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Expression of ion transporters in gill mitochondrion-rich cells in Japanese eel acclimated to a wide range of environmental salinity

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ABSTRACT

We examined morphological changes and molecular mechanisms of ion regulation in mitochondrion-rich (MR) cells of Japanese eel acclimated to different environmental salinities. Electron microscopic observations revealed that the apical membrane of MR cells appeared as a flat or slightly projecting disk with a mesh-like structure on its surface in eel acclimated to freshwater (FW). In seawater (SW)-acclimated eel, in contrast, the apical membrane of MR cells showed a slightly concave surface without a mesh-like structure. The mRNA expression of Na^+/H^+ exchanger-3 (NHE3) in deionized FW and normal SW was higher than that in normal FW and 30%-diluted SW. Expression of $Na^+/K^+/2Cl^-$ cotransporter-1a (NKCC1a) became higher with increasing environmental salinity. Immunofluorescence staining showed that the apical NHE3 immunoreaction was stronger in deionized FW and normal SW than in the other groups. Basolateral NKCC1 immunoreaction was most intense in normal SW. These results indicate that apical NHE3 is involved in ion uptake in fish acclimated to hypotonic environments, and that basolateral NKCC1 is important for acclimation to hypertonic environments. The relatively high expression of NHE3 in SW further indicates a possible role of NHE3 in acid-base regulation in the gills in SW-acclimated fish.

1. Introduction

Teleost species are distributed all over aquatic environments with a wide range of salinities. Independent of environmental salinity, teleosts maintain their plasma osmolality within narrow physiological ranges, equivalent to about one-third seawater osmolality. In teleosts, the gills, intestine, kidney and other organs (e.g. opecular membranes and skin, and urinary bladder) are responsible for ion and water transports during osmoregulatory processes (Marshall and Grosell, 2006). The gill is a major site of passive ion and water movements owing to its large surface area and direct contract with the external environment. At the same time, the gill is among the most important osmoregulatory organs because of the presence of mitochondrion-rich (MR) cells (often referred to as chloride cells or ionocytes).

Mitochondrion-rich cells in the gills are the major site of ion absorption and secretion, and are thus important in both freshwater (FW) and seawater (SW) adaptation. Subtypes of MR cells with distinct morphological and functional features have been identified in several teleost species. In Mozambique tilapia *Oreochromis mossambicus*, MR cells have been classified into four distinct subtypes according to ion transporters expressed at the apical or basolateral membrane: type I with only basolateral Na^+/K^+-ATPase; type II with basolateral Na^+/K^+-ATPase and apical Na^+/Cl^- cotransporter (NCC); type III with basolateral Na^+/K^+-ATPase and basolateral $Na^+/K^+/2Cl^-$ cotransporter 1a (NKCC1a), apical Na^+/H^+ exchanger-3 (NHE3); and type IV with basolateral Na^+/K^+-ATPase, basolateral NKCC1a, and apical cystic fibrosis transmembrane conductance regulator (CFTR). Type II and Type III MR cells are involved in ion uptake, whereas type IV MR cells are responsible for salt secretion (Hiroi et al., 2008; Inokuchi et al., 2008). A recent series of experiments using *in situ* hybridization and immunocytochemistry have proposed a model for ion regulation in zebrafish (*Danio rerio*) MR cells. In this model, there are four types of MR cells: H^+-ATPase-rich (HR) cell; Na^+/K^+-ATPase-rich (NaR) cell; NCC cell; and K^+-secreting (KS) cell (Hwang et al., 2011; Dymowska et al., 2012).

In our previous study, we examined the morphological changes in MR cells in Japanese eel acclimated to a wide range of environmental salinities (Seo et al., 2009), and identified only two types of MR cells. Although MR cells were first demonstrated in European eel *Anguilla anguilla* by Keys and Willmer (1932), eels fall behind other teleost species in terms of molecular mechanisms of MR cells. The life cycle of Japanese eel is closely associated with migration between FW and SW environments. To tolerate a wide range of salinities during the diadromous migration, the eel is equipped with mechanisms for adaptation to both FW and SW, and thus would be another suitable experimental model species for morphological and functional studies on MR cells.

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In the present study, we aimed to clarify the molecular mechanisms of ion uptake and secretion in MR cells of Japanese eel acclimated to a wide range of environmental salinity. First, we compared morphology of gill MR cells between FW and SW by means of scanning and transmission electron microscopy. Next, we cloned and sequenced cDNAs encoding vacuolar-type H^+-ATPase (V-ATPase) A-subunit, NHE3 and NKCC1a from the gills of Japanese eel. In order to specify ion-transporting proteins importantly involved in FW and SW adaptation, we investigated mRNA expressions of V-ATPase A-subunit, NHE3 and NKCC1a in fish acclimated to different salinities. Finally, we observed immunocytochemical localization of those ion-transporting proteins in MR cells to confirm their protein expressions.

2. Materials and methods

2.1. Experimental animals

Cultured Japanese eel weighing approximately 200 g were purchased from a commercial supplier (Hamamatsu, Shizuoka, Japan) and reared in a stock tank (1 m^3) containing recirculating FW at 20 °C. Those FW eel were separated into four groups: those transferred to 1) deionized FW; 2) normal FW; 3) 30%-diluted SW (diluted SW); and 4) normal SW. The diluted SW was prepared by diluting SW with FW (dechlorinated Tokyo tap water). To prepare eels acclimated to deionized FW, normal FW and diluted SW, eel were transferred from the FW stock tank directly to 100-L plastic tanks containing respective environmental waters, and reared for 1 week. For the preparation of normal SW-acclimated eel, FW eel were first acclimated to 50%-diluted SW for 2 days, and then transferred to full-strength SW. The fish were maintained in normal SW for 1 week. Water temperature was maintained at 20 °C, and fish were not fed during this period. Half the water was changed every other day to ensure optimal water quality. Concentrations of Na^+, Cl^-, K^+, Ca^{2+} and Mg^{2+} in four media were determined with an ion analyzer (IA-200, TOA-DKK, Tokyo, Japan). Table 1 shows ion concentrations on the first and last days of the experiment. The fish were anesthetized with 0.1% 2-phenoxyethanol (Wako, Tokyo, Japan) before blood sampling or removal of tissues. Experiments were conducted according to the principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

2.2. Measurement of plasma osmolality

Plasma osmolality was measured in eels acclimated to different environmental salinities. After the fish were anesthetized, blood was collected from the caudal vessels with a heparinized syringe and needle. The blood plasma was immediately separated by centrifugation and subjected to determination of osmolality. Plasma osmolality was measured with a vapor pressure osmometer (Wescor 5520, Logan, UT, USA).

2.3. Scanning electron microscopy

The gills from normal FW- and normal SW-acclimated eels were fixed in 2% paraformaldehyde (PFA)-2% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB, pH 7.4) for 20 h at 4 °C, and preserved in 70% ethanol. The filaments were then dehydrated in ethanol, immersed in *t*-butylalcohol, and dried in a freeze-drying device (VFD-21S, Vacuum Device, Ibaraki, Japan). Dried samples were mounted on specimen stubs, and coated with platinum palladium in an ion sputter (E-1030, Hitachi, Tokyo, Japan), and examined with a Hitachi S-4000 scanning electron microscope.

Table 1
Ion concentrations of the four experimental media.

Medium	$[Na^+]$	$[Cl^-]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$
deionized freshwater	<0.01/0.02	<0.01/0.02	<0.01/0.02	<0.01/0.01	<0.01/<0.01
freshwater	0.71/0.77	0.53/0.59	0.06/0.08	0.48/0.54	0.16/0.19
30%-diluted seawater	112/116	147/144	3.24/3.68	5.91/6.22	8.33/8.23
seawater	426/400	511/501	9.82/10.4	15.3/16.2	41.2/36.8

Concentrations are in mM; values of the first/last day.

2.4. Transmission electron microscopy

The gills from normal FW- and normal SW-acclimated eels were fixed in 2% PFA–2% GA in 0.1 M PB for 20 h at 4 °C. After rinsing in 0.1 M PB, the filaments were postfixed in 1% osmium tetroxide in 0.1 M PB for 1 h at room temperature. The filaments were dehydrated in ethanol, transferred to propylene oxide, and embedded in Spurr's resin (Polysciences, Warrington, PA, USA). Ultrathin sections were cut with a diamond knife and mounted on grids. The sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM1010, JEOL, Tokyo, Japan).

2.5. Molecular cloning of V-ATPase A-subunit, NHE3 and NKCC1a

Total RNA was extracted from the gill filaments of FW-acclimated eel with Isogen (Nippon Gene, Tokyo, Japan), and treated with DNase I (Invitrogen, Carlsbad, CA, USA). Then, first-strand cDNA was synthesized from the total RNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA fragments of V-ATPase A-subunit, NHE3 and NKCC1a were amplified by degenerate PCR with a cDNA template obtained from eel gills. Degenerate primers used for amplification (Table 2) were designed based on the available information in vertebrate species. PCR was performed in a final volume of 20 μL containing 1x PCR buffer (TaKaRa, Shiga, Japan), 200 μM of dNTPs (TaKaRa), 0.5 U *Taq* DNA polymerase (TaKaRa *LA Taq*, TaKaRa), 5 μM (for V-ATPase A-subunit and NHE3) or 0.3 μM (for NKCC1a) each of the primer pair, and an appropriate amount of the gill cDNA template. The amplified products were electrophoresed on agarose gels, ligated into pGEM-T easy Vector (Promega, Madison, WI), and sequenced using a DNA sequencer ABI Prism 310 (Applied Biosystems, Foster City, CA, USA). Sequence data were analyzed with Sequencher v3.1.1 software (Hitachi, Tokyo, Japan). For V-ATPase A-subunit, to extend sequence information, 5′- and 3′-rapid amplifications of cDNA ends (RACE) were performed using CapFishing Full-Length cDNA Premix Kit (Seegene, Seoul, Korea) and SMART™ RACE cDNA

Table 2
Primers used for cloning, reverse transcription-PCR(RT-PCR) and quantitative real-time PCR (qPCR).

Primers	Sequence (5′-3′)	Notes
V-ATPA-df	GCITTYGGNTGYGGNAARAC	degenerate PCR
V-ATPA-dr	TCIGGRTTICCIARACAYTTNAC	degenerate PCR
V-ATPA-smart-F	GGAAGATTAGCGGAGATGCCAGCTGA	3′-RACE
V-ATPA-cap-R	TCTCAACCTTGCCGTCCACCTCCAT	5′-RACE
RT-PCR-VATPA-F	TGTGATCTCGCAGTCCCTC	RT-PCR, qPCR
RT-PCR-VATPA-R	GCCCATGTCCCTGAAGTAC	RT-PCR, qPCR
NHE3-df	AAYGTNTTYGATGCNTTYGT	degenerate PCR
NHE3-dr	ACNGTRAARTANACNA CDAT	degenerate PCR
NHE3-3RACE-F	GAGGACATCAACACGCACCGCCT	3′-RACE
NHE3-5RACE-R	AGGGAGAGCAGAATGGCGAAGATCA	5′-RACE
RT-PCR-NHE3-F	CGGACACAACTACATGAGGGAC	RT-PCR, qPCR
RT-PCR-NHE3-R	CGTCTTTGAGGTTCAGCTGGTG	RT-PCR, qPCR
AA-NKCC1a-F	CAGCAGTTCCAGAAGAAGCAGG	degenerate PCR
AA-NKCC1a-R	TAGAAGGTGAGGACGCTCTGGTG	degenerate PCR
RT-PCR-NKCC1a-F	GAAGAGGTGGAAGGACTGCAGG	RT-PCR, qPCR
RT-PCR-NKCC1a-R	GGACCGTGATGTCGGAGAAGTC	RT-PCR, qPCR
18s-F	CGATGCTCTTAGCTGAGTGT	RT-PCR, qPCR
18s-R	ACGACGGTATCTGATCGTCT	RT-PCR, qPCR