The Journal of Experimental Biology 212, 3994-4001
Published by The Company of Biologists 2009
doi:10.1242/jeb.037275

Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon

S. D. McCormick*, A. M. Regish and A. K. Christensen

USGS, Conte Anadromous Fish Research Center, Turners Falls, MA, USA and Department of Biology, University of Massachusetts, Amherst, MA 01003, USA

*Author for correspondence (mccormick@umext.umass.edu)

Accepted 28 September 2009

SUMMARY

Gill Na^+/K^+-ATPase (NKA) in teleost fishes is involved in ion regulation in both freshwater and seawater. We have developed and validated rabbit polyclonal antibodies specific to the NKA α1a and α1b protein isoforms of Atlantic salmon (*Salmo salar* Linnaeus), and used western blots and immunohistochemistry to characterize their size, abundance and localization. The relative molecular mass of NKA α1a is slightly less than that for NKA α1b. The abundance of gill NKA α1a was high in freshwater and became nearly undetectable after seawater acclimation. NKA α1b was present in small amounts in freshwater and increased 13-fold after seawater acclimation. Both NKA isoforms were detected only in chloride cells. NKA α1a was located in both filamental and lamellar chloride cells in freshwater, whereas in seawater it was present only as a faint background in filamental chloride cells. In freshwater, NKA α1b was found in a small number of filamental chloride cells, and after seawater acclimation it was found in all chloride cells on the filament and lamellae. Double simultaneous immunofluorescence indicated that NKA α1a and α1b are located in different chloride cells in freshwater. In many chloride cells in seawater, NKA α1b was present in greater amounts in the subapical region than elsewhere in the cell. The combined patterns in abundance and immunolocalization of these two isoforms can explain the salinity-related changes in total NKA and chloride cell abundance. The results indicate that there is a freshwater and a seawater isoform of NKA α-subunit in the gills of Atlantic salmon and that they are present in distinct chloride cells.

Key words: osmoregulation, ion transport, mitochondrion-rich cell, *Salmo salar*.

INTRODUCTION

The sodium pump, Na^+/K^+-ATPase (NKA), is present in all animal cells, providing regulation of intracellular ionic gradients used for cellular homeostasis and secondary transport of other compounds (Skou and Esmann, 1992). It is present in especially high concentration in most epithelial cells that regulate ion transport for the whole organism such as the kidney, salt glands and gills (Epstein et al., 1967). In the gills of teleost fishes, NKA is present in specialized ion transport cells known as chloride or mitochondrion-rich cells. These cells are the site of ion uptake in freshwater and salt secretion in seawater (Foskett and Scheffey, 1982), and have the highest known cellular concentration of NKA, with over 100 million molecules per cell (Karnaky, 1986).

All teleosts maintain a nearly constant internal osmotic pressure, approximately one-third that of seawater, irrespective of whether they are in freshwater or seawater (Evans et al., 2005). In freshwater, fish must counteract the passive loss of ions, primarily sodium and chloride, and gain of water. They do this by absorbing ions from their food and actively taking up sodium and chloride across the gill. Excess water is excreted through production of highly dilute urine by the kidney and urinary bladder. Teleosts in seawater are in a constant state of impending dehydration and must counteract the passive gain of ions and loss of water. Teleosts address this challenge by drinking seawater and taking up both salt and water across the gut, while excess sodium and chloride are secreted by the gill. Thus, the transition from freshwater to seawater requires that the gill reverse its function from an ion uptake to a salt secretory organ.

There appear to be several mechanisms for apical absorption of sodium by the fish gill. Sodium–hydrogen exchange appears to be the predominant mechanism, either through sodium–hydrogen exchangers (NHE), or through an apical H^+-ATPase that generates a favorable electrical gradient for the entry of sodium through a sodium channel (ENaC) (Evans et al., 2005). There is also evidence that some teleost fish utilize an apical NaCl cotransporter for sodium uptake (Hiroi et al., 2008). NKA is likely to be involved in the basolateral efflux of sodium into the blood (McCormick, 1995).

Three major ion transport proteins are involved in sodium and chloride secretion by the gill when fish are in seawater. NKA pumps three sodium ions out of the cell while pumping in two potassium ions, making the inside of the chloride cell highly negative and low in sodium. The sodium gradient is then used by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) to bring chloride into the cell. Chloride subsequently leaves the cells on a favorable electrical gradient through an apical chloride channel, which has been shown to be a homolog of the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2002). In addition to providing the electrochemical gradient for chloride secretion, NKA also pumps sodium into the paracellular space where sodium leaves through loose junctions on a favorable electrical gradient. The three major ion transport proteins involved in salt secretion, NKA, NKCC and CFTR, have all been co-localized to chloride cells in several species of teleost fishes (McCormick et al., 2003; Hiroi et al., 2005).

NKA is composed of two essential subunits, α and β. The α-subunit is considered the main catalytic unit and contains all of the major binding sites for ATP, Na^+, K^+ and ouabain (a specific inhibitor

of the enzyme). The β-subunit is a glycosylated polypeptide that assists in folding and positioning of the protein into the basolateral plasma membrane. A third subunit termed γ, also known as FXYD, is not necessary for the catalytic function of NKA, but apparently acts to adapt the kinetic properties of sodium and potassium transport for the functions of different cell types (Garty and Karlish, 2006). Within the α-subunit there are at least four isoforms that are expressed in vertebrates; these have different kinetic properties for Na^+, K^+ and ATP binding, and vary in their inhibition by ouabain and calcium, and their regulation by intracellular second messengers (Blanco and Mercer, 1998). These unique properties along with their cell-specific distribution suggest that the NKA isoforms have distinct physiological function and regulation.

Recently, Richards and colleagues (Richards et al., 2003) provided molecular genetic evidence that there are several isoforms of the α-subunit expressed in the gill tissue of rainbow trout. The mRNA level of the NKA α1a isoform increases after transfer from seawater to freshwater, and that of the α1b isoform increases after freshwater to seawater transfer. Similar effects of salinity on these isoforms have been found in Atlantic salmon (*Salmo salar* Linnaeus); furthermore, the mRNA level of gill NKA α1a decreases and that of NKA α1b increases in freshwater during the parr–smolt transformation when the salinity tolerance of juveniles increases (Nilsen et al., 2007). To date, however, the evidence for these salinity-specific α1 isoforms is based solely on changes in mRNA levels, and there has been no characterization or localization of the proteins themselves. In order to begin characterization of these NKA isoforms we developed and validated antibodies specific for NKA α1a and NKA α1b. We then examined the abundance and localization of each isoform in gill tissue of juvenile Atlantic salmon as a function of environmental salinity.

MATERIALS AND METHODS

Animals and experimental protocols

Juvenile Atlantic salmon (*Salmo salar*) were obtained from the Kensington State Hatchery (CT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in autumn. Fish were reared in 1.7 m diameter tanks supplied with ambient river water at a flow rate of 4 l min^{-1} and provided with supplemental aeration. They were maintained under natural photoperiod conditions and fed to satiation (Zeigler Bros, Gardners, PA, USA) using automatic feeders. For acclimation experiments conducted in the autumn, 0+ parr (10–20 g) were placed in 1.5 m diameter tanks at 10–12°C with particle and charcoal filtration and continuous aeration with either freshwater or seawater. Because Atlantic salmon parr cannot tolerate direct transfer to high salinity, they were first placed in 20 p.p.t. seawater, and salinity was gradually increased to 30 p.p.t. over a 2 week period. Fish were then held at 30 p.p.t. for at least 2 weeks prior to sampling. Fifty per cent water changes occurred at least once per week. During the acclimation experiments fish in freshwater and seawater were fed *ad libitum* once daily.

Food was withheld for 24 h prior to sampling of fish, which occurred between 10:00 and 12:00 h Eastern Standard Time. Fish were anesthetized with tricaine methanesulfonate and blood was drawn from the caudal vessels into a 1 ml ammonium heparinized syringe, spun at 3200 ***g*** for 5 min at 4°C, and plasma was aliquoted and stored at –80°C. Gill arches were removed, the gill filaments trimmed from the ceratobranchials and placed in a 1.5 ml microcentrifuge tube and frozen immediately at –80°C for western blots. Four to six gill filaments were placed in 100 µl of ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen at –80°C within 30 min for measurement of NKA activity. Similarly sized gill samples were placed in 4% paraformaldehyde in 10 mmol l^{-1} phosphate-buffered saline (PBS) at room temperature for 2 h. Fixed gill tissue was then rinsed in PBS, placed in PBS with 30% (w/v) sucrose overnight at 4°C, and subsequently frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA, USA).

Antibody production and purification

To develop isoform-specific antibodies to Atlantic salmon NKA α1a and NKA α1b, we used protein sequence data provided by T. O. Nilsen (University of Bergen, Norway). A ClustalW alignment (EMBL-EBI) of these sequences showed that Atlantic salmon NKA α1a and NKA α1b protein sequences share a high degree of sequence similarity with each other as well as with other NKA isoforms expressed in the gill (NKA α1c and NKA α3). To obtain antibodies for each protein that would not cross-react, we selected epitopes from the central isoform-specific regions of NKA α1a and α1b that would provide both high specificity and sufficient immunogenicity in polyclonal antibody production. The peptides were made from the amino acids IHENNTAGESNHL for NKA α1a and IHKNIMAGESKHL for NKA α1b. The peptide sequences differ in 5–6 of 13 amino acids when compared with NKA α1c and 11 of 13 amino acids for NKA α3, which are also expressed in salmonid gill tissue. A search of the translated sequence of these peptides in BLAST (NCBI) for salmonids and GRASP (Genomic Research on Altlantic Salmon Project; http://grasp.mbb.sfu.ca) databases showed no additional proteins with strong similarities. Peptides were synthesized, purified, and sequenced to verify amino acid composition, and then conjugated to an immune carrier. Two rabbits per antigen were given five immunizations and serum was collected from five production bleeds and a final exsanguination (21st Century Biochemicals, Marlborough, MA, USA). Serum was then affinity purified using NKA α1a- and NKA α1b-specific peptides bound to Sulfolink Coupling Gel (Pierce, Rockford, IL, USA). Each affinity-purified antibody pool was then immunodepleted using Optimized Affinity Resin (OAR, 21st Century Biochemicals) which had been coupled to the peptide corresponding to the other NKA isoform. The unbound (immunodepleted) portion was then used for all experiments.

An antibody to a conserved region of the NKA α-subunit that has been widely used in studies of teleost chloride cells (α5) was obtained from the Iowa Hybridoma Bank. Alexa-labeled goat anti-rabbit and anti-mouse antibodies (Invitrogen Molecular Probes, Carlsbad, CA, USA) were used for immunohistochemistry.

Western blots

Ion transporter abundance was quantified by Western immunoblotting as previously outlined (Pelis and McCormick, 2001). Gill tissue was homogenized in 10 volumes of PBS, containing 30% sucrose (w/v), 2 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and Complete Mini protease inhibitor tablets (Roche, Indianapolis, IN, USA). Homogenates were centrifuged at 5000 ***g*** for 10 min at 4°C. The supernatant was then centrifuged at 20,000 ***g*** for 10 min at 4°C. The resulting supernatant was centrifuged at 48,000 ***g*** for 2 h at 4°C. The final pellet was resuspended in homogenization buffer plus 0.1% Triton X-100 (LabChem, Pittsburg, PA, USA). Protein concentrations were determined by BCA (bicinchoninic acid) Protein Assay (Pierce). Samples were then placed in an equal volume of 2× Laemmli buffer, heated for 15 min at 60°C and stored at –80°C. Samples were thawed and run on a 6.75% SDS-PAGE gel at 10 µg protein per lane for NKA α1a and 5 µg protein per lane for NKA α1b with 10 µg

Precision Plus protein standards in a single reference lane (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dry milk for 1 h at room temperature, rinsed in PBST, and exposed to primary antibody in PBST and 5% non-fat dry milk for 1 h at room temperature. After rinsing in PBST blots were exposed to goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase diluted 1:10,000 in antibody dilution buffer, for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 µmol l^{-1} coumaric acid, 2.5 mmol l^{-1} luminol, 100 mmol l^{-1} Tris-Cl pH 8.5) and ECL B (0.018% H_2O_2, 100 mmol l^{-1} Tris-Cl pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). Digital photographs were taken of individual gels and band staining intensity measured using ImageJ (NIH, Bethesda, MD, USA); protein abundance is expressed as a cumulative 8-bit gray scale value. We evaluated the linearity of western blot quantification by loading protein levels of 2.5, 5, 10 and 15 µg and found a strong linear relationship (R^2=0.99).

Immunohistochemistry

Sections (5 µm thick) were cut in a cryostat at –24°C, parallel to the long axis of primary filaments, and perpendicular to the attachment of secondary lamellae. The tissue was placed on Fisherbrand Colorfrost/Plus slides (Fisher Scientific, Hampton, NH, USA), dried, rinsed with PBS and then incubated in 2% normal goat serum in PBS for 30 min at room temperature. Slides were exposed to primary antibody (anti-NKA α1a, 8 µg ml^{-1}; anti-NKA α1b, 1 µg ml^{-1}; α5, 1:1500) in antibody dilution buffer (0.01% NaN_3, 0.1% BSA, 2% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS) and incubated overnight at 4°C. The slides were rinsed several times with PBS and exposed to fluorescently labeled secondary antibody for 2 h at room temperature. For NKA α1a and NKA α1b colocalization experiments, antibodies were immunolabeled with fluorophore-conjugated Fab fragments from the Zenon Kit according to the manufacturer's instructions (Invitrogen). After incubation the slides were rinsed several times with PBS, covered by a coverslip and examined with a Nikon Diaphot-TMD inverted fluorescence microscope with a mercury lamp. For confocal microscopy the above procedure was modified by labeling 20 µm thick tissue sections, using the following antibody concentrations (anti-NKA α1a, 10 µg ml^{-1}; anti-NKA α1b, 1 µg ml^{-1}; α5, 1:1000) and increasing incubation time in secondary antibody to 4 h. Single *Z*-sections were acquired on a Zeiss 510C META confocal microscope and processed with ImageJ. The labeling intensity of NKA α1a and NKA α1b was obtained by applying the ImageJ Fire LUT (lookup table) to the red channel.

From each fish, immunoreactive chloride cells on the primary filament and secondary lamellae (tallied separately) were counted from sagittal sections of gill filament (700 µm of primary filament/sagittal section) and expressed per millimeter of primary filament. Mean numbers of chloride cells for each group were obtained using the means calculated from each fish. Cell area (µm^2 per cell) was obtained from immunoreactive chloride cells using MetaMorph 4.1.2 (Universal Imaging Corporation, Downingtown, PA, USA). At least 50 immunoreactive chloride cells from several different tissue sections were analyzed from each of at least 6 fish. Fifty cells were not available in some cases (e.g. lamellar NKA α1a-positive chloride cells in seawater) so all available cells were counted and used for analysis.

Gill Na^+/K^+-ATPase activity

Activity in gill homogenates was determined using a temperature regulated microplate method (McCormick, 1993). In this assay, ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presense and absence of 0.5 mmol l^{-1} ouabain. Samples (10 µl) were run in duplicate in 96-well microplates at 25°C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). Protein concentration of the homogenate was determined using a BCA protein assay.

Statistics

Differences between freshwater and seawater-acclimated fish were determined using one-way analysis of variance (ANOVA) for protein abundance and NKA activity, and three-way ANOVA (isoform, salinity, and filamental *vs* lamellar location) for cell number and size, using $P<0.05$ as the criterion for rejecting the null hypotheses. Following detection of significant interaction, the Tukey HSD test was used to examine differences among groups. For all parameters assumptions of normality and equal variance were met.

RESULTS

Three of 20 fish died during the seawater acclimation period, but none died in the week prior to sampling. Plasma chloride levels were 134±0.8 mmol l^{-1} in freshwater and 144±2.7 mmol l^{-1} in seawater, indicating the fish had acclimated to the higher salinity.

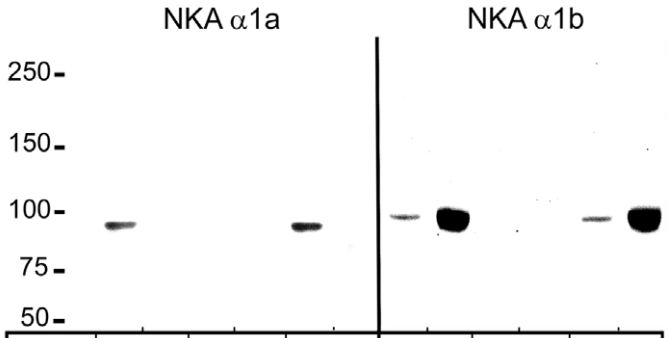
To ensure that the affinity-purified antibodies were isoform specific, we conducted western blot peptide competition assays. The highest working dilution of antibody was determined, and then a 200-fold molar excess of peptide was incubated with the affinity-purified antibodies prior to their use in western analysis. The NKA α1a peptide specifically blocked NKA α1a immunoreactivity, whereas the NKA α1b peptide had no effect (Fig. 1). Similarly, the NKA α1b peptide specifically abolished the NKA α1b immunoreactive band, whereas the NKA α1a peptide did not (Fig. 1). These results demonstrate that both the NKA α1a and NKA α1b affinity-purified antibodies are specific to their intended isoform.

NKA α1a immunoreactivity appeared as one major band at an apparent molecular mass of 91 kDa and occasionally a second less intense band at approximately 86 kDa (Figs 1 and 2). NKA α1a was abundant in Atlantic salmon parr in freshwater and decreased by more than 95% after seawater acclimation, often becoming undetectable (Fig. 3).

NKA α1b immunoreactivity was present as a single band at an apparent molecular mass of 94 kDa in freshwater (Fig. 2). After seawater acclimation the abundance of NKA α1b increased (Figs 1–3). In seawater-acclimated fish, the 94 kDa band was the primary form, while a lesser band of 89 kDa was occasionally detected (Fig. 2).

We also examined the abundance of NKA using an antibody that was made to a conserved region of the α-subunit of NKA. This 'pan-α' antibody detected two or three bands of apparent molecular mass between 86 and 94 kDa. After seawater acclimation of Atlantic salmon parr, the abundance of total NKA α-subunit (detected by the pan-α, α5 antibody) increased 4.1-fold (Figs 2 and 3). Gill NKA activity was 1.9 µmol ADP mg^{-1} protein h^{-1} in freshwater parr and increased to 13.9 µmol ADP mg^{-1} protein h^{-1} after seawater acclimation, a 7-fold increase.

Immunolocalization of NKA with pan-α (α5) antibody showed staining specific to chloride cells present on both the filament and lamellae (Fig. 4). Seawater acclimation resulted in the appearance



FW/SW	FW	SW	FW	SW	FW	SW	FW	SW
Ab	NKA α1a		NKA α1a		NKA α1a	NKA α1b		NKA α1b	NKA α1b
Peptide	-		NKA α1a		NKA α1b	-		NKA α1b	NKA α1a

Fig. 1. Western blot validation of NKA α1a- and NKA α1b-specific antibodies in gill tissue of Atlantic salmon. Each antibody was pre-incubated without peptide (first two lanes), with the target peptide (next two lanes), and with the non-target peptide (final two lanes). Validations were run for gill tissue from both freshwater (FW) and seawater (SW)-acclimated Atlantic salmon. Size markers are in kDa.

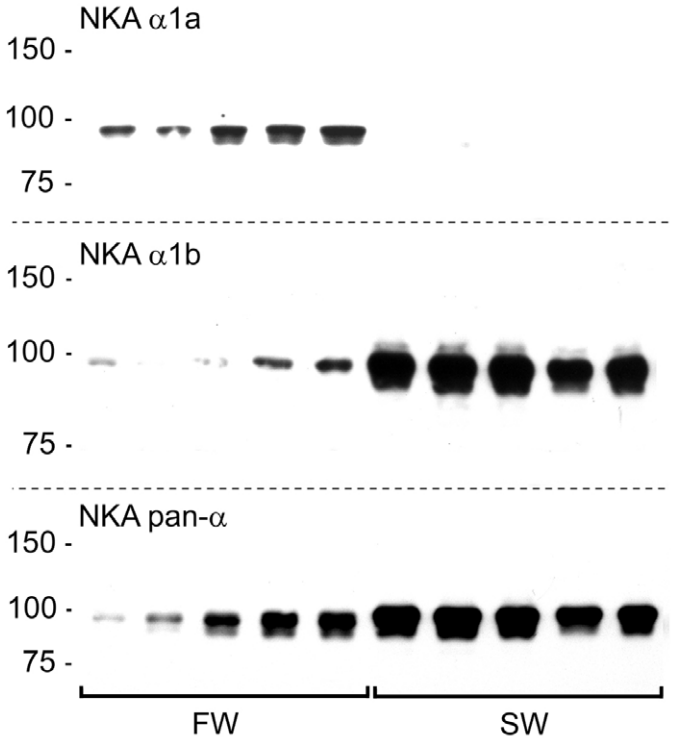


Fig. 2. Representative western blots of NKA α1a (upper), NKA α1b (middle) and NKA pan-α (lower) immunoreactivity in gill tissue of freshwater and seawater-acclimated Atlantic salmon parr. Size markers are in kDa.

of larger chloride cells that were primarily located on the filament. Control sections with no primary antibody had no immunofluorescence signal in gill tissue from freshwater or seawater-acclimated fish.

In freshwater, NKA α1a staining occurred specifically in chloride cells and was not detectable in other cell types within the gill (Fig. 4). Staining occurred in both filamental and lamellar chloride cells. Most chloride cells in freshwater stained positive for both NKA α1a and pan-α (orange cells in merged image, Fig. 4), with the exception of approximately 10% of the filamental chloride cells, which were positive for NKA pan-α but did not stain for NKA α1a (green cells in merged image, Fig. 4). NKA α1a immunoreactivity in gill tissue was largely absent in seawater-acclimated fish; there was a barely detectable immunofluorescence in chloride cells, which was difficult to distinguish from background (Fig. 5). The total number of NKA α1a-immunoreactive chloride cells (filamental and lamellar) decreased by 85% after seawater acclimation.

In freshwater, NKA α1b immunofluorescence occurred in a small number of chloride cells on the filament (Fig. 4). These cells also stained positively for NKA pan-α (orange cells in merged image). After seawater acclimation, the immunofluorescence signal for NKA α1b increased dramatically and was present in all chloride cells; all cells that stained positively for NKA α1b also stained positively for NKA pan-α. Cells that stained positively for NKA α1b in seawater were 2-fold larger in cross-sectional area (Table 1), and stained more intensely, than both NKA α1a- and NKA α1b-positive cells in freshwater (Figs 4 and 6). The number of chloride cells on the lamellae decreased after seawater acclimation, but these were almost all positive for NKA α1b (Figs 4 and 5). Control slides incubated with secondary antibody showed no immunofluorescence.

Co-localization with directly labeled NKA α1a and NKA α1b antibodies indicated that in freshwater these two isoforms were not present in the same chloride cells (Fig. 6). NKA α1a was present in most of the chloride cells of the filament and lamellae, whereas NKA α1b was restricted to a small number of small filamental chloride cells. These cells most often appeared to be covered by pavement cells or other chloride cells. In seawater, NKA α1b was the predominant isoform in all chloride cells, and NKA α1a immunofluorescence was detectable only as a dim staining in chloride cells that were also positive for NKA α1b. There was greater apparent intensity of NKA α1a in seawater using directly labeled antibodies (Fig. 6) relative to the use of secondary antibodies (Fig. 4), probably due to the higher concentration of primary antibody necessary when using directly labeled antibodies.

Using the pan-α antibody, staining was generally more intense in chloride cells in seawater relative to freshwater. For most chloride cells there was an intense and fairly uniform

Table 1. Size (cross-sectional area, μm^2) of NKA α1a- and NKA α1b-immunoreactive chloride cells on the filament and lamellae of freshwater and seawater-acclimated Atlantic salmon parr

	Filament		Lamellae	
	Freshwater	Seawater	Freshwater	Seawater
NKA α1a	45.6±1.7	69.0±5.4*	40.3±1.7	48.0±5.4
NKA α1b	44.1±0.5	87.2±6.3*	21.8±2.9	61.2±5.2*

Values are means ± standard error of 5 fish per salinity, with at least 50 chloride cells measured per fish. Three-way ANOVA indicated a significant effect of salinity and location (filament *vs* lamellae), and a significant interaction between isoform and salinity. Asterisk indicates a significant difference from the freshwater group (Tukey HSD test, $P<0.05$).

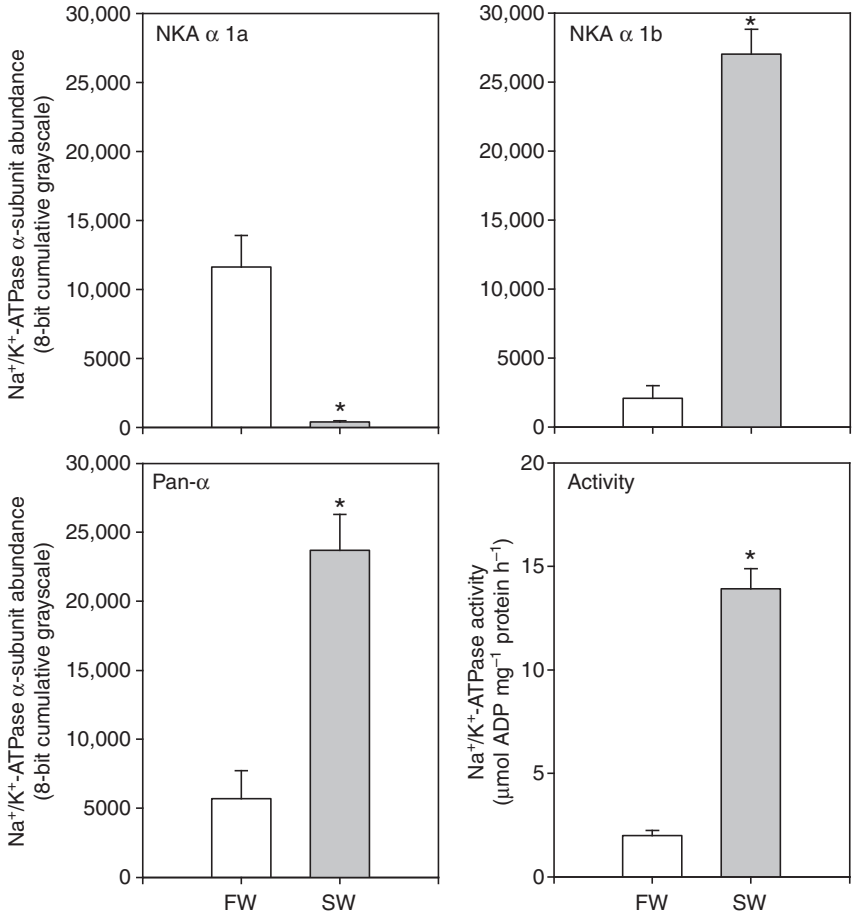


Fig. 3. Abundance of NKA α1a, NKA α1b and NKA pan-α, and NKA activity in gill tissue of freshwater and seawater-acclimated Atlantic salmon parr. Values are means + standard error of 5–6 fish per group. Asterisk indicates a significant change after seawater acclimation (one-way ANOVA, $P<0.05$).

immunofluorescence signal throughout the entire non-nuclear portion of the cell, consistent with localization on the basolateral membrane. In approximately 10–20% of NKA α1b-positive chloride cells in seawater, we observed more intense immunofluorescence in the immediate subapical region (Fig. 7), which was not observed in NKA α1a- or NKA α1b-positive cells in freshwater.

DISCUSSION

Our results indicate that salinity differentially regulates the abundance and localization of the α1a and α1b isoforms of NKA in the gills of Atlantic salmon. In freshwater, NKA α1a is the abundant isoform and is present in most chloride cells. After seawater acclimation the abundance of NKA α1a is greatly reduced and it is largely absent in most chloride cells. In contrast, NKA α1b is present in small quantities in freshwater in a few small filamental chloride cells. After seawater acclimation NKA α1b becomes the dominant isoform and is present in virtually all chloride cells.

These results are consistent with previous studies examining mRNA abundance in rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*) and Atlantic salmon, in which NKA α1a mRNA levels decrease following exposure to seawater, whereas NKA α1b mRNA levels increase (Richards et al., 2003; Bystriansky et al., 2006; Nilsen et al., 2007; Madsen et al., 2009). Previous studies on the influence of salinity on NKA abundance in the gills of teleost fish have used ouabain binding, activity measurements and antibodies that are not isoform specific, none of which can easily distinguish between isoforms of this enzyme. In studies on salmonids and most (though not all) euryhaline fish, gill NKA abundance increases with increasing salinity (McCormick, 1995). If we assume that the isoform-specific antibodies have similar detection efficiencies at the concentrations used in our western blots, then summing their totals results in a 3.4-fold increase in NKA α (for these calculations the values for NKA α1a shown in Fig. 3 were halved because twice as much protein was run on these western blots). This is quite similar to the 4.1-fold increase in NKA abundance after seawater acclimation detected by the NKA pan-α antibody. The mRNA of the other major isoforms of NKA that are expressed in the gill of Atlantic salmon (NKA α1c and α3) is present at much lower levels than both NKA α1a and α1b mRNA (Madsen et al., 2009). Immunohistochemical results indicate that all NKA pan-α-positive chloride cells were detected by antibodies to either NKA α1a or NKA α1b (Fig. 4). Although other NKA isoforms may be present and physiologically relevant, these results indicate that both NKA abundance and NKA-positive chloride cells are explained by the differential regulation of these two isoforms and, by extension, that the bulk of NKA α in fish gills is the sum of NKA α1a and NKA α1b.

The differential regulation of NKA α1a and NKA α1b by salinity suggests that these isoforms may differ in transport activity or regulation relevant to ion uptake and secretion, respectively. One possibility is that the kinetics for transport of sodium and potassium differ between the two isoforms. Previous research has established that NKA α transmembrane regions 4, 5 and 6 are critical to the cation binding and transport characteristics of the enzyme (Mobasheri et al., 2000). In their initial sequencing of these isoforms, Richards and colleagues (Richards et al., 2003) pointed out that the fifth transmembrane region is highly variable and that 7 of 21 amino acids differ between NKA α1a and NKA α1b in this region. Kinetic studies of gill membrane preparations in rainbow trout have found differences between the kinetics of sodium and potassium transport following seawater acclimation. Pfeiler and Kirschner (Pfeiler and Kirschner,

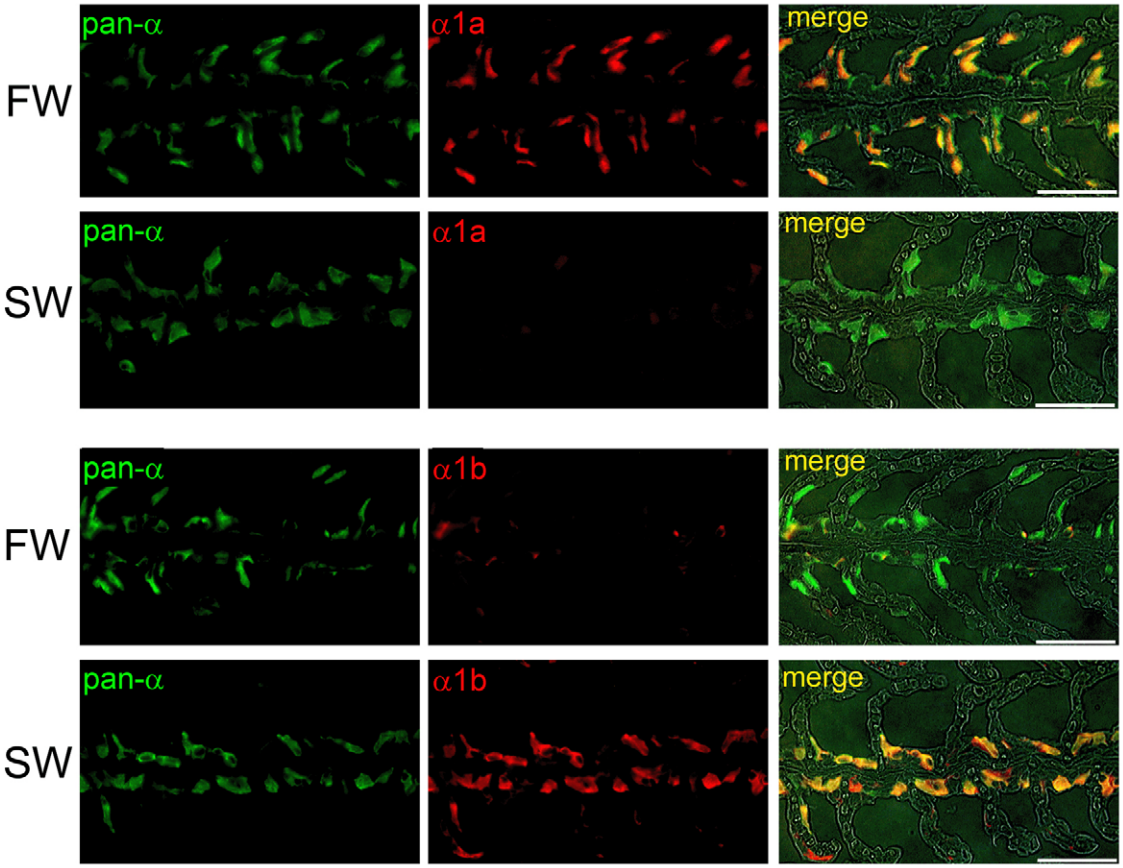


Fig. 4. Localization of NKA pan-α (green), NKA α1a (red, upper panels) and NKA α1b (red, lower panels) immunoreactivity in gill tissue of freshwater and seawater-acclimated Atlantic salmon parr. The merged images include NKA pan-α, NKA α-specific and non-fluorescent images. Co-localization of NKA α-isoforms with NKA pan-α appears as orange cells. Scale bar, 50 μm.

1972) found that potassium was not necessary for activation of sodium-dependent ATPase activity in freshwater, whereas potassium was required in gill tissue from seawater-acclimated rainbow trout. Similarly, Pagliarani and colleagues (Pagliarani et al., 1991) found that the K_m of gill ATPase activity for both sodium and potassium was lower in freshwater than in seawater-acclimated rainbow trout. Recent mutagenic studies using mammalian Na^+/K^+-ATPase as a template found that three amino acid substitutions characteristic of NKA α1a in transmembrane regions 5, 8 and 9 resulted in the promotion of intracellular binding of Na^+ over K^+, which would potentially favor Na^+–H^+ exchange over Na^+–K^+ exchange (Jorgensen, 2008). More research with the native proteins will be necessary to determine whether altered ion transport kinetics characterize the NKA α1a and α1b isoforms.

It has been known for some time that the activity of NKA is dependent on the surrounding lipid environment of the plasma membrane (Crockett and Hazel, 1997). Recently, Lingwood and colleagues (Lingwood et al., 2005) found that 'lipid rafts', which are rich in cholesterol and sphingolipids, are present in high concentrations in gill tissue of seawater-acclimated trout, and are at very low levels in the gills of freshwater trout. The authors utilized the enzyme aryl sulfatase to remove a critical component of lipid rafts, the sphingolipid sulfogalactosylceramide, and found that gill NKA activity was decreased in seawater trout but was unaffected in freshwater trout. Lipid rafts thus appear to be important for the ion secretory function of NKA, but relatively unimportant for its ion uptake function. In a more speculative paper, Lingwood and colleagues (Lingwood et al., 2006) suggest that lipid rafts in seawater chloride cells promote the function of NKA as a transporter for both sodium and potassium, whereas the absence of lipid rafts allows NKA to function primarily as a Na^+-ATPase. They further

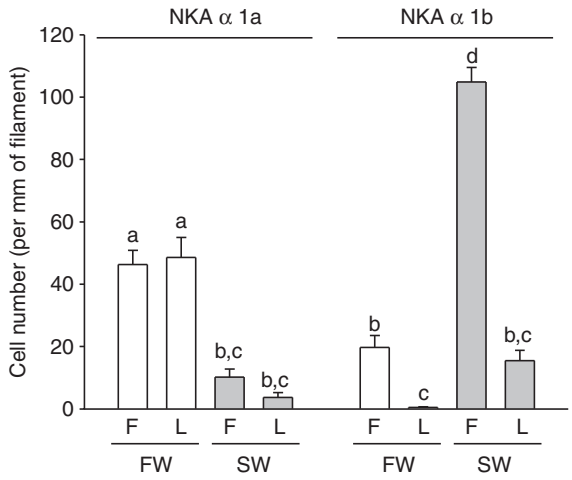


Fig. 5. Abundance of NKA α1a- and NKA α1b-positive chloride cells on the filament (F) and lamellae (L) in freshwater and seawater-acclimated Atlantic salmon parr. Values are means + standard error of 6 fish per group. Three-way ANOVA indicated a significant effect of isoform and location (filament *vs* lamellae), and significant interactions among all combinations of independent variables (isoform, location and salinity). Different letters represent significant differences using the Tukey HSD test (P<0.05).

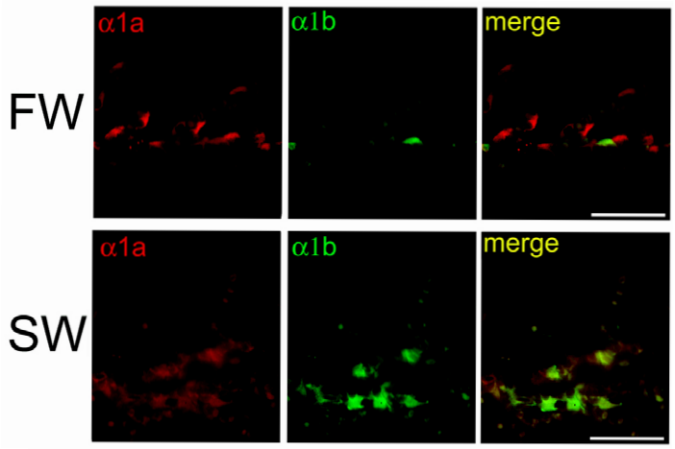


Fig. 6. Localization of NKA α1a and NKA α1b using directly labeled antibodies in freshwater and seawater-acclimated Atlantic salmon parr. Co-localization of the two NKA α-isoforms would appear as orange cells, which are not detectable. Scale bar, 50 μm.

speculate that the genetic difference between NKA α1a and NKA α1b is related to their capacity to function in lipid rafts, i.e. that NKA α1b has evolved to function within lipid rafts as a Na^+/K^+-ATPase, and that NKA α1a functions without lipid rafts as a Na^+-ATPase. It should be possible to utilize the antibodies developed in these studies to demonstrate an association of NKA α1a and NKA α1b with lipid rafts, and to more directly examine the cation transport kinetics of these isoforms.

In the present study we found a 7-fold increase in NKA activity after seawater acclimation of Atlantic salmon parr, compared with a 4-fold increase in total NKA abundance measured by the pan-α antibody. Greater up-regulation in gill NKA enzyme activity compared with abundance (measured by western blots) has been reported in other studies of seawater acclimation of fish (Hiroi and McCormick, 2007; Reis-Santos et al., 2008). One possible explanation for these differences is that the activity per molecule of NKA is greater in seawater than in freshwater. For salmonids, this could be related to differences in phosphorylation or to other functional differences between the NKA α1a and NKA α1b isoforms as discussed above. Alternatively, the method currently employed to measure NKA activity may favor measurement of NKA α1b activity over that of NKA α1a. A study on rainbow trout gills found a much lower ouabain sensitivity of Na^+-ATPase activity compared with Na^+/K^+-ATPase activity (Pfeiler and Kirschner, 1972), though this finding was not confirmed in the same species by others (Pagliarani et al., 1991). If NKA α1a is in fact acting as a ouabain-insensitive Na^+-ATPase, the current use of 0.5 mmol l^{-1} ouabain to detect NKA activity may favor measurement of NKA α1b over α1a. Future research will be necessary to resolve these possibilities and other aspects of the relationship between NKA isoforms and NKA activity.

It was apparent that NKA α1a and NKA α1b were specifically present in chloride cells, and that all NKA-positive chloride cells contained either NKA α1a or NKA α1b. NKA α1b was abundant in all chloride cells in seawater, whereas NKA α1a was either undetectable or present only as a faint background. *In situ* hybridization studies also indicate that the number of chloride cells producing NKA α1a mRNA is reduced in seawater-acclimated Atlantic salmon (Madsen et al., 2009). Although we cannot rule out the possibility that some NKA α1a is present in seawater, it is clear that NKA α1b is the dominant form based on both western blots and immunohistochemistry.

The staining intensity of chloride cells in seawater was generally higher than in freshwater, consistent with previous studies that have found higher concentrations of NKA in chloride cells in seawater than in freshwater (Karnaky et al., 1976). In most chloride cells we saw a similar pattern of uniform immunofluorescence throughout the non-nuclear portion of the cells for NKA α1a and NKA α1b. There were, however, a significant number of NKA α1b-positive cells in seawater that had an asymmetric staining pattern in which the most intense staining was in the subapical region of the cell (Fig. 7). It is possible that this difference in NKA α1b distribution is functionally significant. Greater activity of NKA in the subapical region could result in a more negative charge near the apical membrane, allowing chloride to move more easily through the CFTR on a favorable electrical gradient (Marshall et al., 1995). Subapical NKA could also cause a greater sodium gradient in the region of the loose junctions with accessory cells and thus favor the paracellular efflux of sodium (Evans et al., 2005). More detailed immunolocalization coupled with detection of sodium levels or electrical gradients in the subapical region would help determine whether these observations are physiologically relevant to ion secretion by chloride cells in seawater.

Although the levels of NKA α1b were clearly higher in Atlantic salmon parr acclimated to seawater, there were detectable levels in freshwater, and distinct NKA α1b-positive chloride cells were present on the filament. These cells were smaller than chloride cells in seawater and often appeared to be covered by pavement cells. We hypothesize that these cells are inactive in freshwater and are rapidly uncovered and activated after exposure to seawater. In the euryhaline killifish (*Fundulus heteroclitus*), exposure to freshwater

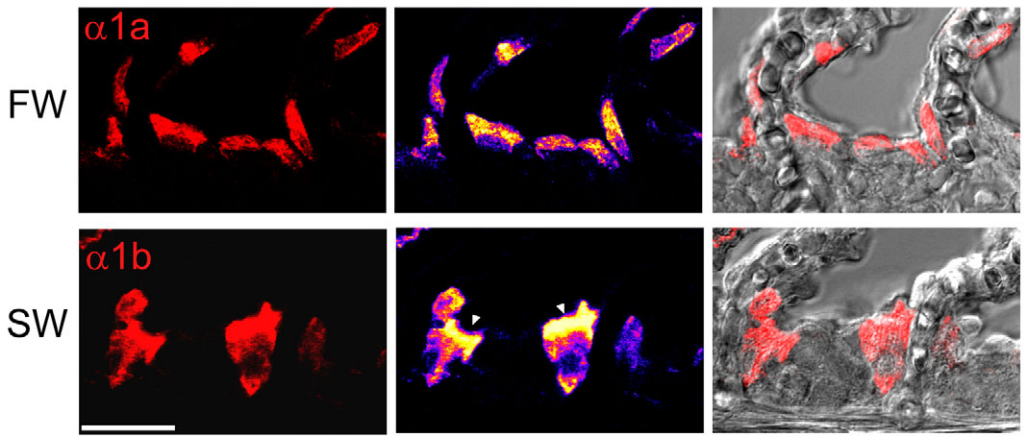


Fig. 7. Confocal image of NKA α1a in freshwater and NKA α1b in seawater-acclimated Atlantic salmon parr. In 10–20% of NKA α1b-positive chloride cells in seawater there was a more intense immunofluorescence in the immediate subapical region (arrowheads), whereas all other cells had a uniform immunofluorescence throughout the non-nuclear cell area. Single *Z*-section; scale bar, 20 μm.

results in the rapid covering of chloride cells by pavement cells and subsequent exposure to seawater results in a rapid uncovering of these cells (Marshall, 2003). The presence of a limited number of inactive NKA α1b-positive chloride cells may explain the partial euryhalinity of parr, which are able to withstand direct transfer to hyperosmotic conditions of up to 24 p.p.t. in autumn but not transfer to full strength seawater. Additional studies will be necessary to test this hypothesis and to determine the fate of both NKA α1b- and NKA α1a-positive chloride cells after seawater transfer. Atlantic salmon develop an even greater seawater tolerance as smolts (the normal downstream migratory stage of salmon), and this is preceded by large increases in gill NKA activity (Zaugg and McLain, 1970). Preliminary results indicate that both NKA α1a and NKA α1b increase in abundance during smolt development (S.D.M., A.M.R. and A.K.C., unpublished results).

Salinity-specific transcription of NKA isoforms has been shown in rainbow trout, Arctic charr and Atlantic salmon (Richards et al., 2003; Bystriansky et al., 2006; Nilsen et al., 2007) and it seems likely that it will be present in all euryhaline salmonids. It is not clear, however, whether such salinity-specific isoforms are widespread among teleost fishes. Cutler and Cramb (Cutler and Cramb, 2001) have suggested that the presence and differential distribution and function of multiple isoforms of ion transport proteins are due to partial chromosomal and whole genome duplication events followed by evolutionary changes in isoforms. The presence of NKA α1a and α1b in salmonids may be related to the tetraploidy that appears to have occurred in this group (Stellwag, 1999). An important area of future research will be to determine whether salinity-specific isoforms of NKA are widespread among teleosts or present only in salmonids.

Our results indicate that NKA α1a is the predominant form of NKA in the gills of Atlantic salmon in freshwater, and that NKA α1b is the predominant form in seawater. Although NKA α1b is only present at low levels in freshwater, it is detectable and present in different chloride cells from those containing NKA α1a. We hypothesize that these cells are inactive in freshwater, but provide a basis for the limited euryhalinity of this life stage of Atlantic salmon. The two isoforms may have distinct functions in freshwater and seawater (such as cation transport affinity), which should be examined in future studies. It will also be of interest to examine the underlying endocrine regulation of these two isoforms, which are likely to be different based on their differential response to salinity.

We thank Steve Gephard, Al Sonski, the staff of the Kensington State Fish Hatchery, and the Connecticut River Atlantic Salmon Commission for supplying juvenile Atlantic salmon. We thank Michael O'Dea for help in rearing fish and running the Na^+/K^+-ATPase activity assay. This project was supported by National Research Initiative Competitive Grant no. 2008-35206-18782 from the USDA Cooperative State Research, Education, and Extension Service. We thank John Kelly and two anonymous reviewers who provided many useful suggestions in review of an early version of this manuscript.

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