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Expression regulation of Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit subtypes in zebrafish gill ionocytes

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Liao BK, Chen RD, Hwang PP. Expression regulation of Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit subtypes in zebrafish gill ionocytes. Am J Physiol Regul Integr Comp Physiol 296: R1897–R1906, 2009. First published April 22, 2009; doi:10.1152/ajpregu.00029.2009.—In zebrafish (Danio rerio), six distinct Na\(^{+}\)-K\(^{+}\)-ATPase (NKA) \(\alpha\)-subunit genes have been identified, and four of them, \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, \textit{zatp1a1a.4}, and \textit{zatp1a1a.5}, are expressed in embryonic skin where different types of ionocytes appear. The present study attempted to test a hypothesis of whether these NKA \(\alpha\) paralogues are specifically expressed and function in respective ionocytes. Double fluorescence in situ hybridization analysis demonstrated the specific expression of \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, and \textit{zatp1a1a.5} in NKA-rich (NaR) cells, Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC)-expressing cells, and H\(^{-}\)-ATPase-rich (HR) cells, respectively, based on the colocalization of the three NKA \(\alpha\) genes with marker genes of the respective ionocytes (epithelial Ca\(^{2+}\) channel in NaR cells; NCC in NCC cells; and H\(^{-}\)-ATPase and Na\(^{+}\)/H\(^{+}\) exchanger 3b in HR cells). The mRNA expression (by real-time PCR) of \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, and \textit{zatp1a1a.5} were, respectively, upregulated by low-Ca\(^{2+}\), low-CI\(^{-}\), and low-Na\(^{+}\) freshwater, which had previously been reported to stimulate uptake functions of Ca\(^{2+}\), Cl\(^{-}\), and Na\(^{+}\). However, \textit{zatp1a1a.4} was not colocalized with any of the three types of ionocytes, nor did its mRNA respond to the ambient ions examined. Taken together, \textit{zATP1a1a.1}, \textit{zATP1a1a.2}, and \textit{zATP1a1a.5} may provide driving force for Na\(^{+}\)-coupled cotransporter activity specifically in NaR, NCC, and HR cells, respectively.

mitochondrion-rich cells; ion regulation; osmoregulation; fish

**In the postgenomic era, functional studies of proteins in specific organs or cell types have become more complicated due to the discovery of additional isoforms of a target protein from bioinformatic inquiries. The zebrafish may be an alternative and suitable in vivo tool to deal with this issue because of the discovery of additional isoforms of a target protein due to the discovery of additional isoforms of a target protein have been identified, and four of them, \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, \textit{zatp1a1a.4}, and \textit{zatp1a1a.5}, are expressed in embryonic skin where different types of ionocytes appear. The present study attempted to test a hypothesis of whether these NKA \(\alpha\) paralogues are specifically expressed and function in respective ionocytes. Double fluorescence in situ hybridization analysis demonstrated the specific expression of \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, and \textit{zatp1a1a.5} in NKA-rich (NaR) cells, Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC)-expressing cells, and H\(^{-}\)-ATPase-rich (HR) cells, respectively, based on the colocalization of the three NKA \(\alpha\) genes with marker genes of the respective ionocytes (epithelial Ca\(^{2+}\) channel in NaR cells; NCC in NCC cells; and H\(^{-}\)-ATPase and Na\(^{+}\)/H\(^{+}\) exchanger 3b in HR cells). The mRNA expression (by real-time PCR) of \textit{zatp1a1a.1}, \textit{zatp1a1a.2}, and \textit{zatp1a1a.5} were, respectively, upregulated by low-Ca\(^{2+}\), low-CI\(^{-}\), and low-Na\(^{+}\) freshwater, which had previously been reported to stimulate uptake functions of Ca\(^{2+}\), Cl\(^{-}\), and Na\(^{+}\). However, \textit{zatp1a1a.4} was not colocalized with any of the three types of ionocytes, nor did its mRNA respond to the ambient ions examined. Taken together, \textit{zATP1a1a.1}, \textit{zATP1a1a.2}, and \textit{zATP1a1a.5} may provide driving force for Na\(^{+}\)-coupled cotransporter activity specifically in NaR, NCC, and HR cells, respectively.

mitochondrion-rich cells; ion regulation; osmoregulation; fish

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R1897
The present study attempted to test the hypothesis that NKA α1 paralogues (zATP1α1.1, zATP1α1.2, zATP1α1.4, and zATP1α1.5) are specifically expressed in distinct subtypes of ionocytes to drive the respective relevant ion transport mechanisms. Experiments were designed to answer the following questions: 1) Are the NKA α1 (α1a.1, α1a.2, α1a.4, and α1a.5) paralogues coexpressed in the same cells? 2) Is each α1 parologue coexpressed with the relevant transporter in distinct ionocytes? and 3) Do changes in environmental ionic compositions, which can induce the transport capacities of various ions, differentially stimulate the expression of each respective α1 parologue?

**MATERIALS AND METHODS**

Experimental animals. Zebrafish (AB strain) brood stocks in the Institute of Cellular and Organismic Biology, Academia Sinica, were kept in FW (local tap water) at 28.5°C under a photoperiod of 14:10-h light-dark before the acclimation experiments. Fish were anesthetized with 100–200 mg/l of buffered MS222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO) before sampling. The experimental protocols were approved by the Academia Sinica Institution Animal Care and Utilization Committee (approval no. RFZOOPH2007040).

Acclimation experiments. Five kinds of artificial FW, high (H)-Na-low (L)-Cl (10 mM Na⁺ and 0.04 mM Cl⁻), L-Na-L-Cl (0.04 mM NaCl), H-Na-H-Cl (10 mM NaCl), H-Ca (2 mM Ca), and L-Ca (0.02 mM Ca) were prepared by adding appropriate amounts of NaCl, Na₂SO₄, MgSO₄, K₂HPO₄, KH₂PO₄, and CaSO₄ to double-deionized water (cat. no. MilliRO60; Millipore, Billerica, MA). The ionic compositions (Table 1) of the five media were confirmed by measuring the Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations with an atomic absorption spectrophotometer (model Z-8000; Hitachi, Tokyo, Japan) and by examining Cl⁻ levels with a spectrophotometer (Hitachi U-2000). The pH of the media was kept between 6.5 and 6.9, and the water temperature was 28.5°C. Twenty-five to thirty zebrafish for each test were acclimated to 30 liters of the different artificial FW media for 2 wk and then were sampled for quantitative real-time RT-PCR (5 individuals for each test). Fish were not fed during the acclimation period. To maintain the water quality, the acclimation media were aerated with a filtered air pump, and newly prepared and mixed media were added every 3 days. The changes in the water ion compositions, pH and ammonia (<0.1 mg/l) were monitored during each water renewing.

Preparation of total RNA and cDNA synthesis. Isolated gill tissues were mixed and homogeneous in Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was purified following the manufacturer’s protocol. The total amount of RNA was determined by spectrophotometry (model ND-1000; NanoDrop Technol, Wilmington, DE), and the RNA quality was checked by running electrophoresis in RNA-denaturing gel. Total RNA pellets were stored at −20°C. For cDNA synthesis, 5 μg of total RNA were reverse-transcribed in a final volume of 20 μl containing 0.5 mM dNTPs, 2.5 μM oligo(dT)₁₈, 5 mM dithiothreitol, and 200 units of superscript reverse transcriptase III (Invitrogen) for 1.5 h at 42°C, followed by a 15-min incubation at 70°C.

Table 2. Probes for in situ hybridization

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank Accession No.</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>atp1a1.1</td>
<td>AF286372</td>
<td>nt 2596 approximately 3420</td>
</tr>
<tr>
<td>atp1a1.2</td>
<td>AF286374</td>
<td>nt 2410 approximately 3216</td>
</tr>
<tr>
<td>atp1a1.4</td>
<td>AY008376</td>
<td>nt 2305 approximately 3120</td>
</tr>
<tr>
<td>atp1a1.5</td>
<td>NM_178909</td>
<td>nt 2939 approximately 3761</td>
</tr>
<tr>
<td>trpv6</td>
<td>NM_001001849</td>
<td>nt 144 approximately 2268</td>
</tr>
<tr>
<td>slc12a102</td>
<td>NM_00145001</td>
<td>nt 639 approximately 2162</td>
</tr>
<tr>
<td>atp6v1a</td>
<td>NM_201135</td>
<td>nt 407 approximately 1143</td>
</tr>
<tr>
<td>slc9a3b</td>
<td>EFS91980</td>
<td>nt 1907 approximately 2745</td>
</tr>
</tbody>
</table>

RNA probe synthesis. Fragments of the target genes (Table 2) obtained by PCR were inserted into pGEM-T Easy vectors. The inserted fragments were amplified with the T7 and SP6 primers by PCR. Digoxigenin- (Dig; Roche, Penzberg, Germany) or biotin-labeled (Roche) RNA probes were synthesized by in vitro transcription with T7 and SP6 RNA polymerase (Takara, Shiga, Japan). The qualities of the probes were examined by using RNA gels, and the concentrations were determined by a dot-blot assay with standard DIG-labeled RNA (100 ng/μl) (Roche).

Fluorescence double in situ hybridization. Excised gills were fixed with 4% paraformaldehyde overnight at 4°C and then washed several times with PBS. After being dehydrated with methanol, whole gills were washed several times with PBS containing 0.1% Tween-20 (PBST). Samples were incubated with hybridization buffer (HyB) containing 50% formamide, 5× SSC, and 0.1% Tween-20 for 5 min at 65°C. Prehybridization was performed for 2 h at 65°C with HyB+, which is the hybridization buffer supplemented with 500 μg/ml yeast transfer RNA and 50 μg/ml heparin. For hybridization, samples were incubated in 100 ng of the RNA probe in 200 μl HyB+ at 65°C overnight. Then, samples were washed at 70°C for 30 min twice in 50% HyB and 50% 2× SSC, 30 min twice in 2× SSC, and 30 min twice in 0.2× SSC. Further washes were performed at room temperature for 5 min in 75% 0.2× SSC and 25% PBST, 5 min in 50% 0.2× SSC and 50% PBST, 5 min in 25% 0.2× SSC and 75% PBST, and 5 min in PBST. Fluorescence staining was conducted with a commercial kit of tyramide signal amplification (TSA) Plus Fluorescence Systems (Perkin-Elmer, Waltham, MA). The hybridization signals detected by the Dig-labeled RNA probes were amplified through fluorescein-TSA, while cyanine 3-TSA was used for the biotin-labeled probes. Images of gill surfaces were acquired with a confocal laser scanning microscope (model TCS-SP5; Leica Lasertechnik, Heidelberg, Germany).

Real-time quantitative RT-PCR. Total RNA was extracted and reverse-transcribed from five zebrafish gill tissues (as a pooled sample) as described above. A total of 25 individuals for each test were sampled (n = 5). mRNA expression of target genes were measured by quantitative (qRT-PCR) with an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA). Primers for all genes were designed (Table 3) using Primer Express software (version 2.0.0; Applied Biosystems). PCRs contained 3.2 ng of cDNA, 100 nM of each primer, and Universal SYBR Green master mix (Applied Biosystems) in a final volume of 20 μl. All qRT-PCR reactions were performed as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min,

Table 1. Ionic compositions (mM) and pH in artificial fresh water

<table>
<thead>
<tr>
<th>Medium</th>
<th>[Na⁺]</th>
<th>[Cl⁻]</th>
<th>[Ca²⁺]</th>
<th>[K⁺]</th>
<th>[Mg²⁺]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Na-L-Cl</td>
<td>0.04–0.05</td>
<td>0.03–0.06</td>
<td>0.18–0.19</td>
<td>0.16–0.17</td>
<td>0.18–0.19</td>
<td>6.6–6.8</td>
</tr>
<tr>
<td>H-Na-L-Cl</td>
<td>10.00–10.5</td>
<td>0.04–0.07</td>
<td>0.17–0.18</td>
<td>0.16–0.18</td>
<td>0.20–0.21</td>
<td>6.7–6.8</td>
</tr>
<tr>
<td>H-Na-H-Cl</td>
<td>9.5–10.1</td>
<td>9.8–10.2</td>
<td>0.19–0.20</td>
<td>0.15–0.17</td>
<td>0.19–0.21</td>
<td>6.7–6.9</td>
</tr>
<tr>
<td>L-Ca</td>
<td>0.46–0.47</td>
<td>0.46–0.48</td>
<td>0.01–0.02</td>
<td>0.22–0.23</td>
<td>0.22–0.23</td>
<td>6.5–6.8</td>
</tr>
<tr>
<td>H-Ca</td>
<td>0.45–0.48</td>
<td>0.45–0.49</td>
<td>1.91–2.00</td>
<td>0.21–0.23</td>
<td>0.22–0.24</td>
<td>6.7–6.9</td>
</tr>
</tbody>
</table>
Table 3. Primer sets for the qualitative RT-PCR analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>atp1a1</td>
<td>F 5'-GCCCTGACCAATGAGAGT-3'</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>R 5'-TACGGTACATCGGACG-3'</td>
<td></td>
</tr>
<tr>
<td>atp1a2</td>
<td>F 5'-TCTGCTGAGTTGAGGAC-3'</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>R 5'-GCTTCTGAGATCAGGAT-3'</td>
<td></td>
</tr>
<tr>
<td>atp1a4</td>
<td>F 5'-TCTGCTGAGTTGAGGAC-3'</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>R 5'-GCTTCTGAGATCAGGAT-3'</td>
<td></td>
</tr>
<tr>
<td>atp1a5</td>
<td>F 5'-GCCCTGACCAATGAGAGT-3'</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>R 5'-GAGCCATCCTGGGAATCCGAC-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F 5'-GCCCTGACCAATGAGAGT-3'</td>
<td>151</td>
</tr>
<tr>
<td>R 5'-AAGGAGGATCCTGGGAATCCGAC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.

followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (the set annealing temperature of all primers). PCR products were subjected to melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with sterile water to determine the levels of background and nonspecific primer dimer noise. The standard curve of each gene was confirmed to be in a linear range with β-actin as an internal control. The amplicons were sequenced to confirm the PCR products.

Phylogenetic analysis. Protein sequences of the NKA α1 genes were retrieved from the Ensembl or NCBI databases, and the accession numbers were listed in Supplementary Table 1 (published with online version of article). Amino acid sequences of NKA α1 from organisms representing different taxa were aligned by ClustalX version 1.81, and the phylogenetic tree was then calculated and drawn with MEGA version 4.1. A rooted phylogenetic tree was built using a Neighbor-joining method with the pairwise deletion gaps calculating option. The bootstrap values showed beside the branches are percentage from bootstrap analysis with 1,000 cycles.

Statistical analysis. Values are presented as means ± SD and were compared using Student’s t-test or one-way ANOVA (Tukey’s pairwise comparison).

RESULTS

Phylogenetic analysis of the ATP1a gene family. As shown in Fig. 1A, there are nine isoforms published in the zebrafish NKA α (ATP1a) gene family, and comparatively fewer isoforms were discovered in other teleost species. To understand the evolutionary relationship of the differences in isoform numbers, an integrated comparison of all published isoforms and those predicted from the entire genome data is essential. So far, the Ensembl database (version 50) has provided the genome data of five teleost species (D. rerio, Oryzias latipes, Tetraodon nigroviridis, Takifugu rubripes, and Gasterosteus aculeatus). Twenty isoforms were retrieved from Ensembl by navigation of the gene family (Ensembl Family ID: ENSF00000000449) in which data of nine genes of zebrafish were annotated sequences, and two medaka isoforms (olATP1a1.1 and olATP1a1.4) were cloned in our unpublished experiments (Lai YW, Liao BK, Hwang PP, unpublished data not shown). Another 11 ATP1a isoforms from other teleostean species were downloaded from NCBI’s GenBank (see online Supplementary Table 1).

According to the phylogenetic analysis (Fig. 1A), three major clades, ATP1a1, ATP1a2, and ATP1a3, were clustered with the teleostean orthologues and corresponding mammalian isoforms. Within the ATP1a1 group, teleostean isoforms were separated into two major clusters, ATP1a1a-like and ATP1a1a.4-like clades. In zebrafish, zATP1a1a.2, zATP1a1a.3, and zATP1a1a.5 were closest to each other in the clade, and their genomic loci also indicated a high possibility of tandem duplication (Fig. 1B). The multiple-species genome comparison from the available genome data showed that all teleostean species have ATP1a1a.1 and ATP1a1a.4, while zATP1a1a.2, zATP1a1a.3, and zATP1a1a.5 are specifically found in zebrafish (Fig. 1B).

Expression of four NKA α1 paralogues in four types of gill cells. According to the in situ hybridization screening of zebrafish embryos (1, 4), four (atp1a1a.1, atp1a1a.2, atp1a1a.4, and atp1a1a.5) of the six α1-like genes are expressed in skin mucous cells, which showed a salt-and-pepper pattern as did the skin ionocytes (20, 29). Therefore, subsequent studies focused on these four genes. Because NKA α1 genes are highly conserved with 78% to 83% identities of the coding region nucleotide sequences among the four genes, the 3′ regions including most 3′ untranslated regions (Table 2) were used to avoid latent cross reactions during in situ hybridization. Fluorescence in situ hybridization results (Fig. 2) showed that each of the four NKA α1 genes was expressed in a specific group of gill cells. Results of double fluorescence in situ hybridization showed no colocalized signal was detected in any combination of two isoforms (Fig. 2), indicating that the four α1 genes were respectively expressed in different types of cells (or ionocytes). Images of in situ hybridization were acquired by scanning the surface of the whole mount gills, which have complicated three-dimensional structures in the gill filaments. Various cell sizes were observed in some images (Figs. 2–4), and this is probably because only a part of a cell, but not a whole cell, was acquired during scanning. Five individuals were used for each in situ hybridization, and the same results were confirmed in all samples. Negative controls in situ-hybridized with respective sense riboprobes were conducted to confirm that no signals were found (data not shown).

NKA α1 paralogues coexpressed with other ionocyte markers. A marker of NaR cells, ECaC (trpv6), which is known to be the apical entry of transepithelial Ca2+ uptake (27, 34), was colocalized with only atp1a1a.1 (Fig. 3A). A cation-chloride cotransporter, NCC-like 2 (scl2a10.2), which was proposed to be responsible for Cl− uptake in NCC cells (42), was specifically detected only in atp1a1a.2-positive cells (Fig. 3B). Furthermore, all signals of HR cell markers examined, including atp6v1a (V-ATPase subunit A) and scl9a3b (NHE3b, sodium/proton exchanger 3b), were detected only in atp1a1a.5-positive cells (Figs. 3, C and D). For any other permutation combinations of the three NKA α1 genes (atp1a1a.1, atp1a1a.2, and atp1a1a.5) with the other ionocyte markers used above, non-colocalization signals were detected (Fig. 4, A–F) by double fluorescence in situ hybridization. However, none of colocalization signals were found between atp1a1a.4 and all the ionocyte marker genes (Fig. 4, G–I). Five individuals were used for each in situ hybridization, and the same results were confirmed in all samples.

Environmental effects on NKA α1 paralogue mRNA expression. Adult zebrafish were acclimated to artificial FWs with different ion concentrations, which could simulate the uptake
capacities of various ions. Comparisons of the effects of ion compositions on the gene regulations of transcription levels of NKA α1 paralogues by qRT-PCR were conducted to examine the relationships among NKA α1 paralogues and the uptake functions of various ions. Of the four NKA α1 paralogues examined in FW zebrafish gills, atp1a1a.2 was expressed at the highest level (~0.2-fold relative to β-actin in arbitrary numbers, n = 5), followed by atp1a1a.4, atp1a1a.1, and atp1a1a.5 (Figs. 5 and 6). After acclimation to different media (H-Na-L-Cl, H-Na-H-Cl, L-Na-L-Cl, H-Ca, or L-Ca), NKA α1 paralogues showed different expression patterns (Figs. 5, A–D and 6, A–D), while atp1a1a.4 was found not to respond to environmental factors (Figs. 5C and 6C). The expression of atp1a1a.1 significantly decreased in the low-Na+ condition (by comparing H-Na-L-Cl

Fig. 1. Phylogenetic analysis of ATP1a isoform amino acid sequences (A) and a schematic picture of the genomic loci of teleostean ATP1a1 genes (B). A: percentages of bootstrapping branch corrections are shown beside the branches, and the orthologous relationships of clades are labeled on the right side to indicate different subtree regions. *Hypothetical protein predicted from other studies. B: multigenomic alignments were retrieved from Ensembl paralogue prediction and multiple alignment of contigs. The chromosome numbers or contig names are shown (left), and the locations of the genes in each chromosome are indicated by sequence numbers. The orthologues of ATP1a1a.1 and ATP1a1a.4 were linked with vertical filled colored boxes.
and L-Na-L-Cl media), while there was no significant difference found between the altered Cl⁻ factors (by comparing H-Na-L-Cl and H-Na-H-Cl media) (Fig. 5A). However, atp1a2 mRNA was upregulated in the low-Cl⁻ condition (by comparing H-Na-L-Cl and H-Na-H-Cl media) (Fig. 5B). Furthermore, atp1a5 mRNA showed the highest expression in L-Na-L-Cl FW in response to a low-Na⁺ environment (by comparing H-Na-L-Cl and L-Na-L-Cl media), but not to the low-Cl⁻ condition (by comparing H-Na-L-Cl and H-Na-H-Cl media) (Fig. 5D).

After acclimation to H-Ca and L-Ca media, the mRNA expression of the three NKA α1 paralogues, atp1a1a, atp1a1a.4, and atp1a1a.5, showed no significant differences, indicating no responses to ambient Ca²⁺ concentration changes (Figs. 6, B and C), while only atp1a1 showed an ~2.3-fold higher mRNA expression level in low-Ca²⁺ FW (Fig. 6A). No

Fig. 2. Representative images of 4 distinct expression patterns of ATP1a isoforms in zebrafish gills. Whole mount double fluorescence in situ hybridizations were used to identify expression patterns of the 6 different combinations of 2 ATP1a isoforms: atp1a1a.2 + atp1a1a.1 (A), atp1a1a.4 + atp1a1a.1 (B), atp1a1a.5 + atp1a1a.1 (C), atp1a1a.4 + atp1a1a.5 (D), atp1a1a.5 + atp1a1a.2 (E), and atp1a1a.5 + atp1a1a.4 (F). Arrows and arrowheads indicate the positive signals of the 2 NKA riboprobes in different cells. Scale bar = 20 μm.

Fig. 3. Whole mount double fluorescence in situ hybridizations of atp1a1a, atp1a1a.2, or atp1a1a.5 with ionocyte marker genes in zebrafish gills. The representative images show the colocalization for atp1a1a.1 + trpv6 (ECaC) (A), atp1a1a.2 + slc12a10.2 (NCC-like 2) (B), atp1a1a.5 + atp6v1a (V-ATPase subunit A) (C), and atp1a1a.5 + slc9a3b (NHE3b) (D). *Colocalization signals. Scale bar = 20 μm.
fish died or showed abnormal phenotypes during the 2-wk acclimation experiments.

**DISCUSSION**

The present study, for the first time, demonstrates the differential mRNA expression and localizations of the three NKA α-subunit paralogues, \( \text{atp1a1a.1} \), \( \text{atp1a1a.2} \), and \( \text{atp1a1a.5} \), in respective types of ionocytes of gills of zebrafish (\( \text{D. rerio} \)). The results suggest that these three paralogues of the NKA α-subunit may play different roles in \( \text{Ca}^{2+} \), \( \text{Cl}^- \), and \( \text{Na}^+ \) uptake mechanisms in zebrafish gills.

Based on the phylogenetic analysis, six of the zebrafish α-subunit genes are orthologues to the mammalian α1-subunit gene, and except for \( \text{ATP1a1b} \), the other five α1-orthologues are near each other in chromosome 1, implying that they may have been derived from tandem duplication events in addition to genome-wide duplication (36). By comparing multiple genomic loci from five teleosts (Fig. 1B), \( \text{ATP1a1a.1} \) and \( \text{ATP1a1a.4} \) gene clusters were found in all species and were also present at reasonable positions in the phylogenetic tree.

Fig. 4: Whole mount double fluorescence in situ hybridizations of \( \text{atp1a1a.1} \) (A and B), \( \text{atp1a1a.2} \) (C and D), \( \text{atp1a1a.5} \) (E and F), or \( \text{atp1a1a.4} \) (G–I) with ionocyte marker genes in zebrafish gills. No colocalizations between of NKA α1 genes (green fluorescence) and ionocyte markers (red fluorescence) were found: \( \text{atp1a1a.1} + \text{slc12a10.2} \) (NCC-like 2) (A), \( \text{atp1a1a.1} + \text{atp6v1a} \) (V-ATPase subunit A) (B), \( \text{atp1a1a.2} + \text{trpv6} \) (ECaC) (C), \( \text{atp1a1a.2} + \text{atp6v1a} \) (D), \( \text{atp1a1a.5} + \text{atp6v1a} \) (E), \( \text{atp1a1a.5} + \text{slc12a10.2} \) (F), \( \text{atp1a1a.4} + \text{trpv6} \) (G), \( \text{atp1a1a.4} + \text{slc12a10.2} \) (H), and \( \text{atp1a1a.4} + \text{atp6v1a} \) (I). Scale bar = 20 μm.

Fig. 5: Effects of environmental \( \text{Na}^+ \) or \( \text{Cl}^- \) concentration on gill transcriptions by quantitative RT-PCR of \( \text{atp1a1a.1} \) (A), \( \text{atp1a1a.2} \) (B), \( \text{atp1a1a.4} \) (C), and \( \text{atp1a1a.5} \) (D) in zebrafish. The mean and SD (\( n = 5 \)) are shown. One-way ANOVA pairwise comparisons were conducted among treatments. H, high; L, low.

Fig. 6: Effects of environmental \( \text{Ca}^{2+} \) concentration on gill transcriptions (by quantitative RT-PCR) of \( \text{atp1a1a.1} \) (A), \( \text{atp1a1a.2} \) (B), \( \text{atp1a1a.4} \) (C), and \( \text{atp1a1a.5} \) (D) in zebrafish. *Significant difference between the 2 groups (Student’s t-test, \( P < 0.05 \)).
study for the first time provides convincing molecular evidence of the distinct existence of ATP1a1a.1, ATP1a1a.2, and ATP1a1a.5 in different ionocytes in zebrafish gills based on specific colocalizations of the mRNAs of these α-paralogues and the relevant transporters of ionocytes. Isoform-specific heterologous antibodies have been previously used to examine NKA α1-, α2-, and α3-isoforms in fish gills (26); however, no antibody is available so far to specifically detect the respective products of the NKA α1 paralogues. This is a challenging and important issue to be solved in the future.

A central unresolved issue concerning NKA is whether the multiple isoforms of the α-subunits have unique or overlapping functions, and the approaches used so far in studies of mammalian NKA isoforms have limitations for exploring this issue, as addressed by Cheng et al. (8). The mRNA expression of zatp1a1.1, zatp1a1.2, and zatp1a1.5 were differentially stimulated by environmental ion compositions, and were parallel to those of the relevant transporters in each type of respective ionocyte (Table 4).

### Table 4. Comparisons of expression and functions of the relevant transporters/enzymes in zebrafish ionocytes

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<tr>
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<tr>
<td><strong>Proposed Function</strong></td>
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<tr>
<td>Na⁺ uptake acid secretion</td>
<td>Ca²⁺ uptake</td>
<td>Cl⁻ uptake</td>
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<tr>
<td><strong>Specifically-Expressed Transporters/Enzymes</strong></td>
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<td></td>
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<tr>
<td>H⁺-ATPase</td>
<td>ECAc</td>
<td>NCC</td>
<td>Unknown</td>
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<tr>
<td>NHE3b</td>
<td>PMCA2</td>
<td>NCX1b</td>
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<tr>
<td>CA15a</td>
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<tr>
<td>CA2-like a</td>
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<tr>
<td><strong>Na⁺-K⁺-ATPase Isoform</strong></td>
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<tr>
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<td><strong>Artificial Medium to Stimulate Functions of Ionocytes</strong></td>
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<td>Low-Na⁺</td>
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<td>ATP1a1a.5</td>
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*Data are from Lin et al. (29, 30), Yan et al. (44), Pan et al. (34), Liao et al. (27), and Wang et al. (42). CA, carbonic anhydrase; ECAc, epithelial Ca²⁺ channel; HA, H⁺-ATPase; NCC, Na⁺-Cl⁻ cotransporter; NCX, Na⁺/Ca²⁺ exchanger; NHE, Na⁺/H⁺ exchanger; NKA, Na⁺-K⁺-ATPase; PMCA; plasma membrane Ca²⁺-ATPase.
other hand, atp1a1.4 was not colocalized in any type of zebrafish gill ionocyte (HR, NaR, or NCC cells), nor was its mRNA expression stimulated by low-ion (Na\(^+\), Ca\(^{2+}\), and Cl\(^-\)) media, indicating that atp1a1.4-expressing cells may be an unidentified type of ionocyte. These unidentified ionocytes were expressed in both gill filaments and lamella, and were not associated with the uptake of Na\(^+\), Ca\(^{2+}\), or Cl\(^-\). Their functions and relevant transporters are another interesting and challenging issue for future study.

The present results of the specific mRNA expression and regulation of atp1a3.5, atp1a1.1, and atp1a1.2 in zebrafish ionocytes allow us to make a more comprehensive explanation for the proposed model in which these NKA α-subunits may play roles in providing some driving force for ion uptake in the respective ionocytes (Fig. 7). The Na\(^+\) uptake mechanism in zebrafish HR cells has been proposed to be similar to that in mammalian kidney proximal tubular cells (30, 44). In apical HR cells, ambient HCO\(_3\)\(^-\) and H\(^+\) secreted by apical NHE3 (or H\(^-\)-ATPase) actively form H\(_2\)CO\(_3\), and H\(_2\)CO\(_3\) is dehydrated by apical CA15a, thereby enabling the passive diffusion of CO\(_2\) into cells. Then the cytosolic CA2-like a hydrates CO\(_2\) to provide the substrates with H\(^+\) and HCO\(_3\)\(^-\), for the apical NHE3b and basolateral Na\(^+\)/HCO\(_3\) cotransporter (NBC) 1, respectively. ATP1a1.5/ATP1b1b may pump cytosolic Na\(^+\) across the basolateral membrane and also provide an intracellular negative potential to drive the electrogenic Na\(^+\)–H\(^+\) exchange by NCX1b. Cl\(^-\) uptake mechanisms in zebrafish ionocytes are still in debate. A previous pharmacological study with H\(^+\)-ATPase and CA inhibitors proposed a proton pump- and CA-dependent Cl\(^-\) uptake model in zebrafish gills (2). However, this notion does not appear to be supported by a recent loss-of-function study on H\(^+\)-ATPase, in which translational knockdown of H\(^+\)-ATPase subunit A impaired the acid secretion but without a significant effect on the Cl\(^-\) content in zebrafish morphants (19). More recently, a novel NCC-like 2 isoform, zebrafish sle12a10.2, was found to specifically be expressed by a group of ionocytes (NCC cells) in zebrafish, and translational knockdown of NCC-like 2 caused significant decreases in both Cl\(^-\) influx and Cl\(^-\) content, suggesting a role of NCC in zebrafish skin/gill Cl\(^-\) uptake pathways (42). Assuming the zebrafish NCC-like 2 as an electroneutral cotransporter like the mammalian orthologue, ambient Na\(^+\)/Cl\(^-\) levels (local tap water, 0.4–0.7 mM) are unlikely to favor the operation of NCC in apical membranes of NCC cells in zebrafish, since the reported intracellular Na\(^+\)/Cl\(^-\) levels in fish gill cells are 55–62 mM (or predicted as 6–17 mM) (35) and 40–90 mM (40), respectively. The issue of the NCC’s driving force remains to be an open question (21, 42). Nevertheless, ATP1a1.2/ATP1b1b may establish an electrochemical gradient to drive the basolateral transport pathways in NCC cells.

In mammals, NKA α-subunit isoforms have been demonstrated to show differences in the affinities for ouabain and substrates (Na\(^+\) and K\(^+\)) as well as interactions with the β-subunit in vitro (10, 24). Interestingly, some studies demonstrated different substrate affinities for gill NKAs between SW- and FW-acclimated tilapia (28) and rainbow trout (O. mykiss) (33), and both tilapia and rainbow trout were found to express NKAs with different combinations of β-subunits, depending on environmental salinities (14, 23, 37). On the other hand, the different responses to endocrine or environmental factors among NKA α-isofoms (or α paralogues) should also be considered when discussing the physiological significances of the existence of multi-isofoms of NKA α-subunits. In tilapia, plasma levels of ouabain, cortisol, and osmolality showed positive correlations during salinity acclimation, suggesting the involvement of ouabain with cortisol in the maintenance of hydromineral balance (25). In zebrafish, different ionocyte types express respective NKA α paralogues, which may subtly differ in ouabain affinity, implying different signaling potentials in these ionocytes. In mammalian kidneys, combinations of α1–3- and β1–2- subunits were found to be differentially expressed in various segments by RT-PCR analysis on microdissected samples (9). In overexpression experiments of mouse collecting duct mpkCCD cells, the human α1- but not the α2-isoform was stimulated by aldosterone, a steroid con-

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**Fig. 7.** Model of the ion regulation mechanisms in zebrafish gill/skin ionocytes. Most of the proposed mechanisms are derived from Hwang and Lee (23) and Hwang and Tangel (21) with some updates. unidentified ionocytes, transporters, or pathway. CA2, carbonic anhydrase 2-like α; CA15, carbonic anhydrase 15a; CIC, Cl\(^-\) channel; ECaC, epithelial Ca\(^{2+}\) channel; HA, H\(^+\)-ATPase; NBC, Na\(^+\)/HCO\(_3\)\(^-\) cotransporter; NCC, Na\(^+\)/Cl\(^-\) cotransporter; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger 1b; NHE, Na\(^+\)/H\(^+\) exchanger 3b; NKA, Na\(^+\)/K\(^+\)-ATPase; PMCA2; plasma membrane Ca\(^{2+}\)-ATPase.
trolling \(\text{Na}^+\) reabsorption in distal nephrons (38, 39). In the present study, the \textit{atp1a1.1}, \textit{atp1a1.2}, and \textit{atp1a1.5} paralogues in zebrafish gill ionocytes showed distinctive responses to the environmental ion levels. This finding is of physiological significance and interest, and implies that regulatory pathways controlling the function and expression of each \(\alpha_1\) paralogue in respective ionocytes may subtly differ to allow zebrafish to adapt to fluctuating environments.

**Perspectives and Significance**

In mammals, it has been for a long time an important issue to study the expressions of different combinations of NKA \(\alpha\)-and \(\beta\)-subunits and their roles in the functions of the various tissues and/or cell types. This phenomenon is evolutionally conserved in mammalian kidneys and zebrafish skin/gills. Compared with mammals, zebrafish is a more powerful model to provide in vivo molecular physiological evidence to answer if these NKA isoforms conduct respective or overlapping functions in the same organ or tissue. In zebrafish, ion transport functions in respective types of ionocytes have been explored, and the methodologies to assay these functions have also been established recently. The question about the functional redundancy of NKA isoforms in zebrafish ionocytes may be answered by examining if the functional defects in ionocytes caused by knockdown of one NKA \(\alpha_1\) paralogue could be rescued by overexpression of another paralogue(s).

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