Differential regulation of cystic fibrosis transmembrane conductance regulator and Na^+, K^+ -ATPase in gills of striped bass, *Morone saxatilis*: effect of salinity and hormones

Steffen Søndergaard Madsen, Lars Nørholm Jensen, Christian Kølbaek Tipsmark¹, Pia Kiilerich and Russell John Borski¹

Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

¹Department of Zoology, North Carolina State University, Raleigh, North Carolina 27695-7617, USA

(Requests for offprints should be addressed to S S Madsen; Email: steffen@biology.sdu.dk)

Abstract

Effects of salinity and hormones on cystic fibrosis transmembrane conductance regulator (CFTR) and α -subunit Na^+, K^+ -ATPase (α -NKA) mRNA (analysed by semi-quantitative PCR) and protein expression (analysed by western blotting and immunocytochemistry) were investigated in gills of striped bass. Freshwater (FW) to seawater (SW) transfer induced a disturbance in serum $[\text{Na}^+]$. Gill CFTR protein, mRNA level and Na^+, K^+ -ATPase activity were unaffected by SW transfer, whereas α -NKA mRNA increased after transfer. CFTR immunoreactivity was observed in large cells in FW and SW gill filaments at equal intensity. Cortisol decreased serum $[\text{Na}^+]$ in FW fish, but had no effect on gill Na^+, K^+ -ATPase activity, α -NKA and CFTR mRNA levels. Incubation of gill tissue with cortisol (24 h, $>0.01 \mu\text{g/ml}$) and

epidermal growth factor (EGF $10 \mu\text{g/ml}$) decreased CFTR mRNA levels relative to pre-incubation and control levels. CFTR expression was unaffected by IGF-I ($10 \mu\text{g/ml}$). α -NKA mRNA levels decreased by 50% after 24 h control incubation; it was slightly stimulated by cortisol and unaffected by IGF-I and EGF. In isolated gill cells, phosphorylation of extracellular-regulated kinase (ERK) 1/2 was stimulated by EGF but not affected by IGF-I. This study is the first to report a branchial EGF response and to demonstrate a functional ERK 1/2 pathway in the teleost gill. In conclusion, CFTR and Na^+, K^+ -ATPase are differentially regulated by salinity and hormones in gills of striped bass, despite the putative involvement of both in salt excretion.

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Introduction

The gill is an important osmoregulatory organ in teleosts with distinct roles in hypo- and hyperosmotic environments. In freshwater (FW) fish, inevitable salt loss across permeable body surfaces is compensated by active ion uptake in various epithelial cells of the gill. The molecular mechanism of monovalent ion uptake involves apical V-type H^+ -ATPase, Na^+ channels (ENaC) and $\text{Cl}^-/\text{HCO}_3^-$ exchange, and basolateral Na^+, K^+ -ATPase and chloride conductance (see Marshall 2002). The molecular nature of the latter is largely unknown, but has recently been associated with the presence of cystic fibrosis transmembrane conductance regulator-like (CFTR) immunoreactivity in killifish (*Fundulus heteroclitus*) chloride and pavement cells (Marshall & Singer 2002). The gill of sea water (SW) fish is able to secrete monovalent ions against electrochemical gradients. Basolateral Na^+, K^+ -ATPase activity sets up appropriate conditions for uphill Cl^- entry through a basolateral $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter (see Marshall 2002). Chloride diffuses to the apical membrane, where it exits passively through ATP-gated

CFTR Cl^- channels. Sodium is secreted passively by a paracellular path, while K^+ is in electrochemical equilibrium across both apical and basolateral membranes. The role of CFTR in ion secretion by marine teleost chloride cells and its dynamics during salinity transitions have been established in a few teleosts, all of which show increased expression of CFTR in apical membranes in response to SW transfer (killifish: Marshall *et al.* 1999, 2002; Atlantic salmon, *Salmo salar*: Singer *et al.* 2002; Hawaiian goby, *Stenogobius hawaiiensis*: McCormick *et al.* 2003; eel: Wilson *et al.* 2004; tilapia, *Oreochromis mossambicus*: Hiroi *et al.* 2005). Apical CFTR-like channels have also been identified in pavement cells of the gill in European sea bass, *Dicentrarchus labrax* (Duranton *et al.* 1997). Cyclic AMP and phosphorylation by protein kinase A (PKA) stimulate CFTR *in situ* (see Marshall *et al.* 1995, Sheppard & Welsh 1999), thus hormones which affect gill cAMP levels may be involved in short-term activation of salt transport. On the other hand, information on the endocrine regulation of gill CFTR expression is scarce. The promoter region of mammalian and killifish CFTR contains glucocorticoid-binding sites (Schulte & Keir 2003, McCarthy & Harris 2005),

and there is evidence that cortisol is an important component of CFTR regulation in killifish (Marshall & Singer 2002) and Atlantic salmon (Singer *et al.* 2003).

Different acclimation strategies have been identified in euryhaline teleosts facing salinity shifts through life. Some teleosts, such as rainbow trout (*Oncorhynchus mykiss*) and tilapia, take up the challenge once exposed and acclimate gradually over several days. This implies a temporary 'crisis' period with large deflections in ion-osmotic homeostasis (Houston 1959), during which strict endocrine regulation initiates a 'slow' development of hypo-osmoregulatory mechanisms in gill, kidney and intestine. In the well-studied group of anadromous salmonids (genera: *Salmo* and *Oncorhynchus*), a developmental process called smoltification occurs prior to downstream migration and SW entry. Smoltification represents a gradual development of hypo-osmoregulatory mechanisms while the fish is still in FW; thus, the environmentally regulated process may be seen as a preparatory development, even though such phenomenon may be difficult to explain in evolutionary terms. Yet other euryhaline fish, such as striped bass, *Morone saxatilis* and killifish, maintain a high level of preparedness to cope with salinity shifts throughout their life (Madsen *et al.* 1994). This strategy has the advantage that salinity fluctuations may be dealt with a minimum of osmotic stress but presumably requires energy input for simultaneously maintaining hyper- as well as hypo-osmoregulatory mechanisms.

The striped bass is naturally occurring on the east coast of North America (Raney 1952) from New England to Florida and has been introduced as a popular game fish on the Pacific coast of North America. It has an anadromous life cycle and may reside in FW, brackish water (BW) and full salinity SW throughout its life. The major growth period occurs in SW or BW and reproduction in FW (Raney 1952), but there is no evidence that the ability to live in those variable environments is correlated with a particular developmental stage as seen in smoltifying salmonids. By contrast, the species displays an excellent ability to acclimate to both FW and SW at all life stages (King & Hossler 1991, Madsen *et al.* 1994, Tipsmark *et al.* 2004). This has led us to speculate that regulation of ion transport in the gill of this species may rely more on rapid modulation than on expressional regulation. The endocrine regulation of hydro-mineral balance is largely unexplored in striped bass. Plasma cortisol levels increase after FW–SW transfer, but also show a stress-associated increase in the FW–FW sham group (Madsen *et al.* 1994). Insulin-like growth factor-I (IGF-I), a hormone with strong SW-adaptive effects in salmonids (McCormick 2001), has recently been documented to have negative effects on SW acclimation in striped bass (Tipsmark *et al.* 2007).

Epidermal growth factor (EGF) is a polypeptide hormone secreted from various tissues with auto-paracrine effects on cells of epidermal and epithelial origin (see Singh & Harris 2005). In mammals, biological responses induced by EGF *in vitro* include increased amino acid transport, activation of Na^+ , K^+ -ATPase activity, stimulation of prostaglandin

metabolism and DNA synthesis (Gregoriou & Rees 1984). The biological activity of EGF is mediated through a cellular transmembrane receptor which has been demonstrated in fish (Newsted & Giesy 2000). Except for its possible role in ovarian development and oocyte maturation (Pang & Ge 2002), the function of EGF in fish is largely unexplored. There is no information on how either of these hormones affects expression of ion-transport proteins in the gill of striped bass.

This study investigated the localisation and expression regulation of CFTR in gills of striped bass. The response of CFTR protein and mRNA abundance to FW–SW transfer was analysed and the effect of putative osmoregulatory hormones (cortisol, IGF-I and EGF) on CFTR and Na^+ , K^+ -ATPase expression was investigated *in vivo* and *in vitro*. The role of extracellular signal-regulated kinase (ERK) activation in the gill response to IGF-I and EGF was investigated in a separate experiment.

Materials and Methods

We investigated the effect of environmental salinity (experiment 1), cortisol *in vivo* (experiment 2) and cortisol, IGF-1 and EGF *in vitro* (experiments 3 and 4) on gill α -subunit Na^+ , K^+ -ATPase (α -NKA) and CFTR mRNA expression. Striped bass (30–60 g) were obtained from freshwater (FW) ponds at the Edenton National Fish Hatchery (Edenton, NC, USA) in December 2004 and acclimated to experimental conditions for >6 weeks on the North Carolina State University Campus (Raleigh, NC, USA). The fish were held in 500 l tanks supplied with recirculated, filtered FW (experiments 1, 2 and 4; hardness: 100 mg/l; alkalinity: 200 mg/l) or 35 ppt artificial SW (Instant Ocean, Aquarium Systems, Mentor, Ohio, USA; 22 °C, 12 h light:12 h darkness). The fish were fed *ad libitum* with a pelleted feed (Southern States, Richmond, VA, USA) until 2 days prior to and throughout an experiment.

Experiment 1: effect of FW–SW transfer

At time 0, ten control fish were sampled directly out of the FW stock tank. Following this, subgroups ($n=10$) were transferred to separate 60 l tanks supplied with either SW or FW (sham) and sampled 6 h, 24 h and 4 days later.

Experiment 2: effect of cortisol *in vivo*

Ten FW fish were anaesthetised and injected intraperitoneally with 2 µg hydrocortisone-hemisuccinate/g body weight (Sigma) dissolved in 0.9% NaCl. Ten fish were injected with saline (2 µl/g body weight) and served as control. A similar dose of cortisol induces transient hypercortisolism in tilapia (Takahashi *et al.* 2006). After injection, the fish were transferred to two 60 l tanks (FW) and allowed to recover. The injection procedure was repeated every other day for a

total of three injections per fish. Sampling of blood, gill and muscle tissue was performed 24 h after the last injection as outlined below.

Experiment 3: effect of cortisol, IGF-1 and EGF in vitro

Gill tissue was carefully excised from six FW-acclimated fish and used to investigate the effect of 24 h incubation (McCormick & Bern 1989) with or without hormones on mRNA expression. After excision of the gill arches, the cartilage arch was trimmed away and the row of gill filaments was cut into blocks of approximately 2–4 pairs of filaments. The blocks from one fish were pooled in a Petri dish containing oxygenated (99% O₂/1% CO₂) Ringer's solution (140 mmol/l NaCl, 15 mmol/l NaHCO₃, 2.5 mmol/l KCl, 1.5 mmol/l CaCl₂, 1.0 mmol/l KH₂PO₄, 0.8 mmol/l MgSO₄, 5.0 mmol/l D-glucose, 5.0 mmol/l Hepes, pH 7.8) containing penicillin/streptomycin (400 000 IU each). After pre-incubation at 22 °C for 2–3 h, 2–3 gill blocks from each fish were transferred to RNAlater (Ambion, Austin, TX, USA) and used as pre-incubation control samples. The remaining gill blocks were used to test the effect of hormones by transferring random replicates of 5–6 blocks to six-well plates containing 5 ml oxygenated Ringer's solution per well with penicillin/streptomycin (100 000 IU each). Hormones and doses were assayed in two series: series 1: control, cortisol at 0.01, 0.1, 1.0 and 10 µg/ml; series 2: control, cortisol 10 µg/ml, IGF-I 10 µg/ml, cortisol+IGF-I 10/10 µg/ml, EGF 10 µg/ml and cortisol+EGF 10/10 µg/ml. Cortisol was added as hydrocortisone-hemisuccinate. Recombinant human IGF-I was purchased from Gropep (Adelaide, Australia) and recombinant human EGF was a gift from Dr Shigeru Mihara, Technical Research Department, Earth Chemical Company Ltd, Hyogo, Japan.

The tissues were incubated at 22 °C for 24 h with gentle circular shaking in a humidified 99% O₂/1% CO₂ atmosphere. After incubation, gill blocks were transferred to tubes containing RNAlater (Ambion) and stored for a few days at 4 °C until RNA isolation and analysis.

Experiment 4: effect of EGF in vitro on ERK 1/2 activity

Gill cells from FW-acclimated fish were prepared as described in Tipsmark & Madsen (2003) modified from Verbost *et al.* (1994). They were transferred to 96-well plates and allowed to settle in Ringer's solution (see above) for 20 min at room temperature. They were incubated with Ringer's (control), phorbol-12-myristate-13-acetate (PMA, a protein kinase C activator, 1 µmol/l; Calbiochem, San Diego, CA, USA), PMA+chelerythrine chloride (a protein kinase C inhibitor, 4 µmol/l; Calbiochem), EGF (5 µg/ml) or IGF-I (5 µg/ml) for 10 min. The cells were homogenised by sonicating in sample buffer (see western procedure) and kept at –20 °C until western analysis of extracellular signal-regulated kinase (ERK) 1/2 activity (*n* = 4).

Sampling procedure (experiments 1–3)

Fish were netted and anaesthetised in buffered tricain methane sulphonate (MS)-222 (200 mg/l). Blood was sampled directly into Eppendorf tubes from the caudal vessels by caudal puncture and stored on ice. Serum was obtained by centrifugation and stored at –20 °C. The fish were then killed by cutting the spinal cord and pithing of the brain. Gills were excised and individual arches were transferred to tubes containing sucrose–EDTA–imidazole (SEI) buffer (300 mmol/l sucrose, 20 mmol/l Na₂-EDTA, 50 mmol/l imidazole, pH 7.3) for Na⁺,K⁺-ATPase analysis or RNAlater for RNA analyses. SEI buffer tubes were quick frozen in liquid nitrogen and RNAlater tubes were stored at 4 °C. At the end of sampling, a piece of caudal musculature was excised and weighed for determination of muscle water content.

Analytical procedures

Plasma [Na⁺] was measured by flame photometry (Instrumentation Laboratory 243, Lexington, MA, USA) on samples diluted 1:200 with 15 mmol/l LiCl. Muscle samples were weighed after drying at 105 °C for 24 h and muscle water content (MWC) was calculated as percentage of wet weight. Gill samples were homogenised in ice-cold SEIDM buffer (300 mmol/l sucrose, 20 mmol/l Na₂-EDTA, 50 mmol/l imidazole, 10 mmol/l β-mercaptoethanol, 0.1% sodium deoxycholate, pH 7.3). Following centrifugation at 5000 *g* for 30 s, Na⁺,K⁺-ATPase activity was assayed according to McCormick (1993) in the supernatant at 25 °C using a microplate reader (SPECTRAMAX PLUS, Molecular Devices, Sunnyvale, CA, USA). Protein content was measured by a microassay based on the method of Lowry *et al.* (1951), and enzymatic activity was normalised to protein content and expressed as µmol ADP/mg protein/h.

RNA isolation and mRNA analysis by semi-quantitative RT-PCR

To assess the level of gene expression of gill Na⁺,K⁺-ATPase α-subunit and CFTR in striped bass, a semi-quantitative reverse transcription PCR (RT-PCR) approach was used (Harting & Wiesner 1997). Total RNA was isolated from gill tissues fixed in RNAlater using the TRIzol procedure (Invitrogen). The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration measured by reading A₂₆₀ nm on diluted samples. Purity (A₂₆₀/A₂₈₀) ranged from 1.8 to 2.0. The integrity of RNA (5 µg) was assessed by gel electrophoresis using 1% EtBr-stained formaldehyde gel. For each treatment group, total RNA was extracted from 5–6 fish. Using 1 µg RNA, first strand cDNA synthesis was performed using SuperScript II RNase H⁺-Reverse Transcriptase kit (Invitrogen) with oligo dT as primers. A sample of the resultant cDNA template (0.1 µg RNA) was used for the PCR (final concentration: 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP, 100 nM gene-specific primer, 32 nM β-actin control primers (Ambion) and 1 U Taq polymerase (Sigma)) in a total reaction volume of

20 µl. PCRs were carried out as multiplex PCR using two sets of primers within the same reaction (β -actin control primers plus α -subunit Na^+, K^+ -ATPase or CFTR primers). Thermal cycling was performed according to the following protocol: 0.5 min denaturation at 94 °C, 0.5 min annealing at 58 °C and 0.5 min extension at 72 °C for 22 cycles (α - Na^+, K^+ -ATPase) or 30 cycles (CFTR). Before analysing all samples from the experiments, the linear range of the PCR for each individual primer pair in multiplex with β -actin primers was determined using a pooled cDNA sample. The linear range was found to be 27–32 cycles for CFTR and β -actin and 19–23 for α -subunit Na^+, K^+ -ATPase and β -actin. The number of cycles used for routine analyses was chosen to insure that all sets of primers resulted in reactions that were within the exponential amplification phase of the PCR for all templates. PCR products were separated according to size by gel electrophoresis in 1.5% EtBr-stained agarose gels (1×TAE buffer). The PCR products were visualised by u.v. illumination and quantified by densitometric analysis of a digital image (doc-IT, Upland, CA, USA) using QuantiScan software (Biosoft, San Francisco, CA, USA). For each individual sample, the integrated density value obtained for the target gene-specific amplicon was divided by the signal obtained for the β -actin control amplicon producing a relative mRNA abundance value. The use of β -actin for normalisation allows for comparison with the previous studies that used β -actin as a control in northern blotting (Madsen *et al.* 1995, D'Cotta *et al.* 2000) and semi-quantitative PCR (Singer *et al.* 2002). There was no significant change in the expression level of β -actin following experimental treatment.

Primers To amplify striped bass α -subunit Na^+, K^+ -ATPase, we used the primers developed by Singer *et al.* (2002), which are based on a conserved portion of the coding region of the α -subunit of several teleosts. Forward primer: 5'-GAGATCCTGGCCCGIGATGGCCC-3' and reverse: 5'-AACCACATGTGGGCCACTGTTCAT-3'. To amplify striped bass CFTR, we used the primers developed by Singer *et al.* (2002) specific for the 3'-untranslated region (3'-UTR) of the Atlantic salmon CFTR-I isoform (Accession no. AF155237). Forward primer: 5'-GAACCTTCTCCAA-TATGGTTGAAGAGGCAAG-3' and reverse: 5'-GCA-CAGTTTTCTTC CCAACTCCTAAC-3'. Primers for the Atlantic salmon CFTR-II isoform (Singer *et al.* 2002) were also tested but gave no amplification. To amplify the striped bass β -actin, we used the QuantumRNA Competimer kit from Ambion. This kit contains primers for β -actin based on a conserved region of sequence for human, mouse and rat β -actin mRNA. The ratio of β -actin primer-Competimer (QuantumRNA, Ambion) was also optimised in separate multiplex PCRs with each of the gene-specific oligonucleotide primer pairs. The CFTR and Na^+, K^+ -ATPase amplicons were sequenced by The University of Chicago Cancer Research Center DNA Sequencing Facility (Chicago, IL, USA).

Western analysis Gill tissue was homogenised in chilled SEID buffer (300 mmol/l sucrose, 20 mmol/l $\text{Na}_2\text{-EDTA}$, 50 mmol/l imidazole, 0.1% sodium deoxycholate, pH 7.3) with protease inhibitor cocktail (Sigma Aldrich, P8340) using a handheld glass homogeniser. The homogenate was centrifuged at 1000 g for 20 min (4 °C) and the membrane fraction was isolated from the supernatant by a second centrifugation at 50 000 g for 30 min (4 °C). The obtained pellet was resuspended and protein content was measured by a microassay based on a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Sample buffer and sample reducing agent (NuPAGE, Invitrogen; final concentration in the loaded samples: 141 mmol/l Tris base, 106 mmol/l Tris-HCl, 73 mmol/l LDS, 0.5 mmol/l EDTA, 50 mmol/l 1,4-dithiothreitol and 8% glycerol (v/v), 0.019% Serva blue G250 (w/v), 0.006% phenol red (w/v)) were added prior to heating at 80 °C for 10 min. Proteins (10 µg) were resolved by SDS-PAGE using 4–12% Bis-Tris gels (NuPAGE) and MES/SDS-buffer (50 mmol/l 2-(*N*-morpholino)-ethanesulphonic acid, 50 mmol/l Tris, 3.5 mmol/l SDS, 1 mmol/l $\text{Na}_2\text{-EDTA}$; with addition of NuPAGE antioxidant) at 200 V (Xcell II SureLock; Invitrogen). Molecular size was estimated by including a prestained marker (Bio-Rad). Following electrophoresis, the gel was soaked for 30 min in transfer buffer (25 mmol/l Tris and 192 mmol/l glycine) and immunoblotted onto nitrocellulose membranes (0.45 µm; Invitrogen) by submerged blotting for 1 h at 30 V (XCell II; Invitrogen). Membranes were blocked in Tris-buffered saline with Tween (TBS-T) with LI-COR (Lincoln, NE, USA) blocking buffer (1:1) and washed in TBS-T. Immunological detection was obtained by incubating overnight (4 °C) with primary antibodies. The primary monoclonal antibody used for CFTR identification is raised against the carboxy terminus of the human CFTR protein (R&D systems, Minneapolis, MN; dilution 1:1000). For detection of dual phosphorylated (active) extracellular-regulated kinase (ERK) 1/2, monoclonal phospho-p44/42 Mitogen activated protein kinase (MAPK; Thr202/Tyr204) from Cell Signaling (Beverly, MA, USA; dilution 1:2000) was used. For detection of total ERK 1/2, membranes were probed with polyclonal anti-MAPK (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:1000). Following washing, membranes were incubated for 1 h with goat anti-mouse and/or anti-rabbit secondary antibodies conjugated to Alexa IRDye 680 or IRDye 800CW (LI-COR). Phosphorylated and general ERK 1/2 were detected on the same blot at 680 and 800 nm respectively. Active (dual phosphorylated) ERK1 and ERK2 were normalised to total ERK1 and ERK2 content. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

Immunocytochemistry CFTR immunoreactive cells were visualised in gills of FW- and SW-acclimated striped bass. Gills were fixed for 24 h at 5 °C in 4% paraformaldehyde, 0.9% NaCl, 5 mmol/l NaH_2PO_4 , pH 7.8 and then rinsed and stored in 70% ethanol until dehydrating and embedding in paraffin.

Sagittal gill sections of 5 μm were stained with the monoclonal CFTR antibody (1:25) mentioned earlier using the protocol described by Seidelin & Madsen (1999). The monoclonal $\alpha 5$ is directed against the chicken Na^+, K^+ -ATPase $\alpha 1$ subunit, is specific for a cytosolic epitope and reacts with all isoforms of distant species. It was developed by D M Fambrough, Johns Hopkins University, Baltimore, MD, USA (see Takeyasu *et al.* 1988) and used in a 1:80 dilution. A goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was used in a 1:200 dilution (Zymed, InVitrogen).

Statistical analysis

All data were analysed by one- or two-way ANOVA followed by pairwise comparison of treatment means using Tukey's procedure in the case of significant ANOVA effects. Data were analysed using the GraphPad Prism software (San Diego, CA, USA). Statistical significance was accepted when $P < 0.05$.

Results

Amplicons and sequence analyses

The sequences of the amplicons obtained using ssCFTR-I and $\alpha\text{-Na}^+, \text{K}^+$ -ATPase primers were submitted to GenBank under the Accession numbers DQ478441 and DQ522301 respectively. They had the expected sizes of 394 and 950 bp (Fig. 1) and were 99% identical with Atlantic salmon CFTR-I (in 3'-UTR region; Chen *et al.* 2001) and 99% identical with tilapia (*O. mossambicus*, Accession number U82549) Na^+, K^+ -ATPase $\alpha 1$ -subunit respectively. PCR using the β -actin primers produced one amplicon with the expected size of 294 bp (Fig. 1).

Experiment 1: effect of FW–SW transfer

There were overall effects of salinity and time on serum $[\text{Na}^+]$ and MWC. Relative to the FW–FW sham group, transfer to SW induced an increase in serum $[\text{Na}^+]$ and a decrease in

MWC, which lasted for the duration of the experiment. Gill Na^+, K^+ -ATPase activity was overall affected by time but unaffected by salinity. CFTR mRNA expression was unaffected by time and salinity ($P = 0.06$). Time and salinity had an overall effect on $\alpha\text{-NKA}$ mRNA level. SW transfer induced an increase in $\alpha\text{-NKA}$ mRNA level compared with the FW–FW sham group (Fig. 2A–E).

Experiment 2: effect of cortisol

In FW fish, serum $[\text{Na}^+]$ decreased and MWC was unchanged by cortisol treatment *in vivo*. Gill CFTR and $\alpha\text{-NKA}$ mRNA levels and Na^+, K^+ -ATPase enzymatic activity were unchanged after cortisol treatment (Table 1).

Experiment 3: effect of cortisol, IGF-1 and EGF *in vitro*

Cortisol doses at and above 0.1 $\mu\text{g}/\text{ml}$ inhibited gill CFTR mRNA expression *in vitro* (one-way ANOVA, Tukey's test). Gill $\alpha\text{-NKA}$ mRNA levels were unchanged by cortisol. There was a decrease in $\alpha\text{-NKA}$ mRNA expression during the 24 h incubation relative to the pre-incubation control. Gill CFTR expression was unchanged in control and 0.01 $\mu\text{g}/\text{ml}$ cortisol relative to the pre-incubation control. There was an overall inhibitory effect of cortisol (two-way ANOVA, $P < 0.003$) and EGF (two-way ANOVA, $P < 0.001$) on gill CFTR expression, whereas IGF-I had no effect. There was an overall stimulatory effect of cortisol on gill α -subunit NKA expression (two-way ANOVA, $P < 0.004$), whereas IGF-I and EGF had no effect (Figs 3 and 4).

Experiment 4: effect of EGF *in vitro* on ERK 1/2 activity

Western blotting using the ERK 1/2-specific antibody and an antibody specific to the dual phosphorylated kinase revealed two protein bands of 47 (ERK1) and 43 kDa (ERK2). EGF stimulated ERK 1/2 significantly ($P < 0.01$), whereas IGF-I had no effect. Treatment of gill cells with PMA induced ERK 1/2 activation that was completely blocked by chelerythrine (Figs 5 and 6).

Immunoblotting and cytochemistry

Western blotting using an anti-human CFTR antibody revealed one band at 146 kDa (not shown), with no significant difference between FW and SW gill preparations (relative intensities: FW 1.00 ± 0.18 ; SW 1.63 ± 0.37 ; $P = 0.20$, *t*-test, $n = 3$). Immunocytochemistry predominantly stained large epithelial cells located in the interlamellar space (FW and SW) and at the base of the lamellae (FW). Faint staining was also observed in pavement cells of the lamellae. Staining intensity and distribution were equal in cells from FW and SW fish. When parallel gill sections (approximately 30 μm apart) were stained with an anti- Na^+, K^+ -ATPase (α -subunit) antibody, immunoreactivity was observed in the same locations of the filament as observed for the CFTR protein (Figs 7 and 8).

Figure 1 Gel electrophoresis of PCR products using CFTR primers and β -actin (lane B) or α -subunit Na^+, K^+ -ATPase and β -actin primers (lane C). Lane A shows 100 bp DNA ladder.

Discussion

CFTR and salinity change

Previous studies have shown a well-developed ability of striped bass to acclimate to salinities ranging from FW to full-strength SW. Madsen *et al.* (1994) and Tipsmark *et al.* (2004) reported very small and short lasting (<24 h) deflections of ion-osmotic homeostasis, when fish were transferred between FW and SW in either direction. They concluded that rapid, post-transcriptional regulation of ion-transport function was responsible for this ability. In the present study, FW-SW transfer caused deflections in MWC and serum [Na⁺] that were larger than in the previous studies. The difference may be due to differences in the experimental details regarding season (August–December versus January), size of fish (>200, 10–20 vs 30–60 g) and stock of fish. It is also possible that the present experimental set-up using relatively small SW/sham transfer tanks (ten fish per 60 l tank) may have elicited a stronger and longer lasting stress response. Striped bass are known to be extremely stress sensitive and to show haematological responses within minutes to hours after a stressful experience (Young & Cech 1993, Wallin & Vandenavyle 1995). The lack of change in overall gill Na⁺,K⁺-ATPase activity in response to SW transfer is in accordance with the previous studies. However, in the present study, α-NKA mRNA levels were significantly elevated 6–96 h post-transfer. This deviates from Tipsmark *et al.* (2004) in which α-NKA mRNA was unchanged. The difference may be due to the different methods and probes used for analysing α-NKA mRNA levels (RT-PCR using consensus α-subunit primers versus northern blotting using seabass α-subunit cDNA probe). Based on the sequence of the present striped bass α-NKA amplicon, the present primers seem to amplify all known α1 isoforms equally well, judged by identities corresponding to *O. mykiss* α1a, 82%; α1b, 86% and α1c, 83% (Richards *et al.* 2003). This is not surprising, since the primers share high homology with these isoforms in rainbow trout. It is however not known at present to what degree these isoforms are expressed in striped bass. Curiously, the sequenced cDNA sequence shares higher identity to tilapia (99%, *O. mossambicus*) than to goldlined sea bream (90%, *Rhabdosargus sarba*). The European seabass (*D. labrax*) probe used by Tipsmark *et al.* (2004) shows the highest identity with *O. mykiss* α3 isoform (84%) compared with 77, 76 and 74% for α1b, α1c and α1a isoforms respectively. Thus, the present study indicates that even though overall gill Na⁺,K⁺-ATPase activity does not change, there may be a significant re-equipment with α1-subunit isoforms following SW transfer.

Figure 2 Effect of FW-SW (closed bars) and FW-FW sham (open bars) transfer on plasma [Na⁺] (A), muscle water content (MWC) (B), gill Na⁺,K⁺-ATPase activity (C), gill CFTR (D) and α-Na⁺,K⁺-ATPase (E) mRNA levels in striped bass. mRNA levels are normalised to β-actin mRNA levels. Asterisk indicates significant difference from corresponding sham group ($P < 0.05$).

Table 1 Effect of *in vivo* cortisol treatment on plasma $[Na^+]$, muscle water content, cystic fibrosis transmembrane conductance regulator and α -subunit Na^+, K^+ -ATPase mRNA (relative to β -actin) and Na^+, K^+ -ATPase enzymatic activity in the gills of FW striped bass. Values are means $\pm$ S.E.M. (n).

	Control	Cortisol
Plasma $[Na^+]$ (mmol/l)	137.3 $\pm$ 2.3 (10)	125.4 $\pm$ 3.4 (10)*
Muscle water content (%)	77.14 $\pm$ 0.19 (10)	77.32 $\pm$ 0.18 (10)
CFTR: β -actin mRNA ratio	1.00 $\pm$ 0.26 (5)	0.71 $\pm$ 0.06 (5)
α - Na^+, K^+ -ATPase: β -actin ratio	1.00 $\pm$ 0.09 (5)	0.94 $\pm$ 0.08 (5)
Na^+, K^+ -ATPase activity (μ mol/mg per h)	14.1 $\pm$ 0.9 (10)	12.7 $\pm$ 1.5 (9)

Asterisk indicates significant difference from control ($P < 0.05$).

The present study demonstrated that CFTR is expressed in gill tissue of the striped bass in both FW and SW and that there is no difference in mRNA levels, overall protein levels and cellular location of CFTR in the two media. Sequence data showed that the present CFTR amplicon is 99% homologue to the Atlantic salmon CFTR-I isoform. PCR was also attempted using primers specific for the Atlantic salmon CFTR-II isoform (developed by Singer *et al.* 2002) but without success. CFTR mRNA levels were unchanged after SW transfer (< 96 h), whereas α -NKA α -mRNA increased 6 h after transfer. This suggests that *de novo* synthesis of the CFTR protein is not initiated immediately in response to hyperosmotic conditions as reported in both killifish (Marshall *et al.* 1999, Scott *et al.* 2004) and Atlantic salmon

(Singer *et al.* 2002). In killifish, a prolonged increase in CFTR mRNA levels was found between 3 h and 10 days after BW–SW transfer (Scott *et al.* 2004), but CFTR protein was unchanged. In Atlantic salmon, CFTR-I expression increased 96 h after FW–SW transfer (Singer *et al.* 2002) and the protein levels were not analysed.

Western blotting with the CFTR antibody revealed a major band at 146 kDa, which compares well with data from mudskipper, *Periophthalmodon schlosseri* (150 kDa, Wilson *et al.* 2000), and killifish (150 kDa, Marshall *et al.* 2002, 160 kDa, Scott *et al.* 2004) using the same antibody. The lack of difference in the abundance of the CFTR protein in FW and SW gills was supported by the immunocytochemical data,

Figure 3 Effect of 24 h incubation *in vitro* with cortisol (0–10 μ g/ml) on CFTR (A) and α - Na^+, K^+ -ATPase (B) mRNA levels in FW-striped bass gill tissue. mRNA levels are normalised to β -actin mRNA levels. Preinc. refers to mRNA levels in gill tissue pre-incubated without hormone for 2–3 h. Asterisk indicates significant difference from the corresponding control (Con) value ($P < 0.05$). Dagger indicates significant difference from pre-incubation control value ($P < 0.05$).

Figure 4 Effect of 24 h incubation *in vitro* with cortisol (F, 10 μ g/ml), IGF-I (10 μ g/ml), EGF (10 μ g/ml) and combined F + IGF-I or F + EGF on gill CFTR (A) and α - Na^+, K^+ -ATPase (B) mRNA levels in FW-striped bass gill tissue. RNA levels are normalised to β -actin mRNA levels. Preinc. refers to mRNA levels in gill tissue pre-incubated without hormone for 2–3 h. Asterisk indicates significant difference from the corresponding control (Con) value ($P < 0.05$). Dagger indicates significant difference from pre-incubation control value ($P < 0.05$).

Figure 5 Western blot showing detection of dual phosphorylated ERK 1/2 (A) and general ERK 1/2 (B) in gill cells treated for 20 min with control Ringer's (lane 1), PMA (lane 2; 1 μmol/l), PMA + chelerythrine (lane 3; 4 μmol/l), EGF (lane 4; 5 μg/ml) and IGF-I (lane 5; 5 μg/ml) FW and SW gill membrane fractions immunostained with hCFTR antibody. Bars designate molecular weight markers (kDa).

which showed unchanged immunoreactivity in large cells at the base of the lamellae and in the interlamellar space of both FW and SW gills. Strong antigen recognition with the anti-CFTR antibody was observed in the same positions in the filament section where Na^+,K^+ -ATPase immunoreactivity

Figure 6 Effect of 20 min incubation of gill cells with control Ringer's, PMA, PMA + chelerythrine (Chel), EGF and IGF-I on dual phosphorylated (active) ERK1 (A) and ERK2 (B) normalised to total ERK 1/2. Asterisks indicate significant difference from control ($P < 0.05$).

Figure 7 CFTR immunostaining in striped bass gill. (A) FW gill, (B) SW gill. Arrows point to immunostaining at the base of the lamellae and in the interlamellar region of the filament. No difference in staining pattern was observed between FW and SW gills. Bar = 50 μm.

occurred, suggesting that these two proteins are located within the same type of cells, presumably chloride cells. Except for faint staining of lamellar pavement cells, immunocytochemistry using the CFTR antibody confirms that there are virtually no lamellar chloride cells in either FW- or SW-striped bass (Madsen *et al.* 1994). The similar CFTR staining pattern and intensity in FW and SW gills is very different from the situation in killifish (Marshall *et al.* 2002), Hawaiian goby (McCormick *et al.* 2003), eel (*Anguilla anguilla*; Wilson *et al.* 2004) and tilapia (Hiroi *et al.* 2005), where both pattern and intensity of staining change significantly in response to salinity shifts. The lack of change in overall CFTR expression and Na^+,K^+ -ATPase protein abundance and activity (Tipmark *et al.* 2004) during SW acclimation, supports our previous hypothesis that striped bass maintain their capacity to move from FW into SW at any time by maintaining sufficient levels of these ion-transport proteins in the gill. The maintenance of latent ion-transport

Figure 8 CFTR (A) and α -subunit Na^+,K^+ -ATPase (B) immunostaining in two parallel sections of SW-striped bass gill approximately 30 μm apart. Arrows point to similar locations of the two immunoreactions. Bar = 50 μm .

machinery seems an energetically 'expensive' solution to euryhalinity but may be rewarded by an extraordinary good ability to move between salinities without suffering major transient osmotic stress which may impact growth and reproduction. Alternatively, both these proteins could be involved in ion uptake by moving apical CFTR to basolateral membranes where it may work in conjunction with Na^+,K^+ -ATPase to facilitate Cl^- entry into the blood. This hypothesis is compatible with the observation that $\text{Na}^+,\text{K}^+,\text{2Cl}^-$ cotransporter is more abundant in SW gills than in FW gills of striped bass (Tipsmark *et al.* 2004). By removing/inactivating the cotransporter protein and simultaneously moving CFTR from an apical to a basolateral subcellular location, a SW-type chloride cell may efficiently be turned into a FW-type chloride cell (see Marshall 2002). A similar chloride cell shift/'re-use' model has been suggested for the Hawaiian goby, another teleost with a high euryhaline capacity (McCormick *et al.* 2003). Using the present enzymatic immunostaining methodology, however, staining is not necessarily confined to the subcellular site of the targeted antigens, as the reaction product may diffuse away from the antigen-antibody complexes. The present technique therefore did not allow us to reach a conclusion about the presence and redistribution of CFTR protein between subcellular domain(s). In most teleosts studied, gill CFTR protein abundance is downregulated in FW compared with SW, suggesting a pivotal role of the CFTR in salt excretion.

However, Marshall *et al.* (2002) recently gave evidence that in killifish, salinity shifts induced a characteristic redistribution of CFTR protein within existing chloride cells, from an apical location in SW to a more diffuse and basolateral location in FW. This may also be the case in striped bass, and opens the possibility of re-using the same chloride cells in FW and SW with only minor redistribution of the most critical ion-transport proteins.

Cortisol effects

Cortisol is implicated in the endocrine signalling response during both FW and SW acclimation in several fish (see Evans 2002). In the case of striped bass, plasma cortisol increases shortly after SW transfer (Madsen *et al.* 1994) and may thus be implicated as a potential SW-adapting hormone. Therefore, it was unexpected that cortisol had a strong inhibitory effect on gill CFTR mRNA levels when tested *in vitro* but not *in vivo*. The physiological significance of this effect is unknown, but may reflect an increased turnover of the CFTR mRNA. By comparison, cortisol slightly stimulated α -NKA mRNA when tested *in vitro*. CFTR mRNA levels were unchanged after 24 h control incubation indicating high stability of the transcript. This is in contrast to the pronounced decrease in α -NKA mRNA during control incubation and suggests differential stability, dynamics and regulation of the two transcripts in the gill. In other fish studies, cortisol has been assigned a role in stimulation of CFTR expression after SW transfer, even though the evidence is still inadequate. In killifish, the evidence is correlative and based on measurements of plasma cortisol and CFTR expression ($n=1$) during a time course of SW acclimation (Marshall *et al.* 1999). In FW Atlantic salmon, CFTR-I mRNA levels were unaffected by 5-day cortisol implants; however, when fish subsequently were sham transferred to FW, CFTR-I transcript levels increased after 24 h (Singer *et al.* 2003). Interestingly, in mouse endometrium, hyperaldosteronaemia induced by a low-sodium diet depresses CFTR and increases ENaC channel expression (Tsang *et al.* 2004).

Putative glucocorticoid-binding sites have been identified in the promoter region of the *F. heteroclitus* CFTR gene (Schulte & Keir 2003), which suggests that cortisol is a component in the regulation of CFTR expression. However, Schulte and Keir (2003) reported that these binding sites were non-functional when tested in cell culture and transgenic fish. In separate studies, cortisol stimulated proliferation of SW-type chloride cells and associated Na^+,K^+ -ATPase and $\text{Na}^+,\text{K}^+,\text{2Cl}^-$ cotransporter expression both at mRNA and protein level (see McCormick 1995). Cortisol also stimulates ion uptake *in vivo* and across cultured gill epithelia *in vitro* (McCormick 2001, Kelly & Wood 2002, Zhou *et al.* 2003). Since cortisol is the predominant circulating corticosteroid in teleosts, one way of differentiating cortisol signalling into SW- or FW-adaptive effects may be achieved via activation of gluco- and mineralocorticosteroid receptors respectively (Sloman *et al.* 2001).

IGF-I, EGF and the ERK 1/2 signalling pathway

Stimulation of protein kinase C (PKC) with phorbol esters activates the Ras–ERK signalling pathway in mammalian systems (Schonwasser *et al.* 1998), and the PKC–ERK cascade is also functional in teleost lymphocytes (MacDougall *et al.* 1999) and pituitary cells (Gur *et al.* 2002). Our finding that chelerythrine blocks the strong stimulation of ERK 1/2 by PMA indicates a functional ERK pathway in gill cells as well. Transient activation of map kinases (ERK1 and p38) has been reported in killifish gill and opercular membrane during exposure to hypo- and hyperosmotic stress *in vivo* and *in vitro* (Kültz & Avila 2001, Marshall *et al.* 2005). Activation of MAPKs may thus be a central event in transducing osmosensory as well as endocrine signals in the gill. Within the time frame of the present *in vitro* incubations, IGF-I had no effect on gill CFTR or α -NKA mRNA expression. This is consistent with the lack of effect of IGF-I on gill cell ERK 1/2 activity; however, it does not exclude the presence of IGF-I-regulated ERK phosphorylation in fish gills, as the response may be strongly time dependent, as seen in rat myoblasts (Adi *et al.* 2002). IGF-I receptors are present in striped bass gill epithelial cells, where they co-localise with Na^+ , K^+ -ATPase and Na^+ , K^+ , 2Cl^- cotransporter protein (Tipsmark *et al.* 2007), but no previous studies have examined IGF-I effects on gill CFTR expression and ERK 1/2 activity. In mammals, ERK activation is indeed one of the possible intracellular pathways for IGF-I action (LeRoith *et al.* 1995). Recently, IGF-I has been shown to be a hyper-osmoregulatory hormone in striped bass and liver IGF-I expression and plasma IGF-I levels decline when striped bass are transferred to SW (Tipsmark *et al.* 2007). This is in accordance with the observation that IGF-I stimulates PRL release from striped bass pituitaries (Fruchtman *et al.* 2000), which may add to the hyper-osmoregulatory effect (Jackson *et al.* 2005). This is in strong contrast to its hypo-osmoregulatory effect in salmonids, killifish and tilapia (Mancera & McCormick 1998), where IGF-I stimulates gill Na^+ , K^+ -ATPase protein abundance and α -mRNA expression (Madsen *et al.* 1995, Mancera & McCormick 1998, Seidelin & Madsen 1999). The striped bass is thus an interesting additional model to include in studies of teleost osmoregulatory endocrinology.

Only one study has previously reported EGF effects on ion transport in fish. Zadunaisky *et al.* (1995) found that EGF stimulated Na^+ /H $^+$ exchange in killifish opercular membrane which may promote salt absorption. Here, we show that EGF significantly decreased CFTR transcript levels in the striped bass gill, independent of the presence of cortisol. By contrast, α -NKA α -mRNA was unaffected by EGF. The effect is accompanied by strong activation of the ERK 1/2 pathway in gill cells indicating an EGF signalling pathway similar to that for several mammalian systems (Zhang & Liu 2002). In mammals, EGF stimulates active Na^+ reabsorption by alveolar epithelial cells by stimulating both α - and β -subunit Na^+ , K^+ -ATPase mRNA expression (Danto *et al.* 1998). In contrast, EGF receptor activation by

interferon- γ inhibits intestinal Cl^- secretion, in part via downregulation of CFTR and Na^+ , K^+ -ATPase activity and expression (Uribe *et al.* 2002). EGF was recently shown to reduce Na^+ transport in ARPKD collecting duct and in distal kidney cells, to reduce steady-state mRNA levels of epithelial sodium channels (ENaC) by 50–75% (Veizis & Cotton 2005) and to reduce ENaC open probability via tyrosine kinase-mediated events (Tong & Stockand 2005). In addition to that, acute inhibition of ENaC by EGF has been reported to involve ERK phosphorylation in mouse collecting duct cells (Falin & Cotton 2006) and chronic exposure to EGF affects trafficking and decreases surface expression of ENaC in cystic fibrosis cells derived from human airway epithelium (Cao *et al.* 2005). Future studies are warranted in order to clarify whether EGF has overall FW- or SW-adaptive effects on fish osmoregulation and ion transport.

In conclusion, we identified the CFTR protein in gill filament epithelial cells in the striped bass. Overall CFTR protein and transcript levels were unaffected by salinity, confirming that striped bass salinity acclimation largely involves rapid non-transcriptional changes of gill function. Gill CFTR and Na^+ , K^+ -ATPase transcript levels were differentially affected in response to salinity and hormones, despite the putative involvement of both in salt excretion. IGF-I had no effect, whereas cortisol and EGF downregulated CFTR transcript levels in the gill. The study gives evidence of EGF as an ion-transport hormone in striped bass gill and suggests that activation of the ERK 1/2 pathway is a part of the signalling mechanism.

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