Endocrine control of Na^+, K^+ -ATPase and chloride cell development in brown trout (*Salmo trutta*): interaction of insulin-like growth factor-I with prolactin and growth hormone

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Abstract

A 2-factorial (3×3) injection experiment was used to investigate the effect and interaction between different hormones on the initial phase of seawater (SW) acclimation in brown trout (*Salmo trutta*). Each fish was given 4 injections on alternate days in freshwater (FW). Factor 1 was either saline, $2 \mu\text{g}$ ovine prolactin (oPRL)/g, or $2 \mu\text{g}$ ovine growth hormone (oGH)/g. Factor 2 was either 0, 0.01, or $0.1 \mu\text{g}$ recombinant human insulin-like growth factor-I (rhIGF-I)/g. In each of the 9 treatment groups, half of the fish were subjected to a 48-h SW-challenge test, and the remaining fish were sham-transferred to FW one day after the last injection. Hypo-osmoregulatory performance was increased by GH and impaired by PRL treatment as judged by changes in plasma osmolality, $[\text{Na}^+]$, $[\text{Cl}^-]$, total $[\text{Mg}]$ and muscle water content (MWC) after SW transfer. IGF-I reduced plasma osmolality after transfer to SW but had no effect on plasma total $[\text{Mg}]$ or MWC. The effects of the two factors on plasma osmolality, $[\text{Na}^+]$, $[\text{Cl}^-]$, and MWC were additive. In sham-transferred fish, GH and IGF-I, alone and in combination, stimulated Na^+, K^+ -ATPase α -subunit mRNA (α -mRNA) content in the gill. This was paralleled by an overall increase in gill Na^+, K^+ -ATPase activity in fish treated with $0.01 \mu\text{g}$ IGF-I/g. Simultaneous administration of PRL completely inhibited the increase in gill

α -mRNA observed in the IGF-I-injected groups. Combination of GH and IGF-I did not further affect the α -mRNA level relative to the single hormone-injected groups. There was an overall decrease in Na^+, K^+ -ATPase activity in pyloric caeca and middle intestine by the low dose and both doses of IGF-I respectively. No effect was observed in the posterior intestine. PRL and GH treatments did not affect enzyme activity in any intestinal segment. Both doses of IGF-I increased Na^+, K^+ -ATPase-immunoreactive (NKIR) cell density in gill primary filaments. PRL and GH had no effect on primary filament NKIR cell density. GH and both doses of IGF-I reduced secondary lamellar NKIR cell density, whereas PRL had no effect.

The main conclusion is that IGF-I and GH induce an overall redistribution of NKIR cells away from the secondary lamella onto the primary filament of FW-acclimated trout. This is associated with an overall increased α -mRNA level in the gill, which may reflect an increased expression within individual NKIR cells in the primary filament. PRL completely abolished the IGF-I stimulation of α -mRNA levels, suggesting a desensitisation of the gill tissue to IGF-I, which may explain the overall anti-SW adaptive effect of PRL.

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Introduction

Teleosts are hypo-osmotic in seawater (SW) and balance the influx of ions and the efflux of water by excreting excess monovalent ions in the gills, absorbing fluid from ingested SW in the intestine, and excreting excess divalent ions via the kidney. The key enzyme to transport processes in the gill and intestine is the membrane-spanning protein Na^+, K^+ -ATPase. Therefore the regulation of Na^+, K^+ -ATPase expression in these organs is of major importance to fish during SW acclimation.

In the opercular membrane of SW-acclimated fish, chloride cells (CCs) comprise the cellular sites for the

excretion of monovalent ions (Foskett & Scheffey 1982). Even though this cellular function has never been directly demonstrated in the gill, there is ample evidence to suggest that CCs have a similar function in the gills of SW-acclimated fish. On the other hand, the role of CCs in freshwater (FW)-acclimated fish is still debated (reviewed by Perry 1997). Historically, most interest has focused concern on CCs in the gill primary filament (PF) epithelium. These cells increase in size and number during SW acclimation (Laurent & Dunel 1980). Recently, attention has turned to the differential roles and the regulation of CCs on the PF and secondary lamellae (SL). In SW-acclimated salmonids, there are very few CCs on

the SL compared with the densities observed in fish acclimated to FW (Laurent & Dunel 1980, Uchida *et al.* 1996). This suggests that CCs located on the PF may be involved in ion secretion, whereas CCs on the SL may be involved in ion absorption.

Fluid absorption across the intestinal mucosa of SW-acclimated fish is driven by the activity of Na⁺,K⁺-ATPase, and intestinal Na⁺,K⁺-ATPase activity increases during SW acclimation (Collie & Bern 1982). Traditionally, fluid uptake has been measured in distal parts of the intestine (e.g. Collie & Bern 1982), but an increasing number of studies indicate that the anterior/pyloric intestine may be more important in fluid uptake (e.g. Vökler *et al.* 1987, Bogé *et al.* 1988).

Several studies have shown that, in FW-acclimated fish, growth hormone (GH), insulin-like growth factor-I (IGF-I) and cortisol increase salinity tolerance and increase PF CC size and number (Madsen 1990a,b; Laurent *et al.* 1994, Seidelin & Madsen 1997, Seidelin *et al.* 1997). Prolactin (PRL) antagonises the SW-adaptive effect of both GH and cortisol, although different mechanisms may be involved (Madsen & Bern 1992, Seidelin & Madsen 1997). The interaction between PRL/GH and IGF-I in fish osmoregulatory physiology has not yet been studied. The aim of this study was to investigate the interactive effects of IGF-I and PRL/GH on (i) immediate salinity tolerance, (ii) gill Na⁺,K⁺-ATPase activity and α -subunit mRNA content, (iii) the distribution of gill CCs in PF and SL, and (iv) Na⁺,K⁺-ATPase activity in different segments of the intestine. A preliminary experiment on the interaction of GH and IGF-I on SW acclimation in brown trout parr has been presented by Seidelin *et al.* (1997).

Materials and Methods

Fish

Immature pre-smolts of anadromous brown trout, *Salmo trutta* (age 0+, first generation hatchery fish, mixed sex) were obtained in February 1997 from Vestjysk Fiskepark, Denmark, where they had been reared in ponds under natural photoperiod and temperature. A month later, this stock of fish developed good smolt characteristics (increased gill Na⁺,K⁺-ATPase activity and salinity tolerance: C Nielsen & S S Madsen, unpublished observations). The fish were brought to Odense University and acclimated indoors in 2000 l flow-through freshwater tanks (15 °C, 12 h:12 h light:darkness cycle). They were fed 1% body weight with commercial trout pellets once daily. After 14 days, 180 fish (21.1 ± 0.4 g, mean weight ± s.e.m.) were randomly sorted into 9 groups of approximately 20 fish per group and transferred to three 400 l freshwater tanks (15 °C). Three groups were pooled in each tank and tagged by fin-clipping. Food was withheld from this day onwards.

Hormonal treatment

Hormone treatments were initiated 4 days after the fish were divided into groups. Prior to intraperitoneal injections, the fish were slightly anaesthetised in 0.05% phenoxyethanol. Injections were repeated on alternate days for a total of four injections per fish. Half the fish in each group were transferred to another 400 l tank after the last injection and 24 h later challenged with 30 parts per thousand (ppt) SW (400 l tank, 15 °C) for 48 h and sampled. The remaining fish were sham-transferred to FW for 48 h before sampling. The following vehicle and hormones were used: saline (0.9% NaCl, 0.05% NaOH, 0.4% bovine serum albumin), recombinant human (rh) IGF-I (gift from Chiron Corporation, Emeryville, CA, USA), ovine (o) PRL (NIADDK-oPRL-19, Baltimore, MD, USA) and oGH (NIADDK-oGH-15).

We used a 3 × 3-factorial statistical design where factor 1 was either saline, 2 µg oPRL/g body weight (bw) or 2 µg oGH/g bw, and factor 2 was either 0, 0.01, or 0.1 µg rhIGF-I/g bw. Accordingly, fish were injected with the following combinations and doses of hormones: (1) saline, (2) 0.01 µg rhIGF-I/g, (3) 0.1 µg rhIGF-I/g, (4) 2 µg oPRL/g, (5) 2 µg oGH/g, (6) 0.01 µg rhIGF-I+2 µg oPRL/g, (7) 0.01 µg rhIGF-I+2 µg oGH/g, (8) 0.1 µg rhIGF-I+2 µg oPRL/g, or (9) 0.1 µg rhIGF-I+2 µg oGH/g. The injection volume was 3 µl/g bw. Recombinant human IGF-I was dissolved in saline prior to the first injection. Tubes with saline and rhIGF-I solutions were frozen (−80 °C). Prior to each injection, oPRL and oGH were dissolved in the thawed solutions. The use of mammalian GH and PRL is justified by the fact that these hormones are known to bind specifically to their respective teleost receptors, although with lower affinity than their homologous counterparts (oPRL: Auperin *et al.* 1994; oGH: Yao *et al.* 1991). Despite structural differences, several studies have shown that mammalian hormones have similar qualitative osmoregulatory effects as the teleost hormones. Lower binding affinity for the heterologous hormone is, however, reflected in the reduced potency compared with the native hormones (oPRL: Hasegawa *et al.* 1986; oGH: Boeuf *et al.* 1994). Recombinant human IGF-I and recombinant salmon IGF-I have almost equipotent *in vitro* sulphation activity in the coho salmon (*Oncorhynchus kisutch*) gill cartilage assay (Moriyama *et al.* 1993).

Sampling

The fish were stunned by a blow to the head, and blood was drawn from the caudal vessels into heparinised syringes and immediately centrifuged at 5000 g for 3 min. The fish were then decapitated, and additional sampling occurred. From 5 FW-acclimated fish in each group, 2 first and 2 third gill arches were immediately homogenised in 2.5 ml ice-cold denaturing solution (4 M guanidinium

thiocyanate, 25 mmol/l sodium citrate, 0.5% sarcosyl, 0.1 M β -mercaptoethanol, and 0.3% antifoam, pH 7.0). From 3 FW-acclimated fish in each group, a piece of one second gill arch was fixed in 4% paraformaldehyde, 0.9% NaCl, 5 mmol/l NaH_2PO_4 (pH 7.8) for 24 h at 5 °C and stored in 70% ethanol at 5 °C until dehydrating and embedding in paraffin. From all FW-acclimated fish one second gill arch, pyloric caeca from the left ventral side of the anterior intestine, the middle intestine (from the last pyloric caecum to the ileorectal valve), and the posterior intestine (from the ileorectal valve to the anus) were dissected and placed in SEI buffer (300 mmol/l sucrose, 20 mmol/l EDTA, 50 mmol/l imidazole, pH 7.3). All samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysed. From all fish, a piece of paraxial muscle was dissected and weighed for determination of total water content.

Analyses

Plasma osmolality was measured on a vapour pressure osmometer (Wescor, Logan, UT, USA), plasma $[\text{Na}^+]$ by flame photometry (Instrumentation Laboratory 243, Lexington, MA, USA), plasma $[\text{Cl}^-]$ by coulometric titration (Radiometer CMT 10, Copenhagen, Denmark), and plasma total $[\text{Mg}]$ by atomic absorption spectrophotometry (Perkin Elmer 2380, Mountain View, CA, USA). Muscle water content was estimated as percentage weight loss after drying in an oven at 105 °C for 48 h.

Northern analysis of gill RNA from 5 fish in each FW treatment group followed the procedure of Seidelin *et al.* (1999). For hybridisation, a 817 bp PvuII/BamHI fragment of the *Xenopus laevis* α_1 -subunit Na^+, K^+ -ATPase (Verrey *et al.* 1987) was radiolabelled and used as probe. The use of the *Xenopus* cDNA in brown trout gills has been validated by Madsen *et al.* (1995). For control hybridisation, a radiolabelled rainbow trout β -actin probe (Pakdel *et al.* 1989) was used. Radioactivity was detected by phosphor imaging (Storm, Molecular Dynamics, Sunnyvale, CA, USA), and relative band intensities were analysed by the ImageQuaNT 4.1 software (Molecular Dynamics). To adjust for unequal loading, data are presented as the ratio of Na^+, K^+ -ATPase α -mRNA to β -actin mRNA content.

Na^+, K^+ -ATPase immunoreactive cells (NKIR cells) were visualised in gill tissue using a monoclonal antibody initially raised in rabbit against a synthetic peptide corresponding to a part of the highly conserved region of the Na^+, K^+ -ATPase α -subunit (Ura *et al.* 1996). The use of this antibody has been validated in several teleosts (Uchida *et al.* 1996, Ura *et al.* 1996). The primary antibody was kindly provided by Dr S Adachi (Hokkaido University, Hakodate, Japan). Five micron thick rehydrated tissue sections were incubated overnight at 4 °C with the primary antibody (1/600 dilution), 30 min at 37 °C with the secondary antibody (goat anti-rabbit immunoglobulin

G-peroxidase conjugated, 1/100 dilution), and peroxidase reaction was run for 10 min using the substrate 3,3'-diamino-benzidine, creating a brown precipitate. Nuclei were subsequently stained with Meyer's haematoxylin. Gill tissue sagittal sections from 3 fish (middle part of the filament) were coded and analysed by light microscopy using 200–400 $\times$ magnification. Approximately 400 NKIR cells were counted in each gill specimen on the PF (interlamellar regions) and on the SL respectively, and the average number of NKIR cells was calculated per interlamellar region or SL.

Na^+, K^+ -ATPase activity was analysed at 25 °C in crude homogenates of gill, pyloric caeca (homogenate of 1 whole pyloric caecum, stripped of fat), middle and posterior intestine (homogenate of mucosal cell layer scraping) by the method of McCormick (1993) using a plate reader (Ceres 900 UV, Bio-Tek, Winooski, VT, USA). Protein content was measured by the method of Lowry *et al.* (1951) modified for plate reader.

Statistics

We used a 3×3 factorial statistical design where factor 1 was either saline, oPRL or oGH, and factor 2 was either 0, 0.01, or 0.1 μg rhIGF-I/g. Statistical differences were analysed using SYSTAT 5.03 (Systat 1991, Evanston, IL, USA). When necessary, transformation of the data was carried out to meet the assumption of homogeneity of variances (Bartlett test). For individual parameters, data were then analysed by two-way analysis of variance (ANOVA). If at least two main effects inside one factor were significantly different, the main hormonal effects were compared with that of saline-injected controls using Dunnett's test. If the interaction among the two factors was significant, differences among individual groups were analysed by Tukey's Honestly Significant Difference Test. In all cases, differences were considered significant at the $P=0.05$ level.

Results

Plasma osmolality, ions, and muscle water content after 48 h in SW

After 48 h in 30 ppt SW, plasma osmolality (Fig. 1A), $[\text{Na}^+]$, and $[\text{Cl}^-]$ (Table 1) were affected similarly by hormone treatment. Muscle water content (Fig. 1B) was negatively correlated with plasma osmolality (linear correlation analysis: $R = -0.71$ to -0.90 ; $P \leq 0.05$) except for the three GH-injected groups. Overall, GH improved and PRL impaired SW acclimation as judged by their opposing effects on plasma osmolality, $[\text{Na}^+]$ and $[\text{Cl}^-]$, and muscle water content. Overall fish treated with IGF-I had lower plasma osmolality than saline-injected controls (Fig. 1A). The effects of factor 1 and factor 2 on plasma

Figure 1 Effects of saline, oPRL, or oGH combined with saline (open bars), 0.01 µg rhIGF-I/g (hatched bars), or 0.1 µg rhIGF-I/g (solid bars) on plasma osmolality (osm.) (A), muscle water content (B), and plasma total [Mg] (C) in *S. trutta* ($n=8-12$). Fish were given 4 injections on alternate days in FW and transferred to 30 ppt SW for 48 h one day after the last injection. Values are means $\pm$ S.E.M. An asterisk indicates an overall effect of the hormone (two-way ANOVA followed by Dunnett's test, $P\leq 0.05$). There was significant interaction among the two factors only for plasma total [Mg]; values with shared letters are not significantly different ($P>0.05$).

osmolality, [Na⁺] and [Cl⁻] as well as muscle water content were additive. PRL overall increased and GH decreased plasma total [Mg] compared with controls (Fig. 1C). There was a significant interaction between factor 1 and factor 2 on plasma total [Mg], and [Mg] levels showed a complex pattern of regulation.

Gill Na⁺,K⁺-ATPase expression and NKIR cells

There was an overall inhibitory effect of PRL and a stimulatory effect of GH on the level of gill Na⁺,K⁺-ATPase α -subunit mRNA (α -mRNA) (Fig. 2A). IGF-I treatment alone stimulated gill α -mRNA levels. Co-administration of PRL totally abolished the increase induced by IGF-I, whereas GH did not further affect the

Table 1 Effects of saline, oPRL or oGH combined with saline, 0.01 µg rhIGF-I/g or 0.1 µg rhIGF-I/g on plasma [Na⁺] (upper value) and [Cl⁻] (lower value) in *S. trutta*. Fish were given 4 injections in FW and transferred to 30 ppt SW for 48 h one day after the last injection. Values are in mmol/l and are means $\pm$ S.E.M., $n=8-12$

		Factor 1		
		Saline	oPRL*	oGH*
Factor 2	Saline	191 ± 4	200 ± 3	176 ± 3
		161 ± 4	178 ± 4	148 ± 2
rhIGF-I 0.01 µg/g*		171 ± 3	178 ± 5	157 ± 2
		145 ± 3	158 ± 5	130 ± 3
rhIGF-I 0.1 µg/g*		169 ± 4	174 ± 4	161 ± 3
		147 ± 4	158 ± 4	137 ± 2

*Significant overall effect of the hormone analysed by two-way ANOVA, $P\leq 0.05$. All hormone effects were additive.

α -mRNA level. Only treatment with 0.01 µg IGF-I/g resulted in an overall increase in gill Na⁺,K⁺-ATPase activity (Fig. 2B).

The anti-Na⁺,K⁺-ATPase antibody specifically stained epithelial cells in both the primary filament and secondary lamellae of the gill (Fig. 3). GH and IGF-I treatment induced a redistribution of NKIR cells in the gill (Fig. 4). Both doses of IGF-I caused an overall increase in gill PF (Fig. 4A), and decrease in gill SL and total NKIR cell number (Fig. 4C). Fish treated with GH overall had fewer NKIR cells in the gill SL (Fig. 4B). PRL did not affect gill NKIR cell number and there was no interactive effect with IGF-I.

Intestinal Na⁺,K⁺-ATPase expression

Fish treated with 0.01 µg IGF-I/g had lower Na⁺,K⁺-ATPase activity in pyloric caeca and middle intestine than control fish (Fig. 5A,B). Treatment with 0.1 µg IGF-I/g decreased middle intestine Na⁺,K⁺-ATPase activity. GH and PRL did not affect pyloric caeca or intestinal Na⁺,K⁺-ATPase activity. Activity of Na⁺,K⁺-ATPase (Fig. 5C) was lower in the posterior than in the middle intestine, and the hormonal effects were similar to those observed in the middle intestine, even though not significant.

Discussion

Osmoregulation

The brown trout used in this study showed a relatively good salinity tolerance which was in accordance with their developmental stage (pre-smolt). PRL showed its expected anti-SW effect and GH its SW-adaptive effect (Bolton *et al.* 1987, Madsen & Bern 1992, Seidelin &

Figure 2 Effects of saline, oPRL, or oGH combined with saline (open bars), 0.01 $\mu\text{g rhIGF-I/g}$ (hatched bars), or 0.1 $\mu\text{g rhIGF-I/g}$ (solid bars) on gill $\alpha\text{-Na}^+, \text{K}^+$ -ATPase: β -actin mRNA ratio (A, $n=5$) and Na^+, K^+ -ATPase activity (expressed as $\mu\text{mol ADP/mg protein/h}$) (B, $n=8-12$) in *S. trutta*. Fish were given 4 injections on alternate days in FW and sham-transferred to FW for 48 h one day after the last injection. Values are means $\pm$ S.E.M. An asterisk indicates an overall effect of the hormone (two-way ANOVA followed by Dunnett's test, $P \leq 0.05$). There was a significant interaction among the two factors only for the $\alpha\text{-Na}^+, \text{K}^+$ -ATPase: β -actin mRNA ratio; values with shared letters are not significantly different ($P > 0.05$).

Madsen 1997), as judged by plasma osmolality and muscle water content, after 48 h in SW (Fig. 1A,B). Treatment of FW-acclimated fish with IGF-I improved hypo-regulation of plasma osmolality and major monovalent ions (Table 1). This SW-adaptive effect of IGF-I in FW-acclimated brown trout is consistent with studies by Seidelin *et al.* (1997), whereas in FW-acclimated Atlantic salmon (*S. salar*) IGF-I had either no effect or impaired salinity tolerance (McCormick 1996). Treatment of 10–12 ppt SW-acclimated fish, however, resulted in improved hypo-osmoregulatory ability when tested in full strength SW (rainbow trout, *O. mykiss*: McCormick *et al.* 1991; *S. salar*: McCormick 1996), indicating that a priming effect of the saline environment is necessary for IGF-I's action in these latter species.

In fish transferred to SW for 48 h, PRL treatment increased, whereas GH treatment decreased plasma total

Figure 3 Adjacent sections of a *S. trutta* control gill stained with the antibody to Na^+, K^+ -ATPase α -subunit and haematoxylin (A) and a negative control stained with haematoxylin (B). Arrows indicate positive immunoreaction (NKIR cell). Bar=50 μm .

[Mg] levels (Fig. 1C). This main effect of GH on plasma total [Mg] after SW transfer has also been observed by Bolton *et al.* (1987) and Madsen & Bern (1992), and may be due to stimulation of magnesium excretion in the kidney. In other studies, PRL was shown not to affect magnesium regulation after SW transfer (Madsen & Bern 1992, Seidelin & Madsen 1997). When comparing the

Figure 4 Effects of saline, oPRL, or oGH combined with saline (open bars), 0.01 μg rhIGF-I/g (hatched bars), or 0.1 μg rhIGF-I/g (solid bars) on average number of gill Na^+, K^+ -ATPase immunoreactive cells per interlamellar region in the primary filament (A), secondary lamellae (B), and total number (C) in *S. trutta* ($n=3$). Fish were given 4 injections on alternate days in FW and sham-transferred to FW for 48 h one day after the last injection. Values are means $\pm$ S.E.M. An asterisk indicates an overall effect of the hormone (two-way ANOVA followed by Dunnett's test, $P \leq 0.05$).

different groups, only the oPRL+0.01 μg rhIGF-I/g group had values different from the saline-injected controls. The discrepancy in the regulation of monovalent ions and total [Mg] is most likely due to differential hormonal regulation of the gill and kidney ion-excretory pathways.

Gill Na^+, K^+ -ATPase expression

Fully mature gill CCs contain large amounts of Na^+, K^+ -ATPase enzymes in their basolateral tubular network compared with other gill epithelial cell types (see review by McCormick 1995). Chloride cells, therefore, appear selectively stained when using an anti- Na^+, K^+ -ATPase antibody on gill micro-sections (Fig. 3; Witters *et al.* 1996). Similarly, Na^+, K^+ -ATPase activity levels in crude

Figure 5 Effects of saline, oPRL, or oGH combined with saline (open bars), 0.01 μg rhIGF-I/g (hatched bars), or 0.1 μg rhIGF-I/g (solid bars) on Na^+, K^+ -ATPase activity (expressed as $\mu\text{mol ADP/mg protein/h}$) in the pyloric caeca (A), middle (B), and posterior intestine (C) in *S. trutta* ($n=8-12$). Fish were given 4 injections on alternate days in FW and sham-transferred to FW for 48 h one day after the last injection. Values are means $\pm$ S.E.M. An asterisk indicates an overall effect of the hormone (two-way ANOVA followed by Dunnett's test, $P \leq 0.05$). There were no hormonal effects on posterior intestine Na^+, K^+ -ATPase levels ($P > 0.05$).

gill homogenates provide a rough measure of overall gill CC density and/or maturation (see review by McCormick 1995).

The pre-smolts used in this study had gill Na^+, K^+ -ATPase activities close to the maximum level observed in this particular strain of anadromous brown trout during smoltification (C Nielsen & S S Madsen, unpublished observations). There was no main effect by PRL, GH or 0.1 μg rhIGF-I/g on enzyme activity (Fig. 2B). Only fish treated with 0.01 μg rhIGF-I/g showed overall increased gill enzyme activity. GH treatment has been reported to increase gill Na^+, K^+ -ATPase activity in several salmonids (e.g. Madsen 1990a,b). The developmental stage of the fish used in this study may explain why GH did not augment gill enzyme activity, as GH only stimulates gill Na^+, K^+ -ATPase activity outside the peak period of smoltification, at least in *S. salar* (Boeuf *et al.* 1994). Previous experiments

in our laboratory have shown that PRL has either no effect or decreases gill Na^+, K^+ -ATPase activity in FW salmonids (Madsen & Bern 1992, Madsen *et al.* 1995, Seidelin & Madsen 1997). In contrast, a single study has shown that PRL is able to stimulate gill Na^+, K^+ -ATPase activity of *S. salar* despite an impaired hypo-osmoregulatory ability when these fish were challenged with SW (Boeuf *et al.* 1994). Gill Na^+, K^+ -ATPase activity was shown to be either unaffected (McCormick 1996, Seidelin *et al.* 1997) or stimulated (Madsen *et al.* 1995) by IGF-I in FW-acclimated salmonids *in vivo*. Madsen & Bern (1993) demonstrated that, in coho salmon (*O. kisutch*), IGF-I is able to stimulate gill Na^+, K^+ -ATPase activity *in vitro*, and that GH priming is critical for the development of this response. Even though the response to IGF-I seems larger in fish treated simultaneously with GH (Fig. 2B), the present study does not provide statistical support for a synergistic interaction between these two hormones in brown trout. Interestingly, while gill total Na^+, K^+ -ATPase activity was either unaffected or increased slightly by hormonal treatment, significant changes were observed in total Na^+, K^+ -ATPase α -subunit mRNA (Fig. 2A) and distribution of Na^+, K^+ -ATPase immunoreactive cells in gill (Fig. 4A-C). Both IGF-I doses decreased gill total NKIR cell number, which in all cases was due to a reduction in SL CC number. At the same time, fish in these groups had increased levels of α -mRNA. GH was shown to increase PF CC number and size in several studies (e.g. Madsen 1990a,b; Laurent *et al.* 1994), but in this study it had no effect (Fig. 4A). In line with its SW-adaptive role, IGF-I increased PF NKIR cell numbers. These cells are likely to be secretory type CCs similar to those present in SW-acclimated fish. The increase in total α -mRNA in the IGF-I-treated groups may thus reflect an increased expression of this gene in the differentiating and/or maturing PF CCs. Treating pre-smolts with PRL alone did not affect gill α -mRNA content or CC numbers in the PF. In accordance with these observations, PRL had no effect on gill Na^+, K^+ -ATPase expression in brown trout parr (Madsen *et al.* 1995), nor did PRL affect CC number in the opercular membrane of SW-acclimated tilapia (*Oreochromis mossambicus*, Herndon *et al.* 1991).

The differential hormonal regulation of NKIR cells in the PF and the SL may reflect their different putative functions, i.e. ion absorption and ion secretion respectively. This idea has gained support from a few studies of environmentally induced and developmental changes in NKIR cell populations. First, SL NKIR cells seem to disappear when fish are transferred from FW to SW (Uchida *et al.* 1996). Secondly, both PF and SL NKIR cell numbers increase during smoltification in masu salmon (*O. masou*, Ura *et al.* 1997). At the peak of smoltification, however, the CC number decreases in the SL. These dynamics suggest that increased ion uptake by SL CCs is necessary during the

proliferation and possibly maturation of ion-excretory PF CCs. Our study suggests that the disappearance of SL CCs at the peak of smoltification could be a pre-adaptive response to SW induced by hormones such as GH and IGF-I.

Intestinal Na^+, K^+ -ATPase activity

During salmonid smoltification or acclimation from FW to SW, Na^+, K^+ -ATPase activity and fluid uptake capacity of all intestinal segments increase (Collie & Bern 1982, Vökler *et al.* 1987, Bogé *et al.* 1988). Accordingly, the FW-acclimated pre-smolts used in this study most likely had well developed mechanisms for intestinal fluid absorption. Several studies indicate that there may be functional differences between the pyloric, middle, and posterior intestine with regard to nutrient, ion, and fluid transport (Collie & Bern 1982, Buddington & Diamond 1987, Bogé *et al.* 1988). In the present study we analysed the Na^+, K^+ -ATPase activity in all these intestinal segments. PRL treatment did not affect Na^+, K^+ -ATPase activity in any intestinal segment of pre-smolt brown trout. This contrasts with the situation seen in SW-acclimated eels, where PRL diminishes intestinal ion and water permeability (Utida *et al.* 1972). From morphological evidence, Nonnotte *et al.* (1995) suggest a SW-adaptive role of GH in the middle intestine of *S. salar*. In the brown trout, this role of GH is not seen in measures of fluid uptake capacity in the posterior intestine (Seidelin *et al.* 1997) or Na^+, K^+ -ATPase activity in the intestine (Seidelin *et al.* 1997, Fig. 5B,C), and pyloric caeca (Seidelin *et al.* 1997, Fig. 5A). Surprisingly, there was a significant overall inhibition of Na^+, K^+ -ATPase activity by IGF-I in the pyloric caeca and middle intestine (Fig. 5A,B). This pattern was similar in the posterior intestine, even though not significant (Fig. 5C). In a previous study, IGF-I treatment did not alter pyloric caeca Na^+, K^+ -ATPase activity and posterior intestine fluid uptake in brown trout parr (Seidelin *et al.* 1997). The physiological significance of the IGF-I effect in pre-smolts in this study is unclear. Our evidence does not support a SW-adaptive role of IGF-I in this tissue.

IGF-I-GH and IGF-I-PRL interactions

Treatment of brown trout pre-smolt with GH or IGF-I alone or in combination increased gill α -mRNA content in a similar manner (Fig. 2A). In a preliminary experiment using brown trout parr, GH and IGF-I additively increased gill Na^+, K^+ -ATPase activity and α -mRNA levels (Seidelin *et al.* 1997). Therefore, it seems that the higher expression level of gill enzyme in the present pre-smolts may be too high to detect an additive stimulatory effect of the two hormones. Alternatively, the sensitivity of the gill epithelium to IGF-I is at its

maximum level due to priming of the epithelium by endogenous GH at this stage of the parr-smolt transformation (Madsen & Bern 1993).

PRL antagonised the SW-adaptive effect of IGF-I in the present study (Fig. 1B), which is in line with its general anti-SW effect, and its antagonistic action with other SW-adaptive hormones such as GH (Madsen & Bern 1992) and cortisol (Seidelin & Madsen 1997). PRL inhibits the increase in gill Na^+, K^+ -ATPase activity induced by GH (Madsen & Bern 1992), but not cortisol (Seidelin & Madsen 1997). PRL's inhibition of IGF-I-stimulated gill α -mRNA content in the present study (Fig. 2A) indicates that PRL may affect GH- and IGF-I-induced gill Na^+, K^+ -ATPase expression by a similar mechanism.

In conclusion, IGF-I and GH additively increased SW tolerance of FW-acclimated brown trout concurrent with a redistribution of NKIR cells from the secondary lamella to the primary filament. This effect was associated with an overall increase in α -mRNA level in the gill, which probably reflects an increased expression within individual NKIR cells of the PF. PRL completely abolished the IGF-I stimulation of gill α -mRNA levels, suggesting a decreased sensitivity of the gill tissue to IGF-I stimulation, which may explain the overall anti-SW adaptive effect of PRL.

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