (▶) at 205 and 116 kDA.

Na^+,K^+–ATPase activity 48%, whereas cortisol treatment resulted in a 2.5-fold increase. Mean gill Na^+,K^+–ATPase activity among the GH+cortisol-treated group was 7.2 μmol ADP · mg protein^{-1} · h^{-1} and approximately 3-fold higher than levels found in the control group. Gill Na^+,K^+–ATPase activity from GH-treated fish was 38% less than that in the cortisol-treated fish ($P < 0.05$; Tukey's HSD). Gill Na^+,K^+–ATPase activity in the GH+cortisol-treated group was significantly higher (49%) than that in the GH-treated group ($P < 0.05$; Tukey's HSD), but not different from fish treated with cortisol. There was no significant interaction of GH and cortisol on gill Na^+,K^+–ATPase activity ($P > 0.05$; two-way ANOVA).

Based on their size and location, Na^+–K^+–$2Cl^-$ cotransporter immunoreactivity occurred in chloride cells, and immunoreactivity within the gill differed among the treatment groups (Fig. 3). Relative to controls, GH and cortisol treatments alone increased the number of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament fourfold and sevenfold, respectively ($P < 0.05$; Tukey's HSD; Fig. 4). The number of immunoreactive chloride cells on the primary filament of fish treated with cortisol was 44% greater than that of fish treated with GH ($P < 0.05$; Tukey's HSD). The number of immunoreactive chloride cells on the primary filament in GH+cortisol-treated fish was twofold greater than that in the GH-treated fish ($P < 0.05$; Tukey's HSD), but not different from the fish treated with cortisol. There was no interaction of GH and cortisol treatment on the number of immunoreactive chloride cells on the primary filament ($P > 0.05$; two-way ANOVA). GH and cortisol did not have an effect on the number of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the secondary lamellae ($P > 0.05$; two-way ANOVA).

Relative to the control group, GH (43%), cortisol (72%), and GH+cortisol (131%) treatments increased the size of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament ($P < 0.05$; Tukey's HSD; Table 1). The mean size of immunoreactive chloride cells on the primary filament of cortisol- and GH+cortisol-treated fish was significantly greater than that of fish treated with GH alone ($P < 0.05$; Tukey's HSD), but were not different from one another. Only cortisol had a significant effect on the size of immunoreactive chloride cells on the secondary lamellae ($P < 0.05$; two-way ANOVA). GH+cortisol treatment increased the size of immunoreactive chloride cells on the secondary lamellae 30% over that of controls ($P < 0.05$; Tukey's HSD). Cortisol treatment alone did not increase the size of immunoreactive chloride cells on the secondary lamellae over controls or fish treated with GH ($P > 0.05$; Tukey's HSD). There was no significant interaction of GH and cortisol treatments on immunoreactive chloride cell (primary

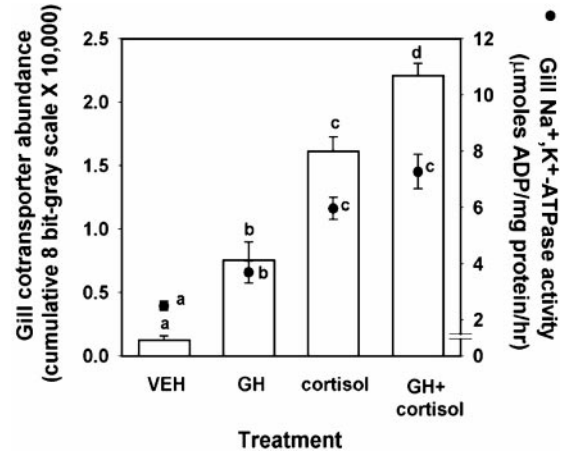

FIG. 2. Effects of growth hormone (GH; 5 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$), and both hormones together (GH+cortisol) on gill Na^+–K^+–$2Cl^-$ cotransporter abundance (□) and Na^+,K^+–ATPase activity (●) in the gills of Atlantic salmon parr. Values are means + standard errors for Na^+–K^+–$2Cl^-$ cotransporter abundance and ± standard errors for Na^+,K^+–ATPase activity. Sample size was eight fish per group. Different letters represent significant differences among treatment groups ($P < 0.05$; Tukey's HSD).

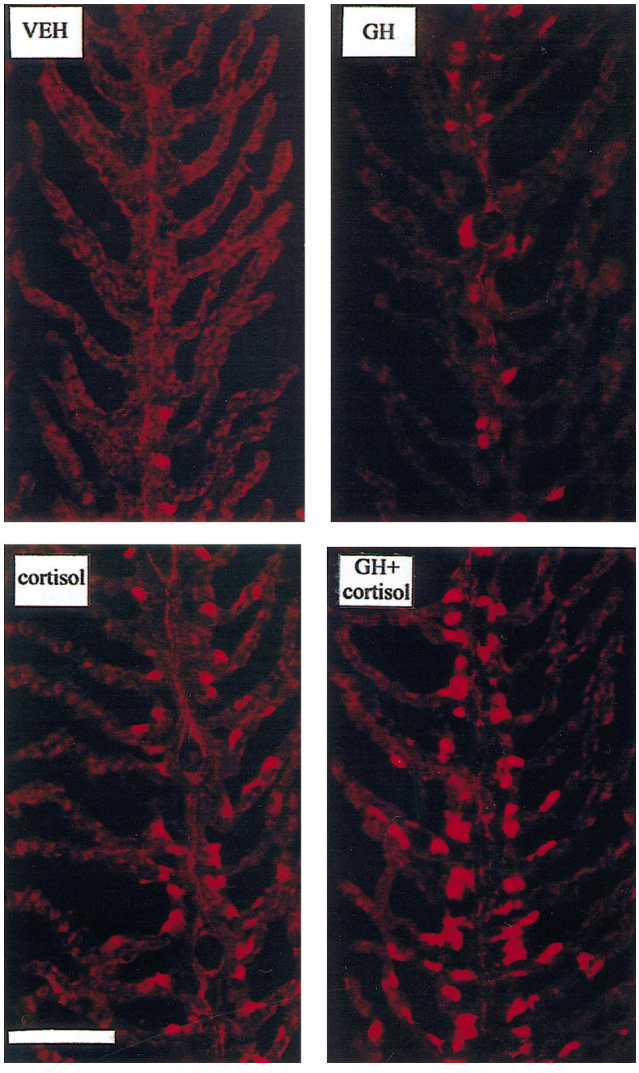

FIG. 3. Immunocytochemical staining of the Na^+–K^+–$2Cl^-$ cotransporter in the gills of Atlantic salmon parr treated with growth hormone (GH; 5 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$), and both hormones together (GH+cortisol) for 2 weeks. The control group (VEH) was injected with vehicle only. Scale bar, 70 μm.

filament and secondary lamellae) size ($P > 0.05$; two-way ANOVA).

Only cortisol had an effect on the shape of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament ($P < 0.05$; two-way ANOVA; Table 1). Immunoreactive chloride cells on the primary filament were more round (larger shape factor) in cortisol and GH+cortisol-treated groups than in controls ($P < 0.05$; Tukey's HSD). GH and cortisol did not have an effect on the shape of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the secondary lamellae ($P > 0.5$; two-way ANOVA).

DISCUSSION

This study provides evidence for the regulation of the teleostean gill Na^+–K^+–$2Cl^-$ cotransporter by GH and cortisol. GH and cortisol treatments each increased Na^+–K^+–$2Cl^-$ cotransporter abundance and the number and size of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament in the gills of Atlantic salmon. Na^+–K^+–$2Cl^-$ cotransporter immunoreactivity occurred exclusively in gill chloride cells (Fig. 3). Increased Na^+–K^+–$2Cl^-$ cotransporter abundance, therefore, is likely the result of chloride cell proliferation and differentiation. The connection between the increased Na^+–K^+–$2Cl^-$ cotransporter abundance and the number and size of immunoreactive chloride cells on the primary filament is apparent when the magnitudes of increase for immunoreactive chloride cell number and size following GH+cortisol treatment are multiplied. Immunoreactive chloride cell (primary filament) number increased 8.1-fold and size increased 2.1-fold, and the product of these suggests a 17-fold increase in Na^+–K^+–$2Cl^-$ co-

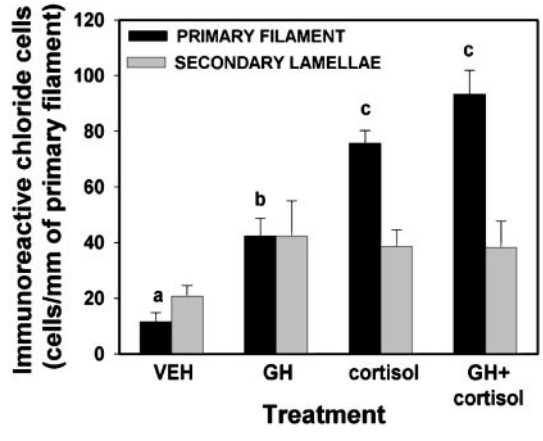

FIG. 4. Effects of growth hormone (GH; 5 $\mu g \cdot g^{-1}$), cortisol (50 $\mu g \cdot g^{-1}$), and both hormones together (GH+cortisol) on the number of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the primary filament and secondary lamellae. Values are means + standard errors ($n = 4$ per group). Different letters represent significant differences among treatment groups ($P < 0.05$; Tukey's HSD).

TABLE 1

Effects of GH (5 $\mu g \cdot g^{-1}$), Cortisol (50 $\mu g \cdot g^{-1}$), and Both Hormones in Combination (GH+Cortisol) on the Size and Shape of Na^+–K^+–$2Cl^-$ Cotransporter Immunoreactive Chloride Cells on the Primary Filament (1° IRCC's) and Secondary Lamellae (2° IRCC's)

	Cell area (μm^2)		Cell shape	
Treatment	1° IRCC's	2° IRCC's	1° IRCC's	2° IRCC's
VEH	40.4 ± 1.3	37.5 ± 2.5	0.526 ± 0.012	0.382 ± 0.02
GH	57.4 ± 3.2*	42.1 ± 1.5	0.541 ± 0.006	0.413 ± 0.01
Cortisol	69.7 ± 2.8*	44.8 ± 3.0	0.566 ± 0.008*	0.398 ± 0.005
GH+cortisol	86.5 ± 5.4*	54.7 ± 6.7*	0.591 ± 0.009*	0.438 ± 0.018

Note. Values are means ± standard errors ($n = 4$ per group). Two-way ANOVA indicated significant effects of GH and cortisol treatments on the size of 1° IRCC's ($P < 0.05$). Cortisol treatment had a significant effect on 2° IRCC size and 1° IRCC shape ($P < 0.05$; two-way ANOVA). There was no significant interaction between GH and cortisol for any of the parameters examined ($P > 0.05$; two-way ANOVA).

* Significantly different from the control group (VEH; $P < 0.05$; Tukey's HSD).

transporter abundance, which closely approximates the 18-fold increase that was observed on Western blots. Chloride cells are three-dimensional, however, and the 17-fold increase that was calculated requires that cell area and volume are changing relative to each other. Similarities were also apparent for the product of immuoreactive chloride cell number and size and for total Na^+–K^+–$2Cl^-$ cotransporter abundance (Western blots) following GH and cortisol treatments alone (GH, 5.3-fold vs 6.1-fold; cortisol, 11.3-fold vs 13-fold). We have previously shown that in Atlantic salmon the gill Na^+–K^+–$2Cl^-$ cotransporter increases in abundance during seawater acclimation and smolting (Pelis *et al.,* 2001). As indicated above, previous research has implicated GH and cortisol as important for seawater acclimation and smolting (see reviews by Hoar, 1988; McCormick, 1995). Previous work with similar hormone treatments in Atlantic salmon demonstrated that GH and cortisol increased whole-animal hypoosmoregulatory performance (McCormick, 1996). The ability of GH and cortisol to increase gill Na^+–K^+–$2Cl^-$ cotransporter abundance and the number and size of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells in Atlantic salmon further implicates GH and cortisol as important seawater-adapting hormones.

In addition to gill Na^+–K^+–$2Cl^-$ cotransporter abundance, Na^+,K^+–ATPase activity was elevated following treatment with GH and cortisol. The stimulatory effect of cortisol on gill Na^+,K^+–ATPase activity was first demonstrated in killifish, *Fundulus heteroclitus* (Pickford *et al.,* 1970), and has since been shown to have the same effect in eels, *Anguilla japonica* (Kamiya, 1972) and *A. rostrata* (Epstein *et al.,* 1971), and numerous salmonids (Richman and Zaugg, 1987; Madsen, 1990a, b, c; McCormick, 1995). *In vivo* treatment with GH was found to increase gill Na^+,K^+–ATPase activity in salmonids (Madsen and Bern, 1993; Almendras *et al.,* 1993), tilapia, *Oreochromis mossambicus* (Borski *et al.,* 1994), and killifish, *F. heteroclitus* (Mancera and McCormick, 1998, 1999). In Atlantic salmon, GH and cortisol treatments increase both Na^+,K^+–ATPase activity and salinity tolerance (McCormick, 1996). In the seawater chloride cell, Na^+,K^+–ATPase creates a sodium gradient which drives Na^+–K^+–$2Cl^-$ cotransport. The increased abundance of both proteins, therefore, is not surprising, given their close relationship in the function of salt secretion by the gill.

GH treatment alone increased gill Na^+–K^+–$2Cl^-$ cotransporter abundance 6-fold, and it increased Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cell (primary filament) number and size 3.7- and 1.5-fold, respectively. The ability of GH to increase Na^+–K^+–$2Cl^-$ cotransporter abundance likely occurs through the stimulation of chloride cell proliferation and differentiation. Treatment with GH has been shown to increase the density of chloride cells on the primary filament of rainbow trout, *Oncorhynchus mykiss* (Madsen, 1990c), and the density and size of chloride cells on the primary filament of brown trout, *Salmo trutta* (Madsen, 1990a). Using bromo-deoxyuridine, a mitotic cell label, Laurent *et al.* (1994) were able to show that GH treatment *in vivo* increases the rate of chloride cell division in the gills of rainbow trout. In tilapia (*Oreochromis niloticus*), GH treatment *in vivo* causes gill chloride cells to develop an extensive tubular system typical of gill chloride cells of seawater-acclimated teleosts (Xu *et al.,* 1997). GH, however, may not act

alone to increase Na^+–K^+–$2Cl^-$ cotransporter abundance and the number and size of immunoreactive chloride cells but may rely on its regulation of and interaction with insulin-like growth factor I (IGF-I). In teleosts, IGF-I is present in many tissues including the gill, liver, kidney, pancreas, and brain (Reinecke *et al.*, 1997). In the gills of salmonids, IGF-I mRNA levels increase during smolting (Sakamoto *et al.*, 1995) and following seawater exposure and *in vivo* treatment with GH (Sakamoto and Hirano, 1993). Treatment of coho salmon (*Oncorhynchus kisutch*) with GH (*in vivo*) followed by *in vitro* treatment of their gills with IGF-I stimulates Na^+,K^+–ATPase activity (Madsen and Bern, 1993). *In vivo* treatment with IGF-I alone does not increase gill Na^+,K^+–ATPase activity in Atlantic salmon (McCormick, 1996), but has a stimulatory effect on gill Na^+,K^+–ATPase activity and the number of Na^+,K^+–ATPase immunoreactive chloride cells on the primary filament of brown trout (Seidelin *et al.*, 1999). Establishment of a direct role for GH in regulating the gill Na^+–K^+–$2Cl^-$ cotransporter would require experiments examining the *in vitro* effects of GH and IGF-I on the Na^+–K^+–$2Cl^-$ cotransporter.

Na^+–K^+–$2Cl^-$ cotransporter abundance (13-fold) and the number (6.8-fold) and size (1.75-fold) of immunoreactive chloride cells on the primary filament increased following cortisol treatment by itself. Although this study indicates that cortisol causes the proliferation and differentiation of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells, other studies looking at the effects of cortisol on chloride cell development have produced contrasting results. The number and size of chloride cells on the primary filament of brown trout (Madsen, 1990a) and rainbow trout (Laurent and Perry, 1990) increase following *in vivo* treatment with cortisol. In contrast, treatment of the yolk-sac membrane of tilapia (*O. mossambicus*) with cortisol was found to increase chloride cell number without changing their size (Ayson *et al.*, 1995). These conflicting results may indicate that the responsiveness of chloride cells to cortisol is species and (or) tissue specific. Within the opercular membrane of tilapia, cortisol treatment (*in vivo*) increases the density of chloride cells (Foskett *et al.*, 1981) but when administered *in vitro* only increases chloride cell size (McCormick, 1990), perhaps suggesting that, *in vivo*, some other factor is working with cortisol to cause the proliferation of chloride cells.

GH and cortisol together (GH+cortisol) increased gill Na^+–K^+–$2Cl^-$ cotransporter abundance 18-fold and the number (8.1-fold) and size (2.1-fold) of immunoreactive chloride cells on the primary filament. Although there was no significant interaction of GH and cortisol, both hormones together (GH+cortisol) had a greater effect than either treatment by itself. The additive effect of GH and cortisol on Na^+–K^+–$2Cl^-$ cotransporter abundance suggests that both hormones may be acting through different mechanisms to increase gill Na^+–K^+–$2Cl^-$ cotransporter abundance. The extracellular binding of GH and intracellular binding of cortisol to their respective receptors may activate different pathways for increased Na^+–K^+–$2Cl^-$ cotransporter transcription and/or chloride cell proliferation and differentiation. GH, however, may play a role in mediating some of the actions of cortisol. Gill corticosteroid receptors increase in number following *in vivo* GH treatment of coho salmon (Shrimpton *et al.*, 1995) and Atlantic salmon (Shrimpton and McCormick, 1998). Furthermore, *in vivo* and *in vitro* treatment of the coho salmon interrenal gland with GH has been shown to increase the gland's responsiveness to adrenocorticotropin hormone (Young, 1988). GH and cortisol in combination have been found to have a synergistic effect on gill Na^+,K^+–ATPase activity in Atlantic salmon (McCormick, 1996) and brown trout (Madsen, 1990a). GH+cortisol treatment in this study, however, acted additively on gill Na^+, K^+–ATPase activity and Na^+–K^+–$2Cl^-$ cotransporter abundance. Differences in the responsiveness of the gill to cortisol change during salmonid development (McCormick *et al.*, 1991) and may explain why GH+cortisol treatment did not have a synergistic effect on gill Na^+,K^+–ATPase activity in the current study.

Chloride cells on the secondary lamellae increase in number during exposure to ion-poor water (Avella *et al.*, 1987), suggesting an important function for these cells in ion absorption. The Na^+–K^+–$2Cl^-$ cotransporter has been hypothesized only to be in seawater chloride cells and involved in ion secretion. We have shown, however, that Na^+–K^+–$2Cl^-$ cotransporter immunoreactivity occurs in chloride cells along the secondary lamellae and that the size of these cells increases during smolting and seawater acclimation, providing evidence against the presence of two cell types (Pelis *et al.*, 2001). Hiroi *et al.* (1999) demon-

strated that individual chloride cells increase in size following freshwater to seawater transfer, suggesting that the same cell may have the capacity to change its function from ion absorption to ion secretion. GH and cortisol did not have an effect on the number or size of Na^+–K^+–$2Cl^-$ cotransporter immunoreactive chloride cells on the secondary lamellae. The sizes of immuoreactive chloride cells on the secondary lamellae, however, were elevated when the two hormones were administered together. GH+cortisol, which has seawater-adapting effects, may increase the size of chloride cells on the secondary lamellae in preparation for seawater exposure. GH+cortisol could also have a freshwater-adaptive effect on chloride cell development and may be an alternate reason that immunoreactive chloride cells on the secondary lamellae increase in size. Laurent and Perry (1990) have shown that cortisol treatment of rainbow trout increased the apical surface areas of chloride cells on the primary filament along with whole-body Na^+ and Cl^- influxes. These data suggest that cortisol promotes ion uptake in freshwater, and that chloride cells on the primary filament, which have been suggested to function in ion secretion only, may function in ion uptake also. Immunoreactive chloride cells among cortisol and GH+cortisol-treated fish were more round than controls, which may be a result of increased chloride cell proliferation and differentiation.

The current study provides evidence for the regulation of the gill Na^+–K^+–$2Cl^-$ cotransporter by GH and cortisol in Atlantic salmon. The increase of the gill Na^+–K^+–$2Cl^-$ cotransporter was likely caused by the proliferation and differentiation of chloride cells on the primary filament. The Na^+–K^+–$2Cl^-$ cotransporter increased in parallel with increases in gill Na^+,K^+–ATPase activity. Na^+–K^+–$2Cl^-$ cotransporter abundance and immunoreactive chloride cell number and size increase during the acclimation of Atlantic salmon parr to seawater (Pelis *et al.*, 2001). The ability of GH and cortisol to increase gill Na^+–K^+–$2Cl^-$ cotransporter abundance in freshwater Atlantic salmon parr provides further evidence that GH and cortisol regulate ion transporters in the gill that are important for seawater entry. Further work will be necessary to determine whether this control of the Na^+–K^+–$2Cl^-$ cotransporter by GH and cortisol is a common feature among euryhaline and anadromous teleosts.

ACKNOWLEDGMENTS

We thank Mike O'Dea, Joe Zydlewski, and Amy Moeckel for their assistance. Dr. J. Larry Renfro made many helpful comments in review of the manuscript. Ovine GH was obtained through NHPP, NIDDK, and Dr. A. F. Parlow.

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