A Peptide-gated Ion Channel from the Freshwater Polyp Hydra*

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Chemical transmitters are either low molecular weight molecules or neuropeptides. As a general rule, neuropeptides activate only slow metabolic receptors. To date, only one exception to this rule is known, the FMRFamide-activated Na⁺ channel (FaNaC) from snails. Until now FaNaC has been regarded as a curiosity, and it was not known whether peptidogated ionotropic receptors are also present in other animal groups. Nervous systems first evolved in cnidarians, which extensively use neuropeptides. Here we report cloning from the freshwater cnidarian Hydra of a novel ion channel (Hydra sodium channel, HyNaC) that is directly gated by the neuropeptides Hydra-RFamides I and II and is related to FaNaC. The cells expressing HyNaC localize to the base of the tentacles, adjacent to the neurons producing the Hydra-RFamides, suggesting that the peptides are the natural ligands for this channel. Our results suggest that neuropeptides were already used for fast transmission in ancient nervous systems.

The DEG/ENaC gene family comprises ion channels with various functions and diverse gating mechanisms (1, 2): the epithelial sodium channel (ENaC) is a constitutively open channel, acid-sensing ion channels (ASICs) are gated by extracellular H⁺, and MEC/degenerin (DEG) channels are mechanically activated channels. The function of many other family members, like the intestinal sodium channel (INaC) (3), is unknown. Arguably the most curious member of this gene family is FaNaC from snails, the only known peptide-gated ion channel, gated by the peptide neurotransmitter Phe-Met-Arg-Phe-NH₂ (FMRFamide) (4, 5). Our study was driven by an interest in defining the original properties and gating mechanism of the primitive ancestor of this gene family. BLAST homology searches revealed that the sequenced genomes of bacteria, yeast, and unicellular eukaryotes do not contain genes for DEG/ENaC channels. Thus, it seemed that this gene family evolved later in evolution, perhaps first in multicellular animals, suggesting that the primitive ancestor had a role in intercellular communication.

Hydra belongs to the phylum Cnidaria that is characterized by a radial symmetry and a primitive nervous system that extensively uses neuropeptides for transmission (6). Because all other DEG/ENaC family members characterized to date are from animals with a bilateral symmetry, features common to channels from Hydra and other family members are likely to be primitive features of an ancestral channel that was present in metazoan animals that lived 600–700 million years ago and that were at the base of the Radiata-Bilateria dichotomy. Here we report cloning and characterization of members of the DEG/ENaC gene family from Hydra magnipapillata, revealing a novel peptide-gated ion channel. The presence of related peptide-gated ion channels in Bilateria and Radiata shows that such channels have been present in organisms in which nervous systems first evolved.

EXPERIMENTAL PROCEDURES

Cloning of HyNaC cDNAs—Several partial sequences for proteins showing sequence homology to channels of the DEG/ENaC gene family were identified from the on-line Hydra Expressed Sequence Tag data base and used to design primers for rapid amplification of 5’- and 3’-cDNA ends (RACE). RACE was performed with cDNA prepared from poly(A) + RNA, isolated from adult one-day starved budding stage H. magnipapillata (strain 105). This strain was a kind gift from Dr. T. Fujisawa (National Institute of Genetics, Mishima, Japan). It was cultured as described previously (7). Full-length HyNaCs were assembled from the 5’- and 3’-RACE products. These sequence data have been submitted to the DDBJ/EMBL/GenBank™ databases under accession Nos. AM393879 (HyNaC1), AM393878 (HyNaC2), AM393880 (HyNaC3), and AM393881 (HyNaC4).

The cDNA for HyNaC1 did not contain a methionine to initiate protein translation upstream of the predicted first transmembrane domain, but it did contain a stop codon there. Moreover, a highly conserved HG motif that is essential for
The gating of these channels (8) was missing. The absence of an initiator methionine was confirmed by expressed sequence tags from the public database. Therefore, we conclude that hynac1 is an inactive pseudogene.

Analysis of Phylogenetic Relationship—Amino acid sequences of members of the DEG/ENaC gene family were aligned using ClustalX; highly divergent sequences at the N and C termini and in the proximal part of the extracellular loop had been deleted. Phylogenetic trees were constructed by neighbor-joining and parsimony analysis with the program package PAUP*4.0 and by maximum likelihood analysis with the program TREE-PUZZLE.

In Situ Hybridization—Fragments of HyNaC cDNAs, 770-090 bp in length, were subcloned in the vector pBluescript KS. Whole mount in situ hybridization was carried out as previously described (9) by using BMP Purple as substrate for the antibody-conjugated alkaline phosphatase. Three overlapping probes were used for the detection of each transcript at a total probe concentration of 0.39 ng/μl for 60 h.

Electrophysiology—For expression studies in Xenopus oocytes, the entire coding sequences of HyNaC2–4 were amplified by PCR from cDNA of whole Hydras and subcloned. Clones were entirely sequenced to exclude PCR errors. Synthesis of cRNA, maintenance of Xenopus laevis oocytes, and recordings of whole cell currents were done as previously described (10). For co-expression of HyNaC subunits, we injected equal amounts of cRNAs of the individual subunits; the total amount was 5–10 ng.

RESULTS AND DISCUSSION

Cloning of HyNaCs from H. magnipapillata—We isolated four cDNAs with homology to DEG/ENaC gene family were aligned using ClustalX; highly divergent sequences at the N and C termini and in the proximal part of the extracellular loop had been deleted. Phylogenetic trees were constructed by neighbor-joining and parsimony analysis with the program package PAUP*4.0 and by maximum likelihood analysis with the program TREE-PUZZLE.

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A Peptide-gated Ion Channel from Hydra

loop containing 12 conserved cysteines between the two hydrophobic domains (Fig. 1). For FaNaC, four subunits assemble into the functional channel (11). Three methods were applied to reveal the phylogenetic relationship of HyNaCs to other DEG/ENaC channels: neighbor-joining, parsimony analysis, and maximum likelihood analysis. All three methods consistently placed HyNaCs on a common branch with ASICs and INaC. The tree obtained with maximum likelihood analysis is shown in Fig. 2. The close relationship of HyNaCs to vertebrate genes is in line with a general trend that vertebrate genomes have diverged much less from the bilaterian ancestor than have the genomes of Drosophila and Caenorhabditis elegans (12). This implies that the sequence of the primordial DEG/ENaC gene was similar to HyNaCs/ASICs/INaC. The predicted

![Diagram of phylogenetic tree showing relationships between different ion channels and gene families.](image-url)
Hydra neurons are known to make synaptic junctions with epitheliomuscular cells and have dense cored vesicles that contain Hydra-RFamides (16, 17). Although the precise location of HyNaCs is unknown, the above observations suggest that HyNaCs are expressed at the basolateral face of epitheliomuscular cells and that their activation by neuronally released Hydra-RFamides leads to tentacle contractions, possibly during feeding behavior of the animals.

**Functional Expression of HyNaCs**—We investigated the functional properties of HyNaCs by expression in *Xenopus* oocytes; cRNA coding for HyNaC2–4 were injected in oocytes either alone or in combination. Expression of HyNaCs did not significantly increase the leak current; rapidly raising the extracellular H^+ concentrations also did not activate the channels (not shown). Thus, HyNaCs do not form constitutively active channels like ENaC or H^+-ated channels like ASICs. We then tested activation by Hydra-RFamides (18). Rapid application of the structurally similar Hydra-RFamides I or II induced a robust (1–10 μA) inward current in oocytes co-expressing HyNaC2 and HyNaC3 (Fig. 4A). Hydra-RFamides, however, never induced a current in uninjected control oocytes, in oocytes expressing the two subunits individually, or in oocytes expressing other subunit combinations; Hydra-RFamides III and IV never induced any current. Thus, HyNaC2 and 3 form a heteromeric channel gated by Hydra-RFamides I and II. Expressing HyNaC4 in addition to HyNaC2 and 3 did not change the characteristics of the current; the inclusion of HyNaC4 in a heteromeric channel can therefore not be excluded but is not necessary for the formation of the active channel. The effect of Hydra-RFamides I and II was concentration-dependent (Fig. 4B); half-maximal activation was obtained with 34 ± 9 μM (mean ± S.E.; five oocytes) Hydra-RFamide I and 28 ± 4 μM (eight oocytes) Hydra-RFamide II. Thus, the apparent affinity of Hydra-RFamides to their receptor was similar to the affinity of FMRFamide to FaNaC (5, 19) and of low molecular weight ligands to their ionotropic receptors. HyNaC2/3 channels incompletely desensitized (Fig. 4A), similar to FaNaC (5). Repeated application of the peptide induced currents with decreasing amplitudes (Fig. 4C); such a tachyphylaxis is also known from ASIC1a (20).

Currents induced by application of Hydra-RFamide II rose rapidly with a time constant of 170 ms (±50 ms, five oocytes). We estimated the speed of our solution exchange system with
A Peptide-gated Ion Channel from Hydra

A. Top, sequences of the Hydra-RFamide neuropeptides; pQ represents a pyro-Glu. Identical amino acids are highlighted with shaded boxes. Bottom, currents elicited by Hydra-RFamides in Xenopus oocytes injected with HyNaC2 and 3. B, concentration response curve for Hydra-RFamides I and II. C, repeated activation of HyNaC2/3 with Hydra-RFamides led to decreased response; in the example shown, 50 μM Hydra-RFamide I was used. D, replacing Na⁺ by Li⁺ or K⁺ in the bath did not significantly change the current amplitude. Error bars represent S.E. (six oocytes). The composition of the bath solution was as in D. F, concentration response curve for the inhibition of the HyNaC current by amiloride. Results are from seven oocytes, except for the data point at 5 mM, which is from three oocytes. G, top, sequence alignment of the second putative transmembrane domain of HyNaC2 and 3 and of rat ASIC1a and αENaC; the position of the glycine exchanged by a serine in HyNaC3 is indicated by an arrow. Bottom, co-expressing wild-type HyNaC2 with mutant HyNaC3 G444S significantly (p < 0.01; t test) reduced the relative amplitude of the K⁺ current compared with wild-type HyNaC3 (left). Error bars represent S.E. (twelve oocytes). The reversal potential was shifted by ~20 mV to more positive potentials (right); the curves shown have been obtained by subtracting the current before peptide application from the current during peptide application (50 μM Hydra-RFamide I). The composition of the bath solution was as follows (in mM): 140 NaCl (or LiCl or KCl), 0.1 flufenamic acid, 10 HEPES.

FIGURE 4. Electrophysiological characterization of the HyNaC2/3 heteromer. A, top, sequences of the Hydra-RFamide neuropeptides; pQ represents a pyro-Glu. Identical amino acids are highlighted with shaded boxes. Bottom, currents elicited by Hydra-RFamides in Xenopus oocytes injected with HyNaC2 and 3. B, concentration response curve for Hydra-RFamides I and II. C, repeated activation of HyNaC2/3 with Hydra-RFamides led to decreased response; in the example shown, 50 μM Hydra-RFamide I was used. D, replacing Na⁺ by Li⁺ or K⁺ in the bath did not significantly change the current amplitude. Error bars represent S.E. (six oocytes). The composition of the bath solution was as in D. F, concentration response curve for the inhibition of the HyNaC current by amiloride. Results are from seven oocytes, except for the data point at 5 mM, which is from three oocytes. G, top, sequence alignment of the second putative transmembrane domain of HyNaC2 and 3 and of rat ASIC1a and αENaC; the position of the glycine exchanged by a serine in HyNaC3 is indicated by an arrow. Bottom, co-expressing wild-type HyNaC2 with mutant HyNaC3 G444S significantly (p < 0.01; t test) reduced the relative amplitude of the K⁺ current compared with wild-type HyNaC3 (left). Error bars represent S.E. (twelve oocytes). The reversal potential was shifted by ~20 mV to more positive potentials (right); the curves shown have been obtained by subtracting the current before peptide application from the current during peptide application (50 μM Hydra-RFamide I). The composition of the bath solution was as follows (in mM): 140 NaCl (or LiCl or KCl), 0.1 flufenamic acid, 10 HEPES.
the tree (Fig. 2), suggesting that peptide gating is a primitive feature of these channels. The close relation of mammalian ASICs and INaC to HyNaCs then makes these channels candidates for channels directly gated by neuropeptides; the best candidates are the channels with unknown activation mechanism, ASIC4 (23) and INaC (3). Hydra-RFamides did not induce currents in oocytes expressing ASICs or INaC, however, showing that these peptides cannot directly activate the mammalian channels (not shown). FMRFamide also does not directly activate ASICs; pre-application of FMRFamide, however, modulates H⁺-gated ASIC currents by direct binding to the channel (24). This was also the case for Hydra-RFamides; preapplication of Hydra-RFamides I-IV (50 μM) significantly slowed desensitization of ASIC3 currents (Fig. 5), suggesting that ASIC3 retained a binding site for neuropeptide transmitters that are separated by more than 500 million years of evolution. Because Hydra-RFamides III and IV most strongly modulated ASIC3 currents (Fig. 5) the receptor for these two peptides may also belong to the DEG/ENaC gene family. Peptide modulation of ASIC currents reinforces the speculation that more peptide-gated channels may be hidden within the DEG/ENaC gene family and may contribute to synaptic transmission also in vertebrates.

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FIGURE 5. Hydra-RFamides modulate ASIC3 currents. Pre-application of Hydra-RFamides (50 μM) slowed desensitization of ASIC3 activated with pH 5.5. One example representative for seven different measurements is shown.