Location of a ligand recognition site of FMRFamide-gated Na^+ channels

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Abstract The second FMRFamide-gated Na^+ channel (*Ht*FaNaC), from *Helisoma trivolvis*, has been cloned. *Ht*FaNaC has some different pharmacological properties to *Ha*FaNaC, from *Helix aspersa*, which has enabled a rational approach to be made to start to identify the FMRFamide recognition site. Several chimeras were made by switching sections between the channels. The differences in sensitivity to FMRFamide, and amiloride, were assessed after expression in *Xenopus* oocytes. The data suggest that a recognition site for FMRFamide, and the potentiating action of amiloride, resides in a sequence of about 120 amino acids in the extracellular loop proximal to the first transmembrane segment.

Key words: FaNaC; FMRFamide recognition; Amiloride; Na^+ channel

1. Introduction

The only known peptide-gated ion channels are the FMRFamide (Phe-Met-Arg-Phe-NH_2)-gated Na^+ channels (FaNaCs) of snail neurons [1–3]. They are members of a class of channels typified by the epithelial amiloride-sensitive Na^+ channels or ENaCs [4–6]. Members of this family of channels appear to be abundant in different species. For example, 24 amiloride-sensitive Na^+ channels are predicted from analysis of the *Drosophila* genome, and 22 in *Caenorhabditis* [7]; several neuronal members of the family have been identified in mammals [8–10]. Available evidence suggests that subunits of all these channels possess only two transmembrane domains (TMs) and an extracellular loop that incorporates several cysteines. In the case of the FaNaCs, the extracellular loop comprises about 450 amino acids; the heterologously expressed channels are probably homotetramers [11].

The ENaCs are constitutively active. On the other hand, the FaNaCs require activation, as do other neuronal members of this family identified in vertebrate and invertebrate species. The natural ligand for the FaNaCs (FMRFamide) is known, but the natural means of activation of the other related neuronal channels are controversial; some may be mechanosensors [12,13], while others can be activated by acid pH [8,10]. There is considerable interest in identifying the physiological roles of these channels. It is possible that some are activated by precise sequences of amino acids that are either incorporated into specific proteins or free as peptides, yet to be defined. Little is known about the receptive sites of these channel proteins.

Two FaNaCs have been cloned. Although the proteins share 65% identity and highly conserved regions within the extracellular loop, they exhibit different sensitivities to FMRFamide and amiloride-like drugs. When expressed in *Xenopus* oocytes, *Ha*FaNaC (from *Helix aspersa*) has an EC_{50} of 2 μM for FMRFamide and is blocked by amiloride [2], but *Ht*FaNaC (from *Helisoma trivolvis*) has an EC_{50} of 70 μM for FMRFamide and is potentiated by amiloride [3]. These two channels therefore provide a good model for investigating the interaction of an amiloride-sensitive Na^+ channel with its native ligand as well as with amiloride.

Here we report a series of experiments in which several chimeric channels have been made by switching sequences between conserved regions of *Ha*FaNaC and *Ht*FaNaC and then studied electrophysiologically after expression in *Xenopus* oocytes. The results suggest that the receptive site for FMRFamide, and also the site responsible for amiloride potentiation, reside much closer to TM1 than to TM2.

2. Materials and methods

A diagrammatic representation of the predicted primary structure of the FaNaCs and the chimeras made is shown in Fig. 1. Both *Ha*FaNaC and *Ht*FaNaC are cloned into the pXENEX1 vector optimized for *Xenopus* oocyte expression [3]. Master chimeric constructs were created as follows: each cDNA possesses a unique *Ppu*MI site at an identical location in the fully conserved region prior to TM2. A unique *Nde*I restriction site was introduced into a highly conserved domain just prior to the TM1 region in each cDNA by creating a silent mutation with the Stratagene QuikChange kit. The fidelity of these and all ensuing constructs was confirmed by dideoxy sequencing. The entire extracellular loop region of each channel cDNA was then amplified using the Expand High Fidelity polymerase chain reaction (PCR) system kit (Boehringer Mannheim); the PCR primers were also designed to change the single non-conserved residue within TM1 of each channel (I79 in *Ht*FaNaC, A76 in *Ha*FaNaC) to that of the counterpart channel, which ensured that the sequence of TM1 was consistent with that of the N-terminus. Each amplified product was cut with *Nde*I and *Ppu*MI and ligated into the similarly digested plasmid for the other channel. The resulting chimeras comprised either the *Helisoma* (*Ht*) intracellular termini and TMs and the *Helix* (*Ha*) sequence of the extracellular loop (labeled *Ht*(*Ha*89–536)*Ht* or *tat*1; see Fig. 1B), or the *Helix* termini and TMs and the *Helisoma* extracellular loop (*Ha*(*Ht*92–534)*Ha* or *ata*1). Each construct is identified by the portion of the extracellular domain from one channel

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Abbreviations: Na^+, sodium ion; FMRFamide, Phe-Met-Arg-Phe-NH_2; PCR, polymerase chain reaction; TM, transmembrane; ENaC, epithelial sodium channel; ASIC, acid-sensing ion channel

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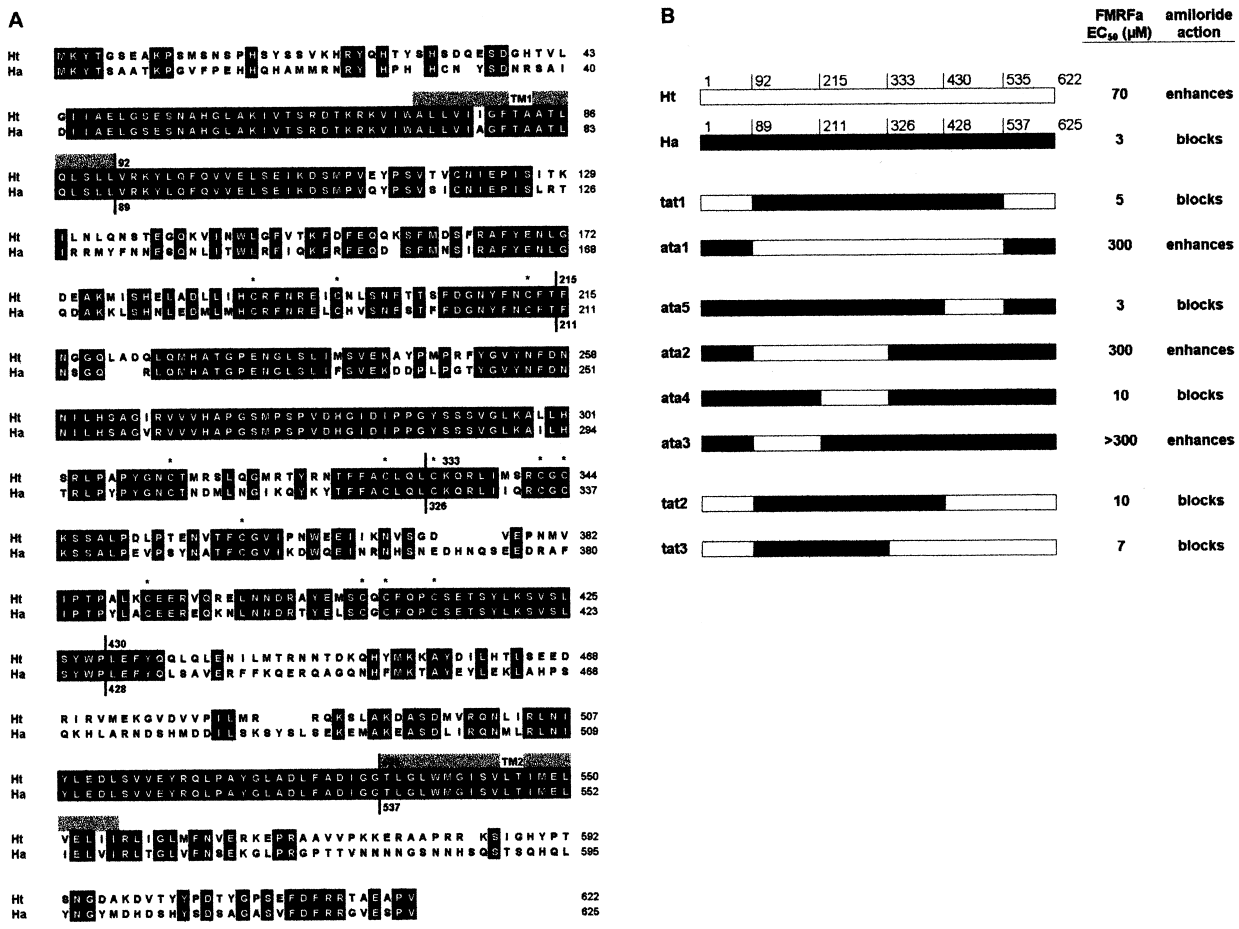

Fig. 1. A: Alignment of the peptide sequences of the FaNaCs from *H. trivolvis* (Ht) and *H. aspersa* (Ha). The locations of the splice sites used in constructing the chimeras are marked by vertical lines, followed by the numerical positions of the succeeding residues. Conserved cysteines are marked by asterisks, and the proposed locations of the two TM segments are indicated by shading. B: Diagrammatic representation of the two wild-type channels and the chimeras constructed and studied in the present experiments. Each chimera is represented by mixtures of black (portions of *Ha*FaNaC) and white (portions of *Ht*FaNaC). A summary of the pharmacological responses of each construction is shown to the right; see Fig. 2 for a more detailed illustration of the FMRFamide responses.

(numbered positions in the peptide sequence) that has replaced the corresponding region in the counterpart channel; see Fig. 1A for a full alignment of the two channel sequences. Subsequent chimeras incorporating smaller regions of the extracellular domain were created from the wild-type channels and/or previously made chimeras using the 'gene splicing by overlapping extension' PCR protocol [14]. All splice sites are embedded within highly conserved regions (see Fig. 1). A point mutation was generated using the Stratagene kit to correct a PCR-induced error in one construct.

cDNAs of *Ha*FaNaC and *Ht*FaNaC and the different chimeras were linearized and transcribed using the T7 version of the mMessage mMachine in vitro transcription kit (Ambion). Samples of cRNAs were denatured, electrophoresed on denaturing formaldehyde gels, and stained with SYBR Green (Molecular Probes) to determine concentration and purity. Oocytes were removed from *Xenopus* under MS-222 anesthesia and treated with 2 mg ml^{-1} collagenase (Sigma Type II or Type I) to remove the follicular cell layer. 0.5–5 ng of cRNA was injected into each oocyte. Oocytes were stored at 18°C in ND96 containing 5% horse, or fetal calf, serum, 2.5 mM Na pyruvate, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma). The sensitivity to FMRFamide (Sigma) and response to amiloride (Sigma) were assessed electrophysiologically with two electrode voltage-clamp 2–10 days after injection.

The voltage-clamp experiments were made with oocytes placed in a 0.5 ml bath that allowed continuous exchange of the physiological solution (ND96). Low resistance (<1 MΩ) micro-electrodes filled with 3 M KCl were used. Flow through the bath was about 5 ml min^{-1}. Test solutions were applied in 1 ml samples and allowed to flow through the bath at the same rate. The effect of amiloride was tested by adding 5–6 ml of a 100 μM solution in ND96 immediately before the peptide dilution. ND96 solution contained: (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, adjusted to pH 7.4 with NaOH.

3. Results

The chimera comprising the intracellular tails and TMs of *Ht*FaNaC with the extracellular loop of *Ha*FaNaC, i.e. *Ht*(*Ha*89–536)*Ht* or *tat*1, had an EC_{50} for FMRFamide of 5 μM and was blocked by amiloride (Figs. 2 and 3). Recordings made under identical conditions with *Ha*FaNaC gave a FMRFamide EC_{50} value of 3 μM and amiloride block. In contrast, the FMRFamide EC_{50} was 300 μM for the construct *Ha*(*Ht*92–534)*Ha* or *ata*1, and amiloride potentiated the FMRFamide response (Figs. 2 and 3). Under the same conditions the FMRFamide EC_{50} was 70 μM for *Ht*FaNaC and amiloride potentiated the peptide response. These experiments confirmed that the approach was generally sound in that the responses of chimeras were similar to those of the channels from which the extracellular loop was taken.

Portions of the extracellular domain preceding TM2 of the

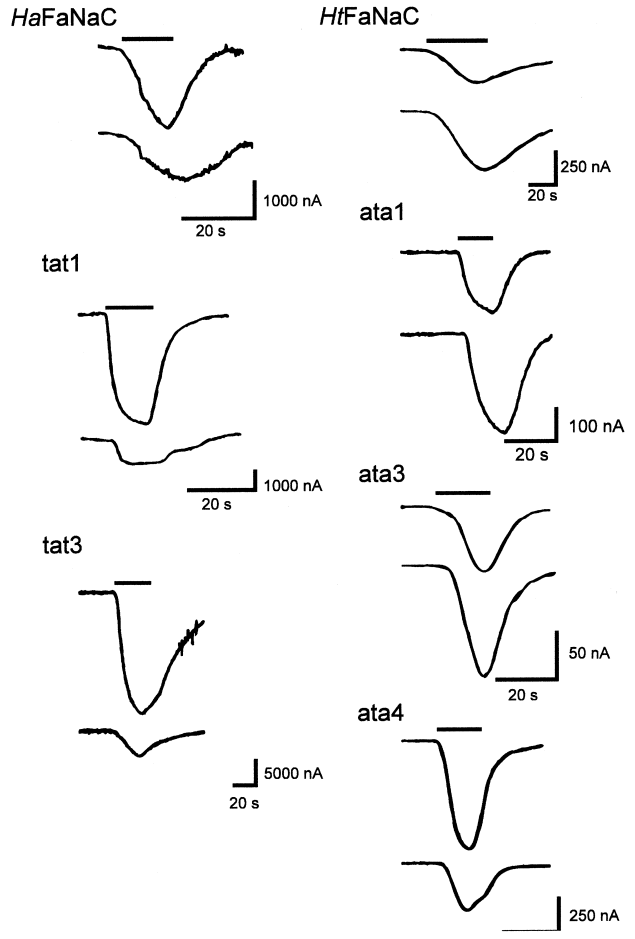

Fig. 2. Examples of FMRFamide responses and the different effects of 100 μM amiloride. The upper recording of each pair shows the response to FMRFamide alone and the lower recording the response of the same concentration of FMRFamide with 100 μM amiloride. In every case the effect of amiloride was reversed with washing (not shown). Application of FMRFamide is indicated by the bar above each pair of recordings. The concentrations of FMRFamide were: 1 μM for *tat*1 and *ata*4, 3 μM for *Ha*FaNaC and *tat*3, 10 μM for *Ht*FaNaC and 100 μM for *ata*1 and *ata*3. As with *Ha*FaNaC, the effect of amiloride on the *tat* chimeras was to reduce the response to FMRFamide, whereas with *Ht*FaNaC, *ata*1, and *ata*3 (and *ata*2, not shown), amiloride enhanced the response to FMRFamide. Inclusion of the extracellular *Helix* sequence adjacent to TM1 abolished the enhancing effect of amiloride on the FMRFamide response.

construct *Ht*(*Ha*89–536)*Ht*, or *tat*1, were then replaced with the corresponding *Ht*FaNaC segments. These new chimeras were *Ht*(*Ha*89–427)*Ht*, or *tat*2, and *Ht*(*Ha*89–325)*Ht*, or *tat*3. The sections switched included a domain that occurs only in the FaNaCs and differs markedly in amino acid sequence between the two channels (see Fig. 1). The expectation was that the responses of such constructs would mimic those of *Ht*FaNaC. However, both *tat*2 and *tat*3 exhibited properties that were very similar to those of *Ha*FaNaC and *tat*1; the FMRFamide EC_{50} values were about 10 μM and 7 μM for *tat*2 and *tat*3, respectively, and the FMRFamide current was blocked with amiloride in each case (Figs. 2 and 3). Similarly, another chimera comprising mainly *Ha*FaNaC with a short section preceding TM2 from *Ht*FaNaC, i.e. *Ha*(*Ht*430–534)*Ha* or *ata*5, responded to FMRFamide with an EC_{50} of 3 μM with amiloride blockade (Figs. 2 and 3). All these data indicate that the differences in pharmacology between *Ha*FaNaC and *Ht*FaNaC cannot be explained by differences in the sequences within the C-terminal section of the extracellular loop. Rather, they suggest that the N-terminal portion is important.

Data from other *HaHtHa* chimeras provided further evidence for this view. Three constructs were tested: *Ha*(*Ht*92–332)*Ha* or *ata*2, *Ha*(*Ht*215–332)*Ha* or *ata*4, and *Ha*(*Ht*92–214)*Ha* or *ata*3. The pharmacology of *ata*2, which essentially has only the N-terminal half of the extracellular loop of *Ht*FaNaC inserted into *Ha*FaNaC, was very similar to that of the chimera comprising the entire extracellular loop of *Ht*FaNaC inserted into *Ha*FaNaC, i.e. *ata*1.

A comparison of *ata*4 and *ata*3, on the other hand, showed marked differences in FMRFamide sensitivity and the effect of amiloride. Whereas the response of *ata*4 was very similar to those of *Ha*FaNaC and chimeras *tat*1, *tat*2, and *tat*3, with a FMRFamide EC_{50} of about 10 μM and amiloride blockade, the response of *ata*3 to FMRFamide was very weak, with an EC_{50} close to 300 μM, and amiloride potentiated the response (Figs. 2 and 3).

4. Discussion

The results show that switching sections of the extracellular loop close to the N-terminal transmembrane region (TM1) of the FMRFamide-gated channel had a profound effect on the FMRFamide EC_{50} values and the response to amiloride, whereas changes in the extracellular loop close to the C-terminal had little or no effect.

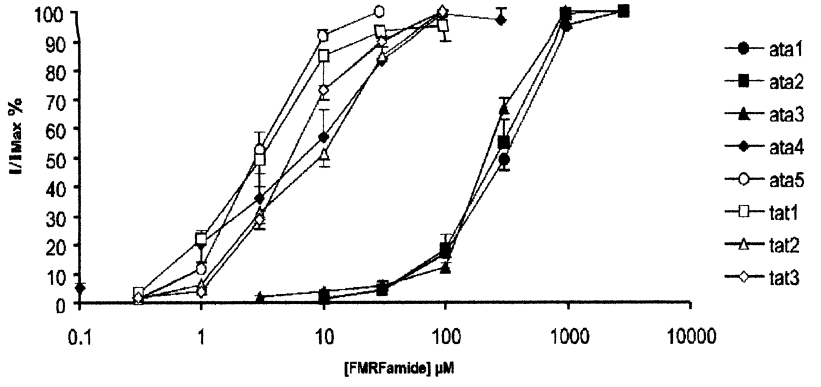

Fig. 3. Dose–response relationships of the different chimeras to FMRFamide. Data are shown as the mean ± S.E.M. of 3–5 experiments.

EC_{50} values depend upon the affinity of a ligand for its receptor and also the degree to which the ligand alters the conformational state of the receptor (gating or efficacy) once bound [15]. Thus, changes in EC_{50} values may reflect a change in affinity alone, a change in gating alone, or a combination of both. The FMRFamide EC_{50} value and amiloride sensitivity of the chimeras *tat*3 and *tat*2 were very similar to *tat*1 (and *Ha*FaNaC), indicating that the changed sequences did not influence either parameter, unless a change in one was precisely counteracted by a change in the other. In view of the significant changes in sequence made (see Fig. 1A), it is most likely that this region is not an important site for FMRFamide recognition. Comparison of the responses of *ata*5 with *Ha*FaNaC also indicates that the C-terminal region of the extracellular loop is not important for FMRFamide recognition. All these data imply that the FMRFamide recognition site(s) resides closer to TM1 than to TM2.

The chimeras *ata*2 and *ata*3 had similar EC_{50} values and amiloride sensitivity to *ata*1 and *Ht*FaNaC. *ata*4, on the other hand, had a FMRFamide EC_{50} value very similar to *Ha*FaNaC and was blocked by amiloride. Comparison of the responses of *ata*4, FMRFamide EC_{50} of 10 mM, and *ata*3, FMRFamide EC_{50} of about 300 mM (see Fig. 2), suggests that the sequence adjacent to TM1 is particularly important in influencing whether the response is similar to *Ht*FaNaC or to *Ha*FaNaC. Our conclusion is that this section, comprising about 120 amino acids of the extracellular loop proximal to TM1, is likely to be an important component of the FMRFamide recognition site. This region also appears to have a major influence on channel gating, which is particularly obvious with the marked enhancing effect of amiloride on the chimeras containing sections of sequence originating from *Ht*FaNaC. Single channel studies on *Ht*FaNaC suggest that amiloride and related drugs influence channel gating (see [3]). An additional effect of amiloride on FMRFamide binding has not been ruled out.

The conclusion that FMRFamide binding is likely to occur close to TM1 is relevant to other members of the family of channels typified by the ENaCs. Although the FaNaCs differ markedly in the C-terminus of the extracellular domain compared to other members, the N-terminal region is similar in size among the various members, albeit the level of conservation is relatively low. FMRFamide and some related peptides have recently been shown to potentiate acid-gated currents in rat dorsal root ganglion neurons, as well as heterologously expressed acid-sensing ion channel (ASIC) α (ASIC1), ASICβ, and DRASIC (ASIC3) channels, an action that appears to be exerted by FMRFamide binding directly to the channel [16]. Based on the contrasting effects of FMRFamide on the ASICα and ASICβ splice variants, which differ in their N-termini for approximately 180 residues, the ligand has been proposed to bind within the N-terminal domain. A similar location is supported by our findings; in fact, the C-termini of the splice sites in the ASIC variants and the *ata*3 chimera differ by only 11 residues.

TM2 and the adjacent extracellular region of ENaCs have been strongly implicated in the formation of the pore and the binding of amiloride, but regions near TM1 also play a role in channel function. Coscoy et al. [17] have obtained evidence that the pre-TM1 region in the ASICs participates in the channel pore, and data of Grunder et al. [18] implicate a region of TM1 of αENaC in channel gating. Our data further demonstrate that the extracellular region adjacent to TM1 in FaNaCs probably plays a role in ligand binding and channel gating. It is noteworthy that the sequence with the highest level of conservation at the nucleotide level (86%) between *Ha*FaNaC and *Ht*FaNaC occurs in the region, corresponding to about 70 amino acids, that forms part of the intracellular N-terminal region, TM1, and a section of the extracellular loop. The domain we have identified in the extracellular loop is 69% identical in peptide sequence between the two channels, and 56% identical in the portion bounded by the first and second conserved cysteines, but is poorly conserved with respect to other members of the amiloride-sensitive Na^+ channel superfamily.

The region that appears to be important in the binding of FMRFamide also helps to determine the action of amiloride. The responses demonstrate that not only can amiloride block be conferred upon chimeras comprising mainly *Ht*FaNaC, but also that an altered form of *Ha*FaNaC can be potentiated by amiloride, supporting our hypothesis of two interaction sites for amiloride with FaNaCs [3,19]. Two distinct amiloride binding sites have also been described in the mammalian BNC1 (MDEG, ASIC2) channel [20], and may exist in other ENaCs. Our data indicate that the response of such channels to amiloride may be modulated by variations in the N-terminus of the extracellular loop.

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