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Distribution and dynamics of branchial ionocytes in houndshark reared in full-strength and diluted seawater environments

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ABSTRACT

In teleost fishes, it is well-established that the gill serves as an important ionoregulatory organ in addition to its primary function of respiratory gas exchange. In elasmobranchs, however, the ionoregulatory function of the gills is still incompletely understood. Although two types of ionocytes, Na^+/K^+-ATPase (NKA)-rich (type-A) cell and vacuolar-type H^+-ATPase (V-ATPase)-rich (type-B) cell, have been found in elasmobranch fishes, these cells were considered to function primarily in acid–base regulation. In the present study, we examined ion-transporting proteins expressed in ionocytes of Japanese-banded houndshark, *Triakis scyllium*, reared in full-strength seawater (SW) and transferred to diluted (30%) SW. In addition to the upregulation of NKA and Na^+/H^+ exchanger type 3 (NHE3) mRNAs in the type-A ionocytes, we found that Na^+, Cl^- cotransporter (NCC, Slc12a3) is expressed in a subpopulation of the type-B ionocytes, and that the expression level of NCC mRNA was enhanced in houndsharks transferred to a low-salinity environment. These results suggest that elasmobranch gill ionocytes contribute to NaCl uptake in addition to the already described function of acid–base regulation, and that NCC is most probably one of the key molecules for hyper-osmoregulatory function of elasmobranch gills. The existence of two types of ionocytes (NHE3- and NCC-expressing cells) that are responsible for NaCl absorption seems to be a common feature in both teleosts and elasmobranchs for adaptation to a low salinity environment. A possible driving mechanism for NCC in type-B ionocytes is discussed.

1. Introduction

Ion regulation is one of the most important issues in the maintenance of body-fluid homeostasis. This is particularly vital for fishes in aquatic environments where they are surrounded by waters where the concentrations of ions can change. It is well-established that the teleost fish gill contributes importantly to ion regulation, in addition to the primary function of respiratory gas exchange. In teleost fish, ionocytes, also known as chloride cells or mitochondrion-rich cells, in the branchial epithelium are responsible for ionoregulation (see review, Evans et al., 2005; Kaneko et al., 2008). Ionocytes of seawater (SW) teleost fish have a well-developed tubular system with numerous mitochondria and form a multicellular complex with an accessory cell to provide a paracellular pathway through which Na^+ is thought to be secreted. A set of ion-transporting proteins, namely basolaterally-located Na^+/K^+-ATPase (NKA) and Na^+, K^+, $2Cl^-$ cotransporter isoform 1 (NKCC1) and apically-located cystic fibrosis transmembrane conductance regulator (CFTR), are expressed in ionocytes of SW teleost fish to secrete Cl^- transcellularly (Marshall, 2002). Contributions of multiple membrane proteins, including NKA, vacuolar-type H^+-ATPase (V-ATPase), Na^+/H^+ exchangers (NHEs) and Na^+, Cl^- cotransporter (NCC, slc12a10), to ion uptake have also been described in freshwater (FW) teleosts; however, the molecular mechanisms for ion uptake seem to vary among species (Hwang and Lee, 2007; Hwang et al., 2011, Dymowska et al., 2012). In Mozambique tilapia and zebrafish, multiple types of cells (cell types I to IV for tilapia; and NCC, NaR and HR cells for zebrafish) have been identified according to the membrane transporters expressed in those cells, and on their possible function (Hiroi et al., 2008; Inokuchi et al., 2009; Hwang et al., 2011).

In contrast to teleost fish, the iono-regulatory function of the elasmobranch gill is less well understood. Although ionocytes have also been found in elasmobranch gill epithelia, their function has been considered to be different from that of teleost ionocytes (Wilson and Laurent, 2002; Evans et al., 2005). Marine elasmobranchs have a specialized salt-secreting gland, the rectal gland (Burger, 1965), and the gill ionocytes have consequently been proposed to be involved in acid–base regulation (Edwards et al., 2002; Tresguerres et al., 2006; Choe et al., 2007; Tresguerres et al., 2007). Molecular and histochemical investigations of ion-transporting proteins have revealed that there are two types of ionocytes in elasmobranch gills: type-A ionocytes (NKA-rich ionocytes) and type-B ionocytes (V-ATPase-rich ionocytes) in spiny dogfish *Squalus acanthias* and Atlantic stingray *Dasyatis sabina* (Choe et al., 2005; Choe et al., 2007; Piermarini and Evans, 2001). The

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type-A ionocytes express NHE isoform 3 (NHE3) on the apical membrane, suggesting that low intracellular Na^+ created by the basolaterally-located NKA promotes H^+ excretion to the environment concomitantly with Na^+ uptake (Choe et al., 2005). On the other hand, HCO_3^- excretion is proposed in the type-B ionocytes, since the pendrin-like Cl^-/HCO_3^- exchanger (PDN, slc26a4) is located on the apical membrane (Piermarini et al., 2002). These molecular investigations supported the idea that elasmobranch gill ionocytes are involved in acid–base regulation rather than NaCl excretion in the SW environment.

Recently, we discovered a novel aggregate structure made up of cells with basolaterally-expressed NKA in the inter-filamental space of the gill septum (Takabe et al., 2012). The cell aggregates, named follicularly-arranged NKA-rich cells, express NHE3 and Ca^{2+} transporter 1 (CaT), and thus are most likely involved in Ca^{2+} homeostasis. During the course of this investigation, we also found expression of CaT mRNA in a small number of ionocytes in the branchial filament. These observations imply that elasmobranch ionocytes still have unidentified functions for ion homeostasis. In the present study, to expose the roles of ionocytes of elasmobranchs, we examined expression of ion-transporting proteins in ionocytes of Japanese-banded houndshark *Triakis scyllium*. We found that, in addition to NKA, NHE3, V-ATPase and PDN already found in the gills of spiny dogfish and Atlantic stingray, CaT and NCC (Slc12a3) were expressed in a certain portion of type-A and type-B cells, respectively. Acclimation to a low-salinity environment induced increases in the numbers of NKA- and NHE3-expressing type-A cells and of V-ATPase-expressing type-B cells. After transfer, the proportion of NCC-expressing type-B cells (type-B-II cell) to total type-B cells (V-ATPase expressing cell) rose, implying that elasmobranch branchial ionocytes contribute importantly to hyper-osmoregulatory ability.

2. Materials and methods

2.1. Fish

All animal experiments were conducted according to the Guidelines for Care and Use of Animals approved by the committee of the University of Tokyo. Japanese banded houndshark, *T. scyllium*, (750–1550 g) were collected in Koajiro Bay, Kanagawa, Japan. They were transported to the Atmosphere and Ocean Research Institute and kept in 2×10^3L holding tanks (20–22 °C, aerated) under a constant photoperiod (12 h:12 h, L:D). The fish were fed on squid for at least 2 weeks before experiments. For sampling, houndsharks were anaesthetized with 0.02% (*w*/*v*) 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MO, USA).

2.2. Transfer experiments

The transfer experiments on houndsharks were performed as described in detail by Yamaguchi et al. (2009). In brief, houndsharks were kept separately in two tanks ($1 - 2 \times 10^3$L) filled with full-strength SW. On day 1, the salinity of the water of the experimental group was reduced by adding FW to achieve a salinity of 80% SW. On days 2 and 3, salinity was further reduced to achieve a salinity of 60% SW and 40% SW, respectively. And FW was again added on day 4 to produce a final salinity of 30% SW (10 ppt, $n = 4$). Control fish ($n = 5$) were kept in full-strength SW (34 ppt) during the transfer protocol. Fish were maintained for 1 week in each salinity condition, and then euthanized for sampling.

2.3. cDNA cloning

The gills of houndshark were dissected out, quickly frozen in liquid nitrogen and kept at −80 °C. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan), and cDNAs were then synthesized using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). We already cloned partial sequences of cDNAs encoding NKA α1-subunit, NKCC1, CFTR, NHE3 and CaT (Takabe et al., 2012). In addition, in the present study, degenerate primer pairs were designed to amplify conserved regions of Na^+, K^+, $2Cl^-$ cotransporter isoform 2 (NKCC2), NCC, V-ATPase, PDN, and chloride channel type-3 (CLC3), and their sequences are shown in Table 1. PCR was performed with Ex-Taq DNA polymerase (TaKaRa, Shiga, Japan). The amplified products were electrophoresed on agarose gels, excised and ligated into pGEM-T Easy Vector (Promega Corp., Madison, WI). The nucleotide sequences were determined by an automated DNA sequencer (PRISM 3130, Applied Biosystems, Foster City, CA).

2.4. Molecular phylogenetic analysis

The deduced amino acid sequences of the obtained cDNAs from houndshark were aligned with those from other animals using ClustalX software (http://www. clustal.org/); sequences were obtained from the DDBJ and Ensembl databases. Molecular phylogenetic trees were constructed using ClustalX software by the neighbor-joining method with bootstrap analysis for 1000 cycles. The rat K^+–Cl^- cotransporter 1 (KCC1) and human CLCKa were used as outgroups for the phylogenetic trees of the NKCC/NCC and CLC protein families, respectively.

2.5. Real-time quantitative PCR assay and RT-PCR

The expression levels of mRNAs were determined by real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection

Table 1
Primer sets used in the present study.

Gene name	Primer sequence 5′ to 3′	
Primer sets for real-time PCR		
NKA	Sense	TGCTTACACTTTAACCAGCAATATCC
	Antisense	GGCTGTCTCTTCATTATATCACTTTC
NHE3	Sense	CGAGAGGCGATTATTTGTCAGTAC
	Antisense	ATCGTCAGCCCCTGGAAGAT
V-ATPase	Sense	ACCATCGAACGCATCATCACT
	Antisense	TCTCACATTGATACGCCAGGAA
PDN	Sense	CGGCTGTTTCTCAAGCTTTTTG
	Antisense	GAGTTTTGCCACCTGTGCTTTC
NCC	Sense	GGGAACTGTCACCAAAAACCAC
	Antisense	TGATGTGTCCACTAGAGCATATCTCAG
CLC3	Sense	TAGCTGTTGCCTCAGGGCTTAG
	Antisense	GAGAAAATATTACCGCAGCAACATG
β-Actin	Sense	CACACTGTGCCCATCTACGAA
	Antisense	GCCAAATCCAGACGCAGAA
Primer sets for molecular cloning		
NKCC2	Sense	ACNGTNGCNGGNATGGARTGGGAR
	Antisense	GCCCACCARTTDATNACRAACAT
NCC	Sense	GGNTAYGGNAARAAYAAYGARCC
	Antisense	TTNCKYTCYTCRTCCATNCKRTT
V-ATPase	Sense	CAYAAYGARATHGCNGCNCARATHTG
	Antisense	GGRTGNGTDATRTCRTCRTTNGGCAT
PDN	Sense	GGNAAYCARGARTTYATHGCNTTY
	Antisense	SWRTTCCARTCNACYTGDATYTC
CLC3	Sense	ATHGAYTGGGTNMGNGARAARTGY
	Antisense	RAANARNACNARNCK
Primer sets for *in situ* hybridization		
NKA	Sense	GGTGCCATTGTAGCTGTGAC
	Antisense	TATAAGGGAAGGCGCAGAACCACCA
NHE3	Sense	TTTTCGAGGAGGTTCATGTG
	Antisense	TGTAATTGTGGCCGATCTGC
V-ATPase	Sense	CACAACGAAATAGCCGCTCA
	Antisense	GGGTGCGTGATGTCGTCGTT
PDN	Sense	GGGAACCAGGAGTTTATTGC
	Antisense	GTGTTCCAGTCGACTTGGAT
NCC	Sense	GGGTACGGGAAGAACAACGA
	Antisense	CTTACGCTCTTCGTCCATCC
CaT	Sense	GGGAACACTGTTATGTTTCA
	Antisense	CATTTGGAAACCCCGAGCGA
CLC3	Sense	ATTGATTGGGTGCGGGAGAA
	Antisense	AAACAGGACCAGACGGCTGT