The Journal of Experimental Biology 209, 3440-3447
Published by The Company of Biologists 2006
doi:10.1242/jeb.02384

Na^+/H^+ antiporter, V-H^+-ATPase and Na^+/K^+-ATPase immunolocalization in a marine teleost (*Myoxocephalus octodecemspinosus*)

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Accepted 14 June 2006

Summary

Long-term pH compensation in a marine teleost requires the transepithelial excretion of H^+ across the gill epithelium. H^+ efflux in the longhorn sculpin (*Myoxocephalus octodecemspinosus*) is dependent on external sodium ion concentration and is inhibited by known inhibitors of Na^+/H^+ exchangers. Our model for proton transport suggests acid-excreting cells in the gill with an apical Na^+/H^+ antiporter and basolateral Na^+/K^+-ATPase. This model is similar to mammalian kidney and elasmobranch gill epithelium in which a basolateral electrogenic-vacuolar proton pump (V-H^+-ATPase) localizes to base-excreting cells. The objective of this study was to detect the presence and location of membrane transporters in marine fish gills using immunohistochemical staining. Our data indicate the presence of an apical and subapical Na^+/H^+-exchanger 2 (NHE2) in the sculpin gill. NHE2 is present in large, ovoid chloride cells and often colocalizes in the same cells as Na^+/K^+-ATPase. We also detected V-H^+-ATPase immunoreactivity, predominantly in cells at the base of the lamellae, with staining patterns indicative of a basolateral location. The 85 kDa protein detected on immunoblots with anti-NHE2 antibodies was found in both control and acid-infused animals and did not change following a large acute acidosis over 8 h.

Key words: Na^+/H^+-exchanger 2 (NHE2), Na^+/K^+-ATPase, V-H^+-ATPase, acid–base regulation, *Myoxocephalus octodecemspinosus*, longhorn sculpin, teleost fish.

Introduction

Long-term compensation of pH changes due to the breakdown of carbohydrates, fats and proteins requires proton transfer across an epithelial surface (Claiborne, 1998). Unlike terrestrial vertebrates that utilize the kidney for these transfers, in seawater fish the majority of acid is excreted across the gills. Early models of ion regulation linked Na^+ influx in freshwater fish to H^+ or NH_4^+ efflux (Krogh, 1939; Maetz, 1971; Smith, 1930). A similar exchange in seawater fish would exacerbate ionic problems in an already hypo-osmotic fish. Evidence for the electroneutral exchange of environmental Na^+ for intracellular H^+ in seawater fish (Claiborne et al., 1999; Evans, 1984), despite the salt load, emphasizes the importance of acid–base regulation.

The Silva model of chloride excretion (for reviews, see Marshall, 2002; Silva et al., 1977) proposes an inwardly directed electrochemical gradient for Na^+ that could be used by apical Na^+/H^+ exchangers (NHEs) for the electroneutral exchange of extracellular (seawater) Na^+ for intracellular H^+. Pharmacological and physiological studies support this apical placement of NHE. For example, Claiborne et al. (Claiborne et al., 1994) showed that acid excretion rates decrease, and eventually reverse to a net influx, as external Na^+ concentrations are lowered. Acid efflux rates behave similarly in the presence of amiloride and 5-(*N*,*N*-hexamethylene)-amiloride, known as NHE inhibitors (Claiborne et al., 1997). These data suggest linked sodium–hydrogen exchange driven by NHEs. In some freshwater-adapted species [supported by work mainly in salmonids and tilapia (reviewed by Evans et al., 2005)], there is also evidence for uncoupled Na^+/H^+ exchange driven by vacuolar H^+-ATPase (Perry et al., 2003). In this model, electrogenic transport of H^+ across the apical plasma membrane *via* H^+-ATPase creates a negative intracellular potential that drives Na^+ uptake through sodium channels in the apical membrane. Some evidence suggests that this mechanism plays less of a role in marine fish, as gill H^+-ATPase expression has been shown to decrease in salmonids when exposed to higher salinities (Lin et al., 1994; Lin and Randall, 1993).

Na^+/H^+ exchangers have been identified in several freshwater and seawater fishes. Studies using heterologous (mammalian) NHE antibodies have had mixed success in

detecting and localizing the protein. For example, using rabbit polyclonal antibodies raised against the C-terminal 85 amino acid residues of mammalian NHE3 (Hoogerwerf et al., 1996), immunoreactive cells were detected at the base of lamellae in freshwater rainbow trout (*Oncorhynchus mykiss*) and seawater blue-throated wrasse (*Pseudolabrus tetrious*) gills (Edwards et al., 1999). By contrast, no specific binding using those same antibodies in freshwater tilapia (*Oreochromis mossambicus*) was reported (Wilson et al., 2000), but rabbit polyclonal antibodies raised against mammalian NHE2 (Tse et al., 1994) cross-reacted with cells in both the interlamellar and lamellar epithelia of freshwater tilapia gill. Edwards and coworkers (Edwards et al., 2005) used heterologous antibodies to demonstrate the adjustment of NHE2 and NHE3 following acidosis in freshwater- and seawater-adapted *Fundulus heteroclitus*, respectively. The first fish-specific NHE antibodies made against NHE3 in the Osorezan dace (*Tribolodon hakonensis*) have recently been reported (Hirata et al., 2003) and immunohistochemical studies demonstrated an apical localization for NHE3 in chloride cells of dace gill. Fish exposed to acidic water showed a marked increase in NHE3.

The marine longhorn sculpin has proved to be a useful model for the *in vivo* and molecular study of systemic acid–base regulation and the gill transfers involved (Claiborne and Evans, 1988; Claiborne et al., 1997; Claiborne et al., 1994). Although we have been able to clone the cDNA for NHE2 from gill tissue of the sculpin (Claiborne et al., 1999; Gunning et al., 2001), immunological detection of the protein using available mammalian antibodies has proved unsuccessful. It was the purpose of the present study to use sculpin-specific antibodies to examine the expression of the NHE2 transporter in branchial epithelial cells. We developed species-specific polyclonal antibodies against putative epitopes on the sculpin gill NHE2 cDNA sequence. Our immunohistochemical data support the presence of Na^+/H^+-exchanger 2 protein, sometimes located near the apical surface, in the sculpin gill. NHE2 is found in large, ovoid chloride cells and often colocalizes in the same cells as Na^+/K^+-ATPase. We also detected V-H^+-ATPase staining, predominantly in cells at the base of the lamellae. Staining patterns of V-H^+-ATPase indicate basolateral localization. Western analysis showed that NHE2 was expressed in both control fish and those exposed to a chronic acidosis over 8 h.

Materials and methods

Animal collection and holding conditions

Marine longhorn sculpin, *Myoxocephalus octodecemspinosus* (Mitchell 1814), were supplied by commercial fishermen at the Mount Desert Island Biological Laboratory (MDIBL) in Salisbury Cove, ME, USA. Fish weighing between 90–195 g were maintained in seawater (15–18°C) pumped from Frenchman Bay and were fed until a few days before experiments. All protocols were approved by the GSU and MDIBL Animal Care and Use Committees.

Acid infusion

Animals were anaesthetized by immersion in MS-222 (1/10,000; 3-aminobenzoic acid ethyl ester; Sigma-Aldrich, St Louis, MO, USA). Following anesthesia, fish were surgically fitted with an intraperitoneal catheter and allowed to recover for 12 h (Claiborne and Evans, 1988). Chronic internal acidosis was induced in animals following a modified protocol (Claiborne et al., 1997) using four sequential intraperitoneal infusions of 2 meq kg^{-1} of 0.1 mol l^{-1} HCl each over 8 h (infused over ~1 min, ~5 ml volume for a typical 250 g animal). Control animals were injected with an equivalent volume of deionized water. During recovery and experimental periods, fish were kept in a 1.5 liter dark box with constantly running seawater (15–18°C) pumped from Frenchman Bay.

Tissue collection

Sculpin were brain and spinally pithed before perfusion of the bulbous arteriosus with heparinized Ringers solution to clear red blood cells. Gill arches were randomly chosen to be excised and placed in fixative [3% paraformaldehyde, 0.05% glutaraldehyde, and 4% picric acid in 10 mmol l^{-1} phosphate-buffered saline (PBS) solution, pH 7.3] for 4 h, transferred to PBS for removal of fixative, dehydrated in an ethanol–CitriSolv series, and embedded in paraffin wax. The remaining arches were snap-frozen for immunoblot analysis.

Antibodies

The antibodies used to detect NHE2 were affinity purified rabbit polyclonal antibodies (BioSource International, Camarillo, CA, USA) made against synthetic peptides (A94-APS: Ac-CVDNEHGSADNFRDGH-amide; sculpin NHE2 amino acid #694-708; GenBank accession number: AF159879; 541-AP: Ac- NENQVKEILIRRHESLREC-amide; from a putative dogfish NHE2, amino acid #636–653 (J. Claiborne, K. Choe and S. Edwards, manuscript in preparation); which is 100% homologous to sculpin #560-577). Mouse monoclonal antibody α5 was developed by Dr Douglas Fambrough, and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institutes of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The α5 antibody was made against the alpha subunit of avian Na^+/K^+-ATPase and binds to all isoforms. The antibody recognizes fish Na^+/K^+-ATPase and has been used widely in fish branchial cell studies (Choe et al., 2002; Edwards et al., 2002; Piermarini and Evans, 2001).

The rabbit polyclonal antibodies for V-H^+-ATPase (HAB) was a gift from Dr S. S. Gill, Department of Entomology, University of California Riverside. The antibodies were made against a 279-amino-acid peptide that matches residues 79–357 of *Culex quinquefasciatus* B subunit (Filippova et al., 1998). These antibodies have been successfully used in previous fish studies to localize V-H^+-ATPase in Atlantic stingrays (Piermarini and Evans, 2001).

Immunohistochemistry

Immunohistochemistry was performed on 5 μm thick serial paraffin wax sections, cut parallel to the long axis of the gill filament. Slides were dewaxed in a series of CitriSolv baths and rehydrated in an ethanol series followed by a 5 min water rinse to remove ethanol, and rinsed in PBS. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 (diluted in DIH_2O for 30 min at room temperature). Following a wash in PBS, non-specific binding sites present on the tissue sections were blocked by incubating the sections in 20 μl of blocking solution [2.5% normal horse serum (NHS); RTU Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA] for 30 min at room temperature. Tissue sections were then incubated overnight at 4°C in a humidified chamber in primary antibody (A94-APS: 1/5000–1/7500; α5: 1/5000–1/10000; HAB: 1/5000–1/7500) diluted in blocking solution.

Unbound antibody was removed using a PBS rinse and each tissue section incubated for 30 min in 20 μl of biotinylated horse anti-rabbit/biotinylated horse anti-mouse IgG (Universal 2° IgG; RTU Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) at room temperature. Sections were rinsed in PBS and incubated in 20 μl of an avidin and biotinylated horseradish-peroxidase macromolecular complex (ABC; RTU Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The ABC reagent was washed off with PBS and sections incubated in a chromagen substrate for 5 min to visualize binding. After a 5 min water rinse, the tissue was dehydrated in an ethanol-CitriSolv series and mounted permanently with a coverslip using Permount (Fisher Scientific, Hampton, NH, USA).

Immunofluorescence

Slide preparation for immunofluorescence detection followed a modified immunohistochemical protocol without incubation in 0.3% H_2O_2. The blocking solution used was 2.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Tissue sections were incubated overnight at 4°C in a humidified chamber while in primary antibody (α5: 1/500; A94-APS: 1/500) diluted in blocking solution. Following a PBS rinse, sections were incubated for 2 h in 20 μl of secondary antibody – either goat anti-rabbit IgG and goat anti-mouse IgG labeled with fluorescein isothiocyanate (FITC), or tetrarhodamine isothiocyanate (TRITC) flurochromes (Sigma-Aldrich, St Louis, MO, USA). Cover slips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. Slides were viewed with a Leitz Laborlux 12 and digital pictures taken with a Nikon Coolpix 4500.

Colocalization of antibodies

Light microscopy

A double labeling technique was used to localize NHE2 immunoreactivity to Na^+/K^+-ATPase and vacuolar H^+-ATPase immunoreactivity in the gills of the sculpin. Gill sections were prepared for chemiluminescent visualization using a modified immunohistochemical protocol. After incubation in the first chromagen, sections were rinsed with H_2O and incubated in blocking solution. The remainder of the immunohistochemical protocol was followed with incubation in primary antibodies made against a second transporter and visualization with a complementary chromagen.

Confocal microscopy

Gill sections were prepared for immunofluorescence detection using a modified protocol. Sections were deparaffinized, rinsed in PBS, then incubated overnight in a mixture containing dilutions of each primary antibody (α5: 1/500 and A94-APS: 1/500 or α5: 1/500 and HAB: 1/5000) at 4°C. Sections were washed in PBS and then incubated in a secondary antibody mixture, TRITC or Alexa Fluor® 488 goat anti-mouse IgG (1:3000) and FITC or Alexa Fluor® 568 goat anti-rabbit IgG (1:3000; Sigma-Aldrich, St Louis, MO, USA/Molecular Probes, Carlsbad, CA, USA). The remainder of the protocol follows the immunofluorescence technique as described above.

Slides were viewed with an Olympus Fluoview 300 laser scanning point source confocal microscope.

SDS–PAGE

Membrane enrichments were prepared from sculpin gill filaments by disruption with a polytron homogenizer for 30 s in ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 100 μg ml^{-1} phenyl methylsulfonyl fluoride and 30 mmol l^{-1} Tris–HCl at pH 7.4). Cell debris was removed by centrifugation (3000 ***g*** for 5 min), and membranes were pelleted by high-speed centrifugation (40000 ***g*** for 30 min). Pellets were resuspended in 200 μl of homogenization buffer. Samples of the final pellet and supernatant fractions were solubilized by the addition of a modified Laemmli sample buffer, without Bromophenol Blue or β-mercaptoethanol (Laemmli, 1970) and used to determine the total protein concentration with a detergent-compatible assay (BCA Protein Assay Kit; Pierce, Rockford, IL, USA). Samples were than added to Laemmli sample buffer (1:2 ratio; Bio-Rad Laboratories, Hercules, CA, USA) and 5% β-mercaptoethanol. 20 μg of total protein was separated in a 4–20% gradient polyacrylamide gel (45 min at 200 V) and transferred to a polyvinylidene difluoride membrane (PVDF; Bio-Rad Laboratories, Hercules, CA, USA) for 5 h at 60 V. After transfer, blots were washed with two rinses of deionized H_2O for 5 min each.

Immunoblotting

The PVDF membrane was blocked with Blotto [non-fat milk in Tris-buffered saline (TBS) pH 7.4; Bio-Rad Laboratories, Hercules, CA, USA] for 50 min at room temperature. Next, the membrane was incubated in primary antibody (541-AP: 1/1000-1/5000) diluted in 0.01% Tween-20 in TBS (TTBS) overnight at 4°C. After five consecutive washes in TTBS for 5 min, the PVDF membrane was incubated in secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit

IgG in TTBS; Pierce, Rockford, IL, USA) for 45 min at room temperature. Excess secondary antibody was removed by four consecutive washes in TTBS and one wash in TBS, each for 5 min. Antibody binding was detected by exposing Hyperfilm ECL imaging film (Amersham Biosciences, Piscataway, NJ, USA) to a chemiluminescent signal (Immuno-Star ECL kit; Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

Longhorn sculpin infused with acid were paired with a control fish as the samples were loaded onto the PAGE gels. The optical density of bands was measured using Scion Image software (Scion Corporation, Frederick, MD, USA) and analyzed with a paired Student's *t*-test using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and JMP (SAS Institute, Cary, NC, USA).

Controls

During immunolocalization, negative control sections were incubated in normal horse serum in lieu of primary antibody. Pre-immune serum and peptides were available for anti-NHE2 antibodies (A94-APS and 541-AP). Incubations in pre-immune serum and antibody–peptide competition controls were substituted for anti-NHE2 primary antibody on control sections and blots.

Results

Immunohistochemistry

NHE2 immunoreactivity was present in gill epithelial cells of the long-horned sculpin. NHE2-immunoreactive cells were localized to the interlamellar region of the filament epithelium, with no evidence of staining along the lamellae. NHE2 immunoreactive cells in this region demonstrated a distinct punctate staining pattern along the apical membrane region and within the intracellular compartment (Fig. 1A,B). Sections of gills removed from acid-infused sculpin showed similar staining patterns to their normal counterparts, with no qualitative difference in the number of cells stained or staining intensity.

There was no evidence of immunoreactivity in sections incubated in sculpin NHE2 antibody–peptide competition controls or in control sections incubated in either pre-immune serum or normal horse serum in lieu of anti-NHE2 antibodies. To determine the probable cell type of NHE2 immunoreactivity, gill sections were stained with anti-Na^+/K^+-ATPase antibody (α5). Staining patterns were diffuse with no background staining throughout large, ovoid cells presumed to be mitochondria-rich chloride cells (Fig. 1C,D). Na^+/K^+-ATPase immunoreactive cells were present along the filament epithelium in the interlamellar region and were not detected in cells of the lamellae. Controls with normal horse serum instead

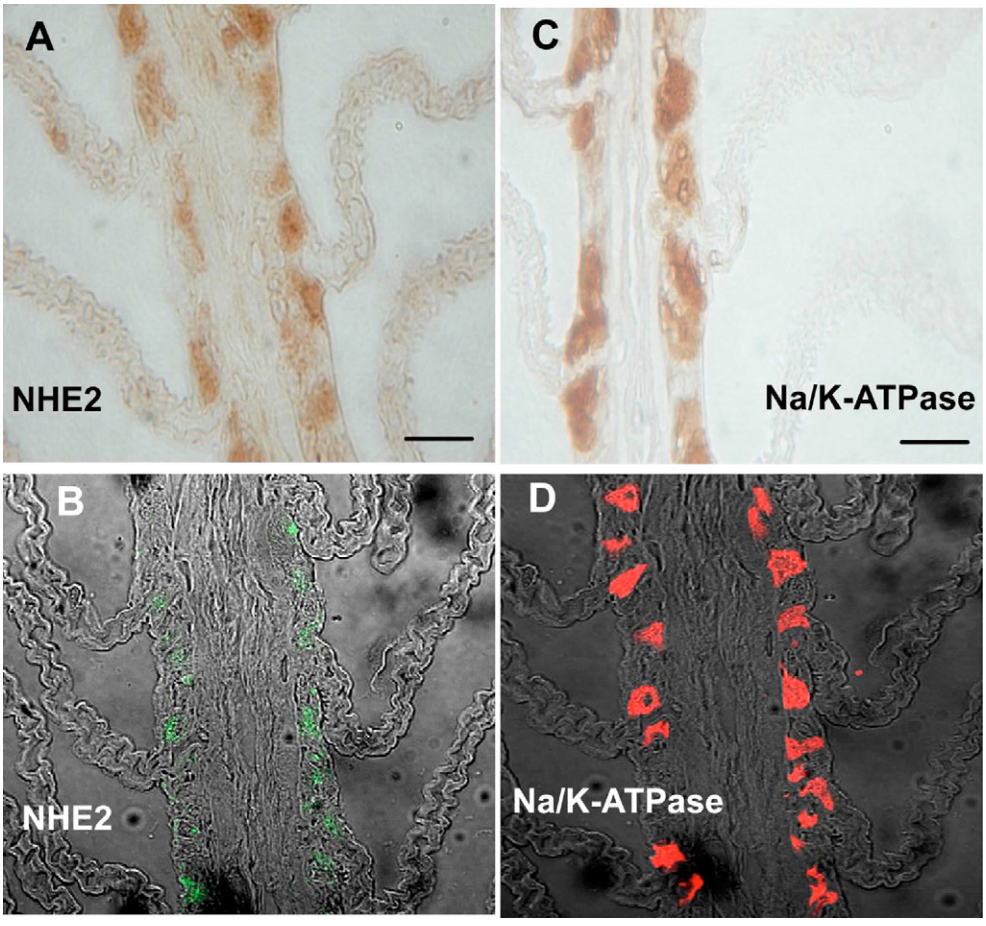

Fig. 1. Longhorn sculpin gill sections from seawater controls with chromagenic and fluorescent staining against NHE2 and Na^+/K^+-ATPase. Gill sections stained with (A) DAB and (B) green fluorescein against NHE2 with sculpin-specific antibodies. Controls (lacking primary antibody, antigen–antibody competition and pre-immune serum) were all negative (not shown). Staining is punctate in the apical and subapical regions of large cells in the filament. The cell distribution is similar to the pattern for Na^+/K^+-ATPase-rich cells. Serial sections stained with (C) DAB and (D) red–rhodamine against Na^+/K^+-ATPase show intense immunoreactivity throughout the cell which is consistent with staining along the tubular infoldings of the basolateral membrane in cells that are probably mitochondria-rich chloride cells. Scale bars, 20 μm.

of primary antibody were negative with no background staining.

Immunofluorescent labeling for NHE2 and Na^+/K^+-ATPase in the same gill filament (Fig. 1B,D), shows a narrower distribution of NHE2 binding often on, or near, the apical side of the cell when compared with that observed for Na^+/K^+-ATPase. The punctate NHE staining colocalized with Na^+/K^+-ATPase-rich cells along the interlamellar region of the filament epithelium (Figs 2 and 3).

V-H^+-ATPase immunoreactivity was present in a population of large ovoid cells located at the base of the lamellae (Fig. 3). Colocalization of Na^+/K^+-ATPase and V-H^+-ATPase was observed in a number of scattered cells (Fig. 3). However, the majority of epithelial cells demonstrated no evidence of V-H^+-ATPase and Na^+/K^+-ATPase colocalization. NHE2 immunoreactivity was also detected in the cells with V-H^+-ATPase immunoreactivity at the base of the lamellae (Fig. 3), but V-H^+-ATPase was not detected in most cells that were NHE2 immunoreactive. Control sections incubated in normal horse serum instead of primary antibody were negative, with minimal background staining.

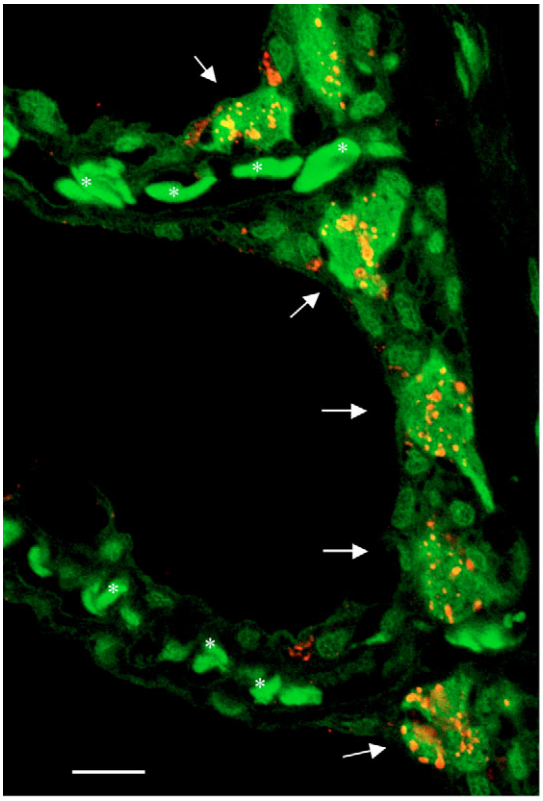

Fig. 2. Confocal merged image of antibodies against NHE2 (rhodamine) and Na^+/K^+-ATPase (fluorescein) at higher magnification (60×; scale bar, 20 μm). Arrows indicate interlamellar cells expressing both NHE2 and Na^+/K^+-ATPase. Autofluorescent erythrocytes in the lamellae are indicated with asterisks. Scale bar, 20 μm.

Western blots

Anti-NHE2 antibodies (541-AP) were specific for a protein of ~85 kDa (Fig. 4). Longhorn sculpin infused with acid showed on average a 28% increase in NHE2 expression over control fish. Increased expression levels were detected in three of four acidotic *vs* control pairs, but there was wide absolute variability between blot densities, and the increase was not significant (paired one-tailed *t*-test; N=4, t=1.61; P>0.10; Fig. 4B).

Discussion

This study has been the first to use fish-specific anti-NHE2 antibodies to demonstrate the localization of NHE2

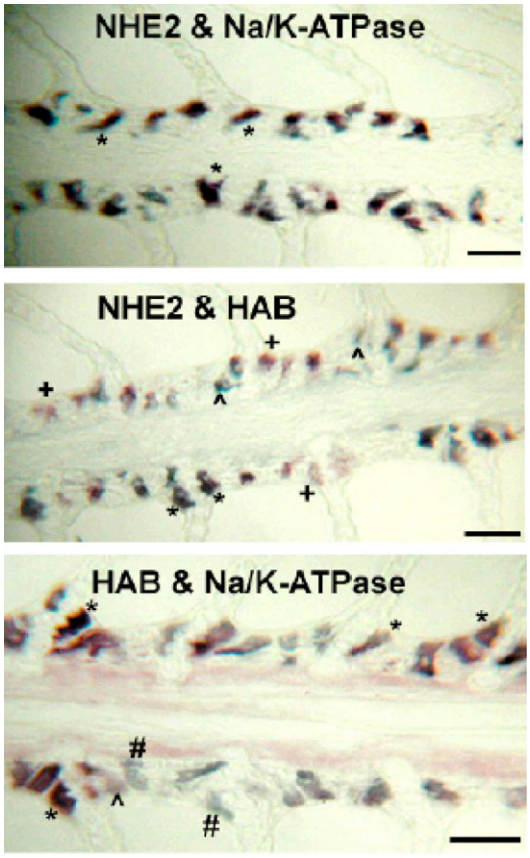

Fig. 3. Colocalization of NHE2, Na^+/K^+-ATPase and V-ATPase. Staining against both NHE2 (A94-APS: 1/10,000, Vector VIP, purple) and Na^+/K^+-ATPase (α5: 1/5000, Vector SG, blue/grey) is evident in the interlamellar region of many gill epithelial cells. No lamellar cells were stained. NHE2 (A94-APS: 1/10,000, Vector VIP, purple) and V-ATPase (HAB: 1/5000, Vector SG, blue/grey) colocalized in some of the same cells. Tissue double-labeled for V-ATPase (HAB: 1/5000, Vector VIP, purple) and Na^+/K^+-ATPase (α5: 1/5000, Vector SG, blue/grey) demonstrated diffuse staining of both. Most stained cells only stained for Na^+/K^+-ATPase, however, some cells stained for both transporters. This could indicate that V-ATPase is present in only a subpopulation of mitochondria-rich cells. *, colocalization in each picture; +, staining of NHE2 only; ^, staining of HAB only; #, staining of Na/K-ATPase only.

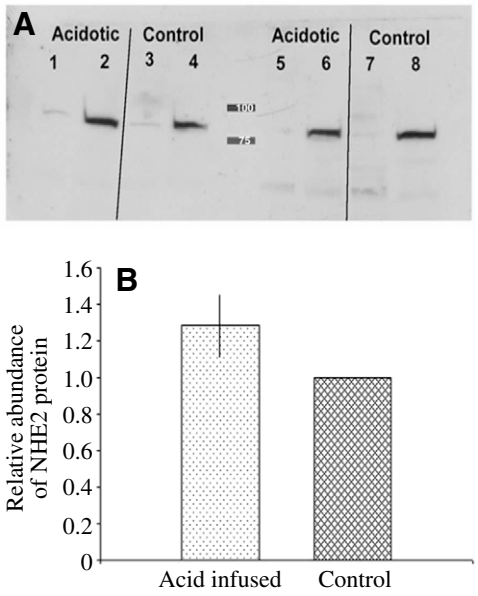

Fig. 4. Relative NHE2 protein expression in control and acidotic fish. (A) This representative western blot, probed with anti-NHE2 antibodies, contains protein from two control (lanes 4 and 8) and two acid-infused (lanes 2 and 6) sculpin. Even-numbered lanes are the membrane fraction and odd-numbered lanes are the cytosolic fraction of homogenized longhorn sculpin gill tissue. An ~85 kDa signal was detected when the immunoblot was probed with anti-NHE2 antibodies (541-AP). Preimmune and peptide-antibody competition controls were negative (not shown). Markers: 100 kDa, 75 kDa. (B) Graph showing differences in relative protein abundance of NHE2 in acid infused *versus* control fish. Values are mean ratios ± s.e.m. (*N*=4).

immunoreactivity to cells in the branchial epithelium of a marine fish, the long horned sculpin. Our results have demonstrated that NHE2 immunoreactivity is present in a punctate staining pattern along the apical region of filament epithelial cells. Colocalization studies with the basolaterally located Na^+/K^+-ATPase suggest that the location of the NHE2 immunoreactive protein is often in, or near, the apical membrane, or possibly located in subapical vesicles. This placement is consistent with a model for seawater teleost acid–base regulation that uses the inward flow of sodium down its electrochemical gradient to drive the electroneutral efflux of protons into the surrounding environment (Claiborne, 1998). Similarly, heterologous mammalian NHE2 antibodies (Tse et al., 1994) have been used to detect a proteins in the membrane fractions from the gills of tilapia (Wilson et al., 2000) and *Fundulus heteroclitus* (Edwards et al., 2005), which were comparable in size to the proteins of our immunological study (~85 kDa). In addition, immunohistochemical studies have shown that tilapia expressed apically located NHE2 in gill interlamellar and lamellar cells (Wilson et al., 2000). NHE2 immunoreactivity has also been located in the interlamellar regions in the gills of several marine elasmobranch species (for reviews, see Claiborne et al., 2002; Edwards et al., 2002).

Seawater longhorn sculpin gill probed with antibodies against the B subunit of V-H^+-ATPase demonstrated diffusely stained cells at the base of some lamellae with few immunoreactive cells in the interlamellar region. The diffuse staining is comparable to the basolateral staining of Na^+/K^+-ATPase (likely along the tubular infoldings of the basolateral membrane). A similar pattern of diffuse basolateral staining in mummichogs probed with anti-V-H^+-ATPase antibodies raised against the A subunit has been described (Katoh et al., 2003). Likewise, a study on the gills of the seawater-adapted euryhaline stingray (*Dasyatis sabina*) demonstrated H^+-ATPase immunoreactive cells localized to the base of the lamellae, showing a diffuse, basolateral staining pattern and lack of colocalization with Na^+/K^+-ATPase (Piermarini and Evans, 2001). The authors concluded that a basolateral V-H^+-ATPase may be involved in bicarbonate excretion in a subpopulation of mitochondria-rich cells. The basolateral V-H^+-ATPase would pump protons out of the cell to set up a favorable electrochemical gradient for Cl^-/HCO_3^- exchange *via* a coupled exchanger. Indeed, there is some evidence for a external Cl^--sensitive, DIDS (4,4′-diisothiocyanatostilbene-2,20-disulfonic acid) inhibitable Cl^-/HCO_3^- exchange in the sculpin (Claiborne et al., 1997).

When utilizing heterologous antibodies there is a possibility that the antibody could be binding non-specifically to the gill cells. Although we did not perform western blots with the heterologous V-H^+-ATPase antibody in the present work, previous studies using the same V-H^+-ATPase antibody in a fish species have demonstrated the presence of a single V-H^+-ATPase-immunoreactive protein of the correct size (60 kDa) in gill protein homogenates (Piermarini and Evans, 2000). In addition, the V-H^+-ATPase antibody has also been successfully used in immunohistochemical studies in the same elasmobranch species (Choe et al., 2004; Piermarini et al., 2002). It is important to also note that the region of the *Culex* H^+-ATPase sequence (residues 79–357) used for the antibody design (Filippova et al., 1998) is highly conserved and exhibits ~90% functional homology to teleost sequences, including a partial sequence we have obtained for the sculpin gill H^+-ATPase (GenBank accession number, DQ520199; corresponding to *Culex* amino acid residues 113–379) which is 91% identical. Thus, there is a high probability that the polyclonal antibody against *Culex* H^+-ATPase is recognizing identical epitopes in the sculpin protein. The sodium pump is widely accepted as a ubiquitous, basolateral transporter, located in mitochondria-rich cells of teleost gill (Hirose et al., 2003; Karnaky et al., 1976; Marshall and Bryson, 1998; Pisam and Rambourg, 1991). Longhorn sculpin gill probed with mouse monoclonal antibody (α5) demonstrated diffuse staining throughout large, ovoid or columnar cells present in the filament epithelium. This staining pattern suggests immunolocalization throughout the infoldings of the basolateral membrane. The immunological probe α5 binds to isoforms a_1, a_2 and a_3 and it has been used to detect basolateral Na^+/K^+-ATPase localization in rainbow trout (Witters et al., 1996), hagfish (Choe et al., 1999), stingray (Piermarini and Evans, 2000), milkfish (Lin et al., 2003) and green puffer fish

(Lin et al., 2004). We found that anti-Na^+/K^+-ATPase antibody consistently stained numerous large, ovoid cells in the interlamellar region of sculpin gill. No staining was ever present in the lamellae. This stain was used as a marker for presumed mitochondria-rich cells. A small population of cells demonstrated V-H^+-ATPase immunoreactivity colocalized with Na^+/K^+-ATPase. By contrast, the majority of the numerous Na^+/K^+-ATPase-immunoreactive interlamellar cells demonstrated colocalization with NHE2. These findings agree with experiments on several marine fish using heterologous antibodies against NHE2 (Edwards et al., 2002).

Mean expression levels of NHE2 detected by western blots were ~30% higher in the acid-loaded sculpin than in control fish (three of four control *vs* acidotic pairs), but this was not a significant difference. We have previously shown that sculpin can recover internal pH from an acute acidosis within 1 h, and animals infused with a chronic acid load begin excreting acid within a few hours and then maintain an elevated net H^+ efflux for eight or more hours, even after the infused acid has been excreted (Claiborne et al., 1997). Thus, we had initially hypothesized that NHE2 protein levels would increase, because preliminary work showed that NHE2 mRNA levels also increase by approximately twofold in acid-infused sculpin over a similar experimental time course (Hair et al., 2002). One explanation for this apparent contradiction is that the changes in protein levels are too small, and too variable to be adequately detected immunologically; a twofold or higher alteration may be necessary (Hirata et al., 2003; Piermarini and Evans, 2000). We might also speculate that baseline levels of NHE2 expression may allow for quick recovery from metabolic acidosis without the need for an immediate increase in protein production. The NHE2 distribution shown in Fig. 2, indicates that the majority of NHE2 is located intracellularly possibly in subapical endosomes and could be 'waiting' for placement on the apical membrane when needed. We used membrane-bound protein fractions when isolating the protein for the western blots, so movement of NHE2 between vesicle and apical membrane would not be distinguished using the present techniques. When expressed in NHE-deficient PS-120 cells, only a fraction of total mammalian NHE2 and NHE3 is found on the cell surface (20% for non-glycosylated NHE2 and 14% for NHE3), with the reminder in the cytoplasm (Cavet et al., 1999). Likewise, mammalian NHE2 may have a short membrane half-life (3 h) when compared to NHE1 and NHE3 (23 h and 15 h, respectively), so changes in protein synthesis and degradation may be an important aspect of NHE2 regulation in the fish as well (Cavet et al., 1999).

It is also probable that additional sodium–hydrogen exchanger isoforms share responsibility for the observed acid excretion. Gill NHE1 is downregulated following acidosis, thus decreasing the presumably basolateral movement of H^+ into the blood and allowing for increased transfer from fish to water (Claiborne et al., 1999). NHE3 has recently been partially cloned in longhorn sculpin (Lanier and Claiborne, 2003) and the mRNA has been detected in northern blots (C. Lanier, C. Cutler, A. Diamanduros and J. Claiborne, unpublished data) and visualized using *in situ* hybridization (A. Diamanduros, S. Edwards and J. Claiborne, unpublished data). Likewise, this isoform appears to be important in Na^+ uptake and acid excretion in *Fundulus heteroclitus* (Edwards et al., 2005; Scott et al., 2005), the freshwater dace (Hirose et al., 2003) and the Atlantic stingray (Choe et al., 2005). *Fundulus* may even predominantly express the different isoforms (NHE2 *versus* NHE3) depending on the salinity of adaptation (freshwater *versus* seawater, respectively) (Edwards et al., 2005). NHE3 protein abundance increases following metabolic acidosis in rat thick ascending limb (Laghmani et al., 1997) and renal brush border (Wu et al., 1996). Unfortunately, mammalian heterologous NHE3 antibodies have proved unsuccessful with the sculpin to date (data not shown), so future investigations will require fish—specific antibodies to NHE3. Likewise, definitive localization of the sculpin NHEs to specific membrane/intracellular compartments await higher resolution imaging approaches such as fluorescence or transmission electron microscopy with immunogold labeling (Varsamos et al., 2002).

In summary, our study has shown that NHE2 proteins are present in the gills of longhorn sculpin. The apical localization of these proteins and colocalization with Na^+/K^+-ATPase in mitochondria-rich cells may indicate a role in ionic and acid–base regulation. The degree of involvement remains to be determined in future studies with comparisons of NHE3 expression over a broader time course. Localization of plasma membrane V-H^+-ATPase indicates that it is present in longhorn sculpin gill. The diffuse, basolateral staining patterns and lack of consistent colocalization with Na^+/K^+-ATPase indicate that V-H^+-ATPase may not be involved with H^+ excretion but could play a role in bicarbonate excretion in a subpopulation of mitochondria-rich cells.

This research was supported by a GSU Professional Development Grant to J.S.C., an MDIBL NIA award to S.L.E. and NSF IBN-0111073 to J.B.C.

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