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Comparative Biochemistry and Physiology, Part A 146 (2007) 155–162
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The putative mechanism of Na^+ absorption in euryhaline elasmobranchs exists in the gills of a stenohaline marine elasmobranch, *Squalus acanthias*

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Received 4 July 2006; received in revised form 11 September 2006; accepted 28 September 2006
Available online 1 December 2006

Abstract

We recently cloned an NHE3 orthologue from the gills of the euryhaline Atlantic stingray (*Dasyatis sabina*), and generated a stingray NHE3 antibody to unequivocally localize the exchanger to the apical side of epithelial cells that are rich with Na^+/K^+-ATPase (A MRC). We also demonstrated an increase in NHE3 expression when stingrays are in fresh water, suggesting that NHE3 is responsible for active Na^+ absorption. However, the vast majority of elasmobranchs are only found in marine environments. In the current study, immunohistochemistry with the stingray NHE3 antibody was used to localize the exchanger in the gills of the stenohaline marine spiny dogfish shark (*Squalus acanthias*). NHE3 immunoreactivity was confined to the apical side of cells with basolateral Na^+/K^+-ATPase and was excluded from cells with high levels of vacuolar H^+-ATPase. Western blots detected a single protein of 88 kDa in dogfish gills, the same size as NHE3 in stingrays and mammals. These immunological data demonstrate that the putative cell type responsible for active Na^+ absorption in euryhaline elasmobranchs is also present in stenohaline marine elasmobranchs, and suggest that the inability of most elasmobranchs to survive in fresh water is not due to a lack of the gill ion transporters for Na^+ absorption.

Keywords: Osmoregulation; Na^+/H^+ exchanger; Na^+/K^+ATPase; Elasmobranch; Acid–base regulation

1. Introduction

In elasmobranchs, the gills are responsible for the majority of ion (Na^+ and Cl^-) absorption and acid or base secretion that are required for systemic acid–base regulation and osmoregulation in fresh water (Claiborne, 1998; Choe and Evans, 2003; Evans et al., 2004). Two populations of mitochondrion-rich cells (MRCs) have been identified in elasmobranch gills that are predicted to mediate these transport processes (Piermarini and Evans, 2001; Tresguerres et al., 2005). Apical immunoreactivity for a Cl^-/HCO_3^- exchanger (pendrin) and basolateral immunoreactivity for vacuolar H^+-ATPase (V H^+-ATPase) characterize one of these MRC populations (Piermarini et al., 2002; Evans et al., 2004). These cells were predicted to secrete base similar to type B intercalated cells of mammalian kidneys, and were named B MRCs. High levels of basolateral Na^+/K^+-ATPase immunoreactivity were discovered in another gill cell type (A MRC) that was hypothesized as the location of transepithelial Na^+ absorption and acid secretion (Piermarini and Evans, 2000). However, the definitive molecular identity of the transporter that mediates Na^+ and acid transport across the apical membranes of these cells was unknown.

Vertebrate Na^+/H^+-exchangers (NHEs) catalyze the electroneutral exchange of Na^+ and H^+ down their respective concentration gradients (Yun et al., 1995; Orlowski and Grinstein, 1997; Wakabayashi et al., 1997; Moe, 1999; Hayashi

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doi:10.1016/j.cbpa.2006.09.027

et al., 2002; Orlowski and Grinstein, 2004). The human genome contains at least nine isoforms (SLC9A1-9) that are expressed in diverse tissues, cells, and membrane locations (Orlowski and Grinstein, 2004; Brett et al., 2005). The physiological functions of epithelial NHEs are best defined in the kidneys of mammals where NHE2 is found in the cortical thick ascending limb, macula densa, distal convoluted tubules, and connecting tubules (Chambrey et al., 2001), and NHE3 is found predominantly in the proximal convoluted tubule and to a lesser extent in the thick ascending limb (Biemesderfer et al., 1993, 1997; Amemiya et al., 1995). In renal proximal tubules apical NHE3 is expressed in cells with basolateral Na^+/K^+-ATPase and Na^+/HCO_3^- cotransporter in a mechanism that is responsible for the majority of Na^+ and HCO_3^- reabsorption in the kidney (Vallon et al., 2000; Wang et al., 2001), and NHE3-knockout mice have systemic acidosis, markedly decreased renal HCO_3^- and fluid absorption, hypotension, and elevated plasma aldosterone (Ledoussal et al., 2001; Woo et al., 2003). The function of NHE2 in the kidney is not yet clear and NHE2-null mice have no measurable phenotypes that suggest renal absorptive malfunction (Ledoussal et al., 2001).

In an attempt to identify the apical transporter responsible for Na^+ absorption and acid secretion in elasmobranch gills, we recently cloned NHE homologues from the gills of the Atlantic stingray, and evaluated their potential roles in acclimation from seawater to fresh water (Choe et al., 2005). The Atlantic stingray is the only North American species of elasmobranch that has permanent populations in both seawater and fresh water (Bigelow and Schroeder, 1953; Johnson and Snelson, 1996; Piermarini and Evans, 1998). It is a small elasmobranch species (300–800 g adults) that can be maintained in either fresh (<5 mM NaCl) or seawater (~500 mM NaCl) aquaria, and therefore it is well suited as a model for gill ion absorption (Piermarini and Evans, 1998). We used real-time PCR to demonstrate that mRNA expression of an NHE3 homologue, but not an NHE2 homologue, increased in the gills of stingrays after they were transferred to from seawater to fresh water (Choe et al., 2005). We then sequenced the complete stingray NHE3 mRNA, and showed that it coded for a protein that is 70% identical to human NHE3 (SLC9A3) (Choe et al., 2005). An antiserum generated against the carboxyl tail of the stingray NHE3 was then used to demonstrate protein expression exclusively in the apical region of Na^+/K^+-ATPase-rich MRCs (A MRCs), and acclimation to fresh water caused a dramatic increase in the number of cells expressing NHE3 (Choe et al., 2005).

The stingray study provided the first NHE3 cloned from an elasmobranch and the first demonstration of an increase in gill NHE3 expression during acclimation to low salinities, suggesting that NHE3 can absorb Na^+ from fresh water. Because the vast majority of elasmobranchs are stenohaline marine and cannot acclimate to fresh water (Evans et al., 2004), it is possible that NHE3 in gills is a derived trait that evolved specifically for Na^+ absorption in elasmobranchs that can osmoregulate in fresh water. Alternatively, NHE3 in gills may be an ancestral trait that exists is all elasmobranchs as a mechanism of acid secretion (Evans, 1975, 1982; Claiborne and Evans, 1992), regardless of external salinity. If this second hypothesis is correct, then the inability of most elasmobranchs to survive in fresh water would not be due to a lack of the appropriate ion transporters for Na^+ absorption. To test this hypothesis, we used our stingray antibody to determine if NHE3 is expressed in the apical region of gills from a stenohaline marine elasmobranch. We also used multi-labeling techniques to determine if NHE3 is expressed in cells with high levels of Na^+/K^+-ATPase, which would be predicted to generate an ion gradient to favor Na^+ absorption in exchange for acid secretion.

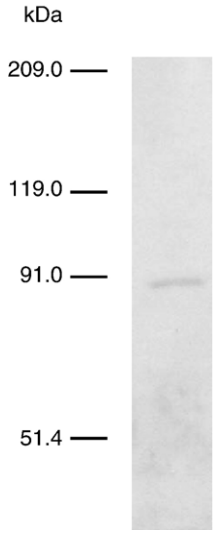

Fig. 1. Western blot of gill cell lysates with the antibody R1B2. A single band of 88 kDa was detected.

2. Materials and methods

2.1. Animals and holding conditions

All procedures were approved by the University of Florida and Mount Desert Island Biological Laboratory Institutional Animal Care and Use Committees. Dogfish sharks (*Squalus acanthias*, approximately 1–3 kg) were captured from waters in the Gulf of Maine. They were transported to the Mount Desert Island Biological Laboratory in Salisbury Cove, ME where they were held in a flow-through 100% seawater tank. The water temperature was between 15 and 18 °C, and the light was on an ambient cycle.

2.2. Antibodies

Antibody R1B2 was generated against a recombinant protein that represents the final 212 amino acids of the carboxyl tail of stingray NHE3. We recently developed this antibody in rats, and demonstrated that it specifically recognizes stingray NHE3 in native tissue and when heterologously expressed in frog oocytes (Choe et al., 2005). Preliminary sequencing of dogfish NHE3 mRNA fragments demonstrates that the protein is over 90% conserved with stingray NHE3 (unpublished observation), and therefore should be recognized by antibody R1B2. 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 Institute of Child

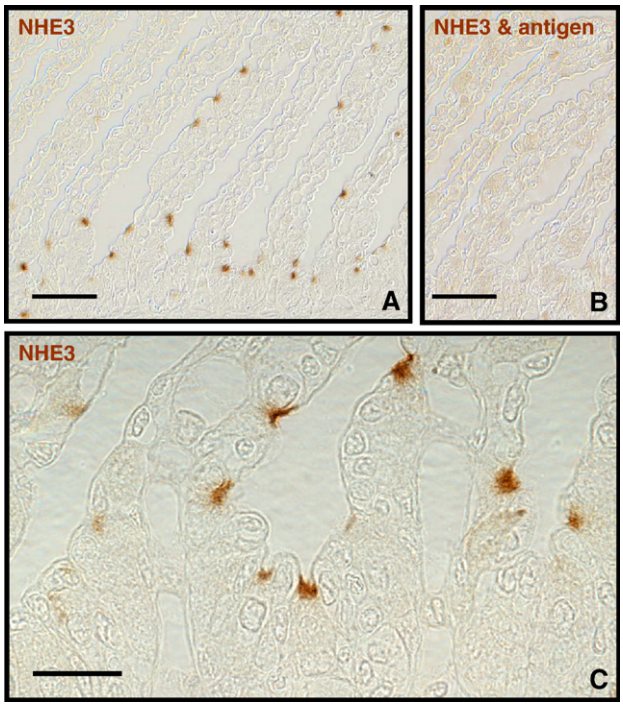

Fig. 2. Representative light micrographs of gill sections from spiny dogfish that demonstrate the localization of NHE3 protein. Gill sections were incubated with antiserum R1B2 (A and C) or antiserum R1B2 and antigen (B). No immunolabeling was observed in negative control sections that were incubated with R1B2 and excess antigen followed by multilink (anti-mouse, rabbit, and donkey) secondary antibodies (B). However, strong immunolabeling occurred in the apical regions of a population of epithelial cells with antiserum R1B2 (A). Scale bars = 100 μm (A and B) or 20 μm (C).

Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. It was made against the avian Na^+/K^+-ATPase α subunit and binds to all isoforms. This antibody recognizes fish Na^+/K^+-ATPase, and is now used widely for studies on fish branchial cells (e.g., Piermarini and Evans, 2000; Wilson et al., 2002b; Choe et al., 2004a). The rabbit polyclonal antibody for vacuolar H^+-ATPase was developed by Filippova et al. (1998), and was a gift from Dr. William Harvey at the Whitney Laboratory, University of Florida (with permission from Dr. Sarjeet Gill, University of California at Riverside). It was made against a 279 amino acid peptide that matches residues 79–357 of *Culex quinquefasciatus* B subunit. This antibody has been used to localize vacuolar H^+-ATPase in Atlantic stingrays (Piermarini and Evans, 2001).

2.3. Western blotting

Immunoblots were prepared from ringer-perfused gill tissue using a procedure modified from Choe et al. (2004b). Briefly, tissues were homogenized in buffer [250 mmol L^{-1} sucrose, 30 mmol L^{-1} Tris, 1 mmol L^{-1} Na_2EDTA, 5 μL/mL protease inhibitor cocktail (Sigma P8340), 100 μg/mL phenylmethylsulfonyl fluoride, pH 7.8] with a mechanical homogenizer for 30 s at maximum speed on ice. Homogenates were then centrifuged at 10,000 *g* for 10 min at 4 °C to remove debris and whole nuclei. The total protein concentration was determined with the Pierce BCA assay, and Laemmli loading buffer was added (Laemmli, 1970). Forty micrograms of protein was loaded and run in 7.5% Tris–HCl precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA), and then transferred onto polyvinylidene difluoride (PVDF) membranes.

The PVDF membranes were blocked in Blotto (5% non-fat milk in TBS) for 1 h at 24 °C and then incubated in Blotto with antibody R1B2 (1:2000). Membranes were then washed with four changes (15 min each) of TBS with 0.1% Tween-20 (TBST), and incubated in goat anti-rat (Promega, Madison, WI, USA) IgG secondary antibody (alkaline-phosphatase conjugated) diluted 1:2500 in Blotto for 1 h at 24 °C. After four more washes in TBST, the membranes were incubated in Immun-Star ECL substrate (Bio-Rad) for 5 min at 24 °C. Luminescent bands were then detected with Amersham-Pharmacia's (Piscataway, NJ, USA) Hyperfilm-ECL, according to manufacturer's protocol. Lastly, western blot results were digitized with a Canon D660U flatbed scanner and Adobe Photoshop 7.0 software.

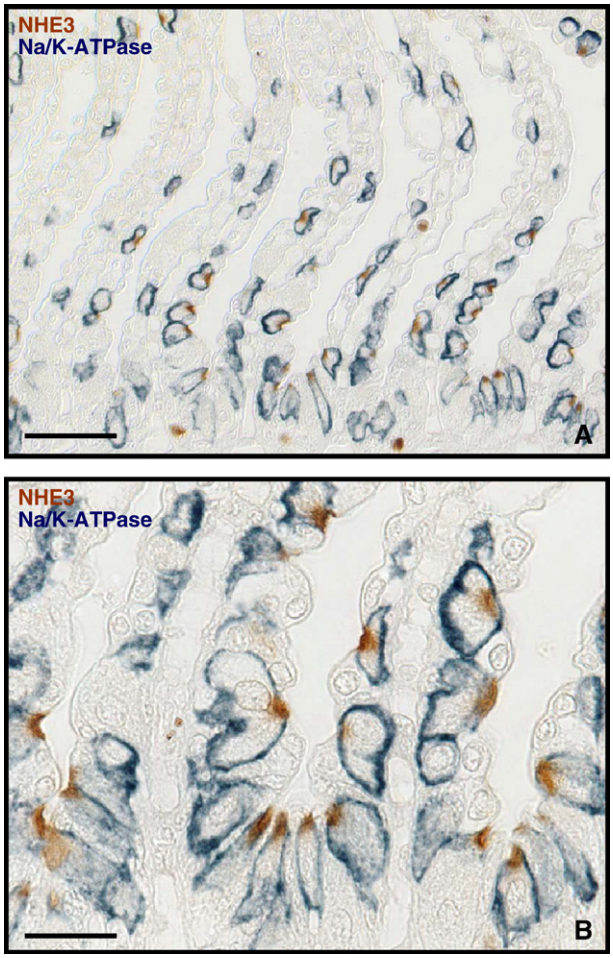

Fig. 3. Representative light micrographs of gill sections from spiny dogfish that demonstrate the localization of NHE3 protein relative to Na^+/K^+-ATPase. Gill sections were incubated with antiserum R1B2 (brown) and antibody α5 (blue). Immunolabeling with antiserum R1B2 was always apical in cells with basolateral Na^+/K^+-ATPase. Scale bars = 100 μm (A) or 20 μm (B).

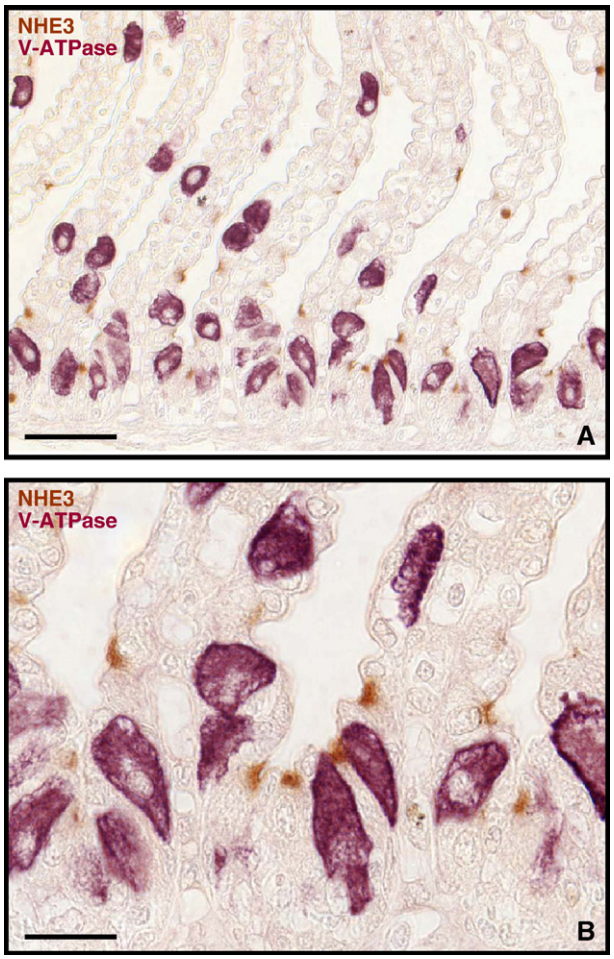

Fig. 4. Representative light micrographs of gill sections from spiny dogfish that demonstrate the localization of NHE3 protein relative to vacuolar H^+-ATPase. Gill sections were incubated with antiserum R1B2 (brown) and an antibody for the vacuolar H^+-ATPase (purple). Immunolabeling with antiserum R1B2 was never in cells with vacuolar H^+-ATPase. Scale bars = 100 μm (A) or 20 μm (B).

2.4. Immunohistochemistry

Immunohistochemistry was completed on paraffin-embedded sections as described previously Choe et al. (2004a,b), with minor modifications. Fixed tissues were dehydrated in an ethanol series and embedded in paraffin wax. Sections were cut at 6 μm and dried onto poly-L-lysine-coated slides. Tissue sections were deparaffinized in Citrisolv (Fisher Scientific, Pittsburgh, PA, USA), and rehydrated in an ethanol series followed by phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited by incubating with 3% H_20_2 for 25 min at 24 °C. Non-specific binding sites on the tissues were blocked by incubating with Biogenex's protein block (San Ramon, CA) (BPB: normal goat serum with 1% bovine serum albumin, 0.09% NaN_3, and 0.1% Tween-20) for 20 min. Sections were then incubated with antibody R1B2 (diluted 1/1000 to 1/2000 in BPB) overnight at 4 °C, in a humidified chamber. Negative control sections were incubated with BPB lacking antibodies, pre-immune serum, or R1B2 that was pre-absorbed with 3.15 nmol L^{-1} antigen. Unbound primary antibodies were removed with a 5 min rinse in PBS. Sections were then incubated with Biogenex's (San Ramon, CA) multilink solution (biotinylated goat anti-mouse, rabbit, guinea pig, and rat antibodies diluted in BPB), followed with Biogenex's horseradish-peroxidase streptavidin solution for 20 min at 24 °C each. After another wash in PBS for 5 min, antibody binding was visualized by incubating with 3,3"-diaminobenzidine tetrahydrochloride (DAB) for 5 min at 24 °C. Sections were then rinsed with running tap water for 5 min, dehydrated in an ethanol-Citrosolv series, and mounted with a coverslip using Permount (Fisher Scientific).

A double labeling technique was used to compare the location of NHE3 immunoreactivity to Na^+/K^+-ATPase and vacuolar H^+-ATPase immunoreactivity in gills (Piermarini and Evans, 2000, 2001). Gill sections were deparaffinized, hydrated, and stained with antibody R1B2 as described above. However, after treatment with DAB and rinsing with water, the sections were again blocked with BPB for 20 min, and incubated with a monoclonal antibody for Na^+/K^+-ATPase (α5) or the polyclonal antibody for the B subunit of vacuolar

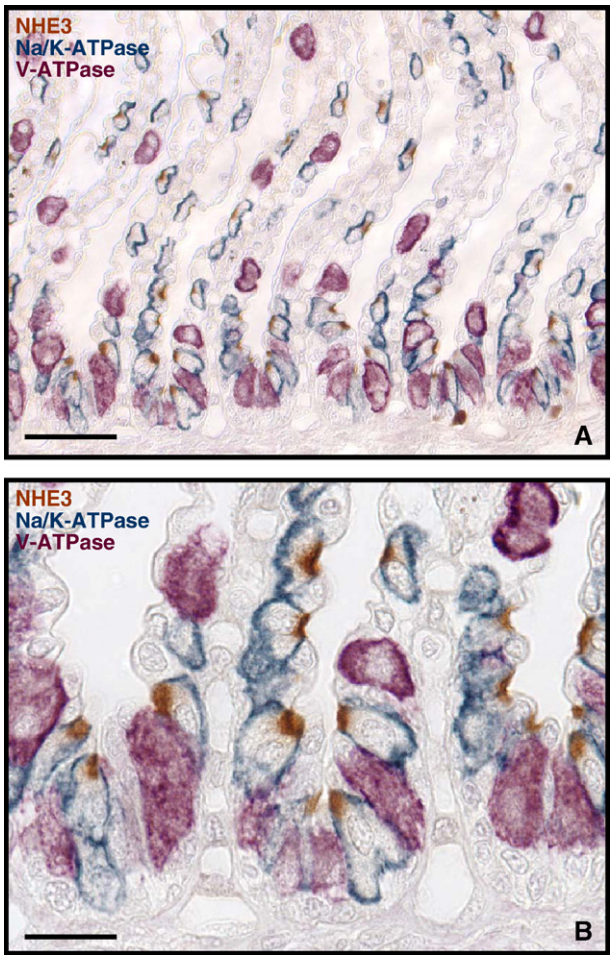

Fig. 5. Representative light micrographs of gill sections from spiny dogfish that demonstrate the localization of NHE3 protein relative to Na^+/K^+-ATPase and vacuolar H^+-ATPase. Gill sections were incubated with antiserum R1B2 (brown), antibody α5 (blue), and an antibody for the vacuolar H^+-ATPase (purple). Immunolabeling with antiserum R1B2 was always apical in cells with basolateral Na^+/K^+-ATPase and never in cells with vacuolar H^+-ATPase. Scale bars = 100 μm (A) or 20 μm (B).

H^+-ATPase. Concentrations were 1/10 or 1/50 for α5 and 1/10,000 for vacuolar H^+-ATPase. Detection of bound antibody was done as described above, except Vector SG, which produced a blue reaction product, was used for Na^+/K^+-ATPase, and Vector VIP, which produced a purple reaction product, was used for vacuolar H^+-ATPase. Lastly, a triple labeling technique was used to simultaneously demonstrate the location of NHE3, Na^+/K^+-ATPase, and vacuolar H^+-ATPase. Gill sections were double labeled for NHE3 and vacuolar H^+-ATPase as above, and then blocked again and labeled for Na^+/K^+-ATPase with 1/10 α5.

3. Results

Antibody R1B2 labeled a single 88 kDa protein on western blots of dogfish shark gill proteins (Fig. 1). Antibody RIB2 also reacted strongly with a subpopulation of epithelial cells in sections of dogfish shark gills (Fig. 2A). Staining was limited to the apical side of cuboidal to columnar epithelial cells in the filaments and lamellae (Fig. 2C). No staining was observed when sections were incubated with BPB, pre-immune serum, or when antibody R1B2 was pre-incubated with antigen (Fig. 2B). We stained for NHE3 and Na^+/K^+-ATPase or NHE3 and vacuolar H^+-ATPase in the same gill sections to determine if NHE3 immunoreactivity colocalized with either of the ATPase ion pumps. NHE3 immunoreactivity was always in the same cells as Na^+/K^+-ATPase immunoreactivity (Fig. 3A). Within these double-labeled cells, Na^+/K^+-ATPase immunoreactivity was confined to the basolateral region and NHE3 staining was always in the apical region (Fig. 3B). Alternatively, NHE3 immunoreactivity was never in the same cells as vacuolar H^+-ATPase immunoreactivity (Fig. 4). In triple-labeled sections that were simultaneously stained for NHE3, Na^+/K^+-ATPase, and vacuolar H^+-ATPase, NHE3 and Na^+/K^+-ATPase colocalized in the same cells, and vacuolar H^+-ATPase labeled an adjacent population of cells (Fig. 5).

4. Discussion

Our study is the first to use an elasmobranch-specific antibody to unequivocally localize NHE3 in the gills of a stenohaline marine elasmobranch, and the first to localize NHE3, Na^+/K^+-ATPase, and vacuolar H^+-ATPase simultaneously in the gills of any fish. Together with our recent identification and characterization of NHE3 in the gills of the euryhaline Atlantic stingray (Choe et al., 2005), this study suggests that marine stenohaline elasmobranchs express the appropriate ion carriers for Na^+ absorption (i.e., NHE3 and Na^+/K^+-ATPase in A MRCs) even though they cannot physiologically acclimate to fresh water. The likely role of these transporters in marine elasmobranchs is acid secretion for acid–base regulation.

4.1. NHE3 in stenohaline marine elasmobranch gills

The apical staining of NHE3 with our elasmobranch specific antibody demonstrates that the exchanger has direct access to environmental water where it could facilitate acid secretion in exchange for Na^+ absorption. The gills of elasmobranchs are clearly the dominant site of whole-animal net-acid secretion during systemic acidosis (Claiborne, 1998; Choe and Evans, 2003; Evans et al., 2004), and a link between Na^+ absorption and H^+ secretion has been established in this tissue (Evans et al., 1979; Evans, 1982). We hypothesize that NHE3 mediates this transport. Attempts to detect changes of NHE3 expression in dogfish gills during systemic acidosis have been unsuccessful with quantitative PCR. Similarly, NHE3 expression did not change in the gills of Atlantic stingrays during hypercapnic acidosis (Choe et al., 2005), and it was proposed that NHE3 transport activity might be regulated by post-translational modifications during acidosis, instead of transcriptional changes. Whole-animal net-acid secretion can increase by almost an order of magnitude within a few hours of experimentally initiating a systemic acidosis (Claiborne and Evans, 1992; Wood et al., 1995). This acute stimulation of acid secretion is likely to occur too rapidly for transcription, translation, folding, and trafficking of new NHE3 protein. Our staining demonstrates that dogfish gills constitutively express high levels of NHE3 in the gills, suggesting that adequate transporters may exist in the absence of acidosis, and that activation of these pre-existing transporters may be responsible for acid secretion during acidosis. Indeed, the activity of NHE3 in mammals is regulated by phosphorylation, protein trafficking, and regulatory proteins via the carboxyl, hydrophilic region of the transporter (Moe, 1999). Most of the regulatory, carboxyl regions of elasmobranch and mammalian NHE3s are well conserved (Choe et al., 2005), suggesting that NHE3 can be regulated by similar pathways in elasmobranchs and that these control mechanisms evolved early in vertebrate evolution. Expression in heterologous cell systems, where transport activity can be tightly monitored, is needed to identify the acute regulatory mechanisms of elasmobranch NHE3 proteins. In addition, the ability to knockdown in vivo gene expression in adult fish could soon be available (Esaki et al., 2006), making it possible to functionally test the hypothesis that NHE3 facilitates acid secretion in gills.

4.2. NHE2/4

A recent study used an antibody for mammalian NHE2 to demonstrate elevated staining on Western blots of gill proteins from acidotic dogfish relative to control dogfish (Tresguerres et al., 2005). However, as Tresguerres et al. (2005) noted in that study, the western blot results must be interpreted cautiously because no molecular sequence for an elasmobranch NHE2 homologue was available. Since that study was published, mRNA for NHE2 homologues has been partially sequenced from the Atlantic stingray (Choe et al., 2005) and completely sequenced from dogfish sharks (NCBI: #DQ324545). As first demonstrated for the Atlantic stingray, elasmobranchs appear to have diverged from the more derived vertebrates (i.e., Tetrapods) before the gene duplication event that resulted in NHE2 and NHE4 of mammals (Choe et al., 2005). In phylogenetic analyses, mammalian NHE2 and NHE4 always group together, separately from the elasmobranch and teleost

NHE2 homologues (Choe et al., 2005). While the dogfish NHE2/4 homologue is 57% identical to mouse NHE2, it is also 48% identical to mouse NHE4, so the elasmobranch NHE2/4 homologue could have functions similar to one or both mammalian isoforms. The exact physiological functions of NHE2 and NHE4, especially in the mammalian kidney, are still being defined. In mammals, NHE2 is expressed in the apical membranes of gastrointestinal cells where it participates in Na^+ absorption (Zachos et al., 2005) and in the apical membranes of renal epithelial cells where it may play a role in secretory functions (Orlowski and Grinstein, 2004). NHE4 is expressed in the basolateral membranes of renal cells that lack detectable levels of NHE1 activity where it may contribute to intracellular pH and volume regulation (Orlowski and Grinstein, 2004). In the dogfish, homologous antibodies recognize the NHE2/4 homologue in the branchial epithelium and it appears to be expressed both in lamellar cells (collocated with H^+-ATPase) and also in cells staining for Na^+/K^+-ATPase in the gill (and likely NHE3; Claiborne et al, unpublished observations). Clearly, further work is needed to understand the role(s) of the elasmobranch NHE2/4 homologue. In addition to increasing our understanding of gill transport mechanisms, characterization of the elasmobranch NHE2/4 homologue would provide a reference point to understand the recent divergence of structure and function between mammalian NHE2 and NHE4.

4.3. Colocalization of NHE3 and Na^+/K^+-ATPase

Na^+/K^+-ATPase and vacuolar H^+-ATPase were previously localized in the gills of the marine spiny dogfish (*S. acanthias*) with heterologous antibodies (Wilson et al., 1997, 2002a; Tresguerres et al., 2005). Serial sections were used to suggest that high levels of the two ATPase ion pumps were predominately expressed in separate cells, with a small proportion of cells expressing both ion pumps (Tresguerres et al., 2005). While we cannot completely rule-out the existence of rare cells that express high levels of both ion pumps, our current multi-labeling results strongly suggest that high levels of Na^+/K^+-ATPase and vacuolar H^+-ATPase are always, or almost always, expressed in two different populations of cells (Fig. 5).

The localization of apical NHE3 only in cells with high levels of Na^+/K^+-ATPase and never in cells with high levels of vacuolar H^+-ATPase is qualitatively identical to the gills of Atlantic stingrays (Choe et al., 2005). We hypothesize that apical NHE3 and basolateral Na^+/K^+-ATPase are functional linked in these cells (A MRC). Na^+/K^+-ATPase in the basolateral membrane is predicted to create a low intracellular [Na^+] that, together with a high [Na^+] in seawater, could provide a gradient for Na^+ to enter through the apical membrane via NHE3 in exchange for secretion of H^+. In fact, an elevation of Na^+/K^+-ATPase protein expression was observed in the gills of dogfish that were systemically infused with HCl for 24 h (Tresguerres et al., 2005), suggesting that increased Na^+ transport is associated with elevated acid secretion. H^+ secretion would lead to generation of HCO_3^- via CO_2 hydration. A yet to be identified, basolateral mechanism for HCO_3^- efflux toward the blood would then provide new base for systemic acid–base regulation. This scenario is similar to renal proximal tubule cells of mammals (Vallon et al., 2000; Wang et al., 2001).

Our group previously demonstrated that pendrin immunoreactivity occurred in the vacuolar H^+-ATPase-rich cells of Atlantic stingrays and spiny dogfish (Piermarini et al., 2002; Evans et al., 2004). In these cells, we predict that vacuolar H^+-ATPase alkalizes the intracellular space by removing H^+ across the basolateral membrane. The alkaline environment would then generate intracellular HCO_3^- that could be secreted across the apical membrane in exchange for Cl^- via a pendrin-like anion exchanger. Interestingly, Tresguerres et al. (2005) observed elevated protein expression, activity, and basolateral localization of vacuolar H^+-ATPase in gills of dogfish that were systemically infused with $NaHCO_3$, suggesting that increased H^+ transport is associated with elevated base secretion.

4.4. The barrier to euryhalinity?

Our previous studies on the euryhaline Atlantic stingray demonstrated that the number of A and B MRCs and the expression of all four ion carriers that they contain (i.e., NHE3, Na^+/K^+-ATPase, pendrin, and vacuolar H^+-ATPase) are greater in fresh water than seawater (Piermarini and Evans, 2000, 2001; Piermarini et al., 2002; Choe et al., 2005). We proposed that the function of these cell types and ion carriers is for Na^+ and Cl^- absorption in hypo-ionic environments. It is now clear that the same cell types with the same ion carriers are expressed in the gills of the stenohaline marine spiny dogfish. This suggests that the appropriate ion carriers and cell types for active ion absorption in fresh water are present in most, if not all elasmobranchs. Therefore, the physiological role of A and B MRCs in most elasmobranchs is likely to be acid and base secretion for systemic pH regulation (Evans et al., 2004). These transport mechanisms then only need to be properly activated in euryhaline species to allow active ion uptake in fresh water. This idea of early, ancestral marine fishes possessing branchial Na^+/acid and Cl^-/base transport mechanisms that "pre-adapt" them for ion absorption in fresh water was first proposed by Evans (1984). However, if most elasmobranchs posses the transport mechanisms for active ion absorption, then why are the vast majority of elasmobranchs (over 95% of extant species) only found in marine environments and believed to be physiologically incapable of osmoregulating in fresh water? We hypothesize that stenohaline marine elasmobranchs lack the ability to properly activate A and B MRCs in fresh water, and/or lack the ability to properly adjust ion and water transport or permeability at other osmoregulatory organs (e.g., kidneys and rectal glands). Coordinated activation of ion absorption in the gills, reduced NaCl secretion from the rectal gland, and increased water secretion and NaCl reabsorption in the kidneys must occur for long-term osmoregulation in fresh water (Hazon et al., 2003). Indeed, in addition to increasing the number of MRCs in the gills, Atlantic stingrays reduce the activity and expression of Na^+/K^+-ATPase in rectal glands (Piermarini and Evans, 2000) and greatly increase urine flow rates and ion reabsorption in kidneys (Janech and Piermarini, 2002) when in fresh water. No obvious differences in the rectal glands, kidneys, or

osmoregulatory hormones of stenohaline and euryhaline elasmobranchs have been observed (Hazon et al., 2003). Therefore, the barrier to euryhalinity in elasmobranchs is likely to reside in subtle differences in the regulation of these osmoregulatory systems by hormonal (e.g., rennin–angiotensin system) (Hazon et al., 1999) or cellular (e.g., phosphorylation and protein–protein interactions) (Moe, 1999) signals. A comprehensive understanding of these regulatory differences will require a careful comparison of the molecular and cellular responses that occur when stenohaline and euryhaline elasmobranchs are exposed to salinity dilutions.

Acknowledgments

We thank Shigehisa Hirose and Akira Kato for assistance with development of the elasmobranch NHE3 antibody. This material is based upon work supported by National Science Foundation grants IBN-0089943 to D. H. E., IBN-0111073 to J. B. C., and 0413427 to K. P. C.

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