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Neuropeptide FF and FMRFamide Potentiate Acid-Evoked Currents from Sensory Neurons and Proton-Gated DEG/ENaC Channels

Candice C. Askwith,* Chun Cheng,† Mutsuhiro Ikuma,* Christopher Benson,† Margaret P. Price,† and Michael J. Welsh*†‡§

*Howard Hughes Medical Institute
†Department of Internal Medicine
‡Department of Physiology and Biophysics
University of Iowa College of Medicine
Iowa City, Iowa 52242

Summary

Acidosis is associated with inflammation and ischemia and activates cation channels in sensory neurons. Inflammation also induces expression of FMRFamide-like neuropeptides, which modulate pain. We found that neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe amide) and FMRFamide (Phe-Met-Arg-Phe amide) generated no current on their own but potentiated H^+-gated currents from cultured sensory neurons and heterologously expressed ASIC and DRASIC channels. The neuropeptides slowed inactivation and induced sustained currents during acidification. The effects were specific; different channels showed distinct responses to the various peptides. These results suggest that acid-sensing ion channels may integrate multiple extracellular signals to modify sensory perception.

Introduction

FMRFamide (Phe-Met-Arg-Phe amide) and related peptides comprise a family of neuropeptides that are abundant in many invertebrates, including *Caenorhabditis elegans* (Nelson et al., 1998), *Aplysia californica* (Greenberg and Price, 1992), and *Drosophila melanogaster* (Schneider and Taghert, 1988). In these organisms, FMRFamide-like neuropeptides act as neurotransmitters and neuromodulators. At least one gene encoding FMRFamide-related peptides is present in mammals; it produces neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe amide) and neuropeptide AF (A18Famide) (Perry et al., 1997; Vilim et al., 1999). Although FMRFamide itself has not been discovered in mammals (Yang et al., 1985), administration of FMRFamide induces a variety of physiologic effects, including alterations in blood pressure, respiratory rate, glucose-stimulated insulin release, and behavior (Mues et al., 1982; Sorenson et al., 1984; Kavaliers and Hirst, 1985; Raffa et al., 1986; Kavaliers, 1987; Telegdy and Bollók, 1987; Thiemermann et al., 1991; Muthal et al., 1997; Nishimura et al., 2000). In mammals, FMRFamide and neuropeptide FF also modify the response to painful stimuli, and neuropeptide FF is induced by inflammation (Tang et al., 1984; Yang et al., 1985; Raffa and Connelly, 1992; Kontinen et al., 1997; Vilim et al., 1999). When FMRFamide or neuropeptide FF is injected intracerebroventricularly, it elicits hyperalgesia and a reduction in morphine-induced analgesia (Tang et al., 1984; Yang et al., 1985; Kavaliers, 1987; Raffa, 1988; Brussaard et al., 1989; Roumy and Zajac, 1998). In addition, FMRFamide-like immunoreactive material is released in mammals following chronic morphine administration, and anti-FMRFamide antibodies can enhance morphine's effects (Tang et al., 1984; Devillers et al., 1995). However, when administered intrathecally, these peptides can have an analgesic effect thought to be mediated through opioid receptors (Raffa, 1988; Raffa and Connelly, 1992; Gouardéres et al., 1993; Roumy and Zajac, 1998).

Some effects of FMRFamide and neuropeptide FF appear to be mediated through opioid receptors; these effects are blocked by the opioid antagonist naloxone (Kavaliers and Hirst, 1985; Kavaliers, 1987; Raffa, 1988; Gouardéres et al., 1993; Roumy and Zajac, 1998). Yet, other effects of FMRFamide and FMRFamide-related peptides are independent of opioid receptors and are insensitive to naloxone (Gayton, 1982; Raffa et al., 1986; Kavaliers, 1987; Raffa, 1988; Allard et al., 1989; Roumy and Zajac, 1998). In mammals, the nonopioid receptor(s) for FMRFamide and related peptides has not been identified, and it is not known how these peptides modulate pain sensation. However, the discovery of a FMRFamide-activated Na^+ channel (FaNaCh) in the mollusc *Helix aspersa* (Lingueglia et al., 1995) provided a clue that similar receptors might exist in mammals.

Unlike many neuropeptide receptors, FaNaCh is an ion channel gated directly by its peptide ligand, FMRFamide (Lingueglia et al., 1995). The neuropeptide receptor FaNaCh is a member of the DEG/ENaC family of channels. DEG/ENaC channels are homo- or heteromultimers composed of multiple subunits (Bassilana et al., 1997; Lingueglia et al., 1997; Coscoy et al., 1998; Waldmann and Lazdunski, 1998). Each subunit contains two transmembrane domains separated by a large, extracellular, cysteine-rich domain and cytosolic N and C termini (Waldmann and Lazdunski, 1998). DEG/ENaC channels are not voltage gated and are cation selective (usually $Na^+ > K^+$). FaNaCh is the only known DEG/ENaC channel that acts as a neuropeptide receptor. Other members of this family are involved in mechanosensation, salt taste, and epithelial Na^+ absorption (Schild et al., 1995; Snyder et al., 1995; Lindemann, 1996; Mano and Driscoll, 1999). Although a mammalian FaNaCh has not yet been isolated, mammals do possess multiple DEG/ENaC family members. Interestingly, one subset of this channel family, the acid-sensing ion channels, has been postulated to play a role in sensory perception and may, like FMRFamide, play a role in pain perception (Waldmann and Lazdunski, 1998). The acid-sensing DEG/ENaC channels respond to protons and generate a voltage-insensitive cation current when the extracellular solution is acidified.

The tissue acidosis associated with inflammation, infection, and ischemia causes pain (Reeh and Steen, 1996). Acidosis also generates proton-dependent transient and sustained Na^+ currents in cultured sensory

§To whom correspondence should be addressed (e-mail: mjwelsh@blue.weeg.uiowa.edu).

neurons (Krishtal and Pidoplichko, 1981; Davies et al., 1988; Akaike et al., 1990; Kovalchuk Yu et al., 1990; Bevan and Yeats, 1991; Akaike and Ueno, 1994). Although the molecular identity of the channels responsible for these currents is unknown, they have been hypothesized to be acid-sensing members of the DEG/ENaC protein family based on their ion selectivity, voltage insensitivity, and expression pattern (Bassilana et al., 1997; Lingueglia et al., 1997; Waldmann et al., 1997a, 1997b; de Weille et al., 1998; Babinski et al., 1999). The acid-sensing ion channels include the brain Na^+ channel 1 (BNC1, also known as MDEG, BNaC1, and ASIC2) and its differentially spliced isoform, MDEG2 (Price et al., 1996; Waldmann et al., 1996; García-Anoveros et al., 1997; Lingueglia et al., 1997); the acid-sensing ion channel (ASICα, also known as BNaC2 and ASIC1) and its differentially spliced isoform, ASICβ (Waldmann et al., 1997b; Chen et al., 1998); and the dorsal root acid-sensing ion channel (DRASIC, also known as ASIC3) (Waldmann et al., 1997a; de Weille et al., 1998; Babinski et al., 1999). BNC1, MDEG2, ASICα, and DRASIC are expressed in the central nervous system (Lingueglia et al., 1997; Waldmann et al., 1997b; Chen et al., 1998; Olson et al., 1998). ASICα, ASICβ, DRASIC, and MDEG2 are expressed in sensory neurons of the dorsal root ganglia (DRG) (Waldmann et al., 1997a; Chen et al., 1998; Olson et al., 1998; Babinski et al., 1999).

We hypothesized that FMRFamide or FMRFamide like-neuropeptides might modulate pH-dependent currents in DRG neurons. This hypothesis was based on the ability of FMRFamide and related peptides to modulate pain perception, the potential connections between painful stimuli and the acid-gated currents in DRG neurons, and the sequence similarity between FaNaCh and the acid-sensing ion channels expressed in sensory ganglia.

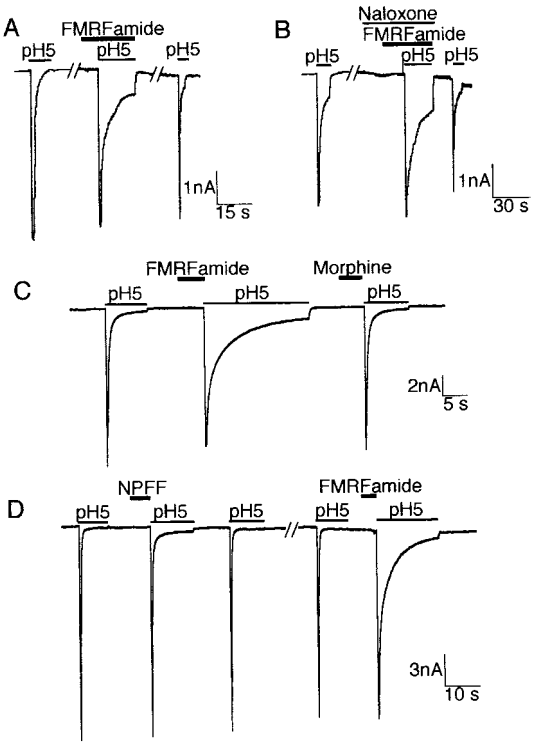

Figure 1. Proton-Gated Currents in Rat DRG Neurons Are Modulated by FMRFamide

(A) Trace of proton-gated whole-cell current; FMRFamide (100 μM) and pH 5 solution were present in bath during time indicated by bars. Unless otherwise indicated, pH was 7.4; n = 8.

(B) Naloxone (100 μM) was present during time indicated by bar; n = 3.

(C) Morphine (50 μM) and FMRFamide (50 μM) were added as indicated; n = 3.

(D) Neuropeptide FF (NPFF) (50 μM) and FMRFamide (50 μM) were present at times indicated by bar; n = 5.

Results

FMRFamide Modulates Proton-Gated Current in Rat DRG Neurons

We used whole-cell patch-clamp recordings to investigate the effect of FMRFamide on proton-gated currents in cultured rat DRG neurons. As previously reported (Krishtal and Pidoplichko, 1981; Akaike et al., 1990; Kovalchuk Yu et al., 1990; Akaike and Ueno, 1994), acidification to pH 5 produced rapidly activating and inactivating currents in the sensory neurons (Figures 1A–1D). FMRFamide added alone generated no response from any of the neurons tested. However, after FMRFamide addition (50–100 μM), the inactivation of proton-dependent currents slowed, and in many neurons there was a sustained current in the continued presence of acid (Figures 1A and 1B). The presence of the neuropeptide immediately before acidification also altered inactivation (Figures 1C and 1D); we examine this further below.

Some effects of FMRFamide are thought to be mediated through activation of opiate receptors (Raffa, 1988; Roumy and Zajac, 1998). To discern whether this might account for potentiation of the proton-gated currents, we tested the effect of naloxone, an opiate antagonist, and morphine, an opiate agonist. Naloxone did not block the effect of FMRFamide (Figure 1B), and morphine did not mimic it (Figure 1C). These results suggested that FMRFamide was not acting through opioid receptors to alter current.

We also tested the mammalian FMRFamide-like neuropeptide FF. Neuropeptide FF modulated currents in a manner similar to that of FMRFamide; it generated no current on its own, but it altered inactivation of proton-gated DRG currents (Figure 1D). The effects, however, were smaller than those generated by FMRFamide (Figure 1D).

Effect of FMRFamide on Acid-Sensing Ion Channels

Members of the DEG/ENaC family are thought to be at least partially responsible for the acid-gated currents in the DRG. This notion is based on the findings that H^+-gated currents from DRG and DEG/ENaC channels, although not identical, share similar pH sensitivity, ion selectivity, and inactivation (Davies et al., 1988; Bevan and Yeats, 1991; Bassilana et al., 1997; Lingueglia et al., 1997; Waldmann et al., 1997a, 1997b; de Weille et al., 1998; Babinski et al., 1999). The differences may be due to as yet unidentified subunits and/or heteromultimeric complexes. Because FMRFamide affected DRG

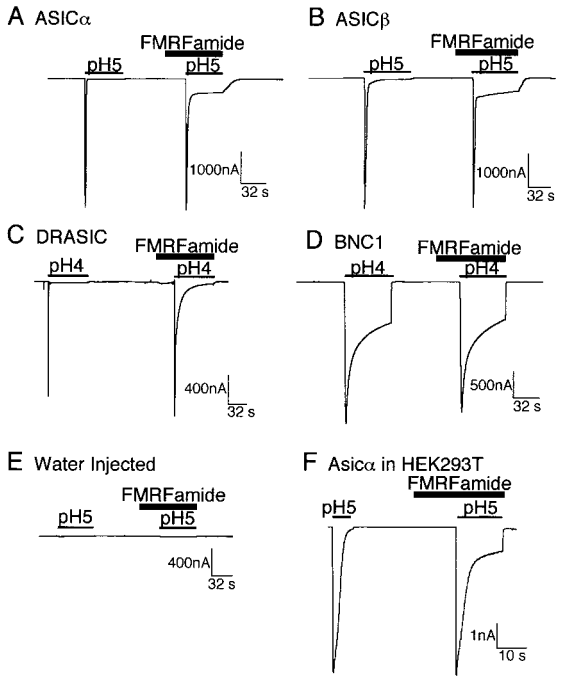

Figure 2. Effect of FMRFamide on H^+-Gated DEG/ENaC Family Members

Data are representative traces from *Xenopus* oocytes expressing ASICα (A), ASICβ (B), DRASIC (C), or BNC1 (D); from water-injected oocyte (E); or from HEK293T cells expressing ASICα (F). Unless otherwise indicated, extracellular pH was 7.4. FMRFamide (50 or 100 μM) and pH 5 solution were present in extracellular solution during time indicated by bars. Experiments were repeated at least seven times.

currents, we reasoned that FMRFamide might alter acid-gated DEG/ENaC channels. To test this hypothesis, we expressed mammalian acid-sensitive ion channels in *Xenopus* oocytes and measured the resulting currents. ASICα and its alternatively spliced variant, ASICβ, generated rapidly inactivating currents when the extracellular pH was lowered from 7.4 to 5 (Figures 2A and 2B). In contrast to its effect on FaNaCh, FMRFamide alone had no effect on either channel. However, subsequently lowering pH in the presence of FMRFamide potentiated the current; Figures 2A and 2B show slowing of inactivation and the appearance of a sustained current at pH 5 in both ASICα and ASICβ. DRASIC showed a similar response in the presence of FMRFamide (Figure 2C); following a reduction in pH, inactivation was slowed, and a sustained current was more apparent. In contrast, the acid-gated currents from oocytes expressing BNC1 were not discernibly altered by FMRFamide (Figure 2D). Neither pH nor FMRFamide in any combination produced current in control, water-injected oocytes (Figure 2E).

FMRFamide also altered the function of ASICα expressed in the human cell line HEK293T (Figure 2F). Acidic extracellular solutions induced rapidly inactivating whole-cell currents. In the presence of FMRFamide, inactivation slowed, and a sustained current was apparent. The effect of FMRFamide on current from acid-gated channels expressed in *Xenopus* oocytes and mammalian cells mimicked that observed in DRG neurons. This similarity suggested that these DEG/ENaC

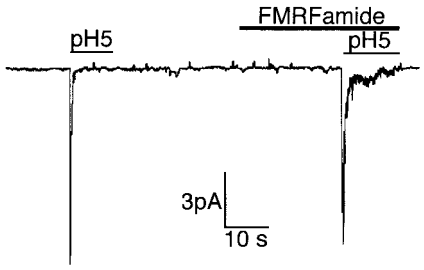

Figure 3. FMRFamide Modulates ASICα Function in Excised, Outside-Out Patches

Tracing is representative of H^+-dependent currents recorded from HEK293T cells transfected with ASICα. FMRFamide (100 μM) and pH 5 solution were present in extracellular solution during time indicated by bars; otherwise, pH was 7.4; n = 6.

channels may be responsible, at least in part, for proton-gated currents in neurons. For further studies, we focused on ASICα since it has been the most extensively studied, it is localized in nociceptive neurons of the DRG (Olson et al., 1998), and it produced a stable sustained current with FMRFamide addition.

We also tested the effect of FMRFamide on ASICα in excised, outside-out patches from HEK293T cells. Figure 3 shows that lowering the extracellular pH activated transient currents. In the presence of FMRFamide, inactivation was slowed substantially. These data suggest that FMRFamide interacts with ASICα.

Sequence of Adding FMRFamide and Acidification

In cells expressing ASICα, the presence of FMRFamide before and during acidification induced a sustained current (Figure 4Aiii). The continued presence of FMRFamide did not prevent channel closure when pH was returned to 7.4 (Figure 4Aiii). Thus, FMRFamide could neither activate nor sustain the current; rather, it modulated acid-activated current. This stands in sharp contrast to FaNaCh, which opens in response to FMRFamide alone and not acid (Lingueglia et al., 1995). The sequence of acid and FMRFamide application was important. The largest sustained currents required FMRFamide addition before lowering the extracellular pH; simultaneous addition of FMRFamide and acid (Figure 4Avi) or addition of FMRFamide after acid (Figure 4Aiv) was much less effective. In contrast, when we first applied FMRFamide at pH 7.4 and then washed away the FMRFamide while simultaneously lowering pH, a sustained current still ensued (Figure 4Av). With ASICα expressed in HEK293T cells, the maximal sustained current also required addition of FMRFamide prior to acidification (Figures 4Bii and 4Biii); application of FMRFamide after the pH reduction failed to induce large, sustained current (Figure 4Biv). Therefore, modulation required FMRFamide addition at pH 7.4, when the channel was closed.

It seemed surprising that FMRFamide could generate a sustained current, even when it was removed while the pH was being lowered (Figures 4Av and 4Biii). We observed similar behavior with acid-evoked currents in DRG cells (Figures 1C and 1D). To investigate this further, we examined the effect of removing FMRFamide from

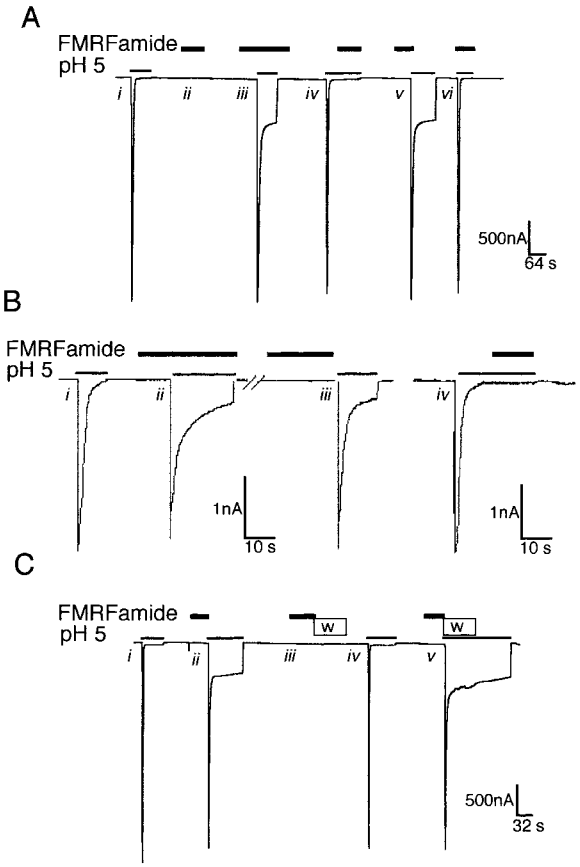

Figure 4. Effect of Order of FMRFamide and Acid Addition

Data are whole-cell currents from *Xenopus* oocytes expressing ASICα (A and C) (n = 5 each) or HEK293T cells expressing ASICα (B) (n = 8). Roman numerals indicate specific interventions referred to in text. pH was 7.4, unless otherwise indicated. FMRFamide (50 or 100 μM) and pH 5 solution were present in bath during times indicated by bars. In (C), cell was continuously perfused with solution, at pH 7.4 or pH 5, for 80 s during time indicated by box.

the bath solution at either pH 7.4 or pH 5. FMRFamide was applied at pH 7.4, and then the bath was continuously washed for 80 s (Figure 4Diii). After this time, acidification generated no sustained current (Figure 4Div). This result suggests that during the 80 s wash, the peptide dissociated or the effect reversed. However, when the pH was reduced while simultaneously removing FMRFamide, the sustained current persisted throughout an 80 s pH 5 wash and beyond (Figure 4Dv). These results suggest that the effect of FMRFamide is only reversible at pH 7.4; once the channel has been activated by acid, the effect of FMRFamide is retained until the pH is returned to 7.4.

Properties of the Current Generated by pH and FMRFamide

FMRFamide concentrations around 1 μM induced detectable sustained currents in cells expressing ASICα (Figure 5A). Maximal levels of sustained current were achieved at ~250 μM FMRFamide. The FMRFamide concentration that induced half-maximal sustained currents was ~33 μM. This concentration is higher than that reported for FaNaCh (2 μM) (Lingueglia et al., 1995).

We asked whether FMRFamide alters the properties of ASICα transient currents and whether the FMRFamide-generated sustained current has properties different from the transient current. Figures 5B and 5C show that the FMRFamide-induced sustained current was inhibited by amiloride in oocytes and HEK293 cells. Figure 5D shows that FMRFamide did not alter the pH sensitivity of the transient current. The FMRFamide-induced sustained current, however, showed sensitivity to a broader pH range compared with the transient current. This broader range of sensitivity might allow a more graded pH response of the FMRFamide-bound channel. This may have implications for the perception of acid-evoked pain, since sustained currents are thought to play a role in pH-dependent nociception (Bevan and Geppetii, 1994).

The current–voltage (I–V) relationship of the H^+-activated transient current of ASICα showed cation selectivity similar to what has been reported previously (Waldmann et al., 1997b); the relative permeabilities were $Na^+/Li^+ = 0.95 \pm 0.06$ and $Na^+/K^+ = 6.76 \pm 0.40$. The slope conductance was similar for all of the cations. The I–V relationship of the peak current was not altered in the presence of FMRFamide (Figure 5E). The sustained current showed a somewhat different ion selectivity (Figure 5F); the relative permeability was $Na^+/Li^+ = 1.05 \pm 0.07$ and $Na^+/K^+ = 1.25 \pm 0.2$, and the slope conductivity sequence was $Na^+ \geq Li^+ > K^+$. The sustained current did not show Ca^{2+} conductance. Thus, FMRFamide did not alter the ASICα response to pH or the properties of the initial transient current. However, the sustained current showed a different cation selectivity and pH response.

Effect of FMRFamide-like Neuropeptides on ASICα

Since FMRFamide itself has not been found in mammals, we asked whether other FMRFamide-like peptides would more potently affect ASICα. We tested FMRFamide-like compounds that have been identified in mammals, including neuropeptide FF and A18Famide, which terminate with the sequence PQRFamide (Yang et al., 1985; Perry et al., 1997), and MERF (met-enkephalin-Arg-Phe), which ends with FMRF but lacks the amide. Neither A18Famide nor MERF altered ASICα current, and neuropeptide FF produced only minor effects on inactivation (Figure 6; see below). We tested several of the many neuropeptides terminating with RFamide that have been discovered in invertebrates (Schneider and Taghert, 1988; Greenberg and Price, 1992; Perry et al., 1997; Nelson et al., 1998). FLRFamide also induced a sustained current in ASICα, albeit less than did FMRFamide (Figure 6). N-terminal extensions of FLRFamide and other RFamide-containing peptides identified in invertebrates did not alter ASICα currents in the presence (Figure 6) or absence of acid. FMRF-OH did not induce a response, indicating that the C-terminal amide is required. These results are similar to the neuropeptide specificity observed for FaNaCh, which has been reported to respond only to FMRFamide and FLRFamide (Cottrell, 1997). We tested morphine to determine whether it could induce a sustained current and naloxone to see if it blocked FMRFamide-induced sustained current in *Xenopus* oocytes. Consistent with our results

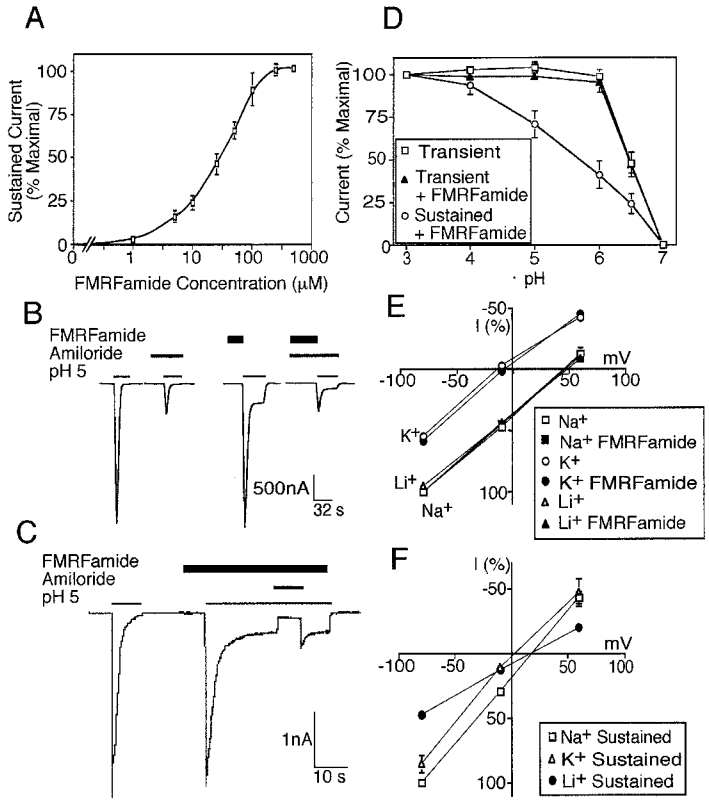

Figure 5. Properties of FMRFamide-Modulated ASICα Current

Data are from *Xenopus* oocytes (A, B, and D–F) or HEK293T cells (C) expressing ASICα.

(A) Effect of FMRFamide concentration on potentiation of H^+-dependent sustained current. Oocytes were exposed to indicated concentrations of FMRFamide prior to and during current activation with pH 5 solution. Measurements were normalized to the value of sustained current obtained with 500 μM FMRFamide. Data are mean ± SEM; n = 6–7.

(B) Effect of amiloride on FMRFamide and acid-induced sustained current. Amiloride (1 mM), FMRFamide (50 μM), and pH 5 are indicated by bars; n = 5.

(C) Amiloride (100 μM), FMRFamide (100 μM), and pH 5 are indicated by bars; n = 3.

(D) pH sensitivity of ASICα current with addition of FMRFamide. FMRFamide (50 μM) was added prior to acidification. Values were normalized to current obtained at pH 3 for the transient and the FMRFamide-modulated sustained current. Data are mean ± SEM; n = 7.

(E and F) I–V relationships of ASICα current measured at pH 5 in the presence and absence of FMRFamide (50 μM); extracellular bath solution containing 116 mM Na^+, K^+, or Li^+, as indicated. In these studies, the solution was changed from 116 mM Na^+ to the other cations ~30 s before acidification, and membrane voltage was stepped from a holding voltage of −60 mV to voltages of −80, −10, or +60 mV immediately before acidification. Currents from each cell were normalized to current obtained in the same cell at −80 mV in the Na^+ solution (100%) (E) or the sustained currents (F). Data are mean ± SEM; n = 8 cells for Na^+ solution and 4 cells for K^+ and Li^+ solutions.

in rat DRG (Figures 1B and 1C), neither morphine nor naloxone altered ASICα current (Figure 6).

Differential Effects of FMRFamide and FRRFamide

In an attempt to learn more about the peptide specificity of acid-gated channel modulation, we tested several FXRFamide peptides. One of these, FRRFamide, showed a pronounced specificity difference between acid-gated channels. With ASICα, equivalent concentrations of FRRFamide generated a sustained current similar to that produced by FMRFamide, although it was smaller in magnitude (Figure 7A). With ASICβ, FRRFamide markedly slowed the rate of inactivation without generating as large a sustained current as FMRFamide (Figure 7B). With DRASIC, both FRRFamide and FMRFamide slowed inactivation of the transient current and increased the sustained current, although equivalent concentrations of FRRFamide had larger effect on transient and sustained currents (Figure 7C).

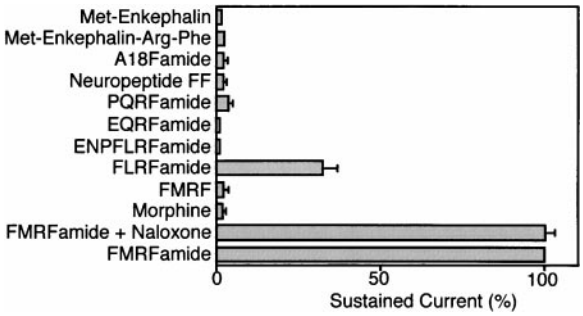

Figure 6. Effect of FMRFamide-like Peptides on ASICα Current

Oocytes expressing ASICα were exposed to indicated peptides, morphine sulphate, or naloxone prior to and during acidification to pH 5. All agents were tested at 50 μM and normalized to the response to FMRFamide (50 μM) obtained in the same cell, except for A18Famide (25 μM) and naloxone (500 μM). Naloxone was applied before the addition of FMRFamide. Data are mean ± SEM for five to eight cells assayed for each condition.

Neuropeptide FF Potentiates DRASIC Current

Differential modulation of the various acid-sensing ion channels by different peptides, and our finding that neuropeptide FF modulated DRG currents, suggested that we should test this mammalian neuropeptide on all of the acid-sensing channels. Figure 8A shows that adding neuropeptide FF prior to acidification slowed the inactivation of H^+-gated DRASIC currents. Interestingly, the kinetics of neuropeptide FF–induced potentiation were different from those induced by FMRFamide. Neuropeptide FF had subtle effects on ASICα currents, slowing inactivation but not generating appreciable sustained current (Figure 8B). ASICβ and BNC1 appeared unaffected by neuropeptide FF addition (data not shown).

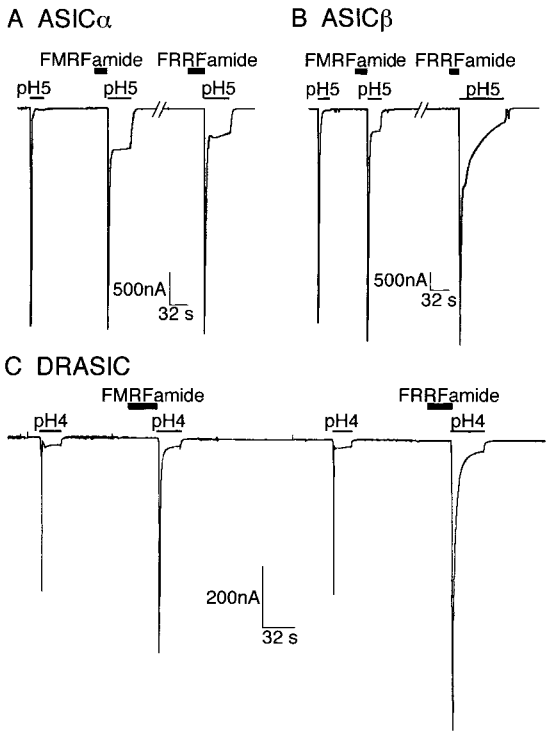

Figure 7. Effect of FMRFamide and FRRFamide on H^+-Gated DEG/ENaC Family Members Expressed in *Xenopus* Oocytes

(A and B) ASICα and ASICβ. FMRFamide (50 μM), FRRFamide (50 mM), and pH 5 solution were present in extracellular solution during time indicated by bars; n = at least 8.

(C) DRASIC. FMRFamide (100 μM), FRRFamide (100 μM), and pH 4 solution were present as indicated by bars; n = 6.

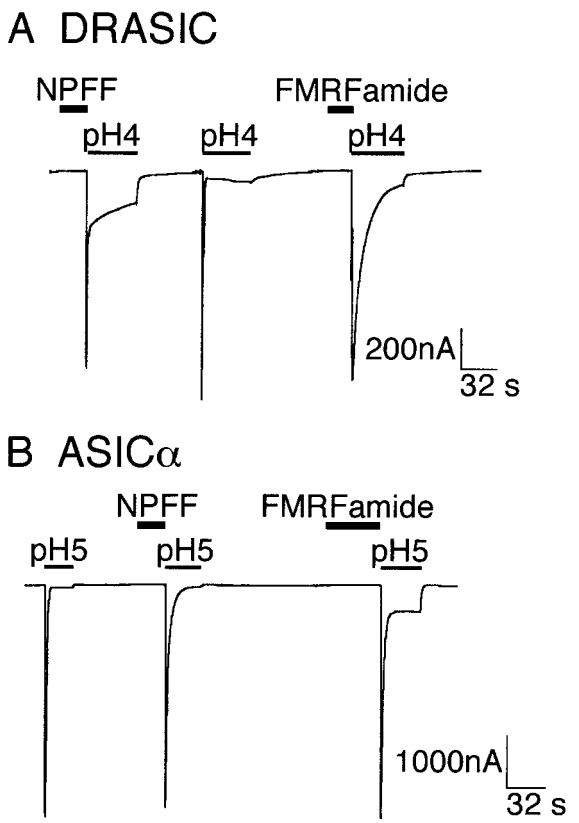

Figure 8. Effect of Neuropeptide FF on DRASIC and ASICα Expressed in *Xenopus* Oocytes

Neuropeptide FF (NPFF) (50 μM) and FMRFamide (50 μM) were present at times indicated by bars; n = 5.

Discussion

Our data indicate that FMRFamide-related neuropeptides potentiate currents from acid-sensing DEG/ENaC channels. Moreover, the localization of acid-sensing ion channels and FMRFamide-like peptides suggest the two may interact in vivo. Both DRASIC and neuropeptide FF are found in the DRG (Waldmann et al., 1997a; Chen et al., 1998; Allard et al., 1999). They are also both localized in the spinal cord and brain (Majane and Yang, 1987; Majane et al., 1989; Chen et al., 1998). Moreover, FMRFamide immunoreactivity that does not appear to be neuropeptide FF is found in DRG and brain (Ferrarese et al., 1986; Majane and Yang, 1987; Vilim et al., 1999). In this regard, it is interesting that FMRFamide was more potent than was neuropeptide FF in activating ASIC and DRG currents. We speculate that additional FMRFamide-related peptides await discovery.

Potentiation of Acid-Gated Currents by FMRFamide-Related Peptides

Earlier studies suggest that FMRFamide-like peptides can activate multiple types of receptors in mammals. These may include an opioid receptor, a G protein–coupled receptor that activates second messenger pathways, and other receptors that so far have remained unidentified (Kavaliers, 1987; Raffa and Connelly, 1992; Payza and Yang, 1993; Gherardi and Zajac, 1997; Nishimura et al., 2000). Our data indicate that mammalian members of the DEG/ENaC channel family also respond to FMRFamide-like peptides. There are at least three possible explanations. FMRFamide-related peptides might bind directly to acid-sensing channels, analogous to their interaction with FaNaCh. FMRFamide-related peptides might bind a receptor that triggers intracellular second messengers that modify the acid-sensing ion channels. Finally, FMRFamide-related peptides might bind a protein associated with acid-sensing ion channels, thereby altering their function. We favor the possibility of a direct interaction for the following reasons. First, the effect of FMRFamide was not mimicked by morphine or blocked by naloxone. Second, FMRFamide had the same effect on ASICα expressed in widely divergent cell types, *Xenopus* oocytes, and a human cell line. If the effect of FMRFamide were indirect, both cell types would have to express similar endogenous receptors coupled to similar second messenger systems. Third, in cells expressing the various individual acid-gated channels, FMRFamide, FRRFamide, and neuropeptide FF generated currents that were not only quantitatively different, but also, more importantly, qualitatively different. If these neuropeptides had different affinities for an unidentified endogenous receptor coupled to a second messenger, then only quantitative differences would be expected. Moreover, such a scenario would predict that

the quantitative effects would be similar for the different channels. This was not the case. Fourth, application of FMRFamide altered ASICα function in excised, outside-out patches of membrane. Experiments to unambiguously determine whether the effect of FMRFamide-related peptides is direct or indirect will require additional work.

The discovery that FMRFamide activated the molluscan FaNaCh showed that a peptide neurotransmitter could directly gate an ion channel (Lingueglia et al., 1995). Our data suggest that the same peptide interacts with the evolutionarily related ASIC and DRASIC channels. However, FMRFamide did not open these mammalian channels on its own; rather, it modulated the response to another agonist, protons. If FMRFamide interacts directly with the channels, these findings suggest that a FMRFamide-binding site has been at least partly conserved in these DEG/ENaC channels but that changes in structure have altered the consequences of the interaction. The alternatively spliced isoforms, ASICα and ASICβ, are identical over most of their length; however, the amino acid sequence from their N termini, through M1, and for a short distance (~100 amino acids) into the extracellular domain is not the same. Differences in the responses of ASICα and ASICβ to FMRFamide and FRRFamide suggest that the more N-terminal portions of ASIC contribute to neuropeptide modulation. That, plus the distinct interactions of FMRFamide and neuropeptide FF with FaNaCh and DRASIC, and the lack of a response with BNC1, provide a strategy and the reagents to investigate where and how these channels interact with FMRFamide and related peptides.

It is intriguing that FMRFamide should be applied before acid. We propose the following model. At pH 7.4, FMRFamide binds and is free to dissociate. However, when FMRFamide is bound at pH 7.4 and then pH is lowered, FMRFamide becomes trapped in the binding site. When the binding site is unoccupied, the channel inactivates rapidly, even in the continued presence of acid. However, when the binding site contains FMRFamide, channel inactivation is slowed and/or partially prevented. This scenario would explain two other observations. The limited ability of the peptide to alter current when applied after acidification could be explained by a conformational change at a low pH that occludes or hides the FMRFamide-binding site (Figures 4A and 4B). Trapping of FMRFamide within an occluded binding site at low pH would explain the continued generation of sustained currents, even after the peptide was removed from the bath (Figure 4D). This interpretation is consistent with our earlier observation that acid pH causes a conformational change in the related BNC1 channel that altered the extracellular solvent accessibility of a specific residue (Adams et al., 1998).

Physiologic Implications

It has been suggested that tissue ischemia and inflammation cause pain by stimulating H^+-gated cation currents (Reeh and Steen, 1996). The sustained component of those currents is thought to be particularly important (Bevan and Yeats, 1991; Lingueglia et al., 1997). Thus, the ability of neuropeptide FF and FMRFamide-related peptides to induce sustained currents suggests that such peptides and the acid-gated channels play a role in nociception. Interestingly, these peptides have been previously linked to pain perception in the spinal cord and brain. For example, chronic inflammation induces neuropeptide FF expression in the spinal cord (Kontinen et al., 1997; Vilim et al., 1999). FMRFamide-related peptides may also contribute to opiate tolerance, in which increasing amounts of opiates are required to achieve the same analgesic effect (Raffa, 1988; Roumy and Zajac, 1998). This may in part be explained by opiate-induced secretion of FMRFamide-related peptides from spinal cord neurons possibly inducing hypersensitivity of the nociceptive neurons (Tang et al., 1984).

Our data may also have implications for DEG/ENaC function in the brain. For example, intracerebroventricular injection of FMRFamide-related peptides induces a variety of physiologic responses (Mues et al., 1982; Sorenson et al., 1984; Tang et al., 1984; Kavaliers and Hirst, 1985; Yang et al., 1985; Raffa et al., 1986; Kavaliers, 1987; Thiemermann et al., 1991; Raffa and Connelly, 1992; Muthal et al., 1997; Roumy and Zajac, 1998). Recently, it was demonstrated that an amiloride analog inhibits FMRFamide-induced regulation of the brain renin–angiotensin system and hypertension (Nishimura et al., 2000). This suggests that these channels are a target of FMRFamide in the brain.

Proton-gated DEG/ENaC channels may function to integrate the response to acid and neuropeptides in the nervous system. Interestingly, another channel thought to be involved in nociception, the capsaicin receptor, also integrates multiple stimuli, heat and acidosis (Caterina et al., 1997; Tominaga et al., 1998). Thus, in neurons H^+-gated currents could vary, depending upon the type and combinations of DEG/ENaC subunits expressed and on the presence of different FMRFamide-like neuropeptides. The diversity of channel subunits and neuropeptides offers rich opportunities for interactions and new targets for pharmacotherapy.

Experimental Procedures

cDNA Constructs

Human ASICα was cloned from brain poly(A) RNA. Rat ASICβ and mouse DRASIC were cloned from DRG RNA. Human BNC1 was cloned as described (Price et al., 1996). Constructs were cloned into pMT3 for expression. The validity of the constructs was confirmed by DNA sequencing.

Cells and Expression Systems

Rat DRG neurons were cultured from Norway rats as described (Benson et al., 1999). Cells were allowed to incubate overnight at room temperature, and studies were done 1–2 days after isolation.

Expression of the cDNA constructs in *Xenopus* oocytes was accomplished by injection of plasmid DNA into the nucleus of defolliculated albino *Xenopus laevis* oocytes (Nasco, Fort Atkinson, WI) as described previously (Adams et al., 1998). Plasmids were injected at concentrations of 100 ng/μl for most experiments. Oocytes were incubated in modified Barth's solution at 18°C for 12–26 hr after injection. Cells injected with DRASIC were allowed to incubate for 24–48 hr before analysis.

HEK293T cells were a gift of Dr. Mark Stinski (University of Iowa). *ASIC*α cDNA was transfected into HEK293T cells using Transfast lipid reagents (Promega, Madison, WI). To identify transfected cells, pGreenlantern vector encoding green fluorescent protein (GIBCO, Gaithersburg, MD) was cotransfected with ASICα at a ratio of 1:6; transfected cells were identified using epifluorescence microscopy. Cells were studied 1–2 days after transfection.

Electrophysiological Analysis

Whole-cell currents in oocytes were measured using two-electrode voltage-clamp as described previously (Adams et al., 1998). Oocytes were bathed in frog Ringer solution containing, in mM: NaCl, LiCl, or KCl, 116; $CaCl_2$, 0.4; $MgCl_2$, 1; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 (pH 7.4). Acidic solutions were buffered with 5 mM 2-(4-morpholino)-ethanesulfonic acid (MES) instead of HEPES. Membrane voltage was held at −60 mV, unless otherwise noted. Most peptides and naloxone were obtained from Sigma (St. Louis, MO) and were added to the extracellular solution. The peptide FRRFamide was synthesized by Research Genetics (Huntsville, AL).

During whole-cell patch clamping of DRG neurons and transfected HEK293T cells, the cells were bathed with an extracellular solution that contained, in mM: NaCl, 128; $MgCl_2$, 5; $CaCl_2$, 1.8; KCl, 5.4; glucose, 5.55; and HEPES, 20 (pH 7.5 or 5). The pipette solution contained, in mM: KCl, 120; NaCl, 10; $MgCl_2$, 2; EGTA, 5; and HEPES, 10. Perfusion of cells with different solutions was done by placing the appropriate outlet in front of the cell. Data were recorded with an Axopatch 200 (Axon Instruments, Foster City, California) and stored on a digital tape recorder. Digitization was executed by acquiring data at 400 Hz using pClamp6 (Axon Instruments).

Excised, outside-out patches were obtained from transfected HEK293T cells. The bath solution contained, in mM: NaCl, 140; $MgCl_2$, 2; $CaCl_2$, 1.8; HEPES, 10 (pH 7.4), or Tris(hydroxymethyl)aminomethane (Tris) or MES (pH 5). The pipette solution contained, in mM: NMDG–Cl, 140; $MgCl_2$, 2; EGTA, 2; and HEPES, 10 (pH 7.4).

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