PEPTIDE POTENTIATION OF ACID-SENSORY ION CHANNEL IN PAIN

Inventors: Michael J. Welsh, Riverside, IA (US); Candice C. Askwith, Iowa City, IA (US)

Assignee: University of Iowa Research Foundation, Iowa City, IA (US)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

Appl. No.: 09/557,506

Filed: Apr. 25, 2000

Int. Cl.7 ......................... G01N 33/566; C12P 21/06; C12N 1/20; C07H 21/04; C07K 1/00

U.S. Cl. .......................... 435/7.21; 435/6; 435/4; 435/69.1; 435/252.3; 435/320.1; 530/300; 530/350; 536/23.5; 436/501

Field of Search ......................... 435/4, 7.21, 6, 435/69.1, 252.3, 320.1; 436/501; 530/300, 350; 536/23.5

References Cited

U.S. PATENT DOCUMENTS

FOREIGN PATENT DOCUMENTS
WO 00/08149 2/2000

OTHER PUBLICATIONS
Catarsi, S. et al., Neuropharmacology 41(592–600)2001.a


* cited by examiner

Primary Examiner—Yvonne Eyler
Assistant Examiner—Michael T Brannock

(74) Attorney, Agent, or Firm—McKee, Voorhees & Sease, P.L.C.

ABSTRACT

An assay for determining agonists, antagonists, or modulators for acid-sensing ion channels. The assay is especially useful for screening analogues. The screening assay can be provided in a kit form. The assay comprises administering the composition to be screened to cells expressing acid-gated channels and then determining whether the composition inhibits, enhances, or has no effect on the channels when acid is introduced. The determination can be performed by analyzing whether a current is sustained by the cells in the presence of the composition and the acid. This current can be compared to that sustained by the FMRFamide and related peptides.

11 Claims, 9 Drawing Sheets
Fig. 3

- pH5
- FMRFamide
- 3pA
- 10 s
Fig. 4A

Fig. 4B

Fig. 4C
**Fig. 8A**

**Fig. 8B**
PEPTIDE POTENTIATION OF ACID-SENSORY ION CHANNEL IN PAIN

BACKGROUND OF THE INVENTION


SUMMARY OF THE INVENTION

The present invention identifies a family of proteins that potentiates the effects of a group of acid-sensing ion channels (DEG/ENaC) which are responsible for pain associated with pain from ischemia and inflammation and certain other physiological effects.

An object of the invention is an assay for screening compositions which effect the acid-sensing ion channels.

Another object of the invention is an assay for screening analogues.

A further object of the invention is a kit which can be used for performing the assay.

Yet another object of the invention is drug compositions identified by the screening assay.
These and other objects, features, and advantages will become apparent after review of the following description and claims of the invention.

FRMFamide and FRMFamide-like peptides modulate acid-activated currents. The present invention provides an assay for screening compositions to identify those which are agonists, antagonists, or modulators of acid-sensing channels of the DEG/ENaC family. This assay can be especially useful for determining analogies. The assay comprises administering the composition to be screened to cells expressing acid-gated channels and then determining whether the composition inhibits, enhances, or has no effect on the channels when acid is introduced. The determination can be performed by analyzing whether a current is sustained by the cells in the presence of the composition and the acid. This current can be compared to that sustained by the FRMFamide and related peptides. This assay can also be provided in kit form.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1.** Proton-gated currents in rat DRG neurons are modulated by FRMFamide.

(A) Trace of proton-gated whole-cell current; FRMFamide (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 FRMFamide (50 μM) were added as indicated. N=3

(D) Neuropeptide FF (NPFF) (50 μM) and FRMFamide (50 μM) were present at times indicated by bar. N=6.

**FIG. 2.** Effect of FRMFamide 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), and from HEK-293T cells expressing ASICα (F). Unless otherwise indicated, extracellular pH was 7.4. FRMFamide (50 or 100 μM) and pH 5 solution were present in extracellular solution during time indicated by bars. Experiments were repeated at least 7 times.

**FIG. 3.** FRMFamide modulates ASICα function in excised, outside-out patches. Tracing is representative of H⁺-dependent currents recorded from HEK-293T cells transfected with ASICα. FRMFamide (100 μM) and pH 5 solution were present in extracellular solution during time indicated by bars; otherwise pH was 7.4. N=6.

**FIG. 4.** Effect of order of FRMFamide and acid addition. Data are whole-cell currents from *Xenopus oocytes* expressing ASICα (A, C) (n=5 each), HEK-293T cells expressing ASICα (B) (n=8). Roman numerals indicate specific interventions referred to in text. pH was 7.4 unless otherwise indicated. FRMFamide (50 or 100 μM), and pH 5 solution were present in bath during times indicated by bars. In panel D, cell was continuously perfused with solution, at pH 7.4 or pH 5, for 80 sec during time indicated by box.

**FIG. 5.** Properties of FRMFamide-modulated ASICα current. Data are from *Xenopus oocytes* (A, B, D-F) or HEK-293T cells (C) expressing ASICα.

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

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

(C) Amiloride (100 μM), FRMFamide (100 μM), and pH 5 are indicated by bars. N=3.

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

(E, F) Current-voltage relationships of ASICα current measured at pH 5 in the presence and absence of FRMFamide (50 μM). Extracellular bath solution containing either 116 mM Na⁺, K⁺, or Li⁺, as indicated. Membrane voltage was stepped from a holding voltage of ~60 mV to voltages of ~80, ~10, or ~40 mV immediately before acidification. 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.

**FIG. 6.** Effect of FRMFamide-like peptides on ASICα current. Oocytes expressing ASICα were exposed to indicated peptides, morphine sulphate, or naltrexone prior to and during acidification to pH 5. All agents were tested at 50 μM and normalized to the response to FRMFamide (50 μM) obtained in the same cell, except for A15Famide (25 μM) and naltrexone (500 μM). Naltrexone was applied before the addition of FRMFamide. Data are mean±SEM for 5 to 8 cells assayed for each condition.

**FIG. 7.** Effect of FRMFamide and FRMFamide on H⁺-gated DEG/ENaC family members expressed in *Xenopus oocytes*. (A, B) ASICα and ASICβ. FRMFamide (50 μM), FRMFamide (50 μM), and pH 5 solution were present in extracellular solution during time indicated by bars. N= at least 8. (C) DRASIC. FRMFamide (100 μM), FRMFamide (100 μM), and pH 4 solution were present as indicated by bars. N=6.

**FIG. 8.** Effect of neuropeptide FF on DRASIC and ASICα expressed in *Xenopus oocytes*. Neuropeptide FF (NPFF) (50 μM) and FRMFamide (50 μM) were present at times indicated by bars. N=5.

**DETAILED DESCRIPTION OF THE INVENTION**

The current invention utilizes the finding that FRMFamide and FRMFamide-like peptides directly modulate the acid-sensing ion channels. This finding can be used to determine compositions that will be useful in altering the response of these channels. Since these peptides and channels appear to have a role in nociception, compositions can be screened for inhibition of acid-sensing ion channels and antagonism of FRMFamide-related peptides to find new analogies. Also, since FRMFamide-related peptides can induce blood pressure effects, behavior effects, and insulin and somatostatin secretion effects, screening of compositions with inhibiting or enhancing effects of acid-sensing ion channels is expected to provide useful drugs which can regulate these physiological responses as well.

FRMFamide-related neuropeptides potentiate currents from acid-sensing DEG/ENaC channels. The localization of acid-sensing ion channels and FRMFamide-like peptides suggest the two may interact in vivo. Both DRASIC and neuropeptide FF are found in the DRG (Allard, M., Roussclot, P., Lombard, M. C., and Theodosius, D. T. (1999).

The discovery that FMRFamide activated the molluscan FaNaCh showed that a peptide neurotransmitter could directly gate an ion channel (Linguegila, et al. (1995)). Several studies suggested 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 (Gherardi, N., and Zajac, J. M. (1997). Neuropeptide FF receptors of mouse olfactory bulb: binding properties and stimulation of adenylyl cyclase activity. Peptides 18, 577–583; Kavaliers (1987); Nishimura, et al. (2000); Payza, K., and Yang, H. Y. (1993). Modulation of neuropeptide FF receptors by guanine nucleotides and cations in membranes of rat brain and spinal cord. J Neurochem 60, 1894–1899; Raffa and Connelly (1992). The data of the Examples below are the first indicating that mammalian members of the DEG/ENaC channel family also respond to FMRFamide-like peptides.

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. Neuropeptide FF and FMRFamide generate no current on their own, but potentiate H+-gated currents from cultured sensory neurons and heterologously expressed ASIC and DRASIC channels. The neuropeptides slow inactivation and induce sustained currents during acidification. The effects are specific; different channels show distinct responses to the various peptides. The results suggest that acid-sensing ion channels may integrate multiple extracellular signals to modify sensory perception. Evidence that FMRFamide directly modulates acid-sensing channel function includes the following:

(a) The effect of FMRFamide was not mimicked by morphine or blocked by naloxone.

(b) FMRFamide had the same effect on ASIC 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.

(c) In cells expressing the various individual acid-gated channels, FMRFamide, FRVRamide, and neuropeptide FF generated currents that were not only quantitatively different, but, more importantly, were also 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 receptor would predict that the quantitative effects would be similar for the different channels. This was not the case.

(d) Application of FMRFamide altered ASIC function in excised, outside-out patches of membrane in which the cytosol is not present.

The current data show that the FMRFamide or FMRFamide-like peptides interact with the ASIC and DRASIC channels which are evolutionarily related to the molluscan FaNaCh. However, FMRFamide did not open these mammalian channels on its own, rather it modulated the response to an agonist, protos. These findings show 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, ASICx 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 (approximately 100 amino acids) into the extracellular domain is not the same. Differences in the response of ASICx and ASICβ to FMRFamide and FRFRFamide suggest that the more N-terminal portions of ASIC contribute to the interaction with FMRFamide, whereas 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.


The data indicates that for the ASIC1a tested FMRFamide, FLRFamide, and FRFRFamide were the only FMRFamide-like peptides which could induce sustained currents. Therefore, the channels have neuropeptide specificity. FRFRFamide showed a marked specificity difference when tested with other channels. For ASIC3, FRFRFamide slowed the rate of inactivation without as large a sustained current as FMRFamide. For DRASIC, FRFRFamide and FMRFamide increased the sustained current though at equivalent concentrations FRFRFamide had a larger effect. The neuropeptide FF only had significant effects with DRASIC.

Though the details of the interactions of these peptides with the channels is not entirely clear, the following is suggested in addition to the above information regarding specificity. N-terminal extensions of RFamide-containing peptides did not appear to alter currents, and results indicated that the C-terminal amide is required for a response.

Additional FMRFamide-related peptides are expected to modulate acid-gated ion channels.

The foregoing and following information indicates an assay for screening compositions to identify those which are agonists, antagonists, or modulators of acid-sensing channels of the DEG/ENaC channel family. The determination of enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. In addition to the ASIC and DRASIC channels, it is expected that FMRFamide or FMRFamide related peptides will potentiate acid-evoked activity of other members of the DEG/ENaC cation channel family. The determination of enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. The determination of enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. Therefore, the assay can be used to determine composition(s) which inhibit enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. Therefore, the assay can be used to determine composition(s) which inhibit enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. Therefore, the assay can be used to determine composition(s) which inhibit enhancement or inhibition can be carried out by administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide orFMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family. Therefore, the assay can be used to determine composition(s) which inhibit
tivity and treat conditions such as pain associated with touch (e.g., pain associated with Herpetic neuralgia); modulate blood pressure; alter respiration; and alter tolerance to opioids to treat opioid addiction. Additionally, these channels are potentially involved in taste, particularly sour and salt taste. Agonists, antagonists, or modulators of these channels and FMRFamide-related agents could be used to inhibit or enhance specific taste sensations. Taste sensations could be altered in response to temperature as well since the activity of these channels is enhanced by cold temperature, or generally, agonists could be used to alter the perception of cold temperature. One of ordinary skill in the art would be able to determine how to screen for the desired effects.

Compositions which bind to the channels can be identified or designed (synthesized) based on the knowledge of FMRFamide potentiation of the channels and determination of the three-dimensional structure of the channels. These compositions could act as agonists, antagonists, or modulators affecting nociception or other physiological responses.

EXAMPLES

Methods and Materials
cDNA Constructs
Human ASICα was cloned from brain polyRNA. Rat ASICβ and mouse DRASIC were cloned from DRG RNA. Human BNC1 was cloned as described in Price et al. (1996) (Price, M. P., Snyder, P. M., and Welsh, M. J. (1996). Cloning and expression of a novel human brain Na⁺ channel. J Biol Chem 271, 7879–7882). Constructs were cloned into pMT3 for expression. The details of the constructions was confirmed by DNA sequencing.

Cells and Expression Systems

eDRG neurons were cultured from Norway rats as described in Benson et al. (1999) (Benson, C. J., Eckert, S. P., and McCleeskey, E. W. (1999). Acid-evoked currents in cardiac sensory neurons: A possible mediator of myocardial ischemic sensation. Circulation Research 84, 921–928). Cells were allowed to incubate overnight at room temperature and studies were done 1 to 2 days after isolation.

Expression of the cDNA constructs in Xenopus laevis oocytes was accomplished by injection of plasmid DNA into the nucleus of defolliculated albino Xenopus laevis oocytes (Nasco, Fort Atkinson, Wis.) as described previously (Adams, C. M., Snyder, P. M., Price, M. P., and Welsh, M. J. (1998). Protons activate brain Na⁺ channel 1 by inducing a conformational change that exposes a residue associated with neurodegeneration. J Biol Chem 273, 30204–30207). 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.

HEK-293T cells were a gift of Dr. Mark Stinski (Univ. of Iowa). ASICα cDNA was transfected into HEK-293T cells using Transfast lipid reagents (Promega, Madison, Wis.). To identify transfected cells, pGreenlantern vector encoding green fluorescent protein (Gibco, Gaithersburg, Md.) was co-transfected 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 a two-electrode voltage-clamp as previously described (Adams, C. M. et al. (1998)). Oocytes were bathed in frog Ringers solution containing, in mM: 116 NaCl, LiCl or KCl, 0.4 CaCl₂, MgCl₂, 5 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES), 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 Chemical Co. (St. Louis, Mont.) and were added to the extracellular solution. The peptide FMRFamide was synthesized by Research Genetics (Huntsville, Ala.).

During whole-cell patch-clamping of DRG neurons and transfected HEK-293T cells, the cells were bathed with an extracellular solution that contained, in mM: 128 NaCl, 5 MgCl₂, 1.8 CaCl₂, 5.4 KCl, 5.55 glucose, 20 HEPES, pH 7.4 or 5. The pipette solution contained, in mM: 120 KCl, 10 NaCl, 2 MgCl₂, 5 EGTA, 10 HEPES. 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, Calif.) and stored on a digital tape recorder. Digitization was executed by acquiring data at 400 Hz using pClamp6 (Axon Instruments, Foster City, Calif.).

Excised, outside-out patches were obtained from transfected HEK-293T cells. The bath solution contained, in mM: 140 NaCl, 2 MgCl₂, 1.8 CaCl₂, 20 HEPES at pH 7.4, or Tris(hydroxymethyl)aminomethane (Tris) or MES at pH 7.4. The pipette solution contained: 140 NMDG-Cl, 2 MgCl₂, 2 EGTA, 10 HEPES, pH 7.4.

Example 1

FMRFamide Modulates Proton-gated Current in Rat DRG Neurons

Whole-cell patch-clamp recordings were used to investigate the effect of FMRFamide on proton-gated currents in cultured rat DRG neurons. As previously reported (Akaike, N., and Ueno, S. (1994). Proton-induced current in neuronal cells. Prog Neurobiol 43, 73–83), acidification to pH 5 produced rapidly activating and inactivating currents in the sensory neurons of the DRG (FIGS. IA–D). FMRFamide added alone generated no response from any of the neurons tested. However, after FMRFamide addition (50–100 μM), the inactivation of proton-dependent current slowed, and in many neurons, there was a sustained current in the continued presence of acid (FIGS. IA and B). The presence of the neuropeptide immediately before acidification also altered inactivation (FIGS. IC, ID).

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, the effect of naloxone, an opiate antagonist, and morphine, an opiate agonist, were used. Naloxone did not block the effect of FMRFamide (FIG. 1B), and morphine did not mimic it (FIG. 1C). These results suggested that FMRFamide was not acting through opioid receptors to alter current.

The mammalian FMRFamide-like neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide) was also tested. Neuropeptide FF modulated currents in a manner similar to FMRFamide; it generated no current on its own, but it altered inactivation of proton-gated DRF currents (FIG. 1D). The effects, however, were smaller than those generated by FMRFamide (FIG. 1D).

Example 2

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. Therefore, it was reasoned that FMRFamide might have a direct effect on acid-gated DEG/ENaC channels. Mammalian acid-sensitive ion channels in Xenopus oocytes were expressed and the resulting currents were measured. ASICα and its alternatively spliced variant ASICβ generated rapidly inactivating currents when the extracellular pH was lowered from 7.4 to 5 (Figs. 2A, B). 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: Figs. 2A and 2B show slowing of inactivation and the appearance of a sustained current at pH 5 in both ASICα and ASICβ. DRAAC showed a similar response in the presence of FMRFamide (Fig. 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 (Fig. 2D). Neither pH nor FMRFamide in any combination produced current in control, water-injected oocytes (Fig. 2E).

FMRFamide also altered the function of ASICα expressed in the human cell line, HEK-293T (Fig. 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 channels may be responsible, at least in part, for proton-gated currents in neurons. Further studies focused on ASICα since it had 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.

Example 3

FMRFamide Modulates ASICα Current in Outside-out Membrane Patches

To test whether FMRFamide interacts directly with the channel, ASICα was expressed in HEK293 cells and current from excised, outside-out patches was recorded. Fig. 3 shows that lowering the extracellular pH activated transient currents. In the presence of FMRFamide, inactivation was slowed substantially. These data indicated that FMRFamide directly affects ASICα.

Example 4

Sequence of Adding FMRF Amide and Acidification

In cells expressing ASICα, the presence of FMRFamide before and during acidification induced a sustained current (Fig. 4Aii). The continued presence of FMRFamide did not prevent channel closure when pH was returned to 7.4 (Fig. 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 (Lingeglia, 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 (Fig. 4Av) or addition of FMRFamide at pH 7.4 and then washing away the FMRFamide while simultaneously lowering pH, a sustained current still ensued (Fig. 4Aav). With ASICα expressed in HEK-293T cells, the maximal sustained current also required addition of FMRFamide prior to acidification (Fig. 4Bii and 4Biii); application of FMRFamide after the pH reduction failed to induce large sustained current (Fig. 4Biv). Therefore, modulation required FMRFamide addition at pH 7.4 when the channel was closed.

FMRFamide could generate a sustained current, even when it was removed while the pH was being lowered (Fig. 4Av, Fig. 4Biii). Similar behavior was observed with acid-evoked currents in DRG cells (Figs. 1C and 1D). The effect of removing FMRFamide from the bath solution at either pH 7.4 or pH7.5 was examined. FMRFamide was applied at pH 7.4, and then the bath was continuously washed for 80 sec (Fig. 4Biii). After this time, acidification generated no sustained current (Fig. 4Biv). This result indicates that during the 80 sec wash, the peptide dissociated from the channel. However, when the pH was reduced while simultaneously removing FMRFamide, the sustained current persisted throughout an 80 sec pH 5 wash and beyond (Fig. 4Biv). 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

Example 5

Properties of the Current Generated by pH and FMRF Amide

FMRFamide concentrations around 1 μM induced detectable sustained currents in cells expressing ASICα (Fig. 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) (Lingeglia, et al. (1995)).

Whether FMRFamide alters the properties of ASICα transient currents and whether the FMRFamide-generated sustained current has properties different from the transient current was investigated. Figs. 5B and 5C show that the FMRFamide-induced sustained current was inhibited by amiloride in oocytes and HEK293 cells. Fig. 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 to 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, S., and Geppetti, J. (1994). Protons: small stimuli of capsaicin-sensitive sensory nerves. Trends Neurosci 17, 509–512).

The current-voltage (I–V) relationship of the H⁺-activated transient current of ASICα showed similar cation selectivity to what has been reported previously (Waldmann, et al. (1997)); the relative permeabilities were: Na⁺/Li⁺= 0.95±0.06, and Na⁺/K⁺=6.76±0.40. The slope conductance was similar for all the cations. The I–V relationship of the peak current was not altered in the presence of FMRFamide (Fig. 5E). The sustained current showed a somewhat different ion selectivity (Fig. 5E); the relative permeability was Na⁺/Li⁺=1.05±0.07 and Na⁺/K⁺=1.25±0.02, and the slope conductance selectivity was Na⁺/Li⁺=K⁺. The sustained current did not show Ca²⁺ 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.
Example 6

Effect of FMRFamide-like Neuropeptides on ASICα

Since FMRFamide itself has not been found in mammals, whether other FMRFamide-like compounds would more potently affect ASICα was investigated. FMRFamide-like compounds were tested that have been identified in mammals including neuropeptide FF and A18Famide, which terminate with the sequence PQRFamide (Perry, S. J., Huang, E. Y. K., Cronk, D., Bagust, J., Sharma, R., Walker, R. J., Wilson, S., and Burke, J. F. (1997). A human gene encoding morphine modulating peptides related to NPFF and FMRFamide, FEBS Lett 409, 426–430; Yang, et al. (1985)) and metenkephalin-Arg-Phe (MERF), which ends with FF but lacks the amide. Neither A18Famide nor MERF altered ASICα current, and neuropeptide FF produced only minor effects on inactivation rate but no sustained current (Fig. 6 and see below). Tests were conducted of several of the many neuropeptides terminating with RFamide that have been discovered in invertebrates (Greenberg, M. J., and Price, D. A. (1992). Relationships among the FMRF-amide-like peptides. Prog Brain Res. 92, 25–37; Nelson, L. S., Kim, K., Memmott, J. E., and Li, C. (1998). FMRFamide-related gene family in the nematode, Caenorhabditis elegans. Mol Brain Res 58, 103–111; Perry, et al. (1997). Schneider, et al. (1988). FLRFamide also induced a sustained current in ASICα, albeit less than FMRFamide (Fig. 6). N-terminal extensions of FLRFamide and other RFamide-containing peptides identified in invertebrates did not alter ASICα currents in the presence (Fig. 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 only respond to FMRFamide and FLRFamide (Cottrell, G. A. (1997). The first peptide-gated ion channel. J Exp Biol 200, 2377–2386). Morphine was tested 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 the results in rat DRG (Figs. 1B and 1C), neither morphine nor naloxone altered ASICα current (Fig. 6).

Example 7

Differential Effects of FMRFamide and FRRFamide

In an attempt to learn more about the peptide specificity of acid-gated channel modulation, several FXRFamide pep-

tides were tested. 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 (Fig. 7A). With ASICβ, FRRFamide markedly slowed the rate of inactivation, without generating as large a sustained current as FMRFamide (Fig. 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 (Fig. 7C).

Example 8

Neuropeptide FF Potentiates DRASIC Current

Differential modulation of the various acid-sensing ion channels by different peptides, and the finding that neuropeptide FF modulated DRG currents, suggested that this mammalian neuropeptide should be tested on all the acid-sensing channels. Fig. 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 (Fig. 8B). ASICβ and BNC1 appeared unaffected by neuropeptide FF addition (data not shown).

Having described the invention with reference to particular compositions, theories of effectiveness, and the like, it will be apparent to those of skill in the art that it is not intended that the invention be limited by such illustrative embodiments or mechanisms, and that modifications can be made without departing from the scope or spirit of the invention, as defined by the appended claims. It is intended that all such obvious modifications and variations be included within the scope of the present invention as defined in the appended claims. The claims are meant to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates to the contrary.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 5
<210> SEQ ID NO 1
<211> LENGTH: 4
<212> TYPE: FRM
<213> ORGANISM: Caenorhabditis elegans
<400> SEQUENCE: 1
Phe Met Arg Phe
1
<210> SEQ ID NO 2

What is claimed is:

1. A method for screening compositions to identify agonists, antagonists, or modulators of acid-sensing ion channels comprising administering the composition to be screened to cells expressing acid-gated channels in the presence of acid and FMRFamide or FMRFamide-related peptides, and determining whether the composition enhances or inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family.

2. The method of claim 1 wherein the determination of opening of the acid-sensing ion channels is via electrophysiological analysis.

3. The method of claim 2 wherein the electrophysiological analysis looks for a sustained current in the channels.

4. The method of claim 2 wherein the electrophysiological analysis looks for an inactivation of a current in the channels.

5. The method of claim 1 wherein the determination of opening of the acid-sensing ion channels is via a method selected from the group consisting of voltage-sensitive dyes, ion-sensitive dyes, and cell death assays.

6. The method of claim 1 wherein the acid-gated channels are selected from the group consisting of ASIC and DRASIC channels.

7. The method of claim 1 wherein the cells are selected from the group consisting of DRG neurons, Xenopus oocytes, HEK-293T cells, cultured cell lines, and central nervous system cells.

8. A method for screening compositions to identify analgesics comprising administering the composition to be screened to cells expressing acid-gated channels, and determining whether the composition inhibits the opening of the acid-sensing ion channels of the DEG/ENaC channel family in the presence of acid and FMRFamide or related peptides.

9. The method of claim 8 wherein the determination is via electrophysiological analysis.

10. The method of claim 9 wherein the electrophysiological analysis looks for inhibition or inactivation of sustained current in the channels.

11. The method of claim 8 wherein the determination of opening of the acid-sensing ion channels is via a method selected from the group consisting of voltage-sensitive dyes, ion-sensitive dyes, and cell death assays.

* * * * *