Cloning and expression of a FMRFamide-gated Na^+ channel from *Helisoma trivolvis* and comparison with the native neuronal channel

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1. We have cloned a cDNA encoding a Phe-Met-Arg-Phe-NH_2 (FMRFamide)-gated Na^+ channel from nervous tissue of the pond snail *Helisoma trivolvis* (HtFaNaC) and expressed the channel in *Xenopus* oocytes. The deduced amino acid sequence of the protein expressed by *HtFaNaC* is 65% identical to that of the FMRFamide-gated channel cloned from *Helix aspersa* (HaFaNaC).

2. HtFaNaC expressed in oocytes was less sensitive to FMRFamide ($EC_{50} = 70\ \mu M$) than HaFaNaC ($EC_{50} = 2\ \mu M$). The two had a similar selectivity for Na^+. The amplitude of the FMRFamide response of HtFaNaC was increased by reducing the extracellular concentration of divalent cations.

3. The conductance of the two channels was similar, but the mean open time of unitary events was shorter for expressed HtFaNaC compared to expressed HaFaNaC. Each channel was susceptible to peptide block by high agonist concentrations.

4. In marked contrast to HaFaNaC and other amiloride-sensitive Na^+ channels, amiloride, and the related drugs benzamil and 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), enhanced the FMRFamide response in oocytes expressing *HtFaNaC* cRNA. The potentiating effects of EIPA and benzamil were greater than those of amiloride. Unitary current analysis showed that with such drugs, there was channel blockade as well as an increased probability of channel opening.

5. The similar permeability of the oocyte-expressed HtFaNaC and the *Helisoma* neuronal channel, and the susceptibility of both to agonist blockade and blockade by divalent cations, suggest that the channels are the same. However, neuronal channels were less susceptible to enhancement by amiloride analogues and in some patches were more sensitive to FMRFamide than expressed HtFaNaC.

The fast depolarization activated by the neuropeptide FMRFamide in *Helix aspersa* (subsequently referred to here as *Helix*) neurones is mediated by a ligand-gated ion channel. This response is notable not only because it is uniquely gated by a peptide, but also because the channel is selective for Na^+ and blocked by amiloride (Cottrell *et al.* 1990; Green *et al.* 1994). The cDNA of a channel with similar properties (FaNaC) was cloned from *Helix* neurones and expressed in *Xenopus* oocytes (Lingueglia *et al.* 1995). FaNaC (subsequently referred to here as HaFaNaC) is similar in structure to the amiloride-sensitive epithelial Na^+ channel (ENaC) and the degenerins of *Caenorhabditis elegans* (see North, 1996). Available data suggest that the functional FMRFamide-gated channel is a homotetramer (Coscoy *et al.* 1998). More recently, similar amiloride-sensitive channels have been cloned from mammalian nervous tissue, some of which can be activated by H^+ (see Waldmann & Lazdunski, 1998; Chen *et al.* 1998).

To help define functional domains in this new class of ligand-gated ion channel, we have cloned the cDNA encoding a FMRFamide-gated channel subunit (HtFaNaC) from the pond snail *Helisoma trivolvis* (subsequently referred to here as *Helisoma*) and expressed the protein in *Xenopus* oocytes. The deduced amino acid sequence of the *Helisoma* channel shows many similarities to that of HaFaNaC but there are differences in agonist sensitivity. The properties of the expressed *HtFaNaC* clone are similar to those of the neuronal FMRFamide-gated channel of the giant dopamine neurone (GDN) and the large serotonin neurone (LSN), which are located in the pedal ganglia of *Helisoma* (Harris & Cottrell, 1995).

During the course of the work we observed that amiloride, benzamil and ethylisopropylamiloride (EIPA), which are known to block epithelial Na^+ channels and related channels (see e.g. Benos *et al.* 1997), had an unusual potentiating effect on FMRFamide-activated currents of HtFaNaC expressed

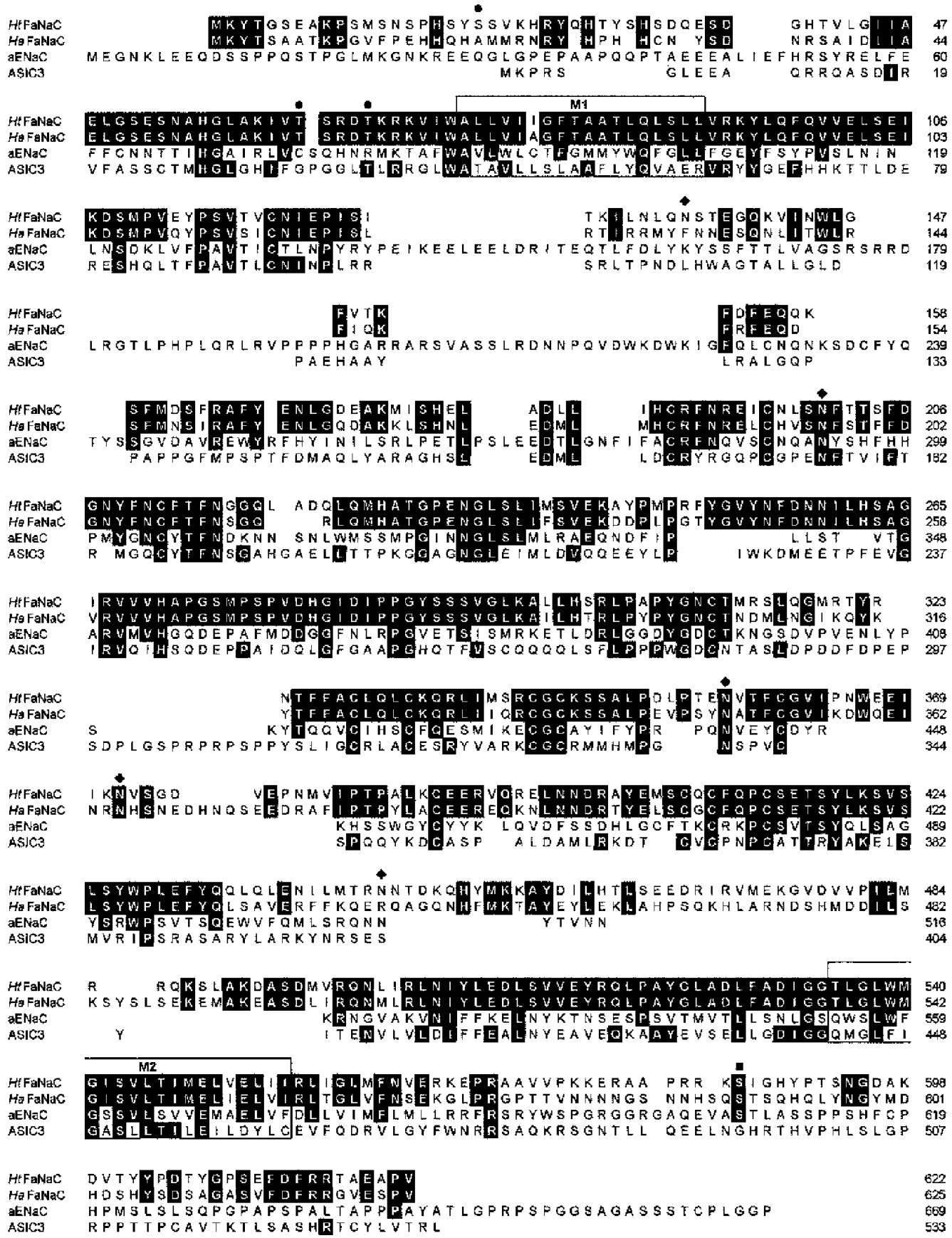

Figure 1. For legend see facing page

in *Xenopus* oocytes. This led to studies designed to compare the effects of amiloride and EIPA in more detail at the whole-cell and unitary current level on expressed HtFaNaC and also on *Helisoma* neurones, the results of which are included here.

METHODS

Cloning of the FMRFamide-gated channel from *Helisoma*

Two degenerate oligonucleotides similar to those designed by Lingueglia *et al.* (1995) were used in a PCR to amplify a fragment of a FMRFamide-gated channel cDNA from a fractionated *Helisoma* neuronal cDNA library (constructed by Dr Erno Vreugdenhil and generously contributed by Drs Andrew Bullock and Garry Hauser). The product was cloned into pGEM-T (Promega) and sequenced using the Sequenase (Amersham) version of the dideoxy method; the Big Dye kit (Perkin-Elmer) was later used to prepare samples for automated sequencing. The 5′ and 3′ termini were amplified from a single library fraction by two rounds of PCR incorporating primers specific for the insert and the vector; the second reaction amplified a sample of the first reaction product using nested primers. The three resulting fragments encompassed 3·6 kb of a cDNA that contained a complete open reading frame with high similarity to the *Helix* clone *HaFaNaC*. The full open reading frame was then amplified from two additional library fractions and from an independent reverse transcription reaction, using RNA extracted from ganglia of *Helisoma*, in order to confirm the cDNA sequence and prepare a full-length clone. Specific primers containing *Bsa*I and *Not*I sites were used to amplify the full open reading frame; the resulting products were digested with *Bsa*I and *Not*I, then ligated into the pXENEX1 vector (Jeziorski *et al.* 1998) cut with *Nco*I and *Not*I. The inclusion of a *Bsa*I site at the 5′ end of the clone allowed generation of an overhang complementary to an *Nco*I site without disrupting the coding of the second amino acid in the open reading frame (Patton *et al.* 1997). The sequences of the new clones were compared to that of the original clone to establish a consensus cDNA sequence. A construct corresponding to the consensus sequence was created in pXENEX1 by minor repair of a PCR-induced error in one clone.

Expression in *Xenopus* oocytes

The *Helisoma* FMRFamide-gated channel construct (p*HtFaNaC*) was linearized with *Hin*dIII, then purified and used to generate cRNA with the T7 version of the mMessage mMachine *in vitro* transcription kit (Ambion). The cRNA was denatured, electrophoresed on a denaturing formamide gel and stained with SYBR Green (Molecular Probes) to determine its concentration and purity. An ovary was removed from *Xenopus* (obtained from Xenopus 1, The Northside, Ann Arbor, MI, USA or from Blades Biological, Cowden, Edenbridge, Kent TN8 7DX, UK) after the frog had been killed by prolonged immersion in MS-222 followed by double pithing. The ovary was then treated with 2 mg ml^{-1} collagenase (Sigma Type II or Type I) for 50 min to remove the follicular cell layer. RNA (0·5–5 ng) was subsequently injected into each oocyte. Oocytes were stored at 17–18 °C in ND96 (see below) containing: 5% horse serum, 2·5 mM sodium pyruvate, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma). Recordings were made 2–5 days after injection.

Solutions and drugs

Patch- and voltage-clamp experiments on oocytes were done using an external solution containing (mM): 96 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$ and 5 Hepes, adjusted to pH 7·4 with NaOH (ND96 solution). For *Helisoma* neurones, the external solution consisted of (mM): 51 NaCl, 1·7 KCl, 1·5 $MgCl_2$, 4·1 $CaCl_2$ and 10 Hepes, with the pH adjusted to 7·3 with NaOH. For patch-clamp experiments on oocytes, a hyperosmotic solution was used to remove the vitelline membrane. The solution consisted of (mM): 200 potassium aspartate, 20 KCl, 1 $MgCl_2$, 10 EGTA and 10 Hepes, adjusted to pH 7·4 with KOH. For outside-out patch recordings the pipette solution contained (mM): 100 CsF or NaF (for *Xenopus* oocytes), 54 CsF or NaF (for *Helisoma* neurones), 3 NaCl, 1 $MgCl_2$, 5 EGTA and 10 Hepes, adjusted to pH 7·4 with CsOH or NaOH .

FMRFamide (Sigma), FLRFamide (Sigma), D-tubocurarine (Sigma) and benzamil (Sigma) were prepared as stock solutions in distilled deionized water and frozen. Amiloride (Sigma) was similarly prepared, but used the same day. EIPA (Sigma) was prepared as a 20 mM stock solution in DMSO. Working dilutions of all drugs were made in ND96 for oocyte recordings, or *Helisoma* saline for neuronal recordings.

Electrophysiological recordings

Whole-oocyte current recordings. Two-electrode voltage-clamp experiments were made with oocytes placed in a small bath that allowed continuous exchange of the physiological solution. Low resistance (< 1 MΩ) microelectrodes filled with 3 M KCl were used with a Warner Oocyte Voltage-Clamp amplifier or an Axoclamp-2B amplifier (Axon Instruments). Flow through the bath was varied from 3 to 6 ml min^{-1} in different experiments. Test solutions were applied in 1 ml samples and then, on most occasions, immediately washed from the bath. An interval of 5 min was used between additions of low to moderate concentrations of peptide solutions; longer periods (10–15 min) were required for recovery from the highest concentrations. The effects of amiloride, benzamil and EIPA were tested by adding 3–6 ml of the required dilution in ND96 solution immediately before addition of the peptide solution. In some cases the peptide solution contained the same concentration of amiloride being tested; in other cases the peptide solution added did not contain amiloride. Similar effects of each drug were observed

Figure 1. Alignment of the peptide sequence of HtFaNaC, the FMRFamide-gated channel from *Helisoma*, with the sequence of the *Helix* channel, HaFaNaC

Also shown are two representative amiloride-sensitive Na^+ channels: the α-subunit of the human epithelial sodium channel αENaC (aENaC; accession no. AAD28355) and the rat acid-sensing channel ASIC3 (also known as DRASIC; accession no. AF069328). Residues that are identical among the sequences shown are shaded in black. The membrane-spanning regions M1 and M2 are boxed. Potential external N-linked glycosylation sites, ◆. Sites for potential phosphorylation by protein kinase C, ●; and by protein kinase A, ■. The alignment was completed using ClustalW (Thompson *et al.* 1994). The accession numbers for HaFaNaC and HtFaNaC are X92113 and AF 254118, respectively.

with the two methods of application. Responses were recorded digitally either with a Gateway PC and Axon pCLAMP software, or using a Macintosh LCII with a MacLab 4S interface and Macintosh Scope software.

Intracellular recordings from *Helisoma* neurones. Recordings were made from two identified neurones (the GDN and the LSN) located in the pedal ganglia of *Helisoma*. A description of the location of these neurones is given in Harris & Cottrell (1995). Ganglia were exposed to 0·1 % trypsin, then the neurones were dissected free from connective tissue and, after further brief exposure to the trypsin solution, thoroughly washed. Currents from perikarya were recorded intracellularly using 1 M potassium acetate-filled microelectrodes and the discontinuous single electrode voltage-clamp method with an Axoclamp-2B. No significant difference was noted between whole-cell and unitary currents recorded from the GDN or the LSN neurones. FMRFamide was transiently applied to whole neurones by pressure ejection from a microelectrode (150–200 kPa). The recording chamber was perfused throughout all experiments with physiological solution. Drug solutions were added to the bath, or applied locally to the neurone immediately prior to FMRFamide.

Outside-out patch recordings from oocyte and neuronal membranes. Unitary currents were recorded in patches from oocytes or neurones using standard techniques with an Axopatch 200 (Axon Instruments) integrating amplifier. Pipettes were pulled from borosilicate glass, fire polished to a final tip diameter of less than 1 μm, then filled with patch pipette solution (see above for composition). They had resistances of 7–10 MΩ and readily formed seals on the oocyte membrane with resistances of 10–20 GΩ. Analog data recordings were filtered at 500 Hz and digitized at 400 μs intervals. For the neuronal patches, recordings were further filtered at 500 Hz with a digital Gaussian filter. FMRFamide was transiently applied to patches by pressure ejection (150–200 kPa). More prolonged applications were also made to patches using a Warner SF-77A Perfusion Fast-Step perfusion system (Warner Instrument Corporation, Hamden, CT, USA), or by local leakage from a separate blunt-tipped micropipette.

RESULTS

Molecular biology

We used a PCR-based cloning strategy to isolate a cDNA that encodes a FMRFamide-gated channel subunit from a *Helisoma* library as described in Methods. The cDNA was 3607 bp in length and contained an open reading frame encoding 622 amino acids, resulting in a calculated molecular mass of 70·4 kDa for the protein. The 5′ end of the cDNA differed at two sites in a second cDNA clone. An insert of 173 bp was found 152 bp downstream of the 5′ terminus of the alternative clone. This insert, which was not flanked by consensus intron–exon boundaries, was far upstream of the open reading frame. A second fragment of 95 bp, lying 20 bp upstream of the apparent start methionine and bounded by the GT and AG residues that signify a possible unprocessed intron, was deleted in the alternative cDNA. Deletion of this fragment, which contains an in-frame stop codon, produced in one clone an extension of the open reading frame 45 codons upstream to an earlier methionine codon. However, additional clones possessing this 95 bp deletion, generated by PCR from a library fraction, contained an in-frame stop codon between the two methionines. We therefore assumed that the downstream methionine, which corresponds closely to the start site proposed for the *Helix* FMRFamide channel and yields a protein of similar length, represents the true amino terminus of the *Helisoma* channel subunit. The deduced amino acid sequence of HtFaNaC is shown in Fig. 1, where it is aligned with that for HaFaNaC and two related sequences.

The amino acid sequence of HtFaNaC is 65 % identical to that of HaFaNaC. The similarity between the two proteins is high in the two proposed transmembrane segments (M1 and M2), where only three conservative substitutions are found in the *Helisoma* channel (see Fig. 1). The putative pore-forming region on the extracellular side of M2 is highly conserved in the *Helisoma* protein channels and other, discrete, regions of the extracellular domain are identical or very similar. Fourteen extracellular cysteines are conserved between the two proteins; thirteen of these cysteines are found in all members of the amiloride-sensitive sodium channel superfamily (e.g. as shown for αENaC and ASIC3). The extracellular domain contains five consensus sites for N-linked glycosylation, of which four are conserved with the *Helix* protein. Three sites for potential phosphorylation by protein kinase C are found near the N-terminus, and one potential cAMP-dependent protein kinase site lies near the C-terminus. Although most of the N- and C-terminal domains are only weakly conserved between the two proteins, a region of more than 25 amino acids immediately preceding M1 is identical in the two.

Response of HtFaNaC expressed in *Xenopus* oocytes to FMRFamide

Inward current responses to FMRFamide, which did not readily desensitize on prolonged exposure to the peptide, were observed in oocytes injected with *HtFaNaC*, but not in uninjected eggs, or eggs injected with water (Fig. 2*A*). Responses were detected 1–2 days after injection of *HtFaNaC* cRNA. The dose–response relationship for FMRFamide is shown in Fig. 2*B*. The EC_{50} value for FMRFamide was about 70 μM, which is considerably higher than the corresponding value of 2 μM for HaFaNaC (Lingueglia *et al.* 1995; Zhainazarov & Cottrell, 1998). The response to Phe-Leu-Arg-Phe-NH_2 (FLRFamide) was very small, being observed only at doses of 100 μM and above. Such high concentrations can cause pronounced channel block (Green & Cottrell, 1999). For this reason, and because such large amounts of peptide were required, a detailed study of the effect of FLRFamide was not made on intact oocytes.

Like the *Helix* clone, expressed HtFaNaC showed preferential permeability for Na^+ compared to K^+ (Fig. 2*C*). Further, using symmetrical Na^+ solution, the FMRFamide-gated unitary currents reversed at 0 mV, compared with a positive extrapolated potential with normal physiological solutions (and see below). The inward current response therefore appears to be mainly due to an increase in Na^+ permeability. Neither Ca^{2+} nor Mg^2 appears to contribute to

the FMRFamide response because FMRFamide did not evoke an inward current in physiological solution containing Ca^{2+} and Mg^2 when Na^+ had been replaced by K^+. Furthermore, FMRFamide-activated unitary currents reversed at 0 mV with symmetrical Na^+ solution but normal levels of divalent cations (see e.g. Fig. 2*C* and *F*). Ca^{2+} and Mg^2 did, however, reduce the amplitude of FMRFamide-activated unitary currents (see below).

FMRFamide activated unitary currents in outside-out patches of membrane from oocytes injected with HtFaNaC

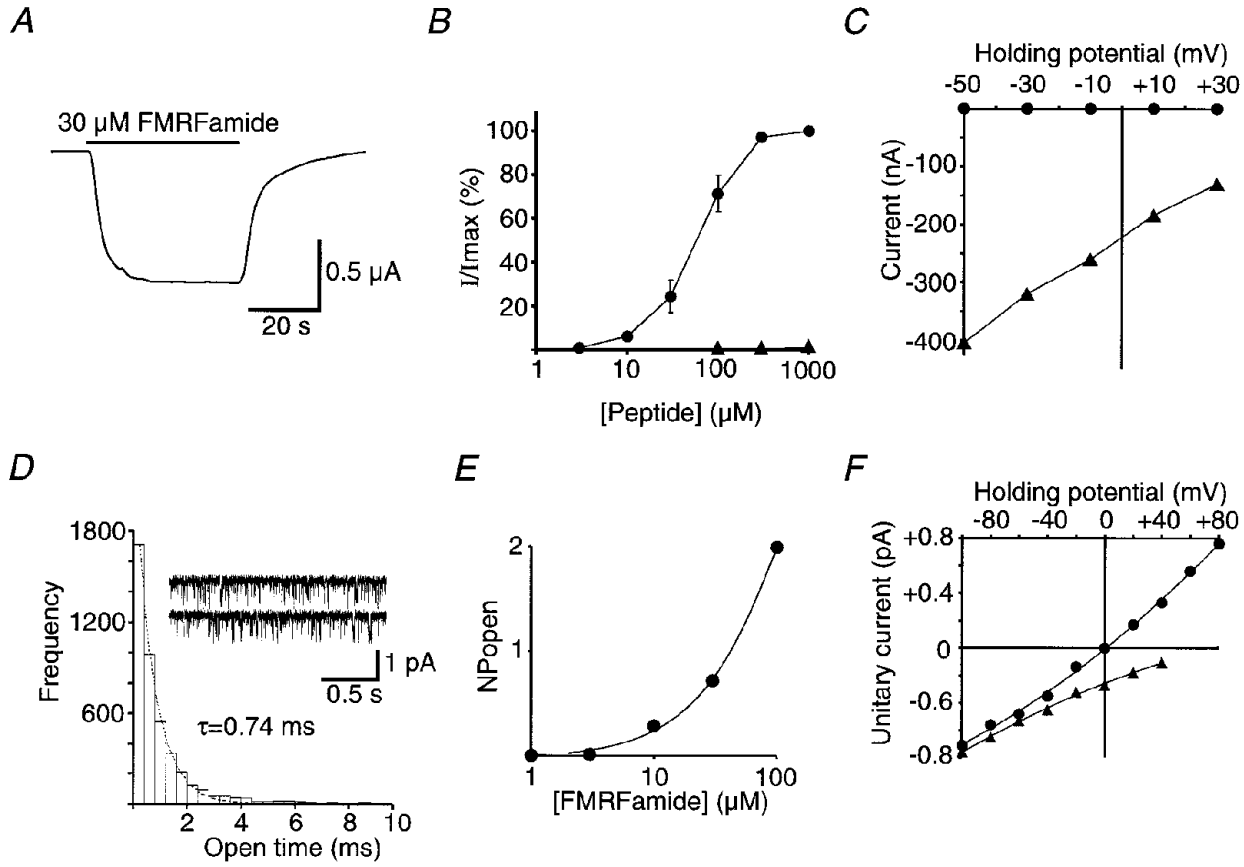

Figure 2. Responses to FMRFamide of intact oocytes, and outside-out patches taken from oocytes, injected with *HtFaNaC* cRNA

A, example of a response showing little desensitization to the applied FMRFamide. *B*, dose–response relationship of HtFaNaC to FMRFamide (●). Also shown are data from responses obtained from one oocyte with FLRFamide (▲). I/I_{max} is the amplitude of the response at a given concentration divided by the maximum response to FMRFamide. The points for FMRFamide represent the mean of responses from six experiments with different oocytes. The EC_{50} value for FMRFamide is approximately 70 μM. Error bars correspond to the S.E.M. *C*, relationship between the holding potential and the amplitude of the FMRFamide response of HtFaNaC recorded in the normal oocyte physiological solution (▲), and in physiological solution with all the Na^+ replaced with K^+ (●). All the points in this graph were obtained from one oocyte and demonstrate a very much higher permeability of the channel to Na^+ than to K^+. *D*, the open time frequency distribution of the unitary currents activated by 10 μM FMRFamide recorded from an outside-out patch of an oocyte that had been injected with *HtFaNaC* cRNA. The data were fitted with a single exponential distribution (dotted line), with $\tau = 0{\cdot}74$ ms. Example traces from the same patch are also shown. The patch potential was −100 mV. In this and all subsequent unitary current recordings, the inward currents are shown as downward deflections from the baseline using the standard convention. *E*, an incomplete dose–response curve showing the relationship between FMRFamide concentration and level of channel activity in a patch assessed by the summed products of the number of channels open in the patch and the probability of that number of channels being open (NP_{open}). The patch contained at least four channels. The holding potential was −100 mV. *F*, the dependency of the amplitude of the unitary currents on the holding potential of the membrane patch. In normal extracellular physiological solution and with CsF in the recording pipette (▲), the currents were inward in sign at +40 mV, suggesting a preferential permeability of the channel to Na^+. With the recording electrode filled with NaF to give the same concentration of Na^+ across the patch (●), the currents reversed at 0 mV, confirming selective permeability to Na^+.

cRNA. Examples of unitary currents evoked by 30 μM FMRFamide are shown in Fig. 2*D*. The time constant of the open time distribution was 0·74 ms. The partial dose–response relationship for a single oocyte patch containing four or more active channels, shown in Fig. 2*E*, was similar to the dose–response curve for the whole oocyte. Agonist blockade by FMRFamide, which was sometimes even detected at 30 μM FMRFamide (see e.g. Fig. 4*A*), made determination of NP_{open} (number of active channels × open probability) values impracticable at higher concentrations. There was no difference in the unitary current when the patch pipette contained KCl or CsCl, but when the patch pipette contained high Na^+ (symmetrical high Na^+) the unitary currents reversed at 0 mV, providing further evidence that the channels were Na^+ selective (Fig. 2*F*). The conductance of expressed HtFaNaC was 6·8 pS at negative holding potentials with symmetrical high Na^+, corresponding closely with the value of 6·1 pS for expressed HaFaNaC measured under similar conditions (not shown).

Response of *Helisoma* neurones to FMRFamide

The fast inward current response elicited by FMRFamide was studied on the GDN and LSN. These neurones responded to locally applied FMRFamide with a biphasic response comprising a fast inward current and a slower outward current. The inward current could be observed in isolation when the neurone was voltage clamped at the K^+ equilibrium potential, approximately −80 to −100 mV (see Cottrell *et al.* 1984; Colombiaoni *et al.* 1995). Repeated application of FMRFamide resulted in some desensitization of the inward current response (Fig. 3*A*), but the degree of desensitization was variable. As with *Helix* neurones, 100 μM D-tubocurarine, known to block many other ligand-gated responses (see Carpenter *et al.* 1977; Green *et al.* 1994), was without effect on the FMRFamide-response (not shown).

The relationship between peptide concentration and response amplitude is shown in Fig. 3*B*, and example traces with three different FMRFamide concentrations tested on unitary

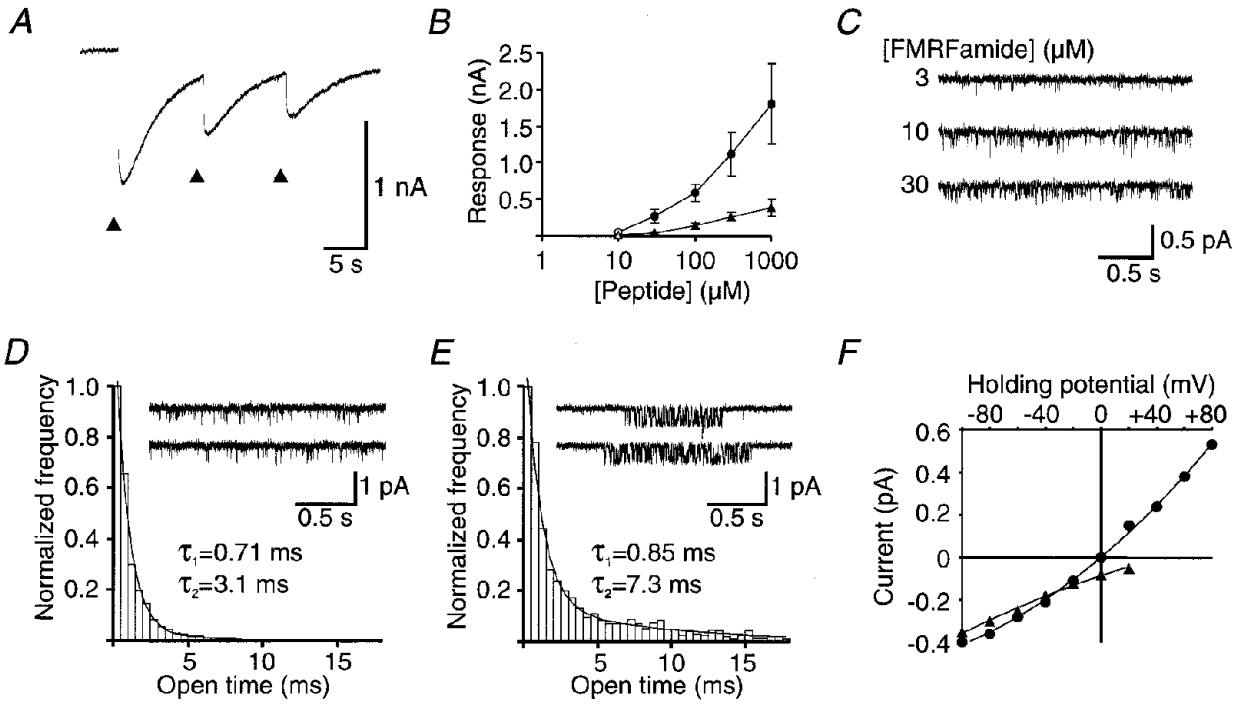

Figure 3. Recordings of FMRFamide-activated whole-cell currents, and unitary currents in outside-out patches, from *Helisoma* neurones

A, inward current responses were observed in isolation when recordings were made at the K^+ equilibrium potential (approximately −100 mV). Repeated application of FMRFamide (arrowheads) resulted in partial desensitization, as with the response in *Helix* neurones (see Green *et al.* 1994). *B*, dose–response relationship for FMRFamide (circles) and FLRFamide (triangles). For filled symbols (with S.E.M. bars), $n = 3$–6 for both FMRFamide and FLRFamide; for open symbols, $n = 1$ or 2. *C*, unitary currents activated by 3, 10 or 30 μM FMRFamide in an outside-out patch from the GDN. The holding potential was −100 mV. *D* and *E*, open time frequency distribution histograms of the unitary currents activated by 10 μM FMRFamide recorded from neuronal outside-out patches, illustrating the two modes of channel opening encountered in neuronal patches. The data of the mode of activity represented in the inset in *D* were fitted with the sum of two time constants: 0·71 ms (97%) and 3·1 ms (3%). Data of the type shown in the inset in *E* were fitted by the sum of two exponential components: 0·85 ms (92%) and 7·3 ms (8%). *F*, current–voltage relationship for unitary currrents recorded with CsF (▲) or NaF (●) in the recording pipette solutions, similar to Fig. 2*F*.

current activity on another patch are shown in Fig. 3*C*. The log dose–response curve (Fig. 3*B*) was similar to that observed with the oocyte-expressed HtFaNaC clone. However, FMRFamide, and to a lesser extent FLRFamide, could occasionally activate unitary currents in neuronal patches at concentrations as low as 1 μM. This suggests that some local factor(s) can influence the activity level of the neuronal channel. Furthermore, FMRFamide was observed to activate two different modes of unitary current activity on the neurone: isolated brief openings (as in Fig. 3*D*) and prolonged clusters of activity (as in Fig. 3*E*). Frequency distribution histograms of open times showed the presence of a brief, major component with $\tau = 0{\cdot}71$ ms and a minor, slower component which was more prominent in recordings that included the prolonged clusters of activity. A persistent downregulation of channel activity was also seen in the neuronal patches after application of 10 μM FMRFamide or higher concentrations, suggesting a process of partial desensitization.

With high Na^+ in the patch pipette, at the same concentration as in the extracellular solution (i.e. symmetical high Na^+ solution), the unitary current response reversed at 0 mV, as with expressed HtFaNaC. The relationship between the amplitude of FMRFamide-activated unitary currents and holding potential in normal physiological solution, and in symmetrical high Na^+ solution, is shown in Fig. 3*F*. The permeability for the neuronal channel was calculated to be $2{\cdot}06e^{-14}$ cm^3 s^{-1} compared with $2{\cdot}01e^{-14}$ cm^3 s^{-1} for HtFaNaC expressed in oocytes, assuming complete selectivity for Na^+.

Effect of divalent cations on the FMRFamide response of expressed HtFaNaC and the *Helisoma* neuronal channel

Reducing the extracellular concentrations of Ca^{2+} and/or Mg^{2+} increased the amplitude of FMRFamide current responses recorded from intact oocytes expressing HtFaNaC (Fig. 4*B*). The amplitude of unitary FMRFamide currents was seen to be progressively decreased with increased concentrations of Ca^{2+} and/or Mg^{2+} from 0·1 to 3 mM

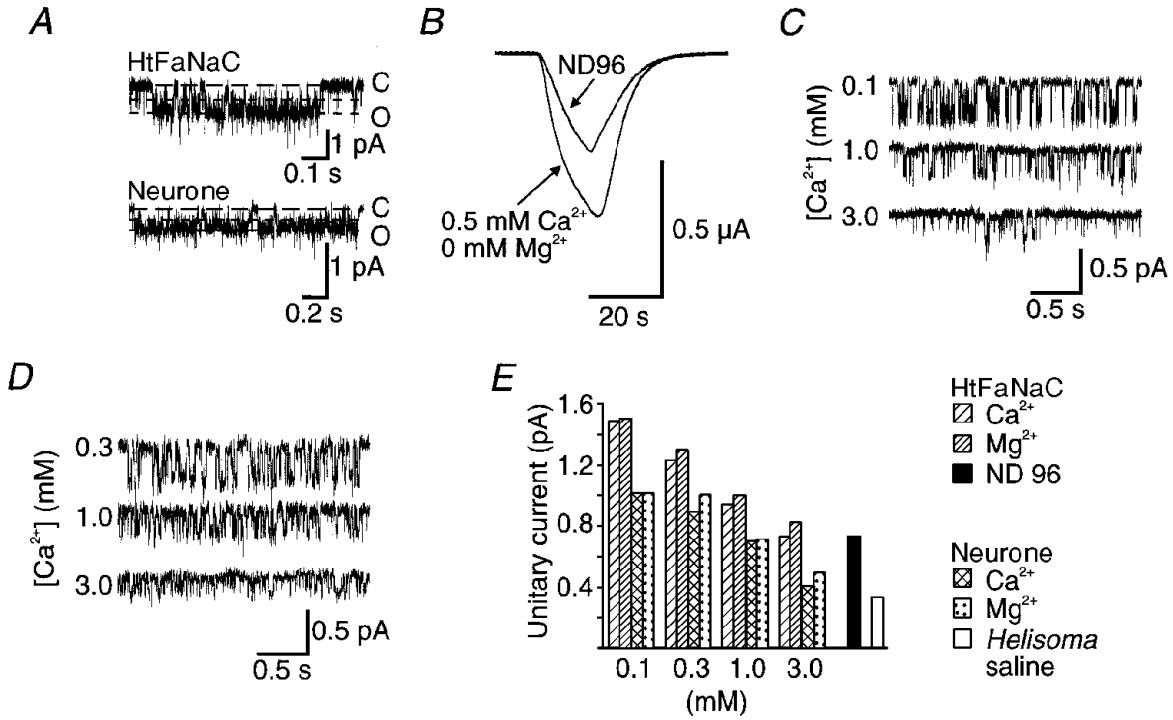

Figure 4. Agonist block, and block by divalent cations of the FMRFamide response of oocyte-expressed HtFaNaC and the neuronal channel

A, flickery block of FMRFamide-induce unitary currents in outside-out patches of oocyte expressing HtFaNaC and of the neurone. In each case, the long-dash line indicates the fully closed level (C), while the short-dash lines indicate the fully open level (O) and the subconductance to which the majority of transitions occur. In these recordings, the concentration of FMRFamide was 30 μM. *B*, an example of the influence of reduced divalent cation levels on the intact oocyte response of HtFaNaC to FMRFamide. The amplitude of the FMRFamide response was markedly increased with 0·5 mM Ca^{2+} and nominally 0 Mg^{2+} ND96 solution. A similar effect was observed in more than 10 preparations. *C* and *D*, FMRFamide-activated unitary currents recorded, respectively, from an oocyte injected with HtFaNaC and a neurone. Recordings are shown for three different external concentrations of Ca^{2+} and with no Mg^{2+}. The holding potential in *A*, *C* and *D* was −100 mV. *E*, histogram showing the amplitude of the FMRFamide-activated unitary currents recorded from HtFaNaC and the neurone. Data are shown for each with four different concentrations of Ca^{2+} or Mg^{2+} from 0·1 to 3 mM, and also for the respective standard external solution.

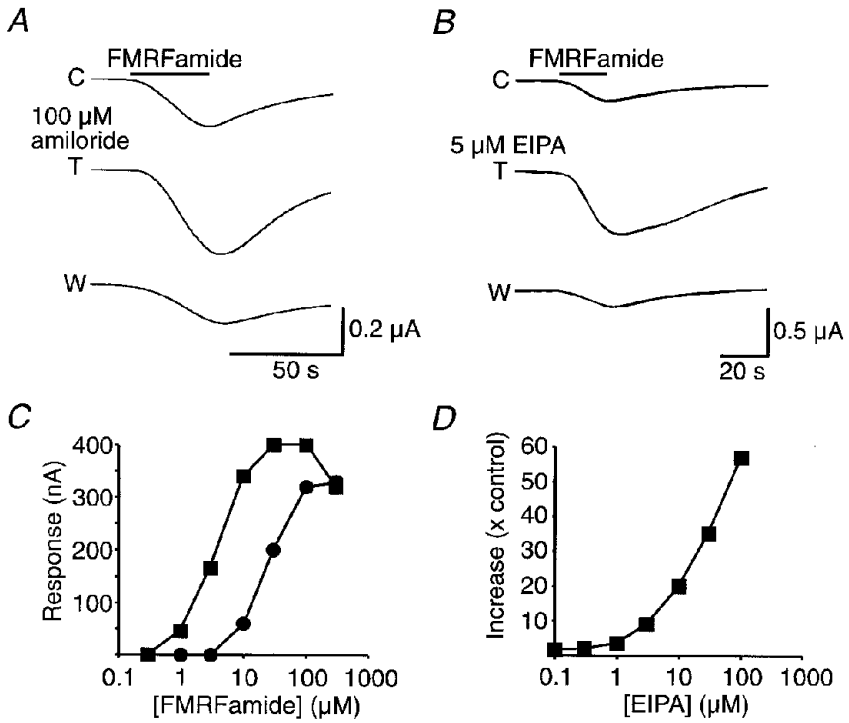

Figure 5. Potentiation by amiloride and EIPA of FMRFamide-activated currents recorded from whole oocytes injected with *HtFaNaC* cRNA

A, effect of 100 μM amiloride on the current response of an oocyte evoked by 10 μM FMRFamide. The oocyte was voltage clamped at −60 mV. C, control response to FMRFamide; T, response in the presence of 100 μM amiloride; and W, response after washing. *B*, effect of 5 μM EIPA on the current response of another oocyte evoked by 30 μM FMRFamide. The oocyte was voltage clamped at −60 mV. C, control response to FMRFamide; T, response in the presence of 5 μM EIPA; and W, response after washing. *C*, dose–response relationship for FMRFamide-activated currents recorded from an oocyte in the absence (●) and presence (■) of 20 μM EIPA. All the recordings were made from the same injected oocyte. *D*, relationship between the increase in amplitude of the response to 10 μM FMRFamide and EIPA concentration. The response to 10 μM FMRFamide was 30 nA at the outset of the experiment. With increasing concentrations of EIPA the size of the response was markedly potentiated, reaching 57 times the amplitude of the response recorded to FMRFamide alone at the outset. The amplitude of responses to FMRFamide alone, made intermittently throughout the experiment, varied between 20 and 50 nA. NB 100 μM EIPA evoked an inward current of about 150 nA both in oocytes injected with *HtFaNaC* cRNA and in uninjected oocytes. Lower concentrations of EIPA did not produce any significant inward current.

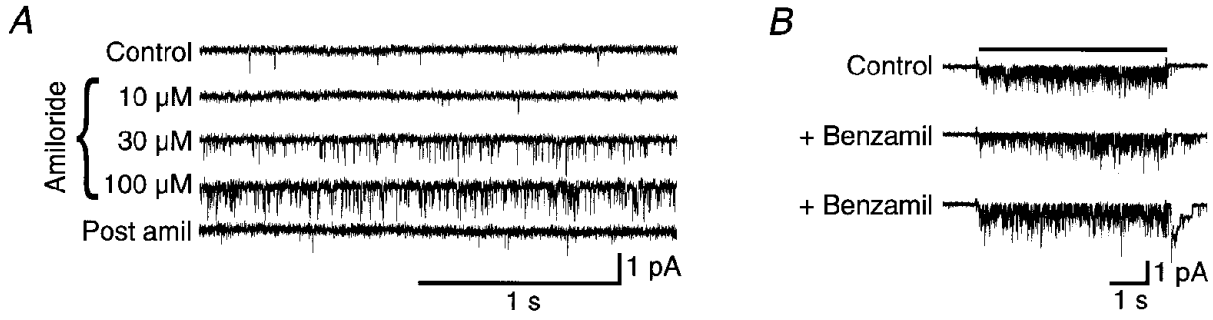

Figure 6. Enhancement of unitary FMRFamide-activated currents recorded in patches from oocytes injected with *HtFaNaC* cRNA

A, unitary currents activated by 10 μM FMRFamide alone (Control and Post amil), and in its combined presence with 10, 30 or 100 μM amiloride. *B*, unitary currents activated by short (5 s) duration applications of 10 μM FMRFamide, and by two consecutive 5 s applications of 10 μM FMRFamide together with 3 μM benzamil.

(Fig. 4*C* and *E*), suggesting that expressed HtFaNaC is sensitive to blockade by extracellular divalent cations. There was also an enhancement in the unitary current amplitude of neuronal channels evoked by FMRFamide with reduced extracellular levels of Ca^{2+} and/or Mg^{2+} (Fig. 4*D* and *E*). Similar effects have been observed with some related channels (Schild *et al.* 1997) and oocyte-expressed HaFaNaC and neuronal *Helix* FMRFamide-gated channels (K. A. Green & G. A. Cottrell, manuscript in preparation).

Effect of amiloride, benzamil and EIPA on HtFaNaC

Unexpectedly, it was found that amiloride markedly potentiated the whole-oocyte response to FMRFamide of oocytes injected with *HtFaNaC* cRNA (Fig. 5*A*). The enhancement was completely reversed with washing. The threshold for amiloride enhancement was about 10 μM. Benzamil and EIPA (Fig. 5*B*) also potentiated the effect of FMRFamide on oocytes expressing HtFaNaC. The threshold for benzamil was 1–5 μM (not shown). At 20 μM, EIPA produced a shift to the left in the dose–response curve of about 1 log unit bringing the EC_{50} of FMRFamide much closer to 2 μM, the corresponding EC_{50} for HaFaNaC (Fig. 5*C*). EIPA was the most potent of the drugs tested in enhancing the action of FMRFamide. Enhancement occurred at sub-micromolar concentations; the effect markedly increased with higher concentrations of EIPA (Fig. 5*D*). The potentiating effect of each of these drugs could also be observed with FMRFamide-gated unitary currents (Figs 6 and 7*A* and *B*). Each increased the probability of channel opening, but EIPA and benzamil could be seen to induce a change in the mode of channel opening from a state with a low probability of openings to one with bursts of longer openings. Each drug also exhibited a blocking effect on the FMRFamide response, which was clearly seen in outside-out patch recordings with for example EIPA (Fig. 7*A* and *C*). The overall effect observed in both whole-oocyte and patch recordings therefore appeared to be a combination of two separate phenomena, blockade and potentiation, each of

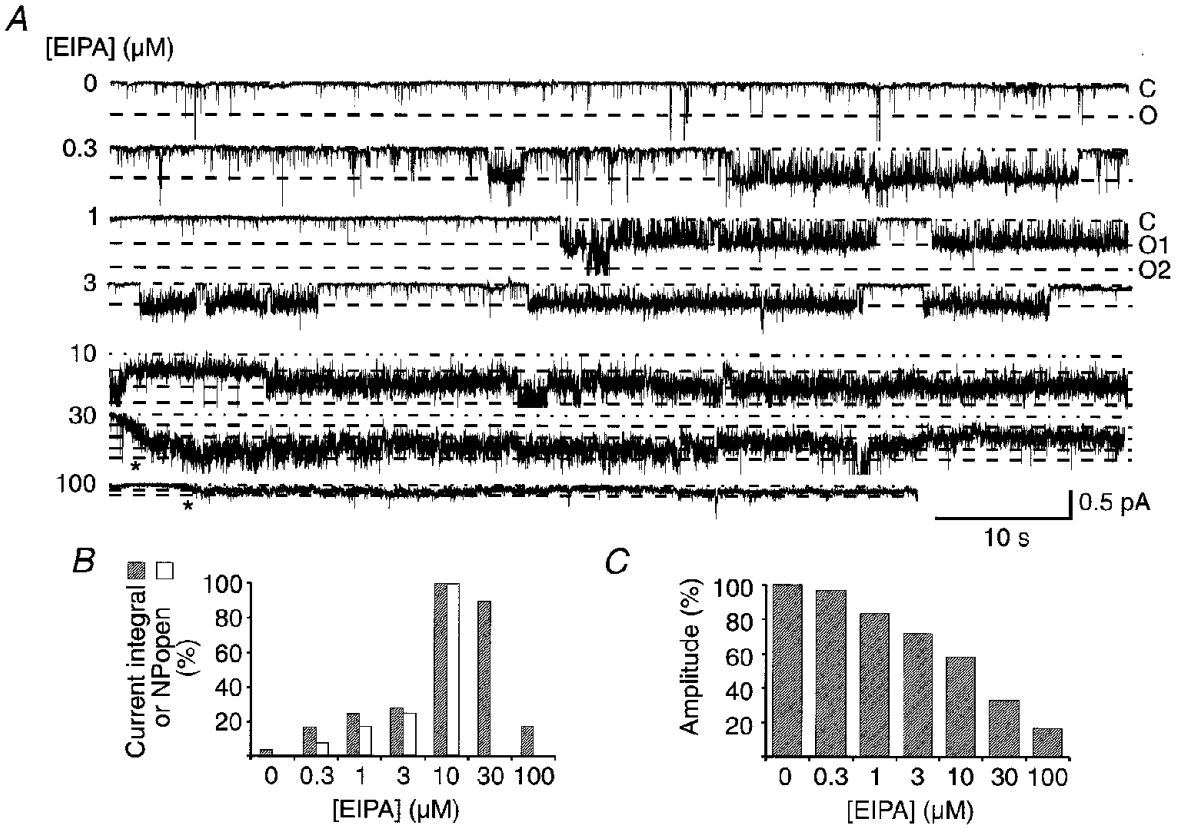

Figure 7. Simultaneous stimulation and block of unitary current activity recorded in a patch from an oocyte injected with *HtFaNaC* cRNA

A, unitary currents activated by 30 μM FMRFamide with co-application of 0–100 μM EIPA. For each trace a dot-dash line indicates the base current level, whilst the dashed lines represent successive unitary current opening levels. FMRFamide was present throughout except for the 30 and 100 μM traces, where the start of application is indicated by an asterisk below the recordings. *B*, unitary current activity as measured by either the integrated current per unit time or the NP_{open} value (which could only be estimated at $\leqslant$ 10 μM EIPA), plotted against the EIPA concentration. Values are percentage of activity with 10 μM EIPA. *C*, histogram of unitary current amplitude plotted as a percentage of amplitudes measured for 30 μM FMRFamide in the absence of EIPA. The holding potential was −100 mV.

which varied in potency with the three drugs tested. For example amiloride was a relatively effective blocker but poor enhancer, whereas EIPA appeared to be a less effective blocker, but was potent in enhancing the action of FMRFamide.

When potentiation and channel blockade were seen in outside-out patch recordings, potentiation appeared to develop more slowly than blockade. For example, when the expressed HtFaNaC was exposed to FMRFamide and then to the same concentration of FMRFamide in the presence of benzamil, a reduction in the FMRFamide-activated current was often initially seen (Fig. 6*B*). This contrasted with the rapid potentiation observed when channels were pre-exposed to benzamil, or the other drugs, and then exposed to FMRFamide in the absence of benzamil. Potentiation usually persisted for several minutes after washing.

Effect of amiloride and related drugs on neuronal FMRFamide responses

Amiloride and related drugs both blocked and enhanced neuronal responses to FMRFamide, but the enhancing effect was less obvious than with HtFaNaC expressed in oocytes. Only blockade of the FMRFamide response was observed with amiloride at the whole-cell level (Fig. 8*A*), but at the unitary current level, amiloride was seen to increase the probability of channel opening in the presence of FMRFamide (Fig. 8*D*). EIPA and benzamil were more effective in producing enhancement. Application of EIPA to intracellularly recorded neurones produced a dose-dependent increase in the amplitude of the response to pressure-applied 300 μM FMRFamide (Fig. 8*B* and *C*), with some prolongation of the response. The effect was readily reversed with washing. EIPA was also observed to stimulate the occurrence of high activity clusters of unitary currents

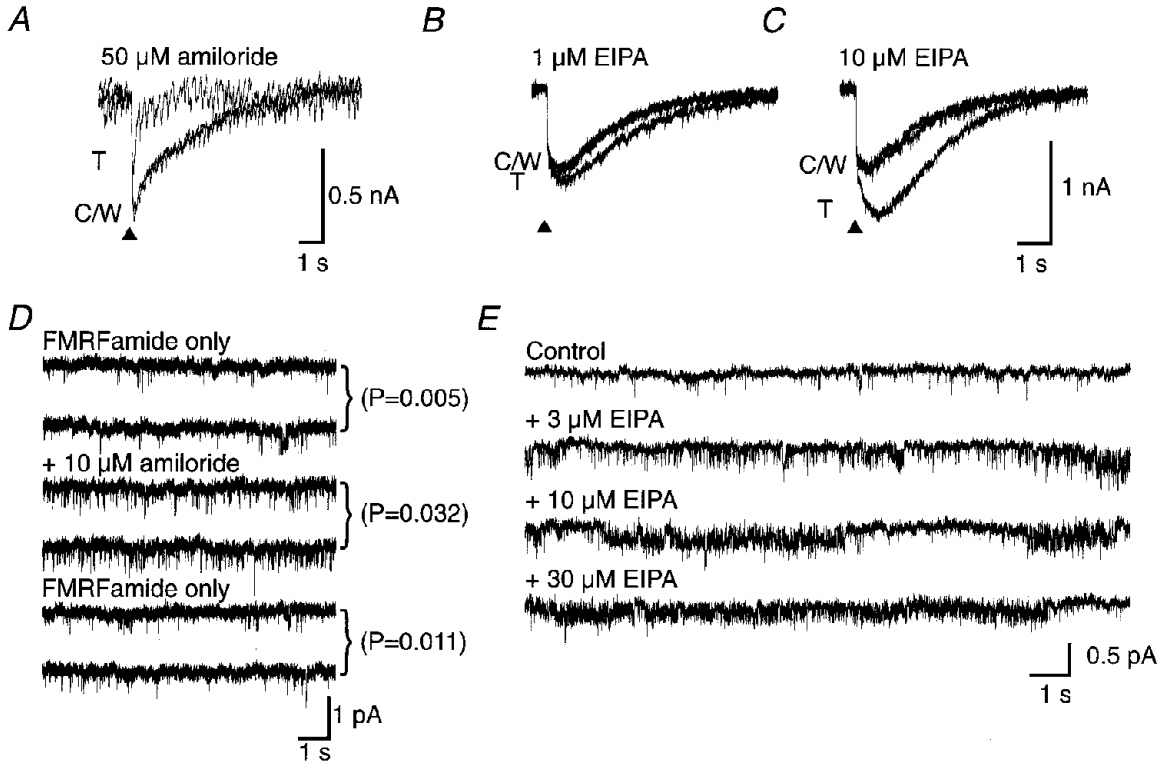

Figure 8. The effect of amiloride analogues on the neuronal responses to FMRFamide

A, the inward current FMRFamide response was blocked by 50 μM amiloride. The superimposed recordings show a control response (C), the test response in the presence of amiloride (T) and the response after washing (W). *B* and *C*, enhancement of whole-cell current responses to pressure-applied 300 μM FMRFamide (▲) by 1 and 10 μM EIPA, respectively. C and W indicate the control and wash traces, respectively, and T is the response in the presence of EIPA. *D*, unlike the whole-neurone response, an enhancing effect of amiloride was observed on unitary currents activated by 10 μM FMRFamide in a patch from the LSN neurone at a holding potential of −100 mV. Two frames are shown for the initial control activity with 10 μM FMRFamide only, the activity in the combined presence of 10 μM FMRFamide and 10 μM amiloride and the final activity after removing the amiloride. P_{open} was reversibly increased in the presence of amiloride. *E*, unitary currents activated by 10 μM FMRFamide recorded in a patch from the LSN neurone in the presence of 0, 3, 10 or 30 μM EIPA. Enhancement and blockade are seen. The holding potential was −100 mV throughout.

(Fig. 8*E*), as seen with oocyte patches with expressed HtFaNaC. At higher EIPA concentrations, however, the stimulatory effect was less continuous than in oocyte patches, possibly due to a greater susceptibility to desensitization in the neuronal patches. The neuronal channels also showed flickery block at higher EIPA concentrations.

DISCUSSION

The primary structure of HtFaNaC

The FMRFamide-gated channel of *Helix* was the first peptide-gated channel to be cloned and heterologously expressed (Lingueglia *et al.* 1995). Although the channel is a member of the superfamily of amiloride-sensitive Na^+ channels, it occupies a branch separate from other superfamily members. Each member of the superfamily contains a large extracellular region, two transmembrane domains (M1 and M2) and intracellular N- and C-terminal regions. The cloning of *HtFaNaC* from *Helisoma* now allows us to make more direct inferences about sequences specific to FMRFamide-gated channels. Like HaFaNaC and the other members of the superfamily, the extracellular domain in HtFaNaC is rich in cysteines. In the extracellular domain, the sequence of amino acids just before M2 and of M2 itself are probably important in forming the ion pore in HaFaNaC (Lingueglia *et al.* 1995), as in other members of the superfamily (see e.g. Waldmann *et al.* 1995). Evidence has also been presented that the region preceding M1 in ASIC participates in the ion pore (Coscoy *et al.* 1999). It is notable that these pre-M regions in the FMRFamide-gated channels of *Helix* and *Helisoma* are identical. Between the last of the extracellular cysteines and the putative pore-forming domain preceding M2, there are about 50 residues in both FMRFamide-gated channels that are not seen in other members of the superfamily. This sequence of amino acids may represent all or part of the peptide-binding moiety of FMRFamide-gated channels. The weak conservation between the sequences of the two channels in this region is intriguing in view of their differing sensitivity to peptide agonists.

In contrast to the epithelial Na^+ channels, which require three distinct subunits to form a fully functional channel (Canessa *et al.* 1994), the *Helix* FMRFamide-gated channel subunit is itself sufficient to generate a response in oocytes to FMRFamide comparable to the neuronal response. Similarly, expression of *HtFaNaC* cRNA alone in oocytes produced a FMRFamide-sensitive current comparable to that of *Helisoma* neurones. It is thought that the functional FMRFamide-gated channel of *Helix* is a tetramer of four identical subunits (Coscoy *et al.* 1998), but it is not yet established that the neuronal channel is homomeric. The *Helix* subunit (HaFaNaC) contains approximately 15 kDa of glycosylation (Coscoy *et al.* 1998). The native *Helisoma* channel may be similarly modified as several consensus sites for N-linked glycosylation are conserved between HtFaNaC and HaFaNaC.

Comparison of the FMRFamide responses of HtFaNaC with those of HaFaNaC

The EC_{50} value of HtFaNaC expressed in oocytes for FMRFamide was about 70 μM compared to about 2 μM for HaFaNaC, possibly indicating a lower affinity of HtFaNaC. However, the difference in EC_{50} values could also be explained in terms of differences in open time constants and P_{open} between the two clones (see Colquhoun, 1998). The amplitudes of the whole-oocyte currents evoked by FMRFamide were usually smaller than those evoked from oocytes injected with a comparable amount of *HaFaNaC* cRNA. FLRFamide, a peptide formed from the same precursor protein (see Lutz *et al.* 1992) was only a very weak agonist and its effect on oocyte-expressed HtFaNaC was not studied in detail. FMRFamide, FLRFamide and related peptides have been shown to exert significant channel block at concentrations higher than 10 μM with the *Helix* channel (Green & Cottrell, 1999); this also occurs with HtFaNaC as well as with the *Helisoma* neuronal channel. Unlike HaFaNaC, amiloride and related drugs had a pronounced enhancing effect on FMRFamide currents evoked in intact oocytes injected with *HtFaNaC* cRNA in addition to having a blocking action. These effects are discussed in more detail below.

Unitary currents from oocyte-expressed HtFaNaC activated by FMRFamide had a conductance similar to that recorded from similarly expressed HaFaNaC (Lingueglia *et al.* 1995; Zhainazarov & Cottrell, 1998). The similar conductance of the two FaNaCs may reflect their high conservation within the putative pore-forming regions (see above). The major open time constant for the HtFaNaC was 0·71 ms. This was consistently shorter than that of 4·84 ms for HaFaNaC (Zhainazarov & Cottrell, 1998). The difference in open times of HtFaNaC and HaFaNaC probably contributes to the smaller whole-cell FMRFamide responses observed with HtFaNaC compared to HaFaNaC, but other factors are also likely to be important (see Zhainazarov & Cottrell, 1998; Green & Cottrell, 1999). The different gating kinetics may be influenced by differences in the terminal regions. Grunder *et al.* (1999) have shown that changes in the amino acid sequence immediately preceding M1 can influence gating of ENaCs. The same region is, however, conserved between HtFaNaC and HaFaNaC, as discussed above. Consequently, if differences in the N-terminal region are important, those described by Grunder *et al.* (1999) cannot explain the differences observed in gating between expressed HtFaNaC and HaFaNaC. The C-terminus is also known to be important in the gating of ENaCs (Fuller *et al.* 1996), but it remains to be seen if this is also the case for FaNaCs.

Effect of amiloride, benzamil and EIPA on HtFaNaC

The results show that amiloride, benzamil and EIPA can markedly enhance the response to FMRFamide of HtFaNaC expressed in *Xenopus* oocytes. The predominant effect of each of the amiloride-related drugs on intact oocytes expressing HtFaNaC was potentiation of the FMRFamide-induced current.

At the unitary current level, it could be seen that the stimulatory effects of each drug were complicated by blockade, most probably open-channel blockade, as assessed by shortened open times (see McNicholas & Cannessa, 1997). The enhancing effect appeared to occur independently of channel blockade, because the two effects could be separated in time. This was particularly obvious with benzamil. In the combined presence of FMRFamide with any of the amiloride analogues tested, the net effect on the current response was the result of a balance between the potentiating and inhibitory effects of the drugs. This varied with the different drugs, EIPA and benzamil being more effective than amiloride in enhancing FMRFamide responses.

The potentiating effect of amiloride on FMRFamide responses of HtFaNaC contrasts with the related channel from *Helix*, where amiloride has so far been observed only to block the FMRFamide response of HaFaNaC (Lingueglia *et al.* 1995; Cottrell, 1997), as well as the neuronal channel (Green *et al.* 1994). The effects of benzamil and EIPA on HaFaNaC or on the neuronal response of *Helix* neurones have not yet been described.

Comparison of response of HtFaNaC expressed in oocytes with neuronal responses of the FMRFamide-gated channel

Although we have not yet established that the HtFaNaC clone obtained from a neuronal cDNA library partly underlies or is solely responsible for the FMRFamide-gated current recorded from the identified *Helisoma* neurones, GDN and LSN, most aspects of the fast inward current elicited in these neurones by FMRFamide were similar to those of expressed HtFaNaC. Responses of *Helisoma* neurones to FMRFamide were very similar to those of expressed HtFaNaC. The sensitivity of the whole-neurone response was comparable to that observed with the expressed clone, but occasionally the neuronal unitary responses appeared to be much more sensitive, with responses being observed with 1 μM FMRFamide (see below). Reduction in the extracellular Ca^{2+} and/or Mg^{2+} levels increased the amplitude of FMRFamide-activated unitary currents of the neurone and the clone similarly in a dose-dependent manner. Furthermore, the permeability of the expressed HtFaNaC was similar to that of the neuronal channel, as was the mean open time of the preponderant open state recorded. The rate of desensitization of the neuronal response was generally faster than that for expressed HtFaNaC, although it is difficult to quantify the difference between the neurone and oocyte because of the different modes of application necessarily used with each preparation. A similar difference in desensitization has been noted between HaFaNaC and the *Helix* neuronal response (Cottrell, 1997). Although the *Helisoma* neuronal response and that of the HtFaNaC were both enhanced and blocked by amiloride, benzamil and EIPA, enhancement was less obvious with the neuronal response than with HtFaNaC. The reason for this discrepancy is not clear; it could relate to differences between the blocking and enhancing activities of each drug on the neuronal channel, or to factors in the natural environment of the channel that increase its initial control responsiveness to FMRFamide, by perhaps evoking a higher activity state (see below).

On some occasions, the neuronal FMRFamide-gated channel did actually show a much higher sensitivity to FMRFamide. The reason for the variation in responsiveness to FMRFamide of the neurone is not known but may relate to different activity states of the channel, as shown in Fig. 3*D* and *E* (and K. A. Green & G. A. Cottrell, manuscript in preparation). The pronounced potentiation of the FMRFamide response of expressed HtFaNaC with EIPA and benzamil has drawn attention to one means by which the activity of HtFaNaC can be dramatically enhanced. EIPA may mimic some endogenous factor(s) that normally regulates activity of the channel. If such factors exist, it may be possible to detect them by comparing the effects of extracts of snail ganglia on FMRFamide responses of HtFaNaC and HtFaNaC expressed in *Xenopus* oocytes.

Do amiloride-like drugs potentiate other 'amiloride-sensitive' channels?

Benzamil has been shown to enhance as was well as block the effect of H^+ on isolated neurones from rat dorsal root ganglia (Green *et al.* 2000). Further, although amiloride at 200 μM reduced the fast component of the H^+ response of the ASIC3 clone expressed in COS7 cells, the small slow component of the H^+ response appeared to be increased in amplitude (Waldmann *et al.* 1997). This effect of amiloride on the ASIC3 channel was not examined further by these workers, however. Adams *et al.* (1999) showed that 100 μM amiloride can potentiate a small inward current activated by 100 μM Zn^{2+} on a mutant channel derived from BNC1 (i.e. BNC1 G430C; BNC1 is also known as MDEG). These workers proposed the existence of two different interaction sites for amiloride. Our data too are best explained in terms of amiloride, EIPA and benzamil each interacting at more than one site on the channel protein because: (a) the enhancing and blocking effects could be separated in time, (b) there was marked variation in the degree of enhancement compared to blockade with each of the different drugs and (c) whereas the enhancing effect is independent of membrane voltage, the blocking effect is voltage dependent being more effective at positive patch-electrode potentials (K. A. Green & G. A. Cottrell, unpublished data; and see McNicholas & Canessa, 1997). The observation that amiloride and related drugs can exert more than one effect on such ENaC/DEG channels may be relevant with respect to the question of whether their persistent anti-hypertensive effect is due to their diuretic action alone (Brody *et al.* 1994).

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