FXYD6, a Na,K-ATPase Regulator, Is Expressed in Type II Taste Cells

Yoichiro Shindo,1 Kana Morishita,2 Eiichi Kotake,1 Hirohito Miura,3 Piero Carninci,4 Jun Kawai,4 Yoshhide Hayashizaki,4 Akihiro Hino,2 Tomomasa Kanda,1 and Yuko Kusakabe2,4

1Research Laboratories for Health and Gustatory Science, Asahi Breweries, Ltd., 1-1-21 Midori, Moriya 302-0106, Japan
2National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba 305-8642, Japan
3Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
4RIKEN Omics Sciences Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

Received October 6, 2010; Accepted March 29, 2011; Online Publication, June 13, 2011
[doi:10.1271/bbb.100718]

Taste buds contain three types of taste cells. Each type can respond to taste stimulation, and type II and III taste cells are electrically excitable. However, there are differences between the properties of type II and III taste cells. In this study, we found that Fxyd6, an Na,K-ATPase regulator gene, is expressed in type II taste cells in the taste buds of mice. Double-labeled in situ hybridization analysis showed that Fxyd6 was coexpressed with transient receptor potential channel cation channel, subfamily M, member 5 (Trpm5), a critical component of the sweet, bitter, and umami taste signal transduction pathways and that it was specifically expressed in type II taste cells. We also found that taste cells frequently coexpressed Fxyd6 and Na,K-ATPase β1. These results indicate the presence of an inherent mechanism that regulated transmembrane Na+ dynamics in type II taste cells.

Key words: Na,K-ATPase; taste cells; gene expression pattern; in situ hybridization; regulation of sodium ions

Vertebrates detect various tastes through taste cells in the taste buds of the oral cavity. There are 3 types of taste cells (type I, type II, and type III) in each taste bud,11 and these taste cells detect 5 basic tastes (sweet, bitter, sour, salty, and umami). Studies have shown that some populations of type I cells respond to the salt taste,2 whereas type I cells have been identified as supporting cells, that do not respond to taste substances.3 Type II cells express G-protein-coupled receptors, T1Rs and T2Rs, and downstream signaling molecules for the sweet, bitter, and umami tastes.3,4,5 Type III cells have synaptic vesicles including synaptic molecules and express the channels for sour taste.2,3,6 Type II and III taste cells are known to be electrically excitable.7,8 Type II cells express Na+ channels (SCNs) SCN2A, SCN3A, and SCN9A, whereas type III cells express only SCN2A. These different expression patterns suggest that each of these taste cell types possesses distinctive Na+ dynamics and its own maintenance system of Na⁺ and potassium ion (K⁺) gradients across the membranes.

The enzymatic molecules that create and maintain the Na⁺ and K⁺ gradients are Na,K-ATPases, also known as Na-K pumps. Na,K-ATPases transport 3 Na⁺ ions out and 2 K⁺ ions into the cell to maintain the gradients across the cell membrane. Na,K-ATPase is necessary in all cells to ensure maintenance of basic cellular homeostasis; it also contributes to specialized tissue functions. The functional variety of Na,K-ATPase depends on the combination of its α and β subunits, and on the FXYD family members that determine its transport properties.9 These combinations are known as isoforms.10 They result from the association of different molecular forms of the α (α1 to α4) and β (β1 to β4) subunits of Na,K-ATPases with members of the FXYD family (FXYD1–7).1,11 All of the members of the FXYD family have recently been found to act as tissue-specific regulators of Na,K-ATPases.11 Most FXYD proteins have single transmembranes, common sequences that contain the FXYD motif at the N-terminus, and conserved residues (2 glycine and 1 serine) in the transmembrane domain.11 All FXYD family members interact with Na,K-ATPases, producing different effects on their kinetics in tissue- and isozyme-specific ways.11,14 Thus, information about the combination of Na,K-ATPase α and β subunits and the subtypes of the FXYD family in a particular tissue is particularly important in predicting tissue function. Little is known about the expression of Na,K-ATPase in taste cells, apart from the findings of one study that found that Na,K-ATPase β1 is expressed in rat taste buds,13 but the role played by Na,K-ATPase in taste signal transduction remains unclear. Moreover, no information is available on Na,K-ATPase regulators such as FXYD proteins in taste cells.

1 To whom correspondence should be addressed. Fax: +81-29-838-7996; E-mail: ykusa@affrc.go.jp
Abbreviations: SCN, sodium channel; RT-PCR, reverse transcription-polymerase chain reaction; CV, circumvallate papillae; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitroblue tetrazolium; Trpm5, transient receptor potential cation channel, subfamily M, member 5
In this study, we investigated the cellular distribution of FXYD6 in a subset of taste cells and its coexpression with Na,K-ATPase β1. Our results indicate that FXYD6 is a type II taste-cell specific regulator of Na,K-ATPase that plays a crucial role in sweet, bitter, and umami taste signal transduction.

Experimental

Experimental animals. Eight- to 20-week-old male C57BL/6N(Cj) mice (Charles River Laboratories Japan, Yokohama, Japan) were treated in accordance with the basic guidelines of the Ministry of Agriculture, Forestry, and Fisheries of Japan for laboratory animal study, and were used for reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization.

Reverse transcription-polymerase chain reaction. First-strand cDNA from circumvallate papillae (CV) was reverse transcribed from 100 ng of total RNA prepared from murine CV epithelium. CV epithelium samples were obtained as previously described.66 Other first-strand cDNAs from control tissues (brain, kidney, lung, muscle, heart, and stomach) were purchased from GenoStaff (Tokyo). The cDNAs of FXYD family members and the Na,K-ATPase subunits were amplified using oligonucleotide primer pairs using previously published sequences (Table 1). For Na,K-ATPase α subunit members, part of each primer was designed from non-coding regions, because they share high homology in their coding regions. PCR was performed using rTaq DNA polymerase (Takara Bio, Ohtsu, Japan). PCR reactions were performed as previously described. 16) CV and fungiform papillae from 8-week-old male mice were frozen in optimum cutting temperature (OCT) compound (Sakura Finetech USA, Los Angeles, CA), and subsequently sectioned into 5-μm thick slices. All hybridization reactions were performed at 65 °C. The in situ hybridization experiments were visualized by gel electrophoresis in 0.8% or 2% agarose gels. The DNA sequences of the PCR products were determined using a Beckman CEQ2000 DNA Analysis System (Beckman Coulter, Brea, CA).

cRNA probes for in situ hybridization. The cDNA clones in the subtracted full-length cDNA library were used in the production of cRNA probes against the full-length cDNAs of Na,K-ATPase β3 (1 to 1,833; GenBank NM_007502) and Fxyd6 (1 to 1,804; GenBank NM_022004). Other cRNA probes were produced from the corresponding RT-PCR products. Each RT-PCR product was subcloned into the pGEM-T or pGEM-T Easy Vector System (Promega, Madison, WI). Transient receptor potential cation channel, subfamily M, member 5 (Trpm5; 88 to 3564; GenBank NM_020277) was also subcloned into the pGEM-T Easy Vector System. The full-length cDNA for mouse polycystic kidney disease 2-like 1 (Pkdl2; 169–2451; GenBank NM_181422) was kindly provided by Dr. Yoshito Ishimaru (The University of Tokyo), and was used for the cRNA probe against Pkdl2. The cRNA probes for single-colored in situ hybridization were prepared using a digoxigenin RNA Labeling Kit (Roche, Mannheim, Germany). For double-colored in situ hybridization, we used a digoxigenin and Fluorescein RNA Labeling Kit (Roche Diagnostics).

In situ hybridization. In situ hybridization experiments were performed as previously described.66 CV and fungiform papillae from 8-week-old male mice were frozen in optimum cutting temperature (OCT) compound (Sakura Finetech USA, Los Angeles, CA), and subsequently sectioned into 5-μm thick slices. All hybridization reactions were performed at 65 °C. In the case of single-colored in situ hybridization, signals were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche; 1:400), in combination with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and

Table 1. Primer Sets for RT-PCR

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<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>GenBank accession no. (coding sequence)</th>
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<tr>
<td>Fxyd1</td>
<td>5′-TCACGATTACCACTCATCCCGCCACAT-3′ (110-129)</td>
<td>NM_019503 (102-389)</td>
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<td>Fxyd2</td>
<td>5′-ATGGGAGGGCTCTCTGTAATACGTTTGGGCTG-3′ (266-247)</td>
<td>NM_007503 (54-266)</td>
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<td>Fxyd3</td>
<td>5′-ATGCAAGATGTCGCTGTCGTCGTA-3′ (92-111)</td>
<td>NM_008557 (92-358)</td>
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<td>Fxyd4</td>
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<td>NM_003648 (118-384)</td>
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<td>Fxyd5</td>
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<td>NM_008761 (234-770)</td>
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<td>NM_020204 (310-594)</td>
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<tr>
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<td>Na,K-ATPase α2</td>
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<td>NM_013415 (589-608)</td>
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<tr>
<td>Na,K-ATPase β2</td>
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<td>NM_020204 (102-389)</td>
</tr>
<tr>
<td>Na,K-ATPase β3</td>
<td>5′-ATGGGAGGGCTCTCTGTAATACGTTTGGGCTG-3′ (146-165)</td>
<td>NM_020204 (102-389)</td>
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<tr>
<td>Na,K-ATPase β4</td>
<td>5′-ATGGGAGGGCTCTCTGTAATACGTTTGGGCTG-3′ (146-165)</td>
<td>NM_020204 (102-389)</td>
</tr>
</tbody>
</table>
Expression of FXYD6 in Taste Cells

Results and Discussion

Cellular distribution of FXYD6 in taste buds

To identify Na,K-ATPase-related genes that function specifically in taste cells, we searched our original subtracted full-length cDNA library prepared from murine CV epithelia.\(^{1,7}\) The annotation data for 1,879 cDNA clones from the library were searched using keywords, which detected cDNAs encoding Na,K-ATPase \(\alpha_1\), \(\beta_3\), and Fxyd6. In situ hybridization analysis indicated that Fxyd6 was distinctly expressed in a specific subset of taste cells (Fig. 1A). Among the seven FXYD family members, we detected expression of Fxyd3–7 in the CV by RT-PCR (Fig. 1B); however, by in situ hybridization we detected only FXYD6, which was specifically expressed in the taste buds (Fig. 1A). In contrast, Fxyd3 was broadly expressed in the epithelium of the CV (Fig. 1A), but was not specifically expressed in the taste buds.

Mammalian taste bud cells are divided into distinct morphological types (basal, type I, type II, and type III), which can be identified by their distinct ultrastructural and immunohistochemical features.\(^{1,5-7}\) To determine which types of cells express Fxyd6 in CV taste buds, we carried out double-labeled in situ hybridization. Trpm5 and Pkd2l1 were used as cell markers in this analysis. Specifically, Trpm5 was used as a marker for type II taste cells that participate in sweet, bitter, and umami taste sensation,\(^{18}\) whereas the candidate sour taste receptor Pkd2l1\(^{19}\) was used as a marker for type III taste cells.\(^{20}\) Double-labeled in situ hybridization indicated that 87.7% of Trpm5-positive cells also expressed Fxyd6, and conversely, that 97.3% of Fxyd6-positive cells expressed Trpm5 (Fig. 2A, Table 2). These results suggest that Fxyd6 is mostly coexpressed with Trpm5. In contrast, the expression of Fxyd6 scarcely overlapped with that of Pkd2l1 (2.9–3.4%) (Fig. 2B, Table 2).

It has been reported that the expression patterns of some taste receptors and G protein \(\alpha\) subunits are different as between the anterior and posterior sides of the tongue.\(^{16,17}\) Accordingly, we examined the expression pattern of Fxyd6 in fungiform papillae. Robust signals for Fxyd6 were observed in the fungiform papillae at the tip of the tongue, similarly to expression in the CV (Fig. 3A). Moreover, most cells that expressed Fxyd6 also expressed Trpm5, as observed for the CV (Fig. 3B).

Expression of the Na,K-ATPase \(\alpha\) and \(\beta\) subunit isoforms in taste cells

We also investigated the expression of the Na,K-ATPase \(\alpha\) and \(\beta\) subunits in taste cells. Na,K-ATPase \(\alpha_1\) and \(\beta_3\) were both in our cDNA library, and Asanomiyoshi et al.\(^{15}\) have reported that Na,K-ATPase \(\beta_1\) is expressed in rat taste buds. In this study, RT-PCR using specific primers for the Na,K-ATPase \(\alpha\) subunit isoforms and fs-cDNA from the CV epithelium showed that only \(\alpha_1\) was expressed in the CV epithelium (Fig. 4A). In the inner ear, FXYD6 perhaps plays a role as a regulator of H,K-ATPase.\(^{20}\) Hence, we also performed RT-PCR using specific primers for H,K-ATPase \(\alpha\) and fs-cDNA from the CV epithelium, and barely detected it (Fig. 4A). In situ hybridization showed that Na,K-ATPase \(\alpha_1\) was broadly expressed in the epithelium of the CV (Fig. 4B), as opposed to being specifically expressed in the taste buds. We did not detect other Na,K-ATPase \(\alpha\) subunit isoforms, besides \(\alpha_1\) and the H,K-ATPase \(\alpha\) subunit in the CV epithelium, by in situ hybridization (Fig. 4B). As for the Na,K-ATPase \(\beta\)

\[\text{Expression of FXYD6 in Taste Cells} \quad 1063\]
subunit isoforms, Na,K-ATPase \(\alpha_1\) and \(\alpha_3\) were detected by RT-PCR (Fig. 5A). In situ hybridization of the CV sections showed that mouse \(\alpha_1\) was expressed in the taste buds (Fig. 5B). In contrast, Na,K-ATPase \(\alpha_3\) was expressed mainly in the basal cells of the CV epithelium, and scarcely in the taste buds (Fig. 5B). Subsequently, we examined the coexpression of Fxyd6 and \(\beta_1\) by double-labeled in situ hybridization (Fig. 6). We observed that 64.5% of \(\beta_1\)-positive cells also expressed Fxyd6, and that 98.3% of Fxyd6-positive cells also expressed \(\beta_1\) (Table 2). This indicates that almost all the Fxyd6-positive cells were also \(\beta_1\)-positive cells (Fig. 6).

Collectively, these observations indicate that type II taste cells express both Fxyd6 and Na,K-ATPase \(\alpha_1\).

Na,K-ATPase isozymes in taste tissue

In view of our results, various isozymes may be present in both the taste cells and the CV epithelial cells around the taste buds. Among the Na,K-ATPase \(\alpha\) subunit isoforms, only Na,K-ATPase \(\alpha_1\) was detected by RT-PCR. We observed its expression in the epithelium of CV, but no specific expression of Na,K-ATPase \(\alpha_1\) was observed in the taste buds. In addition, faint expression of H,K-ATPase \(\alpha\) was detected by RT-PCR. These data suggest that Na,K-ATPase \(\alpha_1\) is the chief \(\alpha\) subunit isoform which functions in both the taste buds and the epithelium of the CV. Furthermore, we observed different expression patterns of Na,K-ATPase \(\beta\) subunit

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**Table 2.** Coexpression Ratios of Fxyd6 and Other Genes in the Murine CV

<table>
<thead>
<tr>
<th>Target gene/ (\text{Fxyd6})</th>
<th>Coexpression ratio (%)(^{a})</th>
<th>(\text{Trp}m5)</th>
<th>(\text{Pk}d21)</th>
<th>Na,K-ATPase (\beta_1)</th>
</tr>
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<tr>
<td>(\text{Fxyd6}/\text{Target gene})</td>
<td>87.7 (257/293)</td>
<td>3.4 (13/378)</td>
<td>64.5 (412/639)</td>
<td></td>
</tr>
<tr>
<td>Target gene/ (\text{Fxyd6})</td>
<td>97.3 (257/264)</td>
<td>2.9 (13/450)</td>
<td>98.3 (412/419)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Numbers of cells counted are given in parentheses.

\(^{b}\)Coexpression ratio of cells expressing \(\text{Fxyd6}\) to cells expressing a target gene.

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**Fig. 3.** Fxyd6 Expression in Type II Taste Cells in Taste Buds of Murine Fungiform Papillae.

Murine fungiform papillae slices were used for double-labeled in situ hybridization. A, Fxyd6 expression in a taste bud of fungiform papillae. B, Coexpression pattern of Fxyd6 and \(\text{Trp}m5\) in the fungiform papillae. Colocalization is indicated in yellow in the merged image. Broken lines indicate the outline of a sample taste bud. Scale bars indicate 50 µm.

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**Fig. 4.** Expression of the Na,K-ATPase \(\alpha\) Subunit in Taste Buds of the Murine CV.

A, RT-PCR analyses of both the Na,K-ATPase \(\alpha\) subunit and the H,K-ATPase \(\alpha\) subunit, using fs-cDNA from the murine CV epithelium (+RT and −RT) and other murine tissues (control). Fs-cDNAs from the brain and kidney were used as controls. The primers for Na,K-ATPase \(\alpha\) subunit members are shown in Table 1. +RT, with reverse transcriptase; −RT, without reverse transcriptase. The expected PCR product sizes ranged from 313 to 490 bp. B, Expression of Na,K-ATPase \(\alpha_1–4\), and H,K-ATPase \(\alpha\) in the CV epithelia by in situ hybridization. The broken lines indicate the outline of a sample taste bud. Scale bars indicate 50 µm.

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**Fig. 2.** Fxyd6 Is Expressed in Type II Taste Cells in Taste Buds of Murine CV.

Murine CV slices were used for double-labeled in situ hybridization. Colocalization of probes is indicated in yellow in the merged image. A, Comparison of Fxyd6 expression with the \(\text{Trp}m5\) probe, which was used as a marker for type II taste cells in taste buds of the CV. B, Comparison of Fxyd6 expression with the \(\text{Pk}d21\) probe, which was used as a marker for type III taste cells. Scale bars indicate 50 µm.

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**Fig. 5.** Expression of Na,K-ATPase \(\beta\) Isoforms in the CV.

A, RT-PCR analyses of both the Na,K-ATPase \(\beta\) subunit and the H,K-ATPase \(\alpha\) subunit, using fs-cDNA from the murine CV epithelium (+RT and −RT) and other murine tissues (control). Fs-cDNAs from the brain and kidney were used as controls. The primers for Na,K-ATPase \(\beta\) subunit members are shown in Table 1. +RT, with reverse transcriptase; −RT, without reverse transcriptase. The expected PCR product sizes ranged from 313 to 490 bp. B, Expression of Na,K-ATPase \(\beta_1–4\), and H,K-ATPase \(\alpha\) in the CV epithelia by in situ hybridization. The broken lines indicate the outline of a sample taste bud. Scale bars indicate 50 µm.

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**Fig. 6.** Expression of Na,K-ATPase \(\beta\) Isoforms in the CV.

A, RT-PCR analyses of both the Na,K-ATPase \(\beta\) subunit and the H,K-ATPase \(\alpha\) subunit, using fs-cDNA from the murine CV epithelium (+RT and −RT) and other murine tissues (control). Fs-cDNAs from the brain and kidney were used as controls. The primers for Na,K-ATPase \(\beta\) subunit members are shown in Table 1. +RT, with reverse transcriptase; −RT, without reverse transcriptase. The expected PCR product sizes ranged from 313 to 490 bp. B, Expression of Na,K-ATPase \(\beta_1–4\), and H,K-ATPase \(\alpha\) in the CV epithelia by in situ hybridization. The broken lines indicate the outline of a sample taste bud. Scale bars indicate 50 µm.

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\(^{a}\)Numbers of cells counted are given in parentheses.

\(^{b}\)Coexpression ratio of cells expressing \(\text{Fxyd6}\) to cells expressing a target gene.

\(^{c}\)Coexpression ratio of cells expressing a target gene to cells expressing \(\text{Fxyd6}\).
isoforms and members of the FXYD family in the CV epithelium. In particular, Fxyd6 was expressed in type II taste cells of the taste buds, and a high percentage of these cells also expressed Na,K-ATPase β1. This indicates that almost all Fxyd6-expressing cells expressed β1 in our study. This in turn suggests the possibility that Na,K-ATPase α1-β1-FXYD6 is the predominant isoform present in type II taste cells, and that it plays an important role in sweet, bitter, and umami taste signal transduction, because type II taste cells express Trpm5, a critical component for sweet, bitter, and umami taste signal transduction. Furthermore, Na,K-ATPase β1 was expressed in taste cells other than Fxyd6-expressing cells; Na,K-ATPase β3 was expressed in tongue epithelial cells but not in taste cells; and Fxyd3 was expressed in both the epithelial cells and taste cells of the CV. This suggests the possible existence of other isoforms besides α1-β1-FXYD6 there. For example, α1-β1-FXYD3 might play a role in both taste cells and tongue epithelial cells, and α1-β3-FXYD3 play a role specific to tongue epithelial cells. These expression patterns indicate that the distribution of Na,K-ATPase isoforms in the tongue epithelium, including the taste buds, is not uniform, and that there is a common Na⁺,K⁺ transport system in sweet-, bitter-, and umami-responsive cells. We detected Fxyd4, Fxyd5, Fxyd7, and H,K-ATPase α by RT-PCR, but we did not observe expression of them by in situ hybridization. Based on these results, we cannot rule out the possibility of the presence of Fxyd4, Fxyd5, Fxyd7, and H,K-ATPase α in the taste buds and/or CV epithelial cells. Further studies are needed to determine their expression and function in taste buds.

**Possible roles of FXYD6 in type II taste cells**

We speculate that FXYD6 can regulate type II cell-specific Na⁺ dynamics. Recently, Gao et al. reported that voltage-gated sodium channels SCN3A and SCN9A were selectively expressed in type II taste cells and might play roles in membrane depolarization. Furthermore, Vandenbeuch and Kinnamon reported that Na⁺ currents in type II taste cells were inactivated at a slower rate than were those currents in type III taste cells, and they concluded that SCN9A might be responsible. Delprat et al. used the Xenopus oocytes expression system, and found that the association of FXYD6 with Na,K-ATPase α1-β1 resulted in a 2-fold decrease in the apparent Na⁺ affinities of the α1 and β1 isoforms, whereas the maximal Na⁺,K⁺-pump currents remained unaltered. They suggested that the decrease in the apparent Na⁺ affinity of the α1-β1-FXYD6 isoform resulted in effective extrusion of Na⁺ from the cells, after an increase in the concentration of Na⁺. Based on these findings, we suggest that FXYD6 is responsible for the longer time required to activate Na,K-ATPase α1-β1 in type II taste cells, contributing to increased concentrations of Na⁺ in the cells, effective extrusion of Na⁺ from the cells, and finally delayed inactivation of the Na⁺ current in the cells. Further investigation using mice lacking FXYD6 should provide insight into the role played by FXYD6 in type II cell-specific Na⁺ dynamics.
Conclusion

In summary, we found that Fxyd6 was expressed in type II taste cells and perhaps associated with Na,K-ATPase β1. Accordingly, we propose that cell-type specific Na+ regulation systems of Na,K-ATPase are present in taste bud cells, and that the Na+ regulation system, especially FXYD6, is an important component of the sweet, bitter, and umami taste signal transduction pathway.

Acknowledgments

We thank Dr. Yoshiro Ishimaru (The University of Tokyo) for the Pkd2l1 cDNA used in double-labeled in situ hybridization, Dr. Tokuda and Dr. Yamaguchi (Kagawa University) for the FXYD6 antibody used in the preliminary experiments, and Ms. Shindo, Ms. Arima, Ms. Okano, and Ms. Tagami for technical help. This work was supported by the Bio-Oriented Technology Research Advancement Institution, JSPS KAKENHI (22580154), and a Research Grant from the RIKEN OSC from MEXT to Y. H.

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