THREE HOMOLOGOUS SUBUNITS FORM A HIGH-AFFINITY PEPTIDE-GATED ION CHANNEL IN HYDRA

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Running title: Subunit composition of a peptide-gated channel

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Recently, three ion channel subunits of the degenerin/epithelial Na⁺ channel (DEG/ENaC) gene family have been cloned from the freshwater polyp Hydra magnipapillata, the Hydra Na⁺ channels (HyNaCs) 2 - 4. Two of them, HyNaC2 and HyNaC3, co-assemble to form an ion channel that is gated by the neuropeptides Hydra-RFamides I and II. The HyNaC2/3 channel is so far the only cloned ionotropic receptor from cnidarians and, together with the related ionotropic receptor FMRFamide-activated Na⁺ channel (FaNaC) from snails, the only known peptide-gated ionotropic receptor. The HyNaC2/3 channel has pore properties, like a low Na⁺ selectivity and a low amiloride affinity, that are different from other channels of the DEG/ENaC gene family, suggesting that a component of the native Hydra channel might still be lacking. Here we report the cloning of a new ion channel subunit from Hydra, HyNaC5. The new subunit is closely related to HyNaC2 and 3 and co-localizes with HyNaC2 and 3 to the base of the tentacles. Co-expression in Xenopus oocytes of HyNaC5 with HyNaC2 and 3 largely increases current amplitude after peptide stimulation and affinity of the channel to Hydra-RFamides I and II. Moreover, the HyNaC2/3/5 channel has altered pore properties and amiloride affinity, more similar to other DEG/ENaC channels. Collectively, our results suggest that the three homologous subunits HyNaC2, 3, and 5 form a peptide-gated ion channel in Hydra that could contribute to fast synaptic transmission.

The DEG/ENaC gene family contains ion channels that have a high selectivity for Na⁺ ions and a rather high affinity for the diuretic amiloride (apparent IC₅₀ ~0.1 – 20 µM). In contrast to these conserved pore properties, activating stimuli and physiological functions of DEG/ENaC channels are strikingly diverse (1): family members from Drosophila are involved in the control of locomotion (2), the liquid clearance from the trachea (3), and in the detection of pheromones (4) and salt (5); members from C. elegans form mechanosensitive ion channels (6); FaNaC from snails is a peptide-gated ion channel (7); and the epithelial Na⁺ channel (ENaC) and acid-sensing ion channels (ASICs) family members from mammals mediate Na⁺ reabsorption (8) and are proton-gated ion channels (9), respectively. The functions of many other DEG/ENaC channels are still unknown.

Recently three DEG/ENaC subunits have been cloned from the freshwater polyp Hydra (10). These subunits, HyNaC2 - HyNaC4, were the first DEG/ENaC channels from Cnidaria, which is an ancient phylum, where the first nervous systems are supposed to have evolved. A fourth HyNaC gene, hynac1, is probably a pseudogene (10). Moreover, it was shown that HyNaC2 and HyNaC3 co-assemble to form a heteromeric ion channel gated by Hydra-RFamides I and II (10), which are neuropeptides isolated from the nervous system of Hydra (11). The mRNA of HyNaC2 and HyNaC3 was localized by in situ hybridization to the base of the tentacles (10), in close
proximity to the nerve cells that express the preprohormone gene that encodes Hydra-RFamides (12), suggesting that these neuropeptides are the natural ligand of the HyNaC2/3 channel. Although the precise location of HyNaCs is not known, it was proposed that they are expressed at the basolateral face of epitheliomuscular cells and it was speculated that release of Hydra-RFamides leads to tentacle contractions, possibly during feeding of the animals (10).

The presence of a peptide-gated channel in the primitive nervous system of a cnidarian suggests that neuropeptides were already used for fast neurotransmission in ancient nervous systems. It also indicates that peptide gating is an ancient feature of DEG/ENaC channels and that this feature has been preserved during evolution in various members of the Protostomia, such as snails, but probably been lost in the Deuterostomia, such as mammals, where other ligands have replaced the peptide ligands.

The HyNaC2/3 channel has pore properties, like a low Na\(^+\) selectivity and a low amiloride affinity (IC\(_{50}\) ~500 \(\mu\)M), which are different from other channels of the DEG/ENaC gene family (10). These results suggest either that a high Na\(^+\) selectivity and a high amiloride affinity are not ancient features of the gene family or that a component of the native Hydra channel is still lacking.

Here we report the cloning of a new ion channel subunit from *Hydra*, HyNaC5. In situ hybridization analysis and functional analysis of HyNaC5 suggest that the three homologous subunits HyNaC2, 3, and 5 form a high-affinity peptide-gated ion channel in *Hydra*. Moreover, preliminary results suggest that this channel could be involved in the coordination of the feeding reaction of *Hydra*.

**EXPERIMENTAL PROCEDURES**

*Cloning of HyNaC5* - Several partial sequences for a protein showing sequence homology to HyNaCs were identified from the online *Hydra* expressed sequence tag (EST) database (www.hydrabase.org) and used to design primers for rapid amplification of 3’-cDNA ends (RACE). Using the Smart RACE cDNA amplification kit (Clontech), two rounds of 3’-RACE were performed with cDNA prepared from polyA+ RNA, isolated from adult one-day starved budding stage *Hydra magnipapillata* (strain 105). Full-length HyNaC5 was assembled from the ESTs and the 3’ RACE products. For expression studies in *Xenopus* oocytes, the entire coding sequence of HyNaC5 was amplified by PCR from cDNA of whole *Hydras* and subcloned. The clone was entirely sequenced to exclude PCR-errors. The consensus sequence of HyNaC5 was assembled from the EST sequences and several independent PCR products. This sequence data has been submitted to the DDBJ/EMBL/GenBank databases under accession No. FN257513.

*In situ hybridization* – A 1435 bp fragment from the coding part of HyNaC5 cDNA was subcloned in the vector pBluescript KS. Whole mount ISH was carried out as previously described (13) by using BMP Purple as substrate for the antibody-conjugated alkaline phosphatase except that blocking of the animals was performed in MAB/1x blocking alkaline phosphatase and that the Alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) were diluted 1:4000. Two different antisense probes of 810 bp and 1435 bp, respectively, were used at concentrations of 0.08 – 0.16 ng/\(\mu\)l for 60 hours. Both probes applied alone or together resulted in the same localization pattern, a combination of both probes yielding a stronger signal than the single probes. Performing ISH with the corresponding sense riboprobes did not display any staining.

*Analysis of the feeding reaction of Hydra* – 5 animals of the species *Hydra magnipapillata* (wildtype strain 105) were placed in 10 ml of Hydra medium (1.0 mM Tris-HCl, 1.0 mM NaHCO\(_3\), 0.1 mM KCl, 0.1 mM MgCl\(_2\), and 1.0 mM CaCl\(_2\)) or Hydra medium supplemented with 100 \(\mu\)M amiloride. Once they were relaxed in the new medium, glutathione (GSH) was added to a final concentration of 10 \(\mu\)M, indicating time 0:00. Every 30 sec we recorded the number of animals moving their tentacles as a response to the application of GSH. The experiments were repeated 4 times. Additional experiments were performed with *Hydra oligactis* without finding any differences (data not shown).
Electrophysiology - Synthesis of cRNA, maintenance of Xenopus laevis oocytes and recordings of whole cell currents were done as previously described (14). For co-expression of HyNaC subunits, we injected equal amounts of cRNAs of the individual subunits; the total amount was 0.3 - 8 ng. In order to avoid artifacts due to tachyphylaxis, for the experiments determining the relative inward currents with different monovalent cations (Fig. 6) and different blockers (Fig. 7), we activated the HyNaC2/3/5 heteromer two times before varying the concentration of the cations or blockers, respectively. To further reduce the impact of tachyphylaxis in both experiments, the sequence of the inhibitors or cations was varied. Oocytes from at least two different frogs were used, except for determination of apparentamiloride affinity of HyNaC2/3, for which oocytes from one frog were used.

Determination of surface expression - The hemagglutinin (HA) epitope (YPYDVPDYA) of influenza virus was inserted in the extracellular loop of HyNaC2 between residues F124 and D125. HyNaC2/3/5 containing tagged HyNaC2 had similar apparent peptide affinity as HyNaC2/3/5 containing untagged HyNaC2 and current amplitude of HyNaC2/3 containing tagged HyNaC2 was more than 10-fold increased by co-expressing HyNaC5 (results not shown).

Oocytes were injected with ~3 ng cRNA of each subunit and surface expression was determined as previously described (15). Briefly, oocytes expressing HyNaCs were placed for 30 min in ND96 with 1% BSA to block unspecific binding, incubated for 60 min with 0.5 μg/ml of rat monoclonal anti-HA antibody (3F10, Roche), washed extensively with ND96/1% BSA, and incubated for 60 min with 2 μg/ml of horseradish peroxidase-coupled secondary antibody (goat anti-rat Fab fragments, Jackson ImmunoResearch). Oocytes were washed six times with ND96/1% BSA and three times with ND96 without BSA. All steps were performed on ice. Oocytes were then placed individually in wells of microplates and luminescence was quantified in a Berthold Orion II luminometer (Berthold detection systems; Pforzheim, Germany). The chemiluminescent substrates (50 μl Power Signal Elisa; Pierce) were automatically added and luminescence measured after 2 sec for 5 sec. Relative light units (RLUs)/s were calculated as a measure of surface-expressed channels. The results are from two independent experiments with oocytes from two different frogs. Eight oocytes were analyzed for each experiment and each condition.

RESULTS AND DISCUSSION

Cloning of HyNaCs from Hydra magnipapillata. - We isolated a new cDNA with high homology to HyNaCs (Fig. 1) from Hydra magnipapillata cDNA; we named the corresponding protein HyNaC5. The predicted amino acid sequence of HyNaC5 is shown together with the sequences of HyNaC2 - 4 in Fig. 1A. The degree of amino acid sequence identities between HyNaC5 and HyNaC2-4 ranges from 28 - 44 %; the closest relative is HyNaC2 (Fig. 1B). Thus, sequence identities between different HyNaCs are similar to sequence identities between the three subunits of the epithelial Na channel, ENaC (16). The open reading frame of the HyNaC5 cDNA codes for a protein of 473 amino acids with a predicted molecular mass of approximately 55 kDa. HyNaC5 shows the structural hallmarks of the DEG/ENaC gene family: two hydrophobic transmembrane domains, short N and C termini, and a large extracellular loop containing 12 conserved cysteines between the two hydrophobic domains (Fig 1A). A topology with two transmembrane domains, a large ectodomain and short intracellular termini is common to all DEG/ENaC subunits (1).

Expression pattern of hynac5 - Whole-mount in situ hybridization for hynac5 revealed expression at the base of the tentacles (Fig. 2A), most likely in epitheliomuscular cells. During budding, hynac5 is expressed at the sites of tentacle formation, immediately before the first tentacle bumps appear (Fig. 2G). Thus, the expression pattern of hynac5 is very similar to the expression patterns of hynac2 and hynac3 (10) (Fig. 2), suggesting that the 3 genes are co-expressed in Hydra cells.

A more careful comparison, however, reveals subtle differences in the expression pattern of the three genes. While hynac 2 and hynac3 are uniformly expressed at the tentacle
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base (Fig. 2B, C), hynac5 expression is strongest at the oral site of each tentacle base with a gradient towards the aboral site (Fig. 2A, Fig. 3A). By comparison, hynac4 expression exhibits a strong restriction to the aboral site of the tentacles (10) (Fig. 3B). The mutually exclusive expression patterns of hynac4 and hynac5 at the base of the tentacles suggest a different function for HyNaC4 and HyNaC5.

Previously it was speculated that activation of HyNaCs leads to tentacle contractions during the "feeding reaction" of Hydra (10). The feeding reaction is induced by physical contact with a prey animal and characterized by an upward movement and contraction of the tentacles, which brings the prey close to the mouth (17). Perhaps HyNaC4 and HyNaC5 have distinct roles in the coordination of this feeding behavior.

HyNaC5 strongly increases current amplitude of the HyNaC2/3 heteromer - We investigated the functional properties of HyNaC5 by expression in Xenopus oocytes; cRNAs coding for HyNaC2 – 5 were injected in oocytes either alone or in combination. Oocytes expressing HyNaC5 alone or in combination with one other HyNaC subunit (2, 3, or 4) could not be activated by any of the four Hydra-RFamides (I – IV; Fig. 4A). In contrast and as reported previously (10), oocytes co-expressing HyNaC2 and 3 exhibited a robust current after stimulation with Hydra-RFamide I (Fig. 4A) and II (not shown); current amplitudes were usually in the range of a few µA. Oocytes expressing HyNaC5 in addition to HyNaC2 and 3 could also be activated by RFamides I and II and showed dramatically increased current amplitudes compared to oocytes co-expressing only HyNaC2 and 3: current amplitudes of oocytes expressing HyNaC2, 3, and 5 were so large (> 50 µA) that they were above the capacity of our amplifier (Fig. 4A, left panel). A quantitative comparison with oocytes, which had been injected with 25-fold diluted RNA, and half-maximal peptide concentrations (see below) revealed a 16-fold increase in the peak current amplitude of HyNaC2/3/5 compared to HyNaC2/3 (p << 0.01; Fig. 4A, right panel), strongly suggesting that HyNaC5 was incorporated in the channel complex to form a HyNaC2/3/5 heteromer. Recently, the crystal structure of an ASIC revealed a number of three subunits in the functional channel (18). Since ASICs are close relatives of HyNaCs in the DEG/ENaC gene family (10), it is reasonable to assume that functional HyNaCs are also composed of three subunits, suggesting a 1:1:1 ratio for the HyNaC2, 3, and 5 subunits in the functional complex.

In the remainder of this study, we usually injected 25-fold diluted cRNAs of HyNaC2/3/5 in order to get current amplitudes in the range of 1 – 30 µA.

Oocytes co-expressing HyNaC5 together with HyNaC2 and 4 could also be activated by Hydra-RFamides I and II; current amplitudes were much smaller than for the HyNaC2/3/5 heteromer, however (Fig. 4A), suggesting that although HyNaC4 is closely related to HyNaC3 (Fig. 1B) it cannot efficiently replace it in the heteromeric complex.

Fig. 4B shows representative current traces of oocytes expressing HyNaC2, 3, and 5. Similar to HyNaC2/3 channels (10) and FaNaC (7), HyNaC2/3/5 heteromers desensitized incompletely: a rapidly declining peak amplitude was followed by a sustained current; the ratio between peak and sustained current was variable. A second application of the ligand induced currents with significantly smaller amplitudes than the first application. Further applications of the ligand, however, induced currents with amplitudes comparable to the second application (Fig. 4B).

HyNaC5 strongly increases the current through surface expressed HyNaCs - Increased current amplitudes of the HyNaC2/3/5 heteromer compared with the HyNaC2/3 heteromer (Fig. 4A), can be due to increased surface expression and/or increased current through surface-expressed channels. To differentiate between these possibilities, we compared the expression of the two heteromers at the oocyte surface. We inserted an HA epitope into the extracellular loop of HyNaC2 and used a monoclonal anti-HA antibody and a luminescence assay to compare the surface expression of HA-tagged channels (15). Luminescence was not significantly different (p = 0.3) between oocytes expressing the HyNaC2/3/5 heteromer and oocytes expressing the HyNaC2/3 heteromer (Fig. 4C). For oocytes expressing the heteromers, luminescence was, however, significantly larger than for oocytes expressing HyNaC2-HA alone.

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(p ≤ 0.01), for which luminescence was not significantly above background (p = 0.2; Fig. 4C).

Assuming a number of three subunits in a functional HyNaC (18), in the 2/3/5 channel only one subunit per channel was tagged, whereas in the 2/3 channel either one or two subunits were tagged, depending on the subunit stoichiometry (2/3/3 or 2/2/3). Therefore, in the case of a 2/2/3 channel our assay would underestimate the surface expression of the 2/3/5 channel relative to the 2/2/3 channel by a factor of 2. Even in this case, however, the difference in surface expression (at most 2-fold) cannot account for the 16-fold difference in current amplitude (Fig. 4A). Thus, HyNaC5 predominantly increases the current through surface-expressed channels. This result suggests that the HyNaC2/3 heteromer either has a small single channel amplitude or that Hydra-RFamides are partial agonists at this channel, leading to a low open probability.

Similar to HyNaC2/3/5, ENaC is also composed of three homologous subunits (α, β, and γ) (16). ENaCs composed of only two different subunits (αβ or αγ), are also active but produce much smaller currents than those composed of three different subunits (αβγ), similar to HyNaCs composed of only two (2/3) different subunits. In contrast to HyNaC2/3, however, the low amplitude of αβ and αγ ENaCs is primarily due to low surface expression (19).

HyNaC5 changes the properties of the HyNaC2/3 heteromer – Similar to HyNaC2/3, HyNaC2/3/5 was activated by Hydra-RFamides I and II. For both peptides, the affinity of HyNaC2/3/5 was increased about 100-fold compared to HyNaC2/3 (Fig. 5): half-maximal activation was obtained with 4.8 ± 2.0 µM (mean ± s.e.m.; 8 oocytes) Hydra-RFamide I and 0.34 ± 0.08 µM (8 oocytes) Hydra-RFamide II compared to 326 ± 108 µM and 38 ± 13 µM (4 oocytes, each), respectively for HyNaC2/3. In each case, the concentration response curve could be well fitted assuming a single population of channels, suggesting that most of the channels in oocytes co-expressing HyNaC2, 3, and 5 were of a single type (2/3/5). Hill coefficients were similar for HyNaC2/3/5 and HyNaC2/3 (0.9 ± 0.1 compared to 0.8 ± 0.1 for Hydra-RFamide I, p = 0.6, and 1.8 ± 0.2 compared to 1.7 ± 0.4 for Hydra-RFamide II, p = 0.8, respectively; n = 8 for HyNaC 2/3/5 and n = 4 for HyNaC 2/3).

Previous evidence suggested that Hydra-RFamides activate HyNaC2/3 directly and not by a second messenger cascade (10). Activation of HyNaC2/3/5 with a strongly increased affinity for Hydra-RFamides further confirms that the binding sites for Hydra-RFamides are on HyNaCs and that these peptides directly activate HyNaCs.

In oocytes expressing the HyNaC2/3/5 heteromer (dilution 1:25), high concentrations (50 µM) of Hydra-RFamides III and IV also elicited a tiny current (< 0.1 µA; not shown), suggesting that these peptides are agonists at this channel with a very low affinity. Hydra-RFamides III and IV did not activate other subunit combinations, also containing HyNaC4. We speculate that a related channel, perhaps containing HyNaC4 in combination with other HyNaC subunits not yet identified, is the high-affinity receptor for Hydra-RFamides III and IV.

Reversal potentials in standard bath solution were not significantly different between HyNaC2/3/5 and HyNaC2/3 (10.1 ± 1.2 mV compared to 8.6 ± 3.5 mV; n = 8; p = 0.69; Fig. 6A), indicating that the permeability ratio PNa/PK is similar for the two channels. The inward rectification of HyNaC2/3/5, however, suggests that for HyNaC2/3/5 outward K⁺ currents are less well conducted than for HyNaC2/3. We further investigated ion permeability by replacing all extracellular Na⁺ by Li⁺ or K⁺ and measuring the amplitude of inward currents at -70 mV. In the case of HyNaC2/3/5, inward currents with K⁺ and Li⁺ as the main charge carrier were significantly smaller than Na⁺ currents (p < 0.01; Fig. 6B). This relative current reduction was especially prominent for K⁺ and is consistent with the inward rectification observed with the I/V curves. In the case of HyNaC2/3, amplitudes of inward K⁺ and Li⁺ currents were also significantly smaller than Na⁺ currents but the reduction in relative amplitude was smaller than for HyNaC2/3/5 (p < 0.01; Fig. 6B). A reduced K⁺ permeability, despite similar permeability ratios PNa/PK, would be expected when for example K⁺ ions permeate more slowly through the HyNaC2/3/5 than through the HyNaC2/3 pore (20). Together these results show that the ion pore of HyNaC2/3/5 has
slightly different permeability properties than the HyNaC2/3 pore.

In addition to a low selectivity between monovalent cations, the HyNaC2/3 pore is characterized by a low apparent affinity for the open channel blocker amiloride (10), which is also uncommon for DEG/ENaC channels. We confirmed that for HyNaC2/3 the IC₅₀ for amiloride is ~500 μM (540 ± 160 μM; five oocytes; Fig. 7A). In contrast, for HyNaC2/3/5 the IC₅₀ for amiloride was significantly reduced (122 ± 9 μM; P = 0.02; six oocytes; Fig. 7A). Since FaNaC is not only activated but also blocked by its peptide ligand, FMRFamide (21), we wondered whether the low apparent amiloride affinity of HyNaCs might be due to a competition with HydraRFamides at the rather high peptide concentrations (30 μM) used to determine amiloride affinity. However, even with a 100-fold lower concentration of the peptide (0.3 μM), apparent amiloride affinity of HyNaC2/3/5 was not significantly increased (IC₅₀ = 98 ± 18 μM; P = 0.2; six oocytes; Fig. 7A), showing that HydraRFamides do not strongly compete with amiloride. Of note, at low concentrations (1 μM) amiloride slightly increased HyNaC current amplitudes. An increased open probability by amiloride has previously also been noted for FaNaC (22,23). Finally, we compared the inhibition of HyNaC2/3 and HyNaC2/3/5 by 100 μM of the two amiloride-analogs benzamil and phenamil. Both blockers more potently blocked HyNaC2/3/5 than HyNaC2/3 (P < 0.05; Fig. 7B). This increased apparent affinity was highly significant for benzamil, for which 100 μM blocked almost 90% of the current. The increased apparent affinity for open channel blockers further support the conclusion that HyNaC5 is incorporated into the HyNaC2/3 channel complex and contributes to its ion pore.

**Amiloride delays the feeding reaction of Hydra** – In situ hybridization suggested that HyNaCs might contribute to the feeding reaction of Hydra. We tried to support this hypothesis by investigating the feeding reaction in the presence of amiloride in the medium. The feeding reaction can be induced by glutathione (GSH) (17). After addition of GSH (10 μM final concentration), *Hydras* responded quickly by moving their tentacles, contracting them and bringing them to their mouth. When the animals were in a medium containing 100 μM amiloride, however, the initiation of tentacles movement was significantly delayed (P < 0.05; Fig. 8). Moreover, the movements did not lead to the characteristic contraction of the tentacles pointing them to the mouth. Similarly, the natural catching reaction after exposing *Hydra* to *Artemia* shrimps was inhibited by 100 μM amiloride (not shown). These effects of amiloride were reversible; when animals were brought back to a medium without amiloride they could feed on *Artemia* like the untreated ones and responded properly to GSH by initiating the feeding reaction. Amiloride did not affect the general behaviour of the animal; animals were still able to contract and move in the medium. However, they were unable to perform the coordinated movements of the tentacles associated with feeding reflex, suggesting the potential importance of amiloride-sensitive ion channels for the feeding reaction.

Brief EPSPs with short synaptic delay have been recorded from the motor giant axon of the jellyfish *Aglantha digitale* (24), providing evidence for fast neurotransmission in Cnidaria. Recently genomic analysis indeed identified 11 ionotropic glutamate receptor subunits in the genome of the cnidarian *Nematostella vectensis* (25), suggesting that they could mediate fast transmission in *Nematostella*. In addition, the existence in cnidarians of channels from the nicotinic acetylcholine receptor/GABAＡ superfamily has been proposed (20); molecular evidence for such channels is so far lacking. Our results show that HyNaC2/3/5 form a peptide-gated ion channel and it should be considered that peptid-gated channels of the DEG/ENaC gene family contribute to fast neurotransmission in cnidarians.

The related FMRFamide-gated FaNaC has been localized to the membrane of snail neurons (26) and the properties of the endogenous current of those neurons match well with the properties of the recombinant FaNaC expressed in heterologous systems (7,22,27), showing that FaNaC carries a fast excitatory current in snail neurons. Defining more clearly the role of HyNaC for fast transmission in *Hydra* similarly requires, in the future, the electrophysiological characterization of the endogenous channel and
localization of HyNaCs by immunocytochemistry.

Given the fact that HyNaC is the only cloned ionotropic receptor of cnidarians, its rather complicated architecture with three different subunits is remarkable. Moreover, each subunit makes a specific contribution to the overall properties of the channel. HyNaC5 for example, as shown in this study, probably contributes to the ligand-binding domain and to the precise structure of the channel pore. This situation is reminiscent of the ENaC (28), an evolutionary recent channel from mammals. Thus, some features of DEG/ENaC channels that seem highly evolved are shared by HyNaC and may have been present already in ancestral channels of this gene family. It can be expected that the investigation of HyNaCs will yield further insights into ancient properties and the evolution of the DEG/ENaC gene family.

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FOOTNOTES

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1 The abbreviations used are: ASIC, acid-sensing ion channel; BLINaC, BrainLiverIntestine Na\(^+\) channel; DEG, degenerin; ENaC, epithelial Na\(^+\) channel; FaNaC, FMRFamide-activated Na\(^+\) channel; HyNaC, Hydra Na\(^+\) channel; RFamide, Arg-Phe-NH\(_2\); RLUs, relative light units.

FIGURE LEGENDS

Fig. 1. A) Sequence alignment of HyNaC5 with HyNaC2-4. Amino acids showing a high degree of identity are shown as white letters on black background. The putative positions of transmembrane domains are indicated by bars, conserved cysteines by circles, consensus sequences for N-linked glycosylation in HyNaC5 by branched symbols. Accession numbers are as follows: HyNaC2, AM393878; HyNaC3, AM393880; HyNaC4, AM393881; HyNaC5, FN257513. B) Phylogram illustrating the relationship of HyNaCs. Amino acid sequences of HyNaC2-5 and rat BLINaC were aligned and the tree for the phylogram established by Neighbor Joining with ClustalX; highly divergent sequences at the N and C termini had been deleted. Branch lengths are proportional to the evolutionary distance. BLINaC is a related channel (10) that was included for comparison; accession number of rat BLINaC is Y19034.

Fig. 2. hynac5 is expressed at the base of the tentacles. Whole mount in situ hybridization reveals strong expression of hynac5 transcripts at the tentacle base in adult animals (A) and buds (D and G). By comparison hynac2 and -3 transcripts are uniformly distributed (B-C). During bud formation hynac5 transcripts appear as soon as tentacles begin to appear (G) similar to hynac2 and hynac3 (E-F, H-I). Expression of hynac5 is strongest at the upper (oral) side of the tentacles (A); primary magnifications are 4x (A-C) and 20x (D-I).

Fig. 3. hynac5 and hynac4 show complementary expression patterns. hynac5 is expressed at the oral site base of the tentacles (A) and in early bud (C), while hynac4 is expressed at the aboral site of the tentacles (B); it also lacks any expression in early bud stages. Arrows indicate the different expression zones of both genes; primary magnifications are 20x (A-C).

Fig. 4. A) HyNaC5 potently increases the current amplitude of the HyNaC2/3 heteromer. Left, bar graphs illustrating the whole oocyte current amplitude after co-expression of different combinations of HyNaC subunits; HyNaCs were activated with 30 µM Hydra-RFamide I. For each condition, a total of ~8 ng cRNA had been injected in oocytes. Note that the amplitude for HyNaC2/3/5 was larger than 50 µA and therefore only a lower limit of the amplitude can be given. Error bars represent s.e.m.; for HyNaC2/3/5, the s.e.m. could not be calculated. n = 10 oocytes. Right, bar graphs illustrating current amplitude of oocytes co-expressing HyNaC2/3 or HyNaC2/3/5; a total of ~0.3 ng cRNA had been injected. Both channels were activated with a concentration of Hydra-RFamide I eliciting half-maximal response: HyNaC2/3 with 33 µM and HyNaC2/3/5 with 0.35 µM.
B) Representative current traces for the HyNaC2/3/5 heteromer. Repeated activation of HyNaC2/3/5 with Hydra-RFamides led to decreased response between first and second application; in the example shown, 30 µM Hydra-RFamide I was used. cRNA for HyNaCs 2, 3, and 5 was diluted 25-fold (a total of 0.3 ng/oocyte). C) HyNaC5 does not increase surface expression of the HyNaC2/3 heteromer. Surface expression of HyNaC2 and HyNaC2/3 in comparison to HyNaC2/3/5 (mean ± S.E.); only the HyNaC2 subunit was HA-tagged. Oocytes injected with untagged HyNaC2/3/5 served as a control (first column). The results are expressed as RLUs/oocyte/s. n = 16.

Fig. 5. HyNaC5 strongly increases apparent affinity for Hydra-RFamides. Top, representative current traces of whole oocytes either expressing HyNaC2 and 3 or HyNaC2, 3, and 5; channels were activated with Hydra-RFamide I. Bottom, concentration-response curves for HyNaC2/3 (open symbols) and HyNaC2/3/5 (closed symbols), each with Hydra-RFamides I (circles) and II (squares). Error bars represent s.e.m. and lines fits to the Hill function. Current amplitudes were normalized to the amplitude obtained with the highest concentration of the ligand (I_{max}). n = 8 for HyNaC2/3/5 and n = 4 for HyNaC 2/3.

Fig. 6. Ion selectivity of HyNaC2/3/5. A) The HyNaC5 subunit does not change reversal potentials. Channels were activated with 30 µM (HyNaC2/3) or 0.3 µM Hydra-RFamide I (HyNaC2/3/5), respectively, and reversal potentials measured by stepping to the indicated holding potentials for 3 sec. B) The HyNaC5 subunit decreases currents carried by K⁺. Left, representative current traces of whole oocytes either expressing HyNaC2 and 3 or HyNaC2, 3, and 5. Channels were activated in the presence of different monovalent cations. Right, bar graphs illustrating the amplitude of the Li⁺ and K⁺ current relative to the Na⁺ current; black bars represent HyNaC2/3/5, white bars HyNaC2/3. Error bars represent s.e.m. (12 oocytes); HyNaCs were activated with 30 µM (HyNaC2/3) or 0.3 µM Hydra-RFamide I (HyNaC2/3/5), respectively. In the presence of HyNaC5, amplitudes of Li⁺ and K⁺ currents were significantly reduced relative to the amplitude of the Na⁺ current. **, p < 0.01; ***, p < 0.001, two-tailed t-test.

Fig. 7. HyNaC5 increases the apparent affinity for amiloride and amiloride analogs. A) Concentration response curve for the inhibition of HyNaC currents by amiloride. Curves were determined with 0.3 µM (HyNaC2/3/5) and 30 µM HydraRFamide II (HyNaC2/3 and HyNaC2/3/5), respectively. n = 5 oocytes for HyNaC2/3 and n = 6 oocytes for HyNaC2/3/5. B) Left, representative current traces of whole oocytes either expressing HyNaC2 and 3 or HyNaC2, 3, and 5. Channels were activated in the presence of different blockers (100 µM). Right, bar graphs illustrating the current remaining in the presence of the respective blockers; black bars represent HyNaC2/3/5, white bars HyNaC2/3. Before and after application of the blockers, channels were activated without blockers; the mean value of these two measurements was used to normalize the currents obtained in the presence of the blockers. Error bars represent s.e.m. (6 oocytes); HyNaCs were activated with 30 µM (HyNaC2/3) or 1 µM Hydra-RFamide I (HyNaC2/3/5), respectively. In the presence of HyNaC5, amiloride and benzamil blocked significantly more current, suggesting an increased affinity for these blockers. *, p < 0.05; ***, p < 0.001, two-tailed t-test.

Fig. 8. Amiloride delays the GSH induced feeding response. Hydra magnipapillata were relaxed in plain medium or medium containing 100 µM amiloride. At time 0:00, GSH was added to a final concentration of 10 µM. Every 30 sec the number of animals moving their tentacles was recorded. A statistically significant delay in the response to GSH was detected in the presence of amiloride. The calculated p-value is < 0.05 as indicated (*), while it is 0.058 for the time point 1:00.
Subunit composition of a peptide-gated channel

Figure 1
Subunit composition of a peptide-gated channel

Figure 2
Figure 3
Subunit composition of a peptide-gated channel

Figure 4
Subunit composition of a peptide-gated channel

Figure 5

[Graph showing the subunit composition of HyNaC2/3, HyNaC2/3/5, RFamide I, and RFamide II at various concentrations of HydraRFamide (μM)].

$I/I_{max}$ vs. HydraRFamide (μM)
Figure 6
Subunit composition of a peptide-gated channel

Figure 7
Subunit composition of a peptide-gated channel

Figure 8
Three homologous subunits form a high-affinity peptide-gated ion channel in hydra

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