Exploration of the Peptide Recognition of an Amiloride-sensitive FMRFamide Peptide-gated Sodium Channel*

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You-Ya Niu1,5†, Yang Yang6†, Yan Liu5, Li-Dong Huang5, Xiao-Na Yang5†, Ying-Zhe Fan*, Xiao-Yang Cheng5, Peng Cao1,5†, You-Min Hu1, Lingyong Li1,‡, Xiang-Yang Li1,‡, Yun Tian1,‡, and Ye Yu1,3,‡

From the 1College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha, 410128, China, 6Institute of Medical Sciences and Departments of Pharmacology and Physiology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, 5Putuo District Center Hospital, Shanghai University of Chinese Traditional Medicine, Shanghai, 200062, China, 4Department of Anesthesiology and Perioperative Medicine, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, 3Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, China, and 2Laboratory of Cellular and Molecular Biology, Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing 210028, China

FMRFamide (Phe-Met-Arg-Phe-NH₂)-activated sodium channel (FaNaC) is an amiloride-sensitive sodium channel activated by endogenous tetrapeptide in invertebrates, and belongs to the epithelial sodium channel/degenerin (ENaC/DEG) superfamily. The ENaC/DEG superfamily differs markedly in its means of activation, such as spontaneously opening or gating by mechanical stimuli or tissue acidosis. Recently, it has been observed that a number of ENaC/DEG channels can be activated by small molecules or peptides, indicating that the ligand-gating may be an important feature of this superfamily. The peptide ligand control of the channel gating might be an ancient ligand-gating feature in this superfamily. Therefore, studying the peptide recognition of FaNaC channels would advance our understanding of the ligand-gating properties of this superfamily of ion channels. Here we demonstrate that Tyr-131, Asn-134, Asp-154, and Ile-160, located in the putative upper finger domain of Helix aspersa FaNaC (HaFaNaC) channels, are key residues for peptide recognition of this ion channel. Two HaFaNaC specific-insertion motifs among the ENaC/DEG superfamily, residing at the putative α4-α5 linker of the upper thumb domain and the α6-α7 linker of the upper knuckle domain, are also essential for the peptide recognition of FaNaC channels. Chemical modifications and double mutant cycle analysis further indicated that those two specific inserts and key residues in the upper finger domain together participate in peptide recognition of HaFaNaC channels. This ligand recognition site is distinct from that of acid-sensing ion channels (ASICs) by a longer distance between

The epithelial sodium channel/Degenerin (ENaC/DEG) superfamily of ion channels. This study was supported by grants from the National Excellent Young Scientist Foundation of China (no. 31222018), National Program on Key Basic Research Project of China (2014CB910302), National Natural Science Foundation of China (nos. 31170787, 31570832, 31400707, 81302694, and 81473377), the Opening Project of National Key Laboratory of Key Incubation Project (12x190030017), Key National S&T Program “Major New Drug Development” Grant (no. 2012ZX09504001-003), and Science and Technology Fund of Shanghai Jiao Tong University School of Medicine (13XJ10042). The authors declare that they have no conflicts of interest with the contents of this article.

†To whom correspondence may be addressed. E-mail: tianyun79616@163.com.
‡To whom correspondence may be addressed. E-mail: yuye@shsmu.edu.cn.

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1 Both authors contributed equally to this work.
2 To whom correspondence may be addressed. E-mail: tianyun79616@163.com.
3 To whom correspondence may be addressed. E-mail: yuye@shsmu.edu.cn.

The abbreviations used are: ENaC/DEG, epithelial sodium channel/Degenerin; FMRFamide, Phe-Met-Arg-Phe-NH₂; FaNaC, FMRFamide-activated sodium channel; HaFaNaC, Helix aspersa FaNaC; HtFaNaC, Helisoma trivolvis FaNaC.
binding sites and the number of bound-protons vary during the allosteric process (10). Additionally, ASIC channels are poly-model receptors responding to various stimuli including acidosis, small molecules, deprivation of extracellular calcium, peptide toxin, and mechano-stimuli, making them more challenging in thoroughly understanding the channel gating of those ion channels. Belonging to the DEG/ENaC superfamily, the FaNaC channel is an FMRFamide (Phe-Met-Arg-Phe-NH₂)-gated ion channel that was discovered in invertebrates and is activated only by peptides rather than acidosis (14) or the deprivation of extracellular calcium (15). In addition, the RFamide peptide ligand control of channel gating may represent an ancient ligand-gating feature in the ENaC/DEG superfamily (16). Thus, the FaNaC channel may be a nice simplified model for studying the ligand-gating mechanism of the ENaC/DEG superfamily, which will advance our understanding of the ligand-gating properties of this superfamily of ion channels.

So far, four FaNaC genes have been identified, namely, HaFaNaC from the Helix aspersa (17), HtFaNaC from Helisoma trivolvis (18), LsFaNaC from Lymnaea stagnalis (14), and AkFaNaC from Aplysia kurodai (19–21). FaNaC is the first discovered peptide-gated ion channel identified in the ENaC/DEG superfamily. Cloned from the freshwater Polyp Hydra, HyNaC is another ancient peptide-gated ion channel, activated by Hydra-RFamides (22). The existence of the peptide-gated channels in both mollusks and coelenterates suggests that an RFamide-gated channel already exists in a common ancestor of mollusks and coelenterates (16). Appearing later than FaNaC and HyNaC in evolution, ASICs are proton-gated acid-sensing ion channels found in chordata. Although ASICs cannot be directly activated by RFamide peptides, they can be regulated by them, including FMRFamide (23), suggesting the presence of peptide-binding pockets in ASICs, by which FMRFamide interacts with and regulates proton activated currents. The close relationship among ASICs, HyNaCs, and FaNaCs suggests that ASICs may have evolved from an RFamide-gated channel (14, 16). It has also been speculated that the entire superfamily of DEG/ENaC has evolved from RFamide-gated ion channels (16). Therefore, studying the FaNaC channels may also provide insights into better understanding of the channel evolution of the ENaC/DEG superfamily.

The sequence homology among ENaC/DEG subfamilies is not high, but around 15–20% (1); However, the sequence identity within a subfamily is much higher, with ~30% for both ENaC subunits and degenerins, ~45–60% for ASICs, and ~65% for the four FaNaC orthologues (1). Despite the high sequence homology among the four FaNaC orthologues, they exhibit different affinities to FMRFamide. For HaFaNaC, the half maximal effective concentration (EC₅₀) of FMRFamide is 2–10 μM (17, 24), while for HtFaNaC the value is 70 μM (18). The amino acid sequence between residues 89 and 211, located within the extracellular domains of HaFaNaC or HtFaNaC, may be responsible for the different affinities to FMRFamide (24, 25), although detailed structural components remain unknown. In addition, alignment of members in the superfAMILY revealed two specific inserts identified in FaNaC channels (Fig. 1A). Analyzing the structure of cASIC1 (Fig. 1B) suggests that insertion I is a ~20 amino acid sequence between α4 and α5 in the thumb domain (Fig. 1, A and C, green) while insertion II is ~50 amino acid long sequence bending over α6 and α7 in the knuckle domain (Fig. 1, A and C, orange). However, whether or not those two specific inserts are involved in the recognition of FMRFamide by HaFaNaC remains unclear.

Here, in a combination of sequence alignment, mutagenesis, electrophysiological recording, Western blot, chemical modification, and double-mutant cycle analysis, we explore the peptide/ligand recognition of FaNaC channels. By comparing ligand recognition and activation mechanisms with other members of the ENaC/DEG superfamily, we aim to provide insights into small peptide-induced channel activation or small molecule-induced channel activation of some members of this superfamily, and thus aim to gain some information about the channel evolution of this superfamily.

**Experimental Procedures**

**Materials and Chemicals**—FMRFamide was synthesized by GL Biochem (Shanghai) Ltd. DTNB and DTT were purchased from Sigma; Hilymax transfection reagents were purchased from DOJINDO Laboratories; sulfo-NHS-LC-biotin, NeutrAvidin agarose resin and ECL solution were all purchased from Pierce, Antibody of EE-tag was purchased from Abcam; antibody of GAPDH and HRP-conjugated secondary antibodies were purchased from Sungen.

**Site-directed Mutagenesis**—The HaFaNaC pHc/CMV was kindly gifted by Drs. Lingueglia, E. and Lazdunski, M. All the constructs had the Glu-Glu (EE) tag (GAATACATGC-CAATGGGA) fused at C-terminal of the channel. Mutations were made using the QuikChange® Mutagenesis Kit following the manufacturer’s instructions and sequenced (by GENEWIZ, Inc.) for verification.

**Cell Culture and Transfection**—CHO cells were cultured in F12 Nutrient Mixture (Gibco) containing 10% fetal bovine serum (Gibco), 1% Glutamycin (Gibco) and 1% penicillin/streptomycin (HyClone). Transient transfection was carried out using the Hilymax transfection reagents (DOJINDO Laboratories) following the manufacturer’s instructions. For each 35-mm dish, CHO cells were transfected with 3 μg of plasmid and 5 μl of transfection reagents. Green fluorescent protein was co-transfected in the cell as a marker with a ratio of 1:6 (GFP: FaNaC) to identify cells expressing HaFaNaC for electrophysiology experiments.

**Electrophysiology**—All electrophysiological recordings were performed at room temperature 24–48 h after cells transfection as we previously described (6, 26). HaFaNaC currents were recorded with the whole cell patch-clamp method using an Axopatch 200B amplifier and Digidata 1440A with Clampex 10.2 software. Currents were sampled at 10 kHz and filtered at 1 kHz. Patch pipettes were pulled from borosilicate glass and had resistances of 3–5 MΩ. The pipette solution contained the following (in mM): 120 KCl, 30 NaCl, 0.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 EGTA, pH 7.4. The bath solution contained the following (in mM): 150 NaCl, 10 glucose, 10 HEPES, 2 CaCl₂, 5 KCl, and 1 MgCl₂, pH 7.4. After establishment of the whole-cell configuration, currents were measured at a holding potential of ~60 mV.
Cell Surface Biotinylation and Western Blot Analysis—Surface biotinylation and Western blots were performed on cultured HEK293 cells according to our previous protocols (26, 27). The HEK 293 cells were transfected with 8 μg plasmids of HaFaNaC or its mutants (in pRc-CMV vector), 1.2 μg plasmid of green fluorescent protein (as a transfection marker), and 15 μl transfection reagent Hylinax. After transfected for 24–48 h, the cells were washed with chilled PBS and incubated in 2 mM sulfo-NHS-LC-biotin (Pierce) at 4 °C in the same buffer for biotinylation of proteins on cell surface. After 30 min, 20 mM glycine was added to stop the reaction. Excessive reagents were then washed out with chilled PBS; and the cells were lysed by 200 μl RIPA buffer (containing protease inhibitors Mixture from Sigma) and centrifuged at 12,000 rpm for 30 min at 4 °C. A small portion of the supernatant (28 μl added with 7 μl of 5×SDS sample buffer) was used as total protein fraction. The remaining lysate was incubated with NeutrAvidin agarose resin (Pierce) overnight in 4 °C. The resin beads were later centrifuged at 5000 rpm for 5 min and rinsed with PBS for 5 times to remove the unbound proteins. The resin-bound proteins were subsequently eluted with 40 μl SDS sample buffer and used as the membrane fraction. The samples (boiled at 98 °C for 5 min, 15 μl per lane) and protein marker (10–170 kDa, purchased from Thermo Scientific Pierce) were separated on a 10% SDS-polycrylamide gel (60V for 30 min and then 100 V for 90 min). The proteins were later transferred to a PVDF membrane by using Trans-Blot S.D. Semi-Dry Transfer Cell (20V for 65 min). After incubation with EE-tag antibody (1:1000; Abcam®) overnight at 4 °C and followed HRP-conjugated secondary antibody, the membrane was visualized with ECL solution (Pierce), overnight at 4 °C and followed HRP-conjugated secondary antibody, and the signals were recorded by using an ImageQuant LAS 4000 mini (GE Healthcare) instrument.

Homology Modeling—A homology model of HaFaNaC was created based on the cASIC1 structure (Protein Data Bank entry 2QTS, 1.9 Å) using Modeller 9.9 as previously described (26, 28). The sequences of HaFaNaC and cASIC1 were retrieved from the GenBank™ (GenBank ID: cASIC1, 63054900, HaFaNaC, 11495511). The alignments of the target sequences were made by Modeller 9.9 and manually adjusted to match published alignments (10). The constructed model was checked and validated by ProCheck (29).

Statistics and Measurements—Data are represented as means ± S.E. Unless otherwise noted, experiments were performed on at least four different cells. Statistical significance between different groups was determined using the Student’s t test, where p < 0.05 (*) or p < 0.01 (**) was considered significant. The data were fit to the Hill equation \( I/I_{\text{max}} = 1/[1+(EC_{50}/[FMRFamide])^nH] \), where \( I \) is the normalized current at a given concentration of FMRFamide, \( I_{\text{max}} \) is the maximum normalized current, \( nH \) is the Hill coefficient. Quantitative analyses of the Western blot results were performed by using Quantity One-4.6.2.

Results

Residues in the Putative Upper Finger Domain Determine the Peptide Recognition of HaFaNaC Channels—Previous studies suggested that residues located near the first transmembrane domain (TM) and the extracellular domain, numbered 89–211, are responsible for different EC_{50} values of FMRFamide between HaFaNaC and HtFaNaC, although the detailed sites remain unclear (24, 25). Sequence alignments and homology models of protein structures suggest that the regions exhibiting high variation in amino acid sequences participate in the formation of the finger domain (Figs. 1C and 2A, unconserved residues are highlighted in blue). Assuming that residues 89–211 contribute to the different FMRFamide affinity between HaFaNaC and HtFaNaC (24, 25), we constructed a series of chimeras by substitution of the sequences of HaFaNaC with corresponding sequences on the HtFaNaC, resulting in 13 chimeras, namely chimera 1 (Ch1, Gln-110–Ile-116), HaFaNaC numbering, similarly hereinafter), chimera 2 (Ch2, Leu-124–Thr-126), chimera 3 (Ch3, Arg-128–Tyr-131), chimera 4 (Ch4, Phe-132–Asn-134), chimera 5 (Ch5, Ser-136–Asn-138), chimera 6 (Ch6, Leu-139–Thr141), chimera 7 (Ch7, Arg-144–Arg-150), chimera 8 (Ch8, D154QK), chimera 9 (Ch9, Asn-158–Ile-160), chimera 10 (Ch10, Gln-169–Leu-174), chimera 11 (Ch11, Asn-177–Met-183), chimera 12 (Ch12, Leu191–Val-194) and chimera 13 (Ch13, Ser-198–Phe-200) (Fig. 2A).

The wild-type (WT) HaFaNaC channel had a saturated concentration and half maximum effective concentration (EC_{50}) for FMRFamide of ~100 μM and ~7 μM (EC_{50} = 6.46 ± 0.15 μM, n = 8, nH = 1.04 ± 0.25, Fig. 2, B and C), respectively. Accordingly, we calculated the ratio of currents (I_{50}/I_{100} μM) induced by 7 μM and 100 μM, to initially simplify the screening of the influence of various mutants or chimeras on the FMRFamide apparent affinity. We then selected mutants with ratio values significantly lower than 0.5 (Fig. 2D), which reflected a significantly decreased affinity rendered by chimera mutants, for the following dose-dependent curve measurements. The chimera mutants Ch3, Ch4, Ch8, and Ch9 decreased the I_{50}/I_{100} μM ratio of currents (Fig. 2D), suggesting that these mutations interrupt FMRFamide recognition of HaFaNaC channels. Whereas the I_{50}/I_{100} μM ratios of chimera mutants Ch1, Ch2, Ch5, Ch7, Ch10, Ch11, Ch12, and Ch13 were comparable with or higher than those of the WT HaFaNaC channels. As revealed by the dose-response curves of the WT HaFaNaC channel (EC_{50} = 6.46 ± 0.15 μM, n = 8, nH = 1.04 ± 0.25) and those of the chimera mutants with altered I_{50}/I_{100} μM values, residues in Ch3 (Arg-128–Tyr-131, EC_{50} = 207 ± 40 μM, n = 5, nH = 1.18 ± 0.06), Ch4 (Phe-132–Asn-134, EC_{50} = 27.3 ± 1.7 μM, n = 4, nH = 1.15 ± 0.07), Ch8 (D154QK, EC_{50} = 91.7 ± 10.6 μM, n = 4, nH = 1.31 ± 0.26), and Ch9 (Asn-158–Ile-160, EC_{50} = 147 ± 19 μM, n = 10, nH = 1.28 ± 0.09) are essential for the peptide recognition of the HaFaNaC channel.

Further studies on single amino acid substitution of Ch3, Ch4, and Ch9 revealed that the mutations Y131Q (in Ch3), N134T (in Ch4), and I160F (in Ch9) significantly decreased the FMRFamide apparent affinity, where the EC_{50} values of the mutants were 53.2 ± 13.9 μM (n = 6, nH = 1.39 ± 0.10), 18.5 ± 3.27 μM (n = 5, nH = 1.22 ± 0.21), and 61.8 ± 10.6 μM (n = 4, nH = 1.29 ± 0.17), respectively (Fig. 2C), suggesting the roles of those amino acids in FMRFamide recognition. Because Ch8 (D154QK) could also decrease the apparent affinity of FMRFamide by 13-fold, we examined the affinity of the single-residue mutants D154Q (23.3 ± 1.7 μM, n = 4, nH = 1.40 ± 0.10) and D154K (84.7 ± 5.4 μM, n = 4, nH = 0.92 ± 0.15). Both significantly
reducing the channel apparent affinity to peptides, suggesting that Asp-154 also participates in the recognition of FMRFamide.

When examining the homology model of HaFaNaC and the location of corresponding residues of the above mutations in the structure of cASIC1a, it is revealed that Tyr-131 (in Ch3) and Asn-134 (in Ch4) reside at a putative H9 helix, while Asp-154 (Ch8) and Ile-160 (Ch9) are located on a putative H2 helix (Figs. 1, A and C and 2A), both of which belong to the upper finger domain (Fig. 1, B and C). In contrast, Ch10, residing at a H3 helix (lower finger domain), had no effect to FMRFamide sensing. Thus, similar to ASIC channels, the finger domain is one of the agonist-recognition sites. Nevertheless, the topmost proton recognition site of ASICs is composed of the lower finger domain (H3 helix, Fig. 1B), the β-ball, and the upper thumb domain (10). Therefore, the structure required for ligand-binding in HaFaNaC may not be exactly the same as that in ASICs, since its ligand-sensing region is located far away from the channel gate (see below).

**The HaFaNaC Specific-Insertion Motif I Between the Putative α-Helices α4 and α5 Is Involved in FMRFamide Recognition**

Given that FMRFamide can only activate peptide-gated channels rather than the other members of the ENaC/DEG superfamily, we therefore speculate that some FaNaC-specific sequences may be related to FMRFamide recognition, besides residues in the upper finger domain mentioned above. By sequence alignments, we observed a HaFaNaC specific-sequence insertion, which is absent in other ENaC/DEG members, that is located in the H4-H5 linker of thumb domain (Figs. 1, A and C and 3A, indicated in green). Interestingly, this HaFaNaC-specific sequence is adjacent to the finger domain identified above (Figs. 1C and 6A, see below). No detectable current was evoked in the cells expressing truncated HaFaNaC/Lys-357–Ala-379, even when 1 mM FMRFamide was applied (Fig. 3B). The Western blot resulted with similar expression levels in plasma membrane and in the cytosol between WT and mutant channels (Fig. 3, D and E), suggesting that the dysfunction of the truncated channel is not due to its deficiency of membrane trafficking. The deletion of residues 357–379 may impair channel function by interrupting the FMRFamide recognition of HaFaNaC, the channel gating, or the channel assembling. Given the fact that the surface expression of the truncated channel was not affected, protein assembly is unlikely to be responsible for the dysfunction of the mutant channel.
To further address this question, we made additional truncation mutants within the sequence Lys-357 to Ala-379 and obtained more truncated mutants. Among these mutants, Δ357–361 (I_{7uM}/I_{100uM} = 0.08 ± 0.02) and Δ362–366 (I_{7uM}/I_{100uM} = 0.22 ± 0.07) altered the I_{7uM}/I_{100uM} ratio of HaFaNaC channel. While Δ367–371 (I_{7uM}/I_{100uM} = 0.60 ± 0.09), Δ372–375 (I_{7uM}/I_{100uM} = 0.58 ± 0.05), and Δ376–379 (I_{7uM}/I_{100uM} = 0.44 ± 0.06) had much smaller impacts on the I_{7uM}/I_{100uM} ratio. Single amino acid truncations were then made only in sequences of 357–361 and 362–366. Among which, ΔTrp-359 (20.4 ± 2.30 μM, n = 6, nH = 2.58 ± 0.15), ΔGln-360 (11.7 ± 0.89 μM, n = 5, nH = 5.07 ± 1.31), ΔGlu-361 (10.15 ± 0.70 μM, n = 4, nH = 2.65 ± 0.36), and ΔIle-362 (18.7 ± 0.37 μM, n = 4, nH = 1.62 ± 0.03) led to rightward shifts of the dose-response curves compared with the WT HaFaNaC channel (Fig. 3F), indicating the role of those residues in FMRFamide recognition. Nevertheless, besides its role in making up the FMRFamide recognition site, it cannot be fully excluded that the insertion motif Lys-357 to Ala-379 may also participate in channel assembly and conformational transition from finger to thumb domains and then to the TM domain during the channel gating process.

Deletion of the HaFaNaC Specific-Insertion II Between Putative α-Helices α6 and α7 Significantly Affects the Recognition of FMRFamide—By sequence alignments, we identified another HaFaNaC specific-insertion motif located at a putative α6-α7 linker of knuckle domain (indicated in orange) from Gln-445 to Ala-493, which is absent from other ENaC/DEG members (Figs. 1, A and C and 4A). It is speculated that this HaFaNaC specific-insertion sequence is spatially adjacent with the recognition site in the upper finger domain identified above (see below). The apparent FMRFamide affinity of the truncated mutant ΔGln-445–493 (EC_{50} = 579 ± 70 μM, n = 8, nH = 1.65 ± 0.23) decreased profoundly (~80-fold decreased compared with WT) (Fig. 4, B and E). By examining mutants with deletions within the sequence from Gln-445 to Ala-493, we found that Δ485–493 is capable of altering the apparent affinity of HaFaNaC (I_{7uM}/I_{100uM} = 0.25 ± 0.03) while truncations Δ445–454, Δ455–464, Δ465–474, and Δ475–484 had no detectable effects (Fig. 4C, I_{7uM}/I_{100uM} = 0.72 ± 0.04, 0.51 ± 0.02, 0.85 ± 0.12 and 0.72 ± 0.07, respectively). Further alanine screening analysis indicated that E489A is able to decrease the apparent affinity of HaFaNaC (EC_{50} = 15.1 ± 0.95 μM, n = 8, nH = 2.12 ± 0.41, Fig. 4, C and E), suggesting the implication of residue Glu-489 in recognition for FMRFamide. Since the change in EC_{50} induced by E489A is far less than that of Δ485–493 (Fig. 4E), segment Gln-445 to Ala-493 may assist the formation of an FMRFamide recognition site as an entity, in which the role of single residue is not crucial.

Meanwhile, induced by saturated FMRFamide, the maximum current of Δ445–493 induced by saturated FMRF-amide (1 mM) is much lower than that of the WT HaFaNaC channel (I_{max, WT} ΔGln-445–493 = 10.8 ± 3.3 pA/pF, versus 232 ± 39 pA/pF; ΔGln-445–493 versus WT, p < 0.01, n = 4–13, Figs. 2B and 4B). Yet there is no apparent difference in intracellular and surface expression levels between Δ445–493 and the
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WT channel (Figs. 4D and 3E), indicating that the Gln-445 to Ala-493 segment not only participates in the recognition of peptides, but also in the channel gating of FaNaC channels.

Covalent Modification of Key Residues Significantly Reduces the Apparent Peptide Affinity of HaFaNaC Channels—To further investigate the roles of the putative upper finger domain, the α4-α5 linker of the upper thumb domain and the α6-α7 linker of the upper knuckle domain in FMRFamide recognition, key residues in those regions were substituted with cysteine, namely I160C, Q360C, and E489C. The effect of covalent modification on binding affinity was examined by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), whose thiobis (2-nitrobenzoic acid) (TNB) group is covalently linked with free cysteine by a disulfide bond (Fig. 5A). The electronegative TNB group would interact with the positively charged arginine of FMRFamide, therefore altering the affinity between FMRFamide and the mutated channel.

Indeed, after the application of DTNB (1 mM) to I160C, 100 μM FMRFamide hardly evoked a detectable inward current of the channel. Only a small current peak was detectable when the concentration of FMRFamide was increased to 1 mM. Even a 5 mM concentration of FMRFamide could not evoke half of the current induced by 100 μM FMRFamide before the modification by DTNB, reflecting a significant decrease in the affinity of the channel with FMRFamide (Fig. 5, F and G). The EC₅₀ of 1160C increased from 34.0 ± 6.93 μM (nH = 1.11 ± 0.12, n = 7) to ~5 mM after the modification by DTNB (Fig. 5F). Application of dithiothreitol (DTT, 10 mM), a reducing reagent, restored the currents to the comparable amplitude of those before DTNB application (Fig. 5B), suggesting that covalent modification may have interrupted the ligand recognition. Similar tests were done on mutants Q360C and E489C, yielding profoundly decreased FMRFamide currents (Fig. 5G), which can be restored by DTT application (Fig. 5, C and D). In contrast, DTNB (1 mM) application has no effect on FMRFamide-induced currents of the WT HaFaNaC channel (Fig. 5, E and G). Taken together, these results indicate that all three domains, the upper finger, the insertion between α4 and α5, and the insertion between α6 and α7 are involved in FMRFamide recognition of the channel. However, the roles of the two inserts are not as important as those of the upper finger domain, reflected by both mutation scanning and covalent modification data.

**FIGURE 3. Mutations in HaFaNaC specific-insertion I between α-helix α4 and α5.** A, sequence alignment of HaFaNaC, ASICs, and ENaC at HaFaNaC-specific insertion I and adjacent residues. B, deletion of 357–379 fully abolished FMRFamide-induced currents. C, pooled data showing the ratio of 7 μM and 100 μM FMRFamide-induced currents of the mutants in HaFaNaC specific-insertion I. Data are means ± S.E. (n = 4). ***, p < 0.01 versus WT, Student’s t test. D, representative images of Western blots showing the total and cell surface expression of HaFaNaC WT channels and Δ357–379 mutant. E, quantitative analysis of total and surface protein expressions of WT and mutated HaFaNaC channels. F, dose-response curves of HaFaNaC WT and mutants in HaFaNaC specific-insertion I. Data points are means ± S.E. (n = 4–6) and fit with Hill equation.
Double Mutant Cycle Analysis Indicates a Cooperative Mechanism of Three Regions in FMRFamide-recognition of HaFaNaC Channels—Although the two specific inserts had relatively fewer effects on peptide recognition when compared with the upper finger domain, ΔGln-445–Ala-493 (α6-α7 linker in knuckle domain) could decrease channel affinity for FMRFamide by ~80 folds; the deletion or partial deletion in Lys-357 to Ala-379 (α4-α5 linker in thumb domain) caused dysfunction or decreased apparent affinity for FMRFamide. Therefore, we speculate that the three regions may work in concert with one another to mediate the FMRFamide recognition of the channel. Indeed, belonging to finger, thumb, and knuckle domains, those three regions in the cASIC1 crystal structure are spatially adjacent to each other (10) (Fig. 1B). Moreover, our HaFaNaC homology model also revealed that the proximity of the three regions (Fig. 6A) is a requisite for a cooperative mechanism in recognition.

To further test whether those regions work in concert with each other, double mutant cycle analysis was performed. First introduced into protein engineering in 1984 (32), double mutant cycle analysis was used to examine intramolecular and intermolecular interactions between proteins or a protein and its ligands (27, 33, 34). A double-mutant cycle involves a WT mutant cycle analysis was performed. First introduced into protein engineering in 1984 (32), double mutant cycle analysis was used to examine intramolecular and intermolecular interactions between proteins or a protein and its ligands (27, 33, 34). A double-mutant cycle involves a WT mutant cycle analysis was performed. First introduced into protein engineering in 1984 (32), double mutant cycle analysis was used to examine intramolecular and intermolecular interactions between proteins or a protein and its ligands (27, 33, 34). A double-mutant cycle involves a WT protein, two single mutants, and the corresponding double mutant protein. If the alteration in free energy of a double mutation is not the sum of changes in free energy of single mutations, the residues at the two positions are coupled (Fig. 6B). If ΔG5 is equal to the sum of ΔG1 and ΔG2 (Fig. 6B), it indicates that the two residues act independently. If ΔG5 is different from the sum of ΔG1 and ΔG2, it indicates that the residues are energetically coupled. We have shown that alanine substitutions of Asp-154 (EC50 = 26.5 ± 5.74 μM, n = 4, nH = 1.48 ± 0.16, Fig. 6C) and Trp-359 (EC50 = 15.1 ± 0.39 μM, n = 4, nH = 2.08 ± 0.48, Fig. 6C) decreased the FMRFamide affinity. Alanine substitutions of both residues (D154A/W359A) further reduced the FMRFamide affinity (EC50 = 102 ± 11.6 μM, n = 4, nH = 1.56 ± 0.15, Fig. 6C). In the double mutant cycle analysis, the interaction energy of Asp-154 and Trp-359 was calculated to be 0.30 kcal/mol, which is considered to be significant. Likewise, the interaction energy between the Asp-154 (upper finger domain, D154A, EC50 = 26.5 ± 5.74 μM, n = 4, nH = 1.48 ± 0.16) and Glu-489 (knuckle domain, E489A, EC50 = 45.0 ± 4.99 μM, n = 7, nH = 1.01 ± 0.35) and the Trp-359 (thump domain, W359A, EC50 = 15.1 ± 0.39 μM, n = 4, nH = 2.08 ± 0.48) pair are calculated to be -0.19 and 0.11 kcal/mol, respectively (Fig. 6, D and E), reflecting comparatively weaker interaction. Therefore, we believe that the three regions interact with each other to some extent.

Taken together, the recognition sites of FMRFamide are possibly composed of the putative upper finger domain, the α4-α5 linker in thumb domain and the α6-α7 linker in knuckle domain from a neighboring subunit, where the α4-α5 linker and the α6-α7 linker may be also implicated in channel activation and conformational transitions.

Discussion

In this study, we identified three regions, namely the putative upper finger domain, the α4-α5 linker in the upper thumb domain, and the α6-α7 linker in the upper knuckle domain that
contribute to FMRFamide recognition. Among these regions, the upper finger domain plays a predominant role, within which the mutations Y131Q, N134T, D154QK, and I160F significantly impair FMRFamide affinity. The thumb and knuckle domains also affect FMRFamide affinity, although to a much smaller extent when compared with the finger domain. These results were further verified by chemical modifications and double mutant cycle analysis. After modification with DTNB at mutation I160C in the finger domain, FMRFamide could not induce current until its concentration was increased to 1 mM, which evoked a small current. The amplitude of current evoked by 5 mM FMRFamide was not up to half of that induced by 100 μM FMRFamide before and after DTNB modification. G, quantitative analysis and significance tests on the FMRFamide-induced currents of I160C, Q360C, E489C, and WT HaFaNaC channels before and after modification by DTNB. *, p < 0.05; **, p < 0.01 versus WT, Student’s t test.

Among the ENaC/DEG superfamily members, the finger domain exhibits the highest diversity (the lowest sequence identity across the ENaC/DEG superfamily, 8.2%) in structure and is the most important domain for sensing extracellular stimuli. For example, mutations within the finger domain of DEG-1 and UNC-8 induced channel superactivation (35, 36). In MEC-4, mutations within the finger domain lead to a disruption of mechanosensation ability in Caenorhabditis elegans (37). In ASIC channels, the lower finger domain associated with the thumb and β-ball forms an acidic pocket, acting as one of the proton sensors (10). In ENaCs, the finger domain has been functionally linked to both shear stress sensitivity (38) and proteolytic regulation (39). The thumb domain is made up of two α-helices, α4 and α5, and several structured loops. Five highly conserved disulfide bridges stabilize the architecture into a rigid rod to facilitate transferring gating motions from the ectodomain to the TM domain. This thumb-dependent gating transmission has been validated by both x-ray structure determination and functional data (10, 40, 41). Both belonging to the ENaC/DEG superfamily, HaFaNaC and ASIC1 may share certain similarities in three-dimensional architectures. In response to the binding of FMRFamide to its recognition sites, the rigid thumb is pulled toward the finger domain, causing the confor-
mational transition to the TM domain and consequently opening the HaFaNaC channels. The activation mechanism is similar to the acidosis-induced channels activation process of ASICs (10). Therefore, the thumb domain may play a significant role in transiting conformational changes after FMRFamide binding. Previous studies suggest that RF-peptides bind inside the pocket of ASIC1a with one end connected to the thumb and finger of a subunit and the other end connected to the knuckle and β-ball of another subunit (42). However, the structural binding mode is based on molecular docking and requires further experimental evidence for verification. A recent study has shown that FMRFamide may stimulate the lower palm domain of ASIC1a and modulate sustained current of the channel (43). Our data suggest that the FMRFamide recognition site may be composed of an upper finger domain and an α4-α5 linker in thumb domain and an α6-α7 linker in the knuckle domain from a neighboring subunit. This may be distinct from the FMRFamide recognition and modulation mechanism of ASICs. In our model, the upper finger domain is a predominant region in FMRFamide recognition, and the two linker regions, the α4-α5 linker in thumb domain and the α6-α7 linker in knuckle domain, exert their roles by maintaining the architecture of the FMRFamide recognition site, rather than by direct contact with FMRFamide. Meanwhile, given the fact that deletions within regions dramatically reduced the maximal response to FMRFamide without apparent changes in surface expression of channels, those two domains, especially the upper thumb domain, may be crucial structural components for the conformational transition from extracellular to TM domains.

Recently, the cASIC1 crystal structure was used as a template to build homology models of ENaC/DEG members, such as ENaC (44–47) and FaNaC (48, 49). The overall sequence identity between the hENaC and cASIC1 is 18.2%, lower than that between HaFaNaC and cASIC1 (18.8%). For the thumb and the palm domain, the sequence identities between the HaFaNaC and cASIC1 are 17.9% and 26.6%, respectively, which may result in a similar architecture with cASIC1. In addition, the HG motif, GXXXG motif, GXS motif, and seven disulfide bonds (indicated in purple in Fig. 1A) are completely conserved among the entire ENaC/DEG superfamily, which are pivotal in maintaining similar architecture among the ENaC/DEG members. Hence, we created a homology model of HaFaNaC based on the cASIC1 structure (Protein Data Bank entry 2QTS, 1.9 Å). The FMRFamide recognition site of HaFaNaC is higher than the topmost proton binding site of the acidic pocket in ASICs, located farther from the TM pore than that of ASICs (Fig. 7, B and C). The difference in ligand recognition sites between HaFaNaC and ASICs, to some extent, may reflect differences in the evolutionary relationship of them. Found in mollusks, HaFaNaC is relatively more primitive than ASICs, with a lower efficiency in its ligand-gating mechanism. In contrast, identified in chordata, ASICs appeared relatively late in evolution with a more advanced and efficient gating mechanism. The ligand binding site is much closer to the ion channel pore in the TM within ASICs, while the other two proton binding sites are located in lower palm domain and wrist domain, which are closer to the channel TM pore (10, 12, 50). The small molecular ligand GMQ can activate some members of ASICs through its binding sites within lower palm domain (6, 50).
Among the three domains required for the recognition of FMRFamide, the two in the thumb and the knuckle domains are HaFaNaC-specific and absent in ASICs (Fig. 1A), which may reflect their status in evolution. It is possible that due to the loss of these specific amino acids, ASICs can only be modulated by FMRFamide rather than being activated. Similar relevance in structure and channel gating was observed in the DEG channel, which was found in nematodes and was involved in mechanoreception. As nematodes appeared earlier than chordates in evolution, the DEG channel contains two conserved DEG-specific domains, namely the cysteine-rich domain (CRDI) and the extracellular regulatory domain (ERD), within its finger domain, and both domains are absent in ASICs (35). Moreover, the positions of DEG-specific insertions are also above the acidic pocket mapped in ASIC and are implicated in sensing mechanical stimuli or regulating the activity of DEG (Fig. 7A). The evolutionary development of the ENaC/DEG superfamily channels faithfully reflects the general law of organism evolution. Firstly, as in forms of activation, the primitive members in evolution, such as HyNaC, FaNaC, and DEG, can only be activated with one factor. For example, HyNaC and FaNaC can be activated by Hydra-RFamide and FMRFamide respectively, while DEG can only sense the mechanical stimulus. In contrast, members that developed later such as ASICs and ENaC are sensitive to diverse stimuli. ASICs can be activated by a proton (51), the small molecular activator GMQ (6), a toxin (8, 9), or deprivation of calcium (52), while ENaCs are constitutively active or can be activated by the small molecular ligand S3969 (5). Accordingly, we propose that an RF-amide like ligand-gating mechanism has been inherited to ENaC/DEG superfamily members from their common ancestor. However, the ligand-gating mechanism of ENaC/DEG remains poorly understood. So far, gating mechanisms of ASICs have been studied thoroughly. There is more than one proton binding site located in different domains of ASICs, resulting in a non-classical ligand-gating mechanism. In contrast, GMQ binding sites are mainly located in the lower palm domain of ASIC (6, 50), similar to hENaC, of which the binding site for the small molecule S3969 is located in the lower palm domain residue Val-348 (5). Our results demonstrate that FMRFamide recognition sites may consist of an upper finger domain, an upper thumb domain, and an upper knuckle domain (Fig. 7C), which is distinct from the GMQ binding site within ASICs as well as the S3969 binding site within ENaC (5, 6). These reflected diverse and complicated gating mechanisms of ENaC/DEG members in response to tissue acidosis, mechanical stimuli, and ligand binding. Our study may gain insights into the characteristics of ligand-gating, the regulation of the gating process, and the evolutionary relationship of ENaC/DEG ion channels. Moreover, it would also provide insight for exploring new activating models of the ENaC/DEG superfamily and lay a molecular mechanism foundation for further ASIC-targeted and ENaC-targeted drug development and disease treatments.

**Author Contributions**—Y. Y. designed the project; Y.-Y. N., Y. Y., and X.-N. Y. performed cell culture, patch-clamp recordings and Western blots; Y.-Y. N., L.-D. H., Y. Y., X.-N. Y., and Y. L. did mutations; Y. Y., Y.-Z. F., and P. C. did protein homology modeling; Y.-Y. N., Y. Y., and Y.-M. H. analyzed data; X.-Y. L. and Y. T. supervised Y.-Y. N. and X.-N. Y.; Y. Y., Y.-Y. N., Y. Y., X.-C., and L.-Y. L. wrote the manuscript. All authors discussed the results and commented on the manuscript.
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**Peptide Recognition of FaNaC**