Comparative Biochemistry and Physiology, Part B 188 (2015) 46–53

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Cloning and characterization of Na^+/H^+ Exchanger isoforms NHE2 and NHE3 from the gill of Pacific dogfish *Squalus suckleyi*

Samuel C. Guffey [a,b,1], Larry Fliegel [c], Greg G. Goss [a,b,*]

[a] Department of Biological Sciences, Z512 Biological Sciences Bldg, University of Alberta, Edmonton, AB T6G 2E9, Canada
[b] Bamfield Marine Sciences Centre, 100 Pachena Road, Bamfield, BC V0R 1B0, Canada
[c] Department of Biochemistry, 347 Medical Sciences Bldg, University of Alberta, Edmonton, AB T6G 2H7, Canada

ARTICLE INFO

Article history:
Received 28 April 2015
Received in revised form 16 June 2015
Accepted 17 June 2015
Available online 23 June 2015

Keywords:
Proton
Homeostasis
Cell
Elasmobranch
Transport

ABSTRACT

Na^+/H^+ Exchanger (NHE) proteins mediate cellular and systemic homeostasis of sodium and acid and may be the major sodium uptake method for fishes. We cloned and sequenced NHE2 and NHE3 from the gill of the North Pacific Spiny Dogfish shark *Squalus suckleyi* and expressed them in functional form in NHE-deficient (AP-1) cell lines. Estimated IC_{50} for inhibition of NHE activity by amiloride and EIPA were 55 μmol l^{-1} and 4.8 μmol l^{-1}, respectively, for NHE2 and 9 μmol l^{-1} and 24 μmol l^{-1}, respectively, for NHE3. Phenamil at 100 μmol l^{-1} caused less than 16% inhibition of activity for each isoform. Although the IC_{50} are similar for the two isoforms, dfNHE2 is less sensitive than human NHE2 to inhibition by amiloride and EIPA, while dfNHE3 is more sensitive than human NHE3. These IC_{50} estimates should be considered when selecting inhibitor doses for fishes and for reinterpretation of previous studies that use these pharmacological agents.

1. Introduction

The majority of sodium and acid/base transfers between the aquatic environment and the body of a fish are mediated by sodium/proton exchangers, or NHEs. Many studies have shown evidence for Na^+/H^+ exchange activity at the level of the whole animal or specific tissues (Krogh, 1939; Evans, 1982; Claiborne et al., 1994, 1997). In fact, this mechanism seems to be responsible for almost all net influx of Na^+ and net efflux of acid (H^+ and/or NH_4^+) across the gills of marine fishes, including elasmobranchs, teleosts, and hagfishes (Bentley et al., 1976; Evans, 1982, 1984; Heisler, 1988; Claiborne et al., 2002; Evans et al., 2005; Parks, Tresguerres and Goss, 2007; Wright and Wood, 2009). To broaden our understanding of this mechanism in marine elasmobranchs, we pursued a molecular functional analysis of dogfish shark NHE proteins (HUGO gene nomenclature: SLC9A subfamily).

In mammals, there are at least nine functional NHE genes, named NHE1–9 (SLC9A1–9) (Donowitz et al., 2013). In chondrichthyan fishes, eight NHE isoforms (NHE1, 2, 3, 5, 6, 7, 8, and 9) have been identified in the draft genome sequence of the holocephalan elephant shark, *Callorhinchus milii* (Venkatesh et al., 2007). All NHE proteins mediate the electroneutral secondary active transport of one extracellular sodium ion for one intracellular proton. The activity of NHEs has been demonstrated to regulate cell volume and intracellular pH (Boron and Boulpaep, 1983; Grinstein et al., 1983) and transepithelial sodium transport (Knickelbein et al., 1983). NHEs are also involved in systemic pH balance and sodium uptake in fishes (Krogh, 1939; Evans, 1982; Claiborne et al., 1997, 2002; Dymowska et al., 2012).

Evidence for NHEs in fish gills comes from physiological, immunohistochemical, and molecular data (see Wright and Wood, 2009 and Dymowska et al., 2012 and references therein). Homologues of human NHE2 and NHE3 have been cloned from the gills of several fish species including the Long-horned sculpin *Myoxocephalus octodecimspinosus*, Atlantic stingray *Dasyatis sabina*, Atlantic spiny dogfish *Squalus acanthias*, Osorezan dace *Tribolodon hakonensis*, and zebrafish *Danio rerio*, among others (Claiborne et al., 1999, 2008; Hirata et al., 2003; Choe et al., 2005; Ito et al., 2014). NHE3 homologues have also been cloned from the kidney and intestine of the banded houndshark *Triakis scyllium* (Li et al., 2013). Interestingly, all of the NHEs cloned from fish gills have been homologues of either NHE2 or NHE3, and attempts to localize other NHE isoforms (e.g., NHE1) to gill cells have returned negative results (Yan et al., 2007). NHE2 and NHE3 are highly expressed in the ionocytes, or mitochondrion-rich cells, of the gill epithelium (Edwards et al., 2002; Choe et al., 2005, 2007; Claiborne et al., 2008; Ballantyne

* Corresponding author at: Department of Biological Sciences, Z512 Biological Sciences, Bldg, University of Alberta, Edmonton, AB T6G 2E9, Canada. Tel.: +1 780 492 1276.
E-mail addresses: guffey0@purdue.edu (S.C. Guffey), lfliegel@ualberta.ca (L. Fliegel), ggoss@ualberta.ca (G.G. Goss).
[1] Present address: Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN 47907, USA.

http://dx.doi.org/10.1016/j.cbpb.2015.06.003
1096-4959/

and Robinson, 2010). Interestingly, a subset of gill ionocytes in the Atlantic Spiny Dogfish *S. acanthias* has been observed to express both NHE2 and NHE3 isoforms on the cell apical surface (Choe et al., 2007; Claiborne et al., 2008), leading to questions regarding the role of each isoform. One of the remaining challenges for fish physiology is to delineate the specific functions of NHE isoforms in these specific tissues.

Inferences into isoform-specific differences have been primarily based on changes in abundance of mRNA or immunoreactive protein. Tresguerres et al. (2005) found that NHE2-like protein in the membrane-enriched fractions of gill homogenates from Pacific dogfish increased in response to systemic acidosis. In the euryhaline stingray, *D. sabina*, Choe et al. (2005) found NHE3 mRNA increased in brackish water versus seawater, and similar results were seen by Reilly et al. (2011) in the bull shark *Carcharhinus leucas*. In *D. sabina*, NHE2 mRNA did not change in response to brackish water while NHE3 mRNA increased, but during acidosis, NHE3 expression did not change (Choe et al., 2005). Experiments in non-elasmobranch fishes show similar results. NHE2-like protein also increased in gill membrane-enriched fractions from hagfish experiencing acidosis (Parks et al., 2007). In rainbow trout, NHE2 mRNA increased during hypercapnic acidosis, while NHE3 mRNA did not (Ivanis et al., 2008). These results are consistent with a role for NHE2 in acid excretion and for NHE3 in sodium absorption.

At the molecular and functional levels, NHE1, NHE2, and NHE3 have been extensively studied (Franchi et al., 1986; Kapus et al., 1994; Brett et al., 2005; Lee et al., 2011; Donowitz et al., 2013). However, these studies have been almost entirely limited to mammalian species. Many experiments on NHEs1–3 cloned from several species and expressed in several systems have revealed some general patterns regarding the functional aspects of these isoforms (Franchi et al., 1986; Borgese et al., 1992; Orlowski, 1993; Kapus et al., 1994). For reviews, see Alexander and Grinstein (2009), Brett et al. (2005), Donowitz and Li (2007), Donowitz et al. (2009, 2013), and Goss and Grinstein (1996). In general, NHE2 is susceptible to inhibition by amiloride and amiloride analogs such as EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride), while NHE3 is around 10–50 times more resistant (Masereel et al., 2003). The few non-mammalian NHEs that have been cloned, expressed and functionally analyzed include a mosquito NHE3 (Pullikuth et al., 2006), a zebrafish gill NHE3b (Ito et al., 2014) and an NHE3 from the freshwater dace *T. hakonensis*, which was found to be slightly more resistant to EIPA than is human NHE3 (Hirata et al., 2003). The other NHEs cloned from fishes and other organisms have not been functionally analyzed, yet physiological studies have proceeded on the assumption that all NHEs function similarly and have similar inhibition profiles to mammalian NHEs.

The goals of this study were to clone NHE2 and NHE3 from the North Pacific Spiny Dogfish (dfNHE2, dfNHE3), to express them in active form in an NHE-deficient cell line, and to examine their functionality and susceptibility to inhibition by common sodium transport inhibiting drugs. In particular, we examined the effects of amiloride (a classical NHE inhibitor), phenamil (a putative sodium channel inhibitor that is assumed not to affect NHEs) and EIPA (supposedly a more potent and specific antagonist of NHEs) on dfNHE2 and dfNHE3 (see Kleyman and Cragoe, 1988). Furthermore, we evaluated the potential to discriminate the activities of dfNHE2 and dfNHE3 through treatment with amiloride or EIPA at varying doses.

2. Materials and methods

2.1. Animals

North Pacific spiny dogfish sharks (*Squalus suckleyi*) were caught by hook and line from the Trevor Channel (Vancouver Island, BC, Canada) and immediately transferred to the Bamfield Marine Sciences Centre, where they were held in a tank provided with flowing seawater. Fish were fed every three days until use. Prior to experimentation, fish were fasted for four days. Fish were euthanized with an overdose of MS-222 (5 g/l), gill tissues were dissected, snap frozen in liquid nitrogen, and held at −80 °C. All experimental procedures were conducted according to Bamfield Marine Sciences Centre animal care protocols RS-11-26 and RS-12-10.

2.2. RNA isolation and cloning of full cDNA

RNA was isolated from freshly thawed tissues using TRIzol Reagent (Life Technologies, Carlsbad, California), treated with DNAse I and purified using RNeasy Mini spin columns (Qiagen Canada, Montreal, Quebec) according to the manufacturer's instructions. Purity was checked by measuring absorbance at 230, 260, and 280 nm on a spectrophotometer and integrity was evaluated using denaturing agarose gel electrophoresis. RNA was converted to cDNA through the Fermentas reverse transcriptase reaction (Thermo Fisher Scientific, Waltham, Massachusetts) using oligo-dT and random hexamer primers.

Several partial sequences of NHE-like transcripts were discovered in a previously constructed dogfish gill transcriptome profile database, and a PCR-based strategy was used to amplify overlapping fragments and deduce the full coding sequences (CDS) of dogfish NHE2 and dogfish NHE3 cDNA (referred to as dfNHE2 and dfNHE3, respectively). Several rounds of 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) were employed. Takara 5′ and 3′ RACE and SMARTer 5′ and 3′ RACE reactions (Clontech Laboratories, Mountain View, California) were employed to amplify several overlapping sequences to span the full CDS up to the 5′ and 3′ UTRs. Finally, two full-length transcripts were amplified using the following primers: 5′-TTAAATACCTGTGACCATGGGCGGTG-3′ and 5′-CGCTTTCATAATGTTGACCGAGATTACCAA-3′ for dfNHE2 and 5′-GCCACGATGGGGAGAGATAGGAGCGAGTGTGC-3′ and 5′-GGACTTGG GATTGACTTAGAGTTACATTGATG-3′ for dfNHE3. The products were then cloned into the pTargeT mammalian expression vector (Promega, Madison, Wisconsin). The constructs were sequenced in both directions by Sanger sequencing using BigDye Terminator reagent v3.1 on an Applied Biosystems 3730 DNA Analyzer. The full insert sequences were identical to the deduced CDS. The entire nucleotide sequence of each cDNA was sequenced to a minimum of 4× sequencing replication using cDNA from two individual sharks.

2.3. Sequence analysis and phylogenetics

A BLASTp search of the NCBI nonredundant protein database revealed high sequence similarity to other NHE2 (SLC9A2) and NHE3 (SLC9A3) sequences in other species. Predicted NHE amino acid sequences were then aligned using CLUSTAL Omega and curated using GBLOCKS with less stringent parameters. Maximum likelihood phylogenetic analysis was conducted using PhyML through the program SEAVIEW using the LG substitution model and an automatically optimized gamma distribution of across-site rate variation. Neighbor-joining methods and maximum parsimony methods also produced optimal tree topologies identical to that produced by the maximum likelihood method. Reliability of separations in the maximum likelihood tree was assessed through 500 bootstrap replicates.

Amino acid sequences of each dfNHE isoform were aligned with select related sequences using CLUSTAL Omega. Canonical phosphorylation sites for protein kinase A (PKA) and serum/glucocorticoid regulated kinase (SGK) were inferred using the GPS 2.1 algorithm with a high threshold value (http://gps.biocuckoo.org).

2.4. Expression in antiporter-deficient cells

The pTargeT/dfNHE2 and pTargeT/dfNHE3 constructs were designed for expression in the AP-1 cell line. This line is a derivative of the Chinese hamster ovary cell line that is completely deficient in NHE activity (Rotin and Grinstein, 1989). The cells were grown in plastic tissue culture dishes at 37 °C in a humidified atmosphere of 5% CO_2/95% air and in a medium of MEM-alpha + 10% fetal bovine serum + 200 units

penicillin/streptomycin per ml + 25 mM HEPES, pH = 7.4. The plasmid constructs were transfected into AP-1 cells using Lipofectamine-2000 reagent (Life Technologies, Burlington, Ontario). Subsequently the growth medium was supplemented with 400 μg/ml geneticin (G418). After resistant colonies developed, secondary selection by acid loading was performed as described by Wang et al. (1998). Cultures were maintained by regular re-establishment from frozen stocks, and cultures were used for experiments within 6–12 passages after transfection.

2.5. Na^+/H^+ exchange assay

NHE activity was calculated from the initial rate of Na^+-induced recovery of intracellular pH (pH_i) after induced cellular acidification as described previously (Murtazina et al., 2001). Cells were grown to 60–100% confluence on glass coverslips, and pH_i was monitored fluorimetrically using BCECF (2-,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; Molecular Probes). The coverslip of BCECF-loaded cells was placed in normal buffer containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH = 7.4 in a cuvette holder at 37 °C with constant stirring. 30 mM ammonium chloride was added for three minutes before the coverslip was transferred to sodium-free buffer in which the NaCl component of normal buffer was replaced by *N*-methyl-D-glucamine HCl. After induction of acidification for 30 s, pH_i recovery was measured in normal buffer containing 135 mM NaCl. The initial rate of pH_i recovery was calculated from the first 20 s of recovery. Calibration of BCECF fluorescence to intracellular pH was conducted by the high K^+/nigericin method (Chaillet and Boron, 1985; Silva et al., 1997). For inhibitor tests, the effect of each treatment was assessed through a double pulse protocol. One cycle of acidification and recovery was measured in the absence of drug. Then another cycle of acidification and recovery was measured with the drug present in both the sodium-free and normal buffers. The measurements were highly repeatable, with activity in the second pulse averaging 100 ± 5% of the first pulse. Application of 0.1% DMSO as a vehicle control caused no significant effect. All drugs and materials were purchased from Sigma-Aldrich (Oakville, Ontario) unless noted otherwise.

2.6. Data presentation and statistical analysis

Activity data are shown as means ± SEM of four replicate cell populations normalized to activity in the absence of drug. Values at zero drug concentration represent the vehicle control treatment. Data for inhibition profiles were fitted to sigmoidal dose–response curves with Hill coefficients using SigmaPlot v11.0.

3. Results

Using PCR and RACE, the full coding sequence of the *S. suckleyi* dogfish NHE2 and dogfish NHE3 (dfNHE2 and dfNHE3, respectively) were deduced and cloned from gill cDNA. Both dfNHE2 and dfNHE3 nucleotide sequences have been deposited in Genbank with accession numbers KR297235 and KR297236, respectively. The *S. suckleyi* dfNHE2 cDNA contains a 2310 bp ORF encoding a 769 amino acid protein, followed by a 150 bp 3′ UTR including the poly-A tail and preceded by a 5′ UTR of at least 225 bp. The *S. suckleyi* dfNHE3 cDNA contains a 2505 bp ORF encoding an 834 amino acid protein, an 887 bp 3′ UTR with poly-A tail and a 5′ UTR of at least 84 bp.

Maximum likelihood phylogenetic inference supports the identification of the cloned sequences as members of the NHE2 (slc9a2) and NHE3 (slc9a3) clades (Fig. 1). The phylogenetic tree obtained here is consistent with the diversification of the NHE (slc9a) family early in the ancestry of the vertebrates.

The deduced sequence of the *S. suckleyi* dfNHE2 differs from the closely related *S. acanthias* NHE2 by seven or eight amino acid residues (Fig. 2). (One residue was unresolved in the published *S. acanthias* NHE2.) The fourth transmembrane domain (TM IV) is almost perfectly

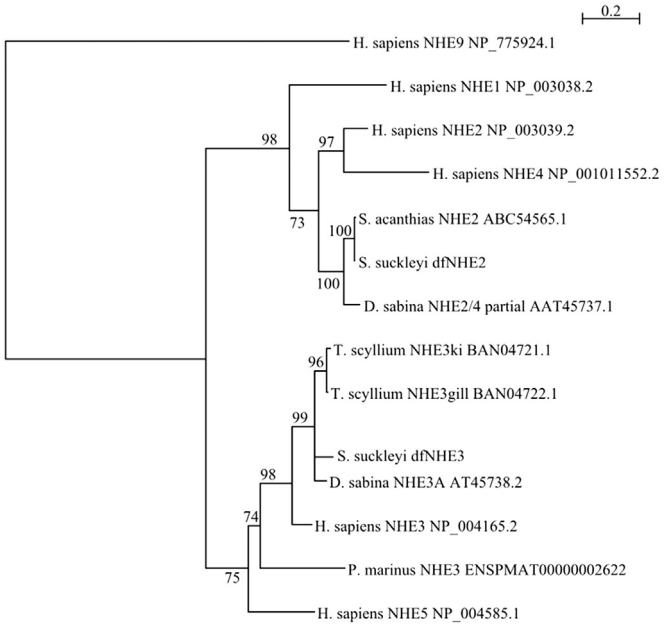

Fig. 1. Maximum likelihood (PhyML) phylogenetic tree of the cloned *S. suckleyi* dfNHE2 and dfNHE3 among other vertebrate NHEs. Numbers indicate the percentage of 500 bootstrap replicates that supported the separation. Branch length represents degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. Divisions with less than 50% bootstrap support have been collapsed. Genbank identification numbers are given at the end of the protein name, except for the *P. marinus* protein, which was retrieved from Ensembl. List of genera: H., *Homo*, S., *Squalus*, D., *Dasyatis*, T., *Triakis*, P., *Petromyzon*.

conserved across all of the examined NHE2s. Two residues in *S. suckleyi* dfNHE2, S602 and S625, are predicted to be SGK (serum- and glucocorticoid-regulated kinase) phosphorylation sites.

The deduced sequence of the *S. suckleyi* dfNHE3 was highly similar to other vertebrate NHE3s (Fig. 3). The dfNHE3 sequence shares 87% amino acid identity with the NHE3 from the gill of the Japanese houndshark, *T. scyllium*. A PKA (protein kinase A) phosphorylation site, S569 in dfNHE3, is conserved across all NHE3s examined. Position S619 in dfNHE3 is predicted to be either an SGK or PKA phosphorylation site. The sequence of TM IV is strongly conserved across vertebrate NHEs, but dfNHE3 differs from the human and *T. scyllium* NHE3 sequence at two positions, N127 and I138.

When expressed in Na^+/H^+ exchange-deficient AP-1 cells, dfNHE2 and dfNHE3 each rescue Na^+/H^+ exchange activity and allow recovery and survival after an induced acid load. Both untransfected AP-1 cells and cells transfected with the empty vector showed no Na^+-induced recovery and did not survive after acid loading. Fig. 4 shows the dose–response curves for inhibition of dfNHE2 by amiloride and EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride) assessed at 135 mmol Na^+ l^{-1}. The inhibitor concentration sufficient for 50% inhibition (IC_{50}) of dfNHE2 was estimated at 55 μmol l^{-1} for amiloride and 4.8 μmol l^{-1} for EIPA. Exposure to 100 μmol l^{-1} phenamil inhibited dfNHE2 activity by only 6 ± 1%. As shown in Fig. 5, dfNHE3 exhibited different sensitivities, with an estimated IC_{50} of 24 μmol l^{-1} for amiloride and 9 μmol l^{-1} for EIPA. Phenamil at 100 μmol l^{-1} inhibited dfNHE3 activity by 16 ± 6%.

4. Discussion

Comparison of the *S. suckleyi* dfNHE2 with the previously reported *S. acanthias* NHE2 reveals either seven or eight amino acid substitutions, as one residue was unresolved in the published *S. acanthias* NHE2 sequence (Claiborne et al., 2008). This evidence corroborates the genetic and life history traits that have been used by Verissimo et al. (2010) and Ebert et al. (2010) to distinguish these two sharks as distinct

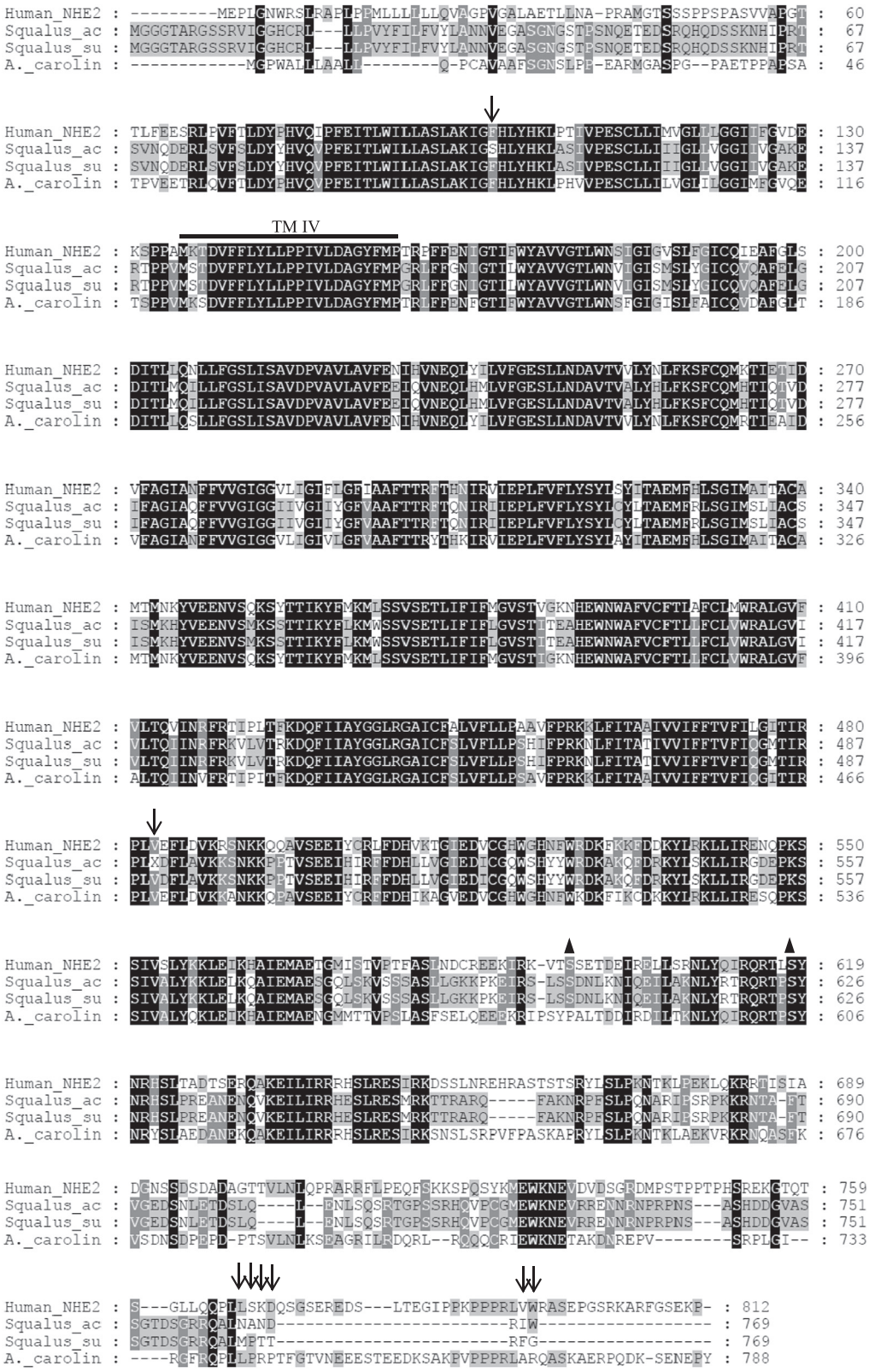

Fig. 2. CLUSTAL Omega alignment of deduced amino acid sequences of *S. suckeyi* gill NHE2 with *S. acanthias* NHE2 and related sequences. Positions highlighted in black are strongly conserved and positions in gray are moderately conserved. Arrows indicate differences between congeneric dogfish sequences. The fourth transmembrane domain, which is critical for transport function, is indicated by an overline. Triangles indicate likely SGK phosphorylation sites in *S. suckleyi* dfNHE2 as predicted by the GPS 2.1 algorithm. Sequence labels: Human NHE2, NP_003039.2; Squalus_ac, *Squalus acanthias* NHE2 ABC54565.1; Squalus_su, *Squalus suckleyi* dfNHE2; A._carolin, *Anolis carolinensis* NHE2-like XP_003224334.1.

species. All but one or two of the amino acid substitutions occur in the carboxyl-terminal tail of the protein, which is known to vary both within and between NHE isoforms and which serves a regulatory role. That said, there have been no structural studies of NHE2 and little is known about the function of various domains except what is inferred based on other NHE isoforms (Donowitz et al., 2013).

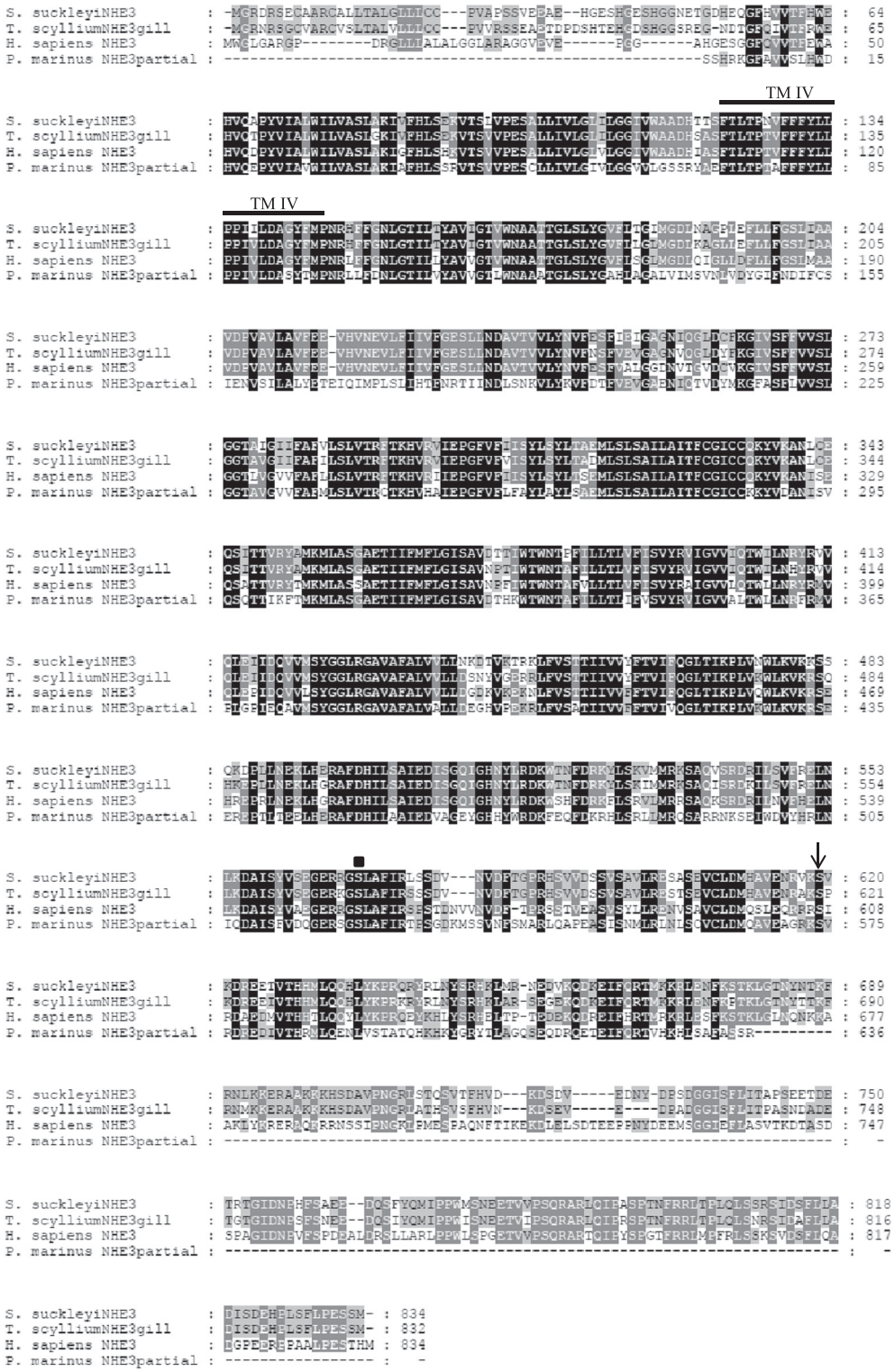

Fig. 3. CLUSTAL Omega alignment of deduced amino acid sequences of *S. suckleyi* gill NHE3 and related sequences. Positions highlighted in black are strongly conserved and positions in gray are moderately conserved. The fourth transmembrane domain, which is critical for transport function, is indicated by an overline. The black square indicates a PKA phosphorylation site that is conserved across all of these NHE3s. An arrow indicates an interesting position, S619 in *S. suckleyi* dfNHE3 that is predicted to be either an SGK or PKA phosphorylation site; the position is an important inhibitory PKA site in rat NHE3. *T. scyllium* NHE3gill BAN04722.1, *H. sapiens* NHE3 NP_004165.2, *P. marinus* NHE3partial ENSPMAT00000002622.

Two residues in *S. suckleyi* dfNHE2, S602 and S625, are predicted to be SGK (serum- and glucocorticoid-regulated kinase) phosphorylation sites. SGK has been cloned from *S. acanthias*, and transcription can be induced in rectal gland cells by exposure to hypertonic medium, presumably playing a role in cell volume regulation (Waldegger et al., 1998). Various studies have shown that NHE2 in several vertebrate species can be induced in response to serum, cellular volume shrinkage, and cellular acidification, with the response most likely mediated by cAMP, protein kinase A, and protein kinase C (Kapus et al., 1994; Kandasamy et al., 1995; McSwine et al., 1998; Gens et al., 2007). Whether these pathways

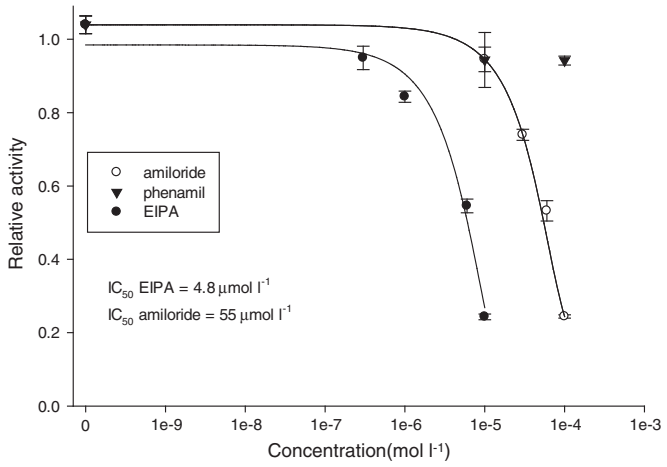

Fig. 4. Dose–response curves showing the effects of select inhibitors on dfNHE2 activity. Values are mean ± SEM, n = 4. The sigmoidal dose–response curves with Hill coefficients were fitted with SigmaPlot v11.0. Phenamil at 100 µmol l^{-1} caused less than 10% inhibition.

regulate dfNHE2 in similar ways to mammalian NHE2 is unknown but could be tested using the expression system we have established.

The dfNHE3 cloned in the present study is very similar to other vertebrate NHE3s, including the recently reported *T. scyllium* kidney NHE3, the first shark NHE3 to be cloned and reported (Li et al., 2013). A protein kinase A phosphorylation site, S569 in dfNHE3, is conserved across all NHE3s examined. The homologous residue in rat NHE3 is one of at least three serines involved in cAMP- and PKA-mediated inhibition (Donowitz and Li, 2007). The residue equivalent to S619 of dfNHE3 in rat NHE3 is S605. S605 is known to be the most important PKA phosphorylation site in rat NHE3 involved in acute downregulation in response to cAMP (Kurashima et al., 1997). In dfNHE3, however, S619 is preceded by the amino acid residue sequence ENRVK, which is interesting because this sequence is predicted to be either an SGK site or a PKA site by the GPS 2.1 algorithm, with the former case being slightly favored by the algorithm. In mammalian NHE3s, SGK stimulates NHE3 activity through phosphorylation at a different site (Wang et al., 2005), as well as by phosphorylation-independent mechanisms (Donowitz and Li, 2007).

The sequence of TM IV is strongly conserved across vertebrate NHEs and is known to be important in Na^+ affinity and in the action of amiloride-based inhibitor drugs (Slepkov et al., 2005; Lee et al., 2011; Tzeng et al., 2011). TM IV is also thought to be part of the novel fold of NHE1 critical in its activity (Lee et al., 2011). Table 1 shows the sequences of TM IV from human NHE1–3 aligned with dfNHE2 and dfNHE3. The sequence of dfNHE2 is nearly identical to human NHE2 (also see Fig. 2), but dfNHE3 differs from the human, *T. scyllium*, and *Petromyzon marinus* NHE3 sequence at two positions, N127 and I138 in dfNHE3 (also see Fig. 3). Under the assumption that the consensus sequence is the ancestral sequence, the V138I substitution is fairly conservative, while the T127N substitution is nonconservative. Although the role of T127 within TM IV is not known, substitution of this position in human NHE1 with cysteine (S158C) greatly reduces transport activity (Slepkov et al., 2005).

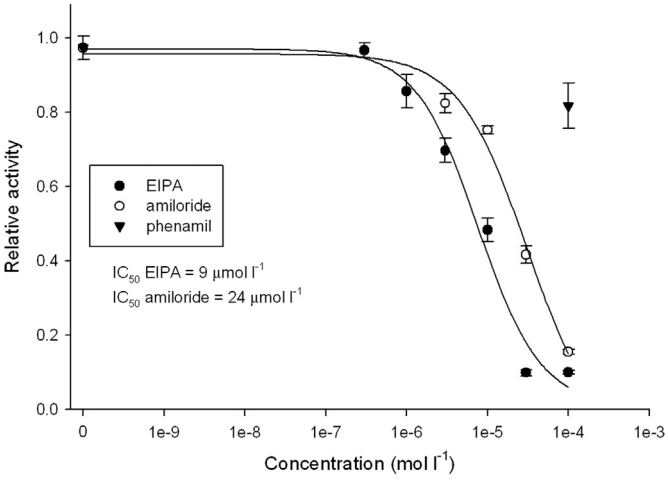

Fig. 5. Dose–response curves showing the effects of select inhibitors on dfNHE3 activity. Values are mean ± SEM, n = 4. The sigmoidal dose–response curves with Hill coefficients were fitted with SigmaPlot v11.0. Phenamil at 100 µmol l^{-1} caused 16 ± 6% inhibition.

The activity of dfNHE2 and dfNHE3 were sensitive to amiloride and EIPA but resistant to phenamil. dfNHE2 is considerably more resistant to amiloride and EIPA than is human NHE2, while dfNHE3 is considerably less resistant than human NHE3 (Kleyman and Cragoe, 1988). Of the very few amino acid residues already known to be important in determining sensitivity to amiloride and its analogs, these residues are identical between human and dogfish isoforms. Given this fact in concert with our observations of considerably different sensitivities between the two species, we conclude that other residues must also be important in Na^+ affinity, amiloride binding, or both. In dfNHE3, substitution T127N may change the shape of the Na^+ and/or amiloride-binding pocket(s), potentially changing the transporter's substrate affinity and sensitivity to inhibitors (Touret et al., 2001; Lee et al., 2011).

This is the first report of IC_{50} for amiloride and EIPA in any non-mammalian NHE2 and NHE3. Other studies have begun to examine pharmacological sensitivity of non-mammalian NHEs. For example, a cloned mosquito NHE3 was rather prematurely labeled as unaffected by amiloride and EIPA due to insufficient data collection (Pullikuth et al., 2006). But in one freshwater cyprinid species, *T. hakonensis*, an NHE3 was cloned, expressed, and tested for EIPA sensitivity in a similar manner to that reported here (Hirata et al., 2003). That study reported an IC_{50} in 1 mmol Na^+ l^{-1} of around 10 µmol EIPA l^{-1}. Accounting for the Na^+ concentration of the assay medium, this implies that the *T. hakonensis* NHE3 exhibits either a much greater affinity for Na^+ or a much lower affinity for EIPA than either dfNHE3 or human NHE3, or some combination of these factors. A recent study by Ito et al. (2014) stated that zebrafish gill NHE3b activity expressed in *Xenopus* oocytes was inhibited by about 65% when exposed to 100 µmol l^{-1} amiloride or 10 µmol l^{-1} EIPA, whereas dogfish NHE3 in the current study was about 85% inhibited by 100 µmol l^{-1} amiloride and 90% inhibited by 10 µmol l^{-1} EIPA, indicating a difference in sensitivity between the two species. In applications of sodium transport inhibitors to fishes, these data should be considered rather than relying on data from mammalian systems to guide experimental design and interpretation.

Future experiments done in a physiological saline or other solutions containing 135 mmol l^{-1} Na^+ may use 100 µmol l^{-1} amiloride to inhibit NHEs, though NHE2 may still exhibit around 20% activity. As little as 30 µmol l^{-1} EIPA should provide maximal inhibition of dogfish NHE2 and NHE3, but this should be confirmed for the species and tissue in question. The action of 100 µmol l^{-1} phenamil on NHEs is low, and it can therefore be used to block sodium channels without significantly affecting NHE2 or NHE3 function. Unfortunately, NHE2 and NHE3 cannot be distinguished through the use of amiloride and EIPA because sensitivities of these isoforms are too similar. In solutions of greater or lesser Na^+ concentration, the concentration of inhibitor should be adjusted, owing to the competitive mode of inhibition exhibited by these drugs (Kleyman and Cragoe, 1988; Masereel et al., 2003). Therefore, examination of inhibitor efficacy in differing Na^+ concentrations is recommended. While K_i values have not been determined in this study, they can be estimated using the relationship $IC_{50} = K_i\ (1 + S/K_m)$, where S is the concentration of Na^+ and K_m is the apparent affinity for Na^+ (Cheng and Prusoff, 1973). Future measurement of apparent K_m values for these NHEs will allow calculation of K_i values and appropriate inhibitor concentrations under any concentration of Na^+ without resorting to

Table 1
Alignment of TM IV sequences from several NHEs and corresponding IC_{50} values at 135 mmol l^{-1} Na^+. TM IV is highly conserved and the underlined residues are known to be involved in Na^+ affinity and the action of amiloride-based drugs (Slepkov et al., 2005). dfNHE2 is considerably more resistant to amiloride and EIPA than is human NHE2, while dfNHE3 is considerably less resistant than human NHE3. Because these differences are present even though the few residues known to be important are identical between the two species, other residues must also be important in Na^+ affinity, amiloride binding, or both. IC_{50} values for dfNHEs at 135 mmol l^{-1} Na^+ are from Figs. 4 and 5. IC_{50} values for human NHEs are calculated at 135 mmol l^{-1} Na^+ from K_i values presented in Masereel et al. (2003) using the relationship $IC_{50} = K_i\,(1 + S/K_m)$, where S is the concentration of Na^+ and K_m is the apparent affinity for Na^+.

Protein	Sequence	$IC_{50}$for amiloride (μmol l^{-1})	IC_{50} for EIPA (μmol l^{-1})
Human NHE1	153PPFLQSDVFFLFLLPPIILDAGYFL	23	0.3
Human NHE2	133PPAMKTDVFFLYLLPPIVLDAGYFM	4	1.8
dfNHE2	140PPVMSTDVFFLYLLPPIVLDAGYFM	55	4.8
Human NHE3	107SFTLTPTVFFFYLLPPIVLDAGYFM	>100	67
dfNHE3	121SFTLTPNVFFFYLLPPIILDAGYFM	24	9

assumptions based on mammalian affinity values. Previous experiments involving the application of these drugs to non-mammalian organisms can now be revisited. Most studies conducted in freshwater have used inhibitor concentrations that seem appropriate in light of the data reported here; however, the conditions used in the present study should be repeated using NHEs from several diverse fish taxa.

Future *in vitro* studies, including testing a broader range of putative NHE inhibitors, substrate affinities and molecular determinants of function are now possible and have not as yet been performed for any fish species. The dfNHE expression system could also be used to test additional inhibitors in hopes of finding some isoform-specific drugs. Promising candidates include cariporide and eniporide for inhibition of NHE3 and S-3226 and BMS284640 for potential inhibition of the NHE2 isoform (Masereel et al., 2003). Also, the sequence data can now be used to create a specific anti-dogfish NHE3 antibody that distinguishes this isoform from other NHEs. In conjunction with the anti-dogfish NHE2 antibody developed by Claiborne et al. (2008), these two antibodies can be tested to determine antibody specificity and investigate NHE functional properties.

5. Conclusions

The dfNHE2 and dfNHE3 cloned and sequenced from the shark *S. suckleyi* are the first NHE2 and NHE3 to be cloned from the same non-mammalian species. Sensitivity to amiloride and EIPA and resistance to phenamil were determined for each isoform stably expressed in AP-1 cells. dfNHE2 was found to be considerably more resistant to these drugs than human NHE2, while dfNHE3 is more sensitive than human NHE3. These data will be useful for studies of sodium transport in fishes, and especially useful in future attempts to discriminate the roles of these two isoforms in systemic pH and sodium homeostasis. The expression system for the dogfish NHEs that we have established provides a very convenient system for investigating many facets of the function and regulation of these two isoforms.

Grants

This work was supported by separate Discovery grants to GGG and LF from the Natural Sciences and Engineering Research Council of Canada (#203736 to GGG; #06564 to LF), and an Alberta Innovates—Technology Futures Graduate Student Scholarship #201100029 to SCG. Funding organizations were not involved in the design, conduct, or reporting of this study.

Acknowledgments

The authors thank Sal Blair and Yongsheng Liu for technical assistance, and we thank two anonymous reviewers, whose comments improved this manuscript.

References

Alexander, R.T., Grinstein, S., 2009. Tethering, recycling and activation of the epithelial sodium/proton exchanger, NHE3. J. Exp. Biol. 212, 1630–1637.
Ballantyne, J., Robinson, J., 2010. Freshwater elasmobranchs: a review of their physiology and biochemistry. J. Comp. Physiol. B. 180, 475–493.
Bentley, P.J., Maetz, J., Payan, P., 1976. A study of the unidirectional fluxes of Na and Cl across the gills of the dogfish *Scyliorhinus canicula* (Chondrichthyes). J. Exp. Biol. 64, 629–637.
Borgese, F., Sardet, C., Cappadoro, M., Pouyssegur, J., Motais, R., 1992. Cloning and expression of a cAMP-activated Na+/H+ exchanger: evidence that the cytoplasmic domain mediates hormonal regulation. PNAS 89, 6765–6769.
Boron, W.F., Boulpaep, E.L., 1983. Intracellular pH in the renal proximal tubule of the salamander. Na+/H+ exchange. J. Gen. Physiol. 81, 29–52.
Brett, C.L., Donowitz, M., Rao, R., 2005. Evolutionary origins of eukaryotic sodium/proton exchangers. Am. J. Physiol. Cell Physiol. 288, C223–C239.
Chaillet, J.R., Boron, W.F., 1985. Intracellular calibration of a pH-sensitive dye in isolated, perfused salamander proximal tubules. J. Gen. Physiol. 86, 765–794.
Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.
Choe, K.P., Kato, A., Hirose, S., Plata, C., Sindic, A., Romero, M.F., Claiborne, J.B., Evans, D.H., 2005. NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R1520–R1534.
Choe, K.P., Edwards, S.L., Claiborne, J.B., Evans, D.H., 2007. The putative mechanism of Na + absorption in euryhaline elasmobranchs exists in the gills of a stenohaline marine elasmobranch, *Squalus acanthias*. Comp. Biochem. Physiol. A 146, 155–162.
Claiborne, J., Walton, J., Compton-Mccullough, D., 1994. Acid–base regulation, branchial transfers and renal output in a marine telost fish (the long-horned sculpin *Myoxocephals octodecimspinosus*) during exposure to low salinities. J. Exp. Biol. 193, 79–95.
Claiborne, J.B., Perry, E., Bellows, S., Campbell, J., 1997. Mechanisms of acid–base excretion across the gills of a marine fish. J. Exp. Zool. 279, 509–520.
Claiborne, J.B., Blackston, C.R., Choe, K.P., Dawson, D.C., Harris, S.P., Mackenzie, L.A., Morrison-Shetlar, A.I., 1999. A mechanism for branchial acid excretion in marine fish: identification of multiple Na+/H+ antiporter (NHE) isoforms in gills of two seawater teleosts. 202, 315–324.
Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid–base regulation in fishes: cellular and molecular mechanisms. J. Exp. Zool. 293, 302–319.
Claiborne, J.B., Choe, K.P., Morrison-Shetlar, A.I., Weakley, J.C., Havird, J., Freiji, A., Evans, D.H., Edwards, S.L., 2008. Molecular detection and immunological localization of gill Na+/H+ exchanger in the dogfish (*Squalus acanthias*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R1092–R1102.
Donowitz, M., Li, X., 2007. Regulatory binding partners and complexes of NHE3. Physiol. Rev. 87, 825–872.
Donowitz, M., Mohan, S., Zhu, C.X., Chen, T.E., Lin, R., Cha, B., Zachos, N.C., Murtazina, R., Sarker, R., Li, X., 2009. NHE3 regulatory complexes. J. Exp. Biol. 212, 1638–1646.
Donowitz, M., Ming Tse, C., Fuster, D., 2013. SLC9/NHE gene family, a plasma membrane and organellar family of Na+/H+ exchangers. Mol. Asp. Med. 34, 236–251.
Dymowska, A.K., Hwang, P.P., Goss, G.G., 2012. Structure and function of ionocytes in the freshwater fish gill. Respir. Physiol. Neurobiol. 184, 282–292.
Ebert, D., White, W.T., Goldman, K.J., Compagno, L.J.V., Daly-Engel, T.S., Ward, R.D., 2010. Resurrection and redescription of *Squalus suckleyi* (Girard, 1854) from the North Pacific, with comments on the *Squalus acanthias* subgroup (Squaliformes: Squalidae). Zootaxa 2612, 22–40.
Edwards, S.L., Donald, J.A., Toop, T., Donowitz, M., Tse, C.M., 2002. Immunolocalisation of sodium/proton exchanger-like proteins in the gills of elasmobranchs. Comp. Biochem. Physiol. A 131, 257–265.
Evans, D.H., 1982. Mechanisms of acid extrusion by two marine fishes: the teleost, *Opsanus beta*, and the Elasmobranch, *Squalus acanthias*. J. Exp. Biol. 97, 289–299.
Evans, D.H., 1984. Gill Na+/H+ and Cl−/HCO3-exchange systems evolved before the vertebrates entered fresh water. J. Exp. Biol. 113, 465–469.
Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.
Franchi, A., Perucca-Lostanlen, D., Pouyssegur, J., 1986. Functional expression of a human Na+/H+ antiporter gene transfected into antiporter-deficient mouse L cells. Proc. Natl. Acad. Sci. U. S. A. 83, 9388–9392.
Gens, J.S., Du, H., Tackett, L., Kong, S.S., Chu, S., Montrose, M.H., 2007. Different ionic conditions prompt NHE2 and NHE3 translocation to the plasma membrane. Biochim. Biophys. Acta Biomembr. 1768, 1023–1035.
Goss, G., Grinstein, S., 1996. Chapter 7 Mechanisms of intracellular pH regulation. In: Neville Bittar, E.E.B.a (Ed.), Principles of Medical Biology. Elsevier, pp. 221–241.

Grinstein, S., Clarke, C.A., Rothstein, A., 1983. Activation of Na/H exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. J. Gen. Physiol. 82, 619–638.
Heisler, N., 1988. Acid–base regulation. In: Shuttleworth, T.J. (Ed.), Physiology of Elasmobranch Fishes. Springer-Verlag, Berlin, pp. 215–252.
Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M.H., Romero, M.F., Hirose, S., 2003. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. Am. J. Physiol. Regul. Integr. Comp. Physiol. 284, R1199–R1212.
Ito, Y., Kato, A., Hirata, T., Hirose, S., Romero, M.F., 2014. Na+/H+ and Na+/NH4+ exchange activities of zebrafish NHE3b expressed in *Xenopus* oocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 306 (5), R315–R327.
Ivanis, G., Esbaugh, A.J., Perry, S.F., 2008. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid/base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 211, 2467–2477.
Kandasamy, R.A., Yu, F.H., Harris, R., Boucher, A., Hanrahan, J.W., Orlowski, J., 1995. Plasma membrane Na+/H+ exchanger Isoforms (NHE-1, 2, and 3) are differentially responsive to second messenger agonists of the protein kinase A and C pathways. J. Biol. Chem. 270, 29209–29216.
Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R., Orlowski, J., 1994. Functional characterization of three isoforms of the Na+/H+ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation. J. Biol. Chem. 269, 23544–23552.
Kleyman, T.R., Cragoe, E.J., 1988. Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. 105, 1–21.
Knickelbein, R., Aronson, P.S., Atherton, W., Dobbins, J.W., 1983. Sodium and chloride transport across the rabbit ileal brush boarder. I. Evidence for Na–H exchange. Am. J. Physiol. 245, G504–G510.
Krogh, A., 1939. Osmotic Regulation in Aquatic Animals. The University press, Cambridge.
Kurashima, K., Yu, F.H., Cabado, A.G., Szabo, E.Z., Grinstein, S., Orlowski, J., 1997. Identification of sites required for down-regulation of Na+/H+ Exchanger NHE3 activity by cAMP-dependent protein kinase phosphorylation-dependent and independent mechanisms. J. Biol. Chem. 272, 28672–28679.
Lee, B.L., Sykes, B.D., Fliegel, L., 2011. Structural analysis of the Na+/H+ exchanger isoform 1 (NHE1) using the divide and conquer approach. Biochem. Cell Biol. 89, 189–199.
Li, S., Kato, A., Takabe, S., Chen, A.P., Romero, M.F., Umezawa, T., Nakada, T., Hyodo, S., Hirose, S., 2013. Expression of a novel isoform of Na+/H+ exchanger 3 in the kidney and intestine of banded houndshark, *Triakis scyllium*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304, R865–R876.
Masereel, B., Pochet, L., Laeckmann, D., 2003. An overview of inhibitors of Na+/H+ exchanger. Eur. J. Med. Chem. 38, 547–554.
McSwine, R.L., Musch, M.W., Bookstein, C., Xie, Y., Rao, M., Chang, E.B., 1998. Regulation of apical membrane Na+/H + exchangers NHE2 and NHE3 in intestinal epithelial cell line C2/bbe. Am. J. Physiol. Cell Physiol. 275, C693–C701.
Murtazina, R., Booth, B.J., Bullis, B.L., Singh, D.N., Fliegel, L., 2001. Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na+/H+ exchanger. Eur. J. Biochem. 268, 4674–4685.
Orlowski, J., 1993. Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. J. Biol. Chem. 268, 16369–16377.
Parks, S.K., Tresguerres, M., Goss, G.G., 2007. Blood and gill responses to HCl infusions in the Pacific hagfish (*Eptatretus stoutii*). Can. J. Zool. 85, 855–862.
Pullikuth, A.K., Aimanova, K., Kang'ethe, W., Sanders, H.R., Gill, S.S., 2006. Molecular characterization of sodium/proton exchanger 3 (NHE3) from the yellow fever vector, *Aedes aegypti*. J. Exp. Biol. 209, 3529–3544.
Reilly, B.D., Cramp, R.L., Wilson, J.M., Campbell, H.A., Franklin, C.E., 2011. Branchial osmoregulation in the euryhaline bull shark, *Carcharhinus leucas*: a molecular analysis of ion transporters. J. Exp. Biol. 214, 2883–2895.
Rotin, D., Grinstein, S., 1989. Impaired cell volume regulation in Na(+)-H+ exchange-deficient mutants. Am. J. Physiol. Cell Physiol. 257, C1158–C1165.
Silva, N.L., Wang, H., Harris, C.V., Singh, D., Fliegel, L., 1997. Characterization of the Na+/H+ exchanger in human choriocarcinoma (BeWo) cells. Pflugers Arch. 433, 792–802.
Slepkov, E.R., Rainey, J.K., Li, X., Liu, Y., Cheng, F.J., Lindhout, D.A., Sykes, B.D., Fliegel, L., 2005. Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na+/H+ exchanger. J. Biol. Chem. 280, 17863–17872.
Touret, N., Poujeol, P., Counillon, L., 2001. Second-site revertants of a low-sodium-affinity mutant of the Na+/H+ exchanger reveal the participation of TM4 into a highly constrained sodium-binding site. Biochemistry 40, 5095–5101.
Tresguerres, M., Katoh, F., Fenton, H., Jasinska, E., Goss, G.G., 2005. Regulation of branchial V-H + -ATPase Na+/K + -ATPase and NHE2 in response to acid and base infusions in the Pacific spiny dogfish (*Squalus acanthias*). J. Exp. Biol. 208, 345–354.
Tzeng, J., Lee, B.L., Sykes, B.D., Fliegel, L., 2011. Structural and functional analysis of critical amino acids in TMVI of the NHE1 isoform of the Na+/H+ exchanger. Biochim. Biophys. Acta Biomembr. 1808, 2327–2335.
Venkatesh, B., Kirkness, E.F., Loh, Y.H., Halpern, A.L., Lee, A.P., Johnson, J., Dandona, N., Viswanathan, L.D., Tay, A., Venter, J.C., Strausberg, R.L., Brenner, S., 2007. Survey sequencing and comparative analysis of the elephant shark (*Callorhinchus milii*) genome. PLoS Biol. 5.
Verissimo, A., Mcdowell, J.R., Graves, J.E., 2010. Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution. Mol. Ecol. 19, 1651–1662.
Waldegger, S., Barth, P., J. Jr., N.F., Greger, R., Lang, F., 1998. Cloning of sgk serine-threonine protein kinase from shark rectal gland: a gene induced by hypertonicity and secretagogues. Pflugers Arch. 436, 575–580.
Wang, H., Singh, D., Fliegel, L., 1998. Functional role of cysteine residues in the Na+/H+ exchanger effects of mutation of cysteine residues on targeting and activity of the Na+/H+ exchanger. Arch. Biochem. Biophys. 358, 116–124.
Wang, D., Sun, H., Lang, F., Yun, C.C., 2005. Activation of NHE3 by dexamethasone requires phosphorylation of NHE3 at Ser663 by SGK1. Am. J. Physiol. Cell Physiol. 289, C802–C810.
Wright, P.A., Wood, C.m., 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J. Exp. Biol. 212, 2303–2312.
Yan, J.J., Chou, M.Y., Kaneko, T., Hwang, P.P., 2007. Gene expression of Na+/H+ exchanger in zebrafish H+-ATPase-rich cells during acclimation to low-Na+ and acidic environments. Am. J. Physiol. Cell Physiol. 293, C1814–C1823.