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