Endogenous Arginine-Phenylalanine-Amide-related Peptides Alter Steady-state Desensitization of ASIC1a*

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The acid-sensing ion channels (ASICs) are proton-gated, voltage-insensitive cation channels expressed throughout the nervous system. ASIC1a plays a role in learning, pain, and fear-related behaviors. In addition, activation of ASIC1a during prolonged acidosis following cerebral ischemia induces neuronal death. ASICs undergo steady-state desensitization, a characteristic that limits ASIC1a activity and may play a prominent role in the prevention of ASIC1a-evoked neuronal death. In this study, we found exogenous and endogenous arginine-phenylalanine-amide (RF-amide)-related peptides decreased the pH sensitivity of ASIC1a steady-state desensitization. During conditions that normally induced steady-state desensitization, these peptides profoundly enhanced ASIC1a activity. We also determined that human ASIC1a required more acidic pH to undergo steady-state desensitization compared with mouse ASIC1a. Surprisingly, steady-state desensitization of human ASIC1a was also affected by a greater number of peptides compared with mouse ASIC1a. Mutation of five amino acids in a region of the extracellular domain changed the characteristics of human ASIC1a to those of mouse ASIC1a, suggesting that this region plays a pivotal role in neuropeptide and pH sensitivity of steady-state desensitization. Overall, these experiments lend vital insight into steady-state desensitization of ASIC1a and expand our understanding of the structural determinants of RF-amide-related peptide modulation. Furthermore, our finding that endogenous peptides shift steady-state desensitization suggests that RF-amides could impact the role of ASIC1a in both pain and neuronal damage following stroke and ischemia.

Tissue acidosis accompanies many injuries such as inflammation, infection, seizures, ischemia, and stroke (1–3). Extracellular acidosis induces neuronal damage and death by activating ASIC1a (4–6). There are four acid-sensing ion channel (ASIC) 2 genes in mammals and at least six ASIC subunits expressed in neurons throughout the nervous system (7–9). ASIC subunits form homomultimeric or heteromultimeric channels with distinct biophysical characteristics (10–14). ASIC1a is the predominant subunit in the brain, and disruption of the Asic1 gene eliminates the vast majority of proton-gated current in central neurons (4, 11, 15). ASIC1a homomultimeric channels are permeable to calcium ions, a property thought to be crucial for ASIC1a-induced neuronal signaling and induction of neuronal death following acidosis (4, 5, 16, 17). In mice, pharmacologically preventing ASIC1a activation or disrupting the Asic1 gene drastically reduces neuronal death caused by cerebral ischemia (4, 6). Thus, ASIC1a represents a novel target for pharmacological design to limit or prevent stroke-induced brain damage in humans (17).

Compounds that alter ASIC function affect neuronal death during prolonged acidosis (4, 18, 19). In particular, PcTx1-containing venom from the West Indies tarantula, Plasmatopus cambridgie prevents ASIC1a activation by promoting steady-state desensitization (20, 21). Mild increases in proton concentration (approximate pH 7.2–6.9) cause ASIC1a channels to undergo steady-state desensitization and become unavailable for subsequent activation when the pH lowers to values that are normally sufficient to induce activation (pH < 6.8) (22). PcTx1 increases the pH sensitivity of steady-state desensitization and causes ASIC1a channels to desensitize at pH 7.4 (20, 21). Because desensitized channels fail to activate when the pH acidifies, PcTx1 limits ASIC1a activity. PcTx1 also limits neuronal damage following cerebral ischemia in mice and inhibits inflammatory pain and hyperalgesia (4, 6, 23). These results indicate that increasing the pH sensitivity of steady-state desensitization of ASIC1a can reduce pain perception, neuronal damage, and death following cerebral ischemia and suggest that modulation of steady-state desensitization can affect ASIC1a-dependent neuronal activity.

RF-amide-related neuropeptides affect ASIC desensitization following activation (14, 24–32). RF-amides represent a small family of neuropeptides that contain a C-terminal “arginine-phenylalanine-amide” consensus. RF-amides have a variety of functions, most of which are mediated through G protein-coupled receptors (33–36). However, some effects of RF-amides are thought to be due to direct action on amiloride-sensitive ASIC-like channels (37, 38). RF-amide-related neuropeptides limit desensitization following proton-induced activation of ASIC1a and acid-activated currents in neurons (14, 24, 28–32). Thus, in the presence of specific RF-amides, ASICs conduct current longer than in the absence of peptide. In the peripheral nervous system, this interaction is thought to accentuate pain perception (39). However, the connection between RF-amide-related peptides and ASIC1a activity in the central nervous system is not well understood.

Most studies of ASIC1a and its functional roles in vivo have focused on rodent ASIC1a. Yet mouse and human ASIC1a have

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2 The abbreviations used are: ASIC, acid-sensing ion channel; MES, 4-morpholineethanesulfonic acid; RF-amide, arginine-phenylalanine-amide.
amino acid differences within the large extracellular region of the channel, which controls pH sensitivity and desensitization (22, 40–43). In this study, we tested the hypothesis that these amino acid differences affect the biophysical characteristics of human and mouse ASIC1a. We determined that there are species-specific differences in the pH dependence and neuropeptide sensitivity of ASIC1a between mouse and human. Furthermore, we determined that not only desensitization following activation but also steady-state desensitization are modulated by exogenous and endogenous RF-amide peptides. Human ASIC1a was affected by a greater complement of endogenous peptides compared with mouse. These results suggest that the biophysical characteristics of human and mouse ASIC1a, although similar, are not identical and that modulation of human ASIC1a activity in vivo may be more complex than in rodents.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Construction and Expression in Xenopus Oocytes**—Human and mouse nucleotide sequence corresponding to GenBank™ accession numbers NM_001095 and NM_009597, respectively, were cloned into the pMT3 mammalian expression plasmid as described previously (24). Point mutations were introduced into the human ASIC1a-pMT3 expression plasmid using the Stratagene QuickChange® site-directed mutagenesis kit (Cedar Creek, TX). Mutagenic primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All ASIC cDNA constructs were confirmed by DNA sequencing at the Plant-Microbe Genomic Facility at the Ohio State University. Qiagen Midi or Maxi prep kits (Valencia, CA) were used to prepare plasmid DNA for oocyte injection.

Unfertilized oocytes were harvested from female *Xenopus laevis* purchased from *Xenopus* I (Dexter, MI), using standard procedures (44). Briefly, frogs were anesthetized with tricaine methane sulfonate; ovarian lobes were surgically isolated, and the follicle membranes were removed from the oocytes by treatment with 1.2 mg/ml of collagenase in Modified Barth’s solution lacking calcium (2.4 mM NaHCO₃, 88 mM NaCl, 15 mM HEPES, 1 mM KCl, 0.8 mM MgSO₄, 125 units/liter penicillin/streptomycin) for 2–3 h at room temperature (44). Healthy stage IV and V oocytes were sorted and stored in Modified Barth’s solution plus calcium (0.4 mM CaCl₂ and 0.3 mM Ca(NO₃)₂)₃. One to 3 h after isolation, oocyte nuclei were injected with pMT3-ASIC1a expression plasmids at 100 ng/μl. Data were acquired using an Oocyte Clamp OC-725 Amplifier (Warner Instruments, Hamden, CT), an AXON Digidata 1200 digitizer, and pCLAMP-8 software (Molecular Devices, Sunnyvale, CA). All experiments were done using frog Ringer’s solution (116 mM NaCl, 2 mM KCl, 5 mM HEPES, 5 mM MES, 2 mM CaCl₂, 1 mM MgCl₂) with a pH adjusted to the indicated levels using 1 N NaOH. Oocyte recordings were done in a modified RC-Z3 250-μl oocyte recording chamber (Warner Instruments, Hamden, CT). The solution exchange rate in the recording chamber was ~1 ml/s. FMRF-amide was purchased from Bachem Biosciences Inc. (King of Prussia, PA). FRRF-amide, NPFF (SQAAFLPQPF-amide), and human QRFP, also known as P518 and 26RFa (TSGPLGNAELNGYS-RKKGGFSFRF-amide), were synthesized by EZBiolab, Inc. (Westfield, IN). PcTx1-containing venom from *P. cambridgii* was purchased from SpiderPharm (Yarnell, AZ). Peptides and venom at the indicated concentrations did not change the pH of the extracellular solutions. Neither acid, peptide, nor venom at the indicated concentrations induced current in oocytes not injected with ASIC1a.

**pH Dose Response**—Basal pH was maintained at pH 7.4, and maximal current was established by applying 1 ml of saturating pH solution (pH 5.0) to the oocytes. During experiments, 1 ml of acidic test pH solution was applied to the oocyte, and protonated current was allowed to decay fully. Oocytes were then washed with 5 ml of pH 7.4 solution and allowed to recover for 2 min between pH applications. This amount of time, determined experimentally, allowed full recovery from desensitization of both mouse and human ASIC1a. Experimental test pH applications were flanked by saturating pH applications (pH 5.0) to minimize the impact of potential tachyphylaxis of proton-gated current. Peptides were applied at the designated concentrations in pH 7.4 solution 2 min before application of the activating pH solutions. For quantification, peak current amplitude was determined for each test pH and normalized to the average of the flanking pH 5.0 current amplitudes.

**Steady-state Desensitization**—Oocytes were maintained in a basal pH solution of 7.9 during experiments to ensure that no steady-state desensitization was occurring prior to pH 5.0 activation (20, 45). To induce steady-state desensitization, 1 ml of “conditioning” test pH was applied to the cells for 2 min followed by application of pH 5.0. Cells were then washed with 5 ml of pH 7.9 solution and allowed to recover for 2 min before the next conditioning and activating (pH 5.0) acid application. Maximal current was determined by application of pH 5.0 from a conditioning pH of 7.9 before and after test conditioning pH recordings to account for any tachyphylaxis of proton-gated current. Peptides were applied in pH 7.9 solution for 2 min, and then in conditioning pH solutions for another 2 min prior to pH 5.0 application. To analyze the concentration-dependent effects of RF-amide-related peptides, steady-state desensitization was studied at a single conditioning pH (6.8 for human ASIC1a, 7.0 for mouse ASIC1a), and the peptide concentration was varied. For experiments using PcTx1-containing venom, cells were maintained in basal pH 7.4. Venom was applied to cells in the conditioning pH at a 1:10,000 dilution for 4 min before and during activation.

**Data Analysis**—Data were analyzed using the Axon Clampfit 9.0 software. To measure the rate of channel desensitization, the decay phase of current was fitted to a single exponential equation, \( I = I_0 + k_1 \cdot e^{-t/\tau_d} \), and the \( \tau_d \) was calculated. Two-tailed Student’s \( t \) tests were done with paired or unpaired data (as indicated in the figure legends). A “p” value less than 0.05 was considered significant. The pH₉₅
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was calculated by fitting the data from individual oocytes using the equation $I/I_{\text{max}} = 1/(1 + \{(EC_{50}/[\text{peptide}])\}^n)$, where $n$ is the Hill coefficient; $EC_{50}$ is the peptide concentration inducing half of the saturating peptide effect ($I_{\text{max}}$).

RESULTS

Differential Sensitivity of Mouse and Human ASIC1a to Protons—Human or mouse ASIC1a was expressed in Xenopus oocytes, and two-electrode voltage clamp was used to compare the biophysical properties of whole-cell proton-gated currents. In oocytes expressing ASIC1a from either species, reducing the extracellular basal pH from 7.4 to 5.0 induced large inward currents (Fig. 1A) that desensitized similarly ($\tau_d = 3.01$ s for human ASIC1a and 3.27 s for mouse ASIC1a, $n = 7–13$, $p = 0.64$). However, application of sub-maximal acidic pH solutions to oocytes expressing either mouse or human ASIC1a revealed that the pH sensitivity of activation was subtly different between species (Fig. 1A). Although application of pH 6.7 solution to human ASIC1a induced only small currents ($5.4 \pm 1.3\%$ of pH 5.0, $n = 10$; $p = 9.7 \times 10^{-6}$ compared with human ASIC1a) (Fig. 1A). Furthermore, pH 6.0 solutions produced maximal currents in human ASIC1a ($101.5 \pm 5.4\%$ of pH 5.0, $n = 7$) but not in mouse ASIC1a ($79.8 \pm 4.1\%$ of pH 5.0, $n = 9$; $p = 0.006$ compared with human ASIC1a) (Fig. 1A). To determine the extent of the difference in pH-dependent activation between mouse and human ASIC1a, a detailed pH dose response was performed. Mouse ASIC1a was activated by smaller acidic shifts in pH than human ASIC1a but required more acidic pH values to plateau (Fig. 1B). Interestingly, the activation curves of mouse and human ASIC1a intersected near the pH of half-maximal activation ($pH_{0.5}$), which was not statistically different ($pH_{0.5}$ of human ASIC1a = 6.46 ± 0.04, $n = 10$, and $pH_{0.5}$ of mouse ASIC1a = 6.57 ± 0.05, $n = 6$; $p = 0.13$). The Hill coefficient for proton-induced activation of mouse ASIC1a was smaller than human ASIC1a, although the data were not statistically significant (Hill coefficient of human ASIC1a = 5.9 ± 1.0, $n = 10$, and mouse ASIC1a = 3.4 ± 1.0, $n = 6$; $p = 0.11$).

The fact that the pH dose-response curve was different between mouse and human ASIC1a suggested that pH sensitivity of other channel properties might also be different. To analyze the pH dependence of steady-state desensitization, we conditioned oocytes for 2 min with extracellular solutions ranging from pH 7.9 to 6.6 (the conditioning pH) and then activated proton-gated currents with the saturating pH of 5.0 (Fig. 1C) (22). Following the application of a conditioning pH of 7.0, the peak current amplitude of human ASIC1a pH 5.0-evoked currents was 78.9 ± 11.5% of maximal ($n = 13$). However, pH 7.0 conditioning of mouse ASIC1a resulted in a dramatic current reduction to 9.9 ± 2.2% of maximal current ($n = 27$; $p = 7.2 \times 10^{-10}$ compared with human ASIC1a). Quantification revealed that the pH sensitivity of steady-state desensitization was significantly different between mouse and human ASIC1a (Fig. 1D). The threshold for induction of steady-state desensitization was close to the normal physiological pH of 7.4 for mouse...
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A. a schematic of human, mouse, and APKDL (A274S/P285S/K291N, ΔD298, ΔL299) ASIC1a subunits. The extracellular domain is located between the two transmembrane domains (black squares with TM). White ovals indicate the location of amino acids in mouse ASIC1a that are different from human ASIC1a. Bottom shows the amino acid sequence surrounding the APKDL residues. Boxed residues in human ASIC1a were mutated to amino acids present in the mouse sequence. B, steady-state desensitization of APKDL. Top, representative traces of APKDL current evoked by pH 5.0 (as indicated by white bars above trace) following a conditioning pH of 7.9 or 7.0 (shaded bars above trace). Bottom, quantification of the pH dependence of steady-state desensitization of APKDL. Human, and mouse ASIC1a. I/I_{max} is the peak current amplitude evoked by pH 5.0 from the test conditioning pH normalized to peak current amplitude evoked by pH 5.0 from conditioning pH 7.9 (n = 6–27). C, pH-dependent activation of APKDL. Top, representative traces of APKDL currents evoked by extracellular acidic solutions of the indicated pH (shaded bars above trace). Bottom, pH dose response of APKDL, human, and mouse ASIC1a. I/I_{max} is the amplitude of peak current evoked by the test pH normalized to maximal peak current evoked by pH 5.0 (n = 6–11). Basal pH was maintained at 7.4 between acid applications. D, quantification of steady-state desensitization of mutant ASIC1a channels. I/I_{max} is pH 5.0-evoked peak current following conditioning at pH 7.0 normalized to pH 5.0-evoked peak current from conditioning pH 7.9 (n = 4–31). E, pH-dependent activation of mutant ASIC1a channels. I/I_{max} is peak current amplitude evoked by pH 6.7 normalized to peak current amplitude evoked by pH 5.0. Basal pH was maintained at 7.4 between acid applications (n = 3–9). *, p < 0.05; **, p < 0.02 compared with mouse ASIC1a using Student’s t test to examine statistical significance with unpaired data. Error bars are the mean ± S.E.

ASIC1a, as conditioning with pH 7.2 decreased pH 5.0-evoked current to 87.8 ± 6.6% (n = 19) maximal current. Steady-state desensitization of human ASIC1a was not observed after conditioning with pH 7.2 (106.2 ± 4.7%, n = 15; p = 0.04 compared with mouse ASIC1a), but a conditioning pH of 7.0 was sufficient to induce steady-state desensitization (Fig. 1D). The calculated half-maximal pH for induction of steady-state desensitization (pH_{0.50}) of mouse ASIC1a was 7.15 ± 0.01 (n = 12) and 6.91 ± 0.02 for human ASIC1a (n = 9; p = 9.3 × 10^{-12}). Taking into account the relative steepness of these curves, this change may represent a large functional difference in the pH sensitivity of steady-state desensitization.

Five Amino Acids Determine the Differences between Mouse and Human ASIC1a—There are 10 amino acid differences between mouse (GenBank™ accession number NM_009597) and human (GenBank™ accession number NM_001095) ASIC1a. Eight of these are located in the extracellular domain of the channel (Fig. 2A), which is known to be important for proton-induced activation and desensitization (22, 40, 43, 45). To determine which amino acids are responsible for the difference in mouse and human characteristics, we mutated residues in the extracellular domain of human ASIC1a to amino acids found in mouse ASIC1a. We found that human ASIC1a with mutations in five amino acids (A274S/P285S/K291N/ΔD298/ΔL299 or APKDL) completely mimicked mouse ASIC1a. Similar to mouse ASIC1a, a conditioning pH of 7.0 induced substantial steady-state desensitization of the APKDL mutant. For example, the pH 5.0 peak current amplitude after conditioning pH 7.0 was 6.0 ± 1.8% maximal current for APKDL (n = 14), 9.9 ± 2.2% for mouse ASIC1a (n = 27; p = 0.24 compared with APKDL), and 78.9 ± 11.5% for human ASIC1a (n = 13; p = 8.0 × 10^{-7} compared with APKDL).
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2B). Furthermore, the steady-state desensitization curve of APKDL overlapped with the mouse ASIC1a steady-state desensitization curve (Fig. 2B). The calculated pH_{50,d} value for APKDL steady-state desensitization was 7.19 ± 0.002 (n = 7), which was not different from mouse ASIC1a (7.15 ± 0.007, n = 12; p = 0.1 compared with APKDL) and was distinct from human ASIC1a (6.91 ± 0.02, n = 9; p = 1.5 × 10^{-7} compared with APKDL). The pH dose response of APKDL activation also mimicked mouse ASIC1a. The APKDL mutant channel displayed abundant current with pH 6.7 application (pH 6.7-evoked current was 40.2 ± 5.9% maximal current for APKDL, n = 7, 41.6 ± 5.8% for mouse ASIC1a, n = 10; p = 0.87 compared with APKDL, and 5.44 ± 1.3% for human ASIC1a, n = 10; p = 5.5 × 10^{-6} compared with APKDL) (Fig. 2C). APKDL also showed a pH-dependent activation curve similar to mouse ASIC1a with a pH_{50} of 6.57 ± 0.06 (n = 8). Mutations of human APKDL residues individually affected the pH dependence of activation and/or steady-state desensitization (Fig. 2, D and E). However, the conversion of any single APKDL residue in human ASIC1a did not completely recapitulate the characteristics of the mouse channel.

PcTx1-mediated Inhibition Is Dependent on the pH Sensitivity of ASIC1a Steady-state Desensitization—PcTx1 inhibits ASIC1a activity by increasing the pH sensitivity of steady-state desensitization, but it can also enhance pH-dependent activation if desensitization is not induced (20, 21, 46). Because we observed differences in the pH sensitivity of steady-state desensitization and activation, we hypothesized that PcTx1 may differentially affect human and mouse ASIC1a. We found that PcTx1 reduced pH 5.0-evoked currents in mouse ASIC1a following conditioning with either pH 7.4 or 7.2 (39.4 ± 16.0% compared with control for conditioning pH 7.4, n = 4, p = 0.004; 13.4 ± 7.3% for conditioning pH 7.2, n = 3, p = 0.02) (Fig. 3A). However, PcTx1 had no significant effect on human ASIC1a following conditioning with pH 7.4 (91.9 ± 12.1% compared with control, n = 3, p = 0.27) but reduced acid evoked current to 62.1 ± 10.1% following conditioning with pH 7.2 (n = 3, p = 0.003) (Fig. 3B). These results indicate that PcTx1 modulated both channels, but the effect of PcTx1 is pH-dependent. To determine whether the APKDL mutations are responsible for the divergence in PcTx1 activity, we examined the effects of PcTx1 on the human ASIC1a APKDL mutant channel. Similar to mouse ASIC1a, PcTx1 significantly reduced acid-evoked currents of APKDL at both pH 7.4 and 7.2 (41.6 ± 4.1% compared with control for conditioning pH 7.4, n = 3, p = 7 × 10^{-5}; 19.2 ± 8.1% for conditioning pH 7.2, n = 3, p = 0.018) (Fig. 3C). Taken together, these data support previous observations that PcTx1-mediated inhibition of ASIC1a is pH-dependent and suggest that PcTx1 action is linked to the proton sensitivity of steady-state desensitization.

RF-amide-related Peptides Differentially Affect Desensitization of Mouse and Human ASIC1a—RF-amide-related peptides modulate ASIC1a activity by limiting ASIC1a desensitization after activation (24). Previous data suggested that mouse and human ASIC1a may be differentially affected by RF-amide peptides (11, 24). To explore potential species differences in RF-amide peptide modulation of ASIC1a, we employed two exogenous peptides, FMRF-amide and FRRF-amide. FMRF-amide is a molluskan peptide that induces many of the same effects of endogenous mammalian peptides in vivo (47, 48). FRRF-amide is a synthetic peptide that strongly modulates proton-gated currents in mouse hippocampal neurons (11). As previously published, both peptides induced a small, persistent, acid-dependent sustained current following activation of human ASIC1a (44, 48). For example, the residual current remaining after activation and desensitization of human ASIC1a was 0.46 ± 0.13% of the peak current amplitude for control (n = 7), 1.95 ± 0.33% with FMRF-amide (n = 6; p = 0.001 compared with control), and 4.78 ± 1.16% with FRRF-amide (n = 6; p = 0.002 compared with control) (Fig. 4C). Neither peptide affected the rate of desensitization following activation of human ASIC1a as measured by the τ_d (Fig. 4D). In contrast, FRRF-amide increased the τ_d of mouse ASIC1a (Fig. 4, B and D) and induced
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RF-amide peptides differentially affect human and mouse ASIC1a desensitization following activation. Representative traces of human (A), mouse (B), and APKDL (E) currents evoked by pH 5.0 (as indicated by white bars above trace) with either no peptide (control), 50 μM FMRF-amide (black bars), or 50 μM FRRF-amide (gray bars). Peptides were added in the extracellular pH 7.4 solution 2 min prior to activation. C, peptide effects on acid-dependent sustained current. Sustained current during the plateau phase of proton-gated current was measured 30 s after pH 5.0-activated current and normalized to the peak current amplitude (n = 6–11). D, effect of peptides on the τd of desensitization (τd) for human, mouse, and APKDL. Data represent the τd of pH 5.0-evoked current in the presence of FMRF-amide or FRRF-amide (50 μM) normalized to the τd in the absence of peptide (n = 7–13). *p < 0.05; ** indicates p < 0.02 compared with control (no peptide) using Student’s t test with paired data. Error bars represent the mean ± S.E.

A pH-dependent sustained current in mouse ASIC1a (Fig. 4, B and C). However, FMRF-amide did not significantly affect the τd of mouse ASIC1a or alter the acid-dependent sustained current from the control (Fig. 4, B–D). Thus, FMRF-amide did not profoundly affect the characteristics of mouse ASIC1a. These results indicate that RF-amides differentially affect mouse and human ASIC1a. As reported previously, extracellular application of either FMRF-amide or FRRF-amide did not impact the peak current amplitude of pH 5.0-evoked currents in mouse or human ASIC1a. Mouse ASIC1a peak amplitude with FMRF-amide was 98.7 ± 8.8% pH 5.0 amplitude of control (n = 8; p = 0.6) and 105.0 ± 6.1% in the presence of FRRF-amide (n = 7; p = 0.6). Human ASIC1a with FMRF-amide was 92.7 ± 4.7% of control (n = 8; p = 0.16) and was 98.7 ± 4.1% with FRRF-amide (n = 17; p = 0.75).

To determine whether the five amino acids mutated in APKDL also confer the difference in peptide sensitivity of mouse and human ASIC1a, we tested the effects of FMRF-amide and FRRF-amide on APKDL (Fig. 4E). Similar to mouse ASIC1a, FRRF-amide increased the τd of APKDL. The acid-dependent sustained current was also increased by FRRF-amide (sustained current amplitude was 62.6 ± 0.18% of the peak current for control, n = 7, and 6.14 ± 2.05% of the peak current with FRRF-amide, n = 7; p = 0.03 compared with control) (Fig. 4, C and D). FMRF-amide did not impact either property of APKDL (Fig. 4, C–E). These results indicate that the APKDL residues in human ASIC1a influence FMRF-amide sensitivity as well as the pH dependence of steady-state desensitization.

RF-amides Modulate ASIC1a Steady-state Desensitization—Because both steady-state desensitization and RF-amide peptide modulation are different between mouse and human ASIC1a, we hypothesized that RF-amide peptides may also impact steady-state desensitization of ASIC1a. We tested the exogenous peptides FMRF-amide and FRRF-amide and found that both peptides inhibited steady-state desensitization of human ASIC1a current (Fig. 5A). For example, a conditioning pH of 6.8 induced steady-state desensitization of human ASIC1a such that only 10.6 ± 2.2% (n = 20) of the maximal current remained. When FRRF-amide or FMRF-amide was present prior to and during the conditioning pH 6.8, pH 5.0-evoked currents were substantially larger (73.6 ± 11.0% for FRRF-amide, n = 9; p = 1.5 × 10−8, and 73.2 ± 13.3% for FMRF-amide, n = 9; p = 3.4 × 10−7 compared with control). Detailed analysis of steady-state desensitization revealed that both FMRF-amide and FRRF-amide shifted the pH dependence of human ASIC1a such that more acidic pH values were required to induce steady-state desensitization (Fig. 5B). The presence of FRRF-amide shifted the pH0.5d of human ASIC1a from 6.91 ± 0.02 (n = 9) to 6.69 ± 0.02 (n = 4; p = 7.2 × 10−5). In the presence of FMRF-amide, the pH0.5d for human ASIC1a shifted to 6.77 ± 0.05 (n = 5; p = 0.003 compared with control).

FRRF-amide also limited steady-state desensitization of mouse ASIC1a (Fig. 5C). The pH 5.0-evoked current following conditioning pH 7.0 was 9.94 ± 2.2% of maximal current (n = 27), and with FRRF-amide it was 69.1 ± 5.5% (n = 12; p = 2.0 × 10−14 compared with control). Similar to human ASIC1a, FRRF-amide shifted the pH dependence of mouse ASIC1a steady-state desensitization to more acidic values (Fig. 5D), and the pH0.5d of mouse ASIC1a shifted from 7.15 ± 0.01 (n = 12) to 6.92 ± 0.04 in the presence of FRRF-amide (n = 8; p = 1.3 × 10−6 compared with control). However, FMRF-amide had no significant effect on steady-state desensitization of mouse ASIC1a (conditioning pH 7.0 with FMRF-amide = 11.3 ± 6.6%, n = 7; p = 0.8 compared with control) (Fig. 5, C and D). The pH0.5d of mouse ASIC1a with FMRF-amide was 7.16 ± 0.04 (n = 6; p = 0.87 compared with control). The APKDL mutations in human ASIC1a recapitulated the mouse ASIC1a response to peptides (Fig. 5, E and F). FRRF-amide decreased the pH sensitivity of steady-state desensitization and facilitated pH 5.0-evoked currents following conditioning pH 6.8 of APKDL (Fig. 5F). FRRF-amide had no affect on steady-state desensitization of APKDL (pH 5.0-evoked currents following conditioning pH 6.8 = 3.1 ± 0.9, n = 6; p = 0.91 compared with control) (Fig. 5F). These data suggest that the region containing the APKDL mutations determines which peptides impact the pH sensitivity of ASIC1a steady-state desensitization.

The concentration dependence of FMRF-amide or FRRF-amide modulation of ASIC1a steady-state desensitization was also assessed (Fig. 6). 10 μM FRRF-amide induced significant changes in peak current amplitude of human ASIC1a following conditioning with pH 6.8 (current amplitude of human ASIC1a following conditioning pH 6.8 conditioning = 22.0 ± 7.5% maximal current, n = 7, and 55.3 ± 11.8% with 10 μM FRRF-amide, n = 7; p = 0.002) (Fig. 6A). Quantification showed that concentrations greater than 1.0 μM of FRRF-amide were required to affect human ASIC1a, and the EC50 of FRRF-amide was 5.5 μM (Fig. 6B). Higher concentrations of FMRF-amide were required for modulation of human ASIC1a (peak current amplitude of
Application of pH 6.5 solutions to human ASIC1a normally induces 50.6 ± 6.1% of the maximal (pH 5.0-evoked) current (n = 11) (Fig. 7A). The pH 6.5-evoked human ASIC1a current following incubation with FMRF-amide was 61.9 ± 6.2% (n = 11; p = 0.2 compared with control) and with FRRF-amide was 38.9 ± 6.5% (n = 4; p = 0.3 compared with control) (Fig. 7A). A complete pH dose response revealed that FMRF-amide or FRRF-amide did not substantially affect the pH dose response of activation (Fig. 7A). The pH0.5 for activation was also not different with peptide incubation (pH0.5 for human ASIC1a = 6.49 ± 0.06, n = 7, pH0.5 with FMRF-amide = 6.53 ± 0.04, n = 10; p = 0.56 compared with control, and the pH0.5 with FRRF-amide was 6.41 ± 0.04, n = 4; p = 0.36 compared with control). Similar results were attained with mouse ASIC1a (pH0.5 for mouse ASIC1a = 6.61 ± 0.04, n = 6, pH0.5 with FMRF-amide = 6.47 ± 0.08, n = 6; p = 0.18 compared with control, and the pH0.5 with FRRF-amide = 6.52 ± 0.03, n = 5; p = 0.68 compared with control) (Fig. 7B). Thus, RF-amide-related peptides specifically affect the pH dependence of steady-state desensitization but have little impact on the pH sensitivity of activation.

FMRF-amide Competes with FRRF-amide to Affect Steady-state Desensitization of Mouse ASIC1a—FMRF-amide decreases the pH sensitivity of steady-state desensitization of mouse ASIC1a. However, FMRF-amide, a peptide that differs only by the presence of a methionine rather than an arginine, is ineffective. The fact that FMRF-amide did not impact steady-state desensitization could be explained by two possibilities. First, FMRF-amide may not bind to mouse ASIC1a. Second, FMRF-amide may bind but not affect steady-state desensitization. To evaluate these hypotheses, we tested whether FMRF-amide could compete with FRRF-amide and diminish the modulatory effect on mouse ASIC1a steady-state desensitization (Fig. 8). When steady-state desensitization was induced by a conditioning pH of 7.0, the pH 5.0-evoked current was 3.4 ± 1.1% of the maximal current (n = 13) (Fig. 8A–I). The application of 100 µM FMRF-amide had no effect on the steady-state desensitization (peak current amplitude following pH 7.0 conditioning pH in the presence of FMRF-amide = 4.9 ± 1.2% of the maximal current, n = 11; p =

The Impact of RF-amide-related Peptides on pH-dependent Activation—Modulators that shift pH sensitivity of steady-state desensitization often change the pH sensitivity of activation (18, 22, 49). Therefore, we tested the impact of FMRF-amide and FRRF-amide on the pH dose response of activation (Fig. 7).
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FIGURE 6. RF-amides impact steady-state desensitization in a concentration-dependent manner. Representative traces showing concentration-dependent effects of FMRF-amide and FRRF-amide (concentration indicated above trace) on steady-state desensitization of human (A) or mouse (C) ASIC1a. Current was evoked by pH 5.0 following conditioning with the indicated pH (shaded bars). Peptides were applied for 2 min at pH 7.9 (not shown) and during the 2-min conditioning pH at the concentrations indicated in the traces. Graphs show the concentration-response curves for peptide effects on steady-state desensitization of human ASIC1a evoked from a conditioning pH of 6.8 (Fig. 6A) or mouse ASIC1a evoked from a conditioning pH of 7.0 (Fig. 6D). Error bars represent the mean ± S.E.

0.39 compared with control) (Fig. 8A–ii). As expected, application of 10 μM FRRF-amide alone limited steady-state desensitization such that pH 5.0-evoked current following pH 7.0 conditioning was 27.8 ± 6.0% of the maximal current (n = 8; p = 8.1 × 10^-5 compared with control) (Fig. 8A–iii). However, when both 10 μM FRRF-amide and 100 μM FMRF-amide were applied, the pH 5.0-evoked current following a conditioning pH of 7.0 was only 3.9 ± 0.7% of the maximal current (n = 6; p = 0.78 compared with control and p = 0.005 compared with 10 μM FRRF-amide alone) (Fig. 8A–iv). Thus, the presence of FMRF-amide prevented FRRF-amide action. The inhibitory effect of FMRF-amide on FRRF-amide modulation of mouse ASIC1a was reduced when FRRF-amide concentration was increased to 50 μM (Fig. 8A–v and -vi). The pH 5.0-evoked current following a conditioning pH of 7.0 in the presence of 50 μM FRRF-amide was 50.8 ± 7.7% of the maximal current (n = 7), and when 100 μM FMRF-amide and 50 μM FRRF-amide were present, the current was reduced to 38.6 ± 10.0% (n = 7; p = 0.05 compared with 50 μM FRRF-amide alone). As a control, we assayed the effects of co-application of both peptides on human ASIC1a, a channel that is normally modulated by both peptides (Fig. 8B). We found that the addition of both FRRF-amide (10 μM) and FMRF-amide (10 μM) caused an additional change in steady-state desensitization of human ASIC1a compared with FMRF-amide alone (Fig. 8B). Thus, co-application of FMRF-amide and FRRF-amide does not nonspecifically prevent peptide modulation of ASIC1a. Together, these results suggest that FMRF-amide and FRRF-amide compete for binding to mouse ASIC1a and that FMRF-amide binding to mouse ASIC1a does not affect steady-state desensitization.

Mouse and Human ASIC1a Respond Differently to Endogenous RF-amide-related Peptides—FMRF-amide and FRRF-amide inhibit steady-state desensitization of human ASIC1a current by shifting the pH sensitivity such that more acidic pH solutions are required for desensitization. FMRF-amide and FRRF-amide are not present in mammals, but they often mimic the effect of endogenous RF-amide-related peptides on ASIC1a (24, 28, 29, 31, 32). To determine whether endogenous RF-amide-related peptides also inhibit steady-state desensitization, we tested the effect of neuropeptide FF (NPFF) and a second endogenous RF-amide called QRFP (50) on human and mouse ASIC1a (Fig. 9). As before, peptides were applied before and during conditioning with a pH chosen to give 80–90% desensitization in the absence of peptide (pH 7.0 for mouse and APKDL and pH 6.8 for human ASIC1a). We found that incubation with NPFF significantly limited steady-state desensitization of both mouse and human ASIC1a (Fig. 9, A and B). Specifically, pH 5.0-evoked currents of human ASIC1a following a conditioning pH of 6.8 were substantially larger when NPFF was applied (pH 5.0-evoked current was 14.4 ± 3.1% of maximal current for control, pH 5.0-evoked current with NPFF was 39.8 ± 5.8%, n = 8; p = 0.0008) (Fig. 9, A and B). Steady-state desensitization of mouse ASIC1a was also affected by NPFF (pH 5.0-evoked currents after conditioning with pH 7.0 were 10.8 ± 3.9% for control and 30.6 ± 8.1% with NPFF, n = 7; p = 0.02 using the paired t test) (Fig. 9, A and B). Treatment with QRFP had a similar effect on human ASIC1a steady-state desensitization (pH 5.0-evoked current following a conditioning pH of 6.8 was 8.6 ± 4.5 for control, n = 6, and with QRFP was 24.8 ± 9.1, n = 6; p = 0.03) (Fig. 9, A and B). However, QRFP did not impact mouse ASIC1a, and the peak current amplitude following a conditioning pH of 7.0 was unchanged (n = 6; p = 0.1) (Fig. 9, A and B). Neither peptide significantly affected the peak current amplitude of pH 6.5 nor pH 6.3-evoked currents suggesting that the pH dose response of mouse and human ASIC1a activation was unaffected by the presence of peptide (Fig. 9C). Endogenous peptides were also tested on the APKDL mutant channel (Fig. 9A). NPFF altered the pH dependence of steady-state desensitization such that 20.5 ± 4.2% of maximal current
**DISCUSSION**

Most studies focus on rodent ASIC1a and/or the role of ASIC1a in rodent models. Here we show that the biophysical properties of mouse and human ASIC1a are not identical. In particular, human ASIC1a requires more acidic pH values to induce steady-state desensitization compared with mouse ASIC1a. The apparent proton sensitivity of steady-state desensitization is further reduced by neuropeptides, and a larger number of neuropeptides affect human ASIC1a compared with mouse ASIC1a. Previous work comparing ASIC1a from fish and rodents found substantial differences in proton-dependent activation and desensitization following activation (41). These two properties were not dramatically different between mouse and human ASIC1a. We determined that substituting five amino acids in human ASIC1a with the amino acids found in mouse ASIC1a (A274S/P285S/K291N/ΔD298/ΔL299) resulted in a channel that mimicked all the characteristics of mouse ASIC1a. Interestingly, alignments of mammalian ASIC1a channels revealed that only primates contain all Ala-274, Pro-285, Lys-291, Asp-298, and Leu-299 residues (Fig. 10). These results suggest that the enhanced proton and neuropeptide sensitivity of human ASIC1a steady-state desensitization may be specific to primate species. These results also suggest caution when translating work from ASIC1a involvement in rodent models of ischemia and pain to humans. In particular, PcTx1 inhibition is pH-dependent (20, 21), and our data suggest that PcTx1-mediated inhibition is affected by the inherent proton sensitivity of ASIC1a. In our studies, PcTx1 did not robustly inhibit human ASIC1a when extracellular pH was 7.4. This difference may have important consequences for the use of PcTx1, or similar compounds, to prevent ASIC-mediated neuronal damage. Because PcTx1 also enhances the pH sensitivity of activation, it has the potential to profoundly potentiate ASIC1a current if desensitization is not induced (20, 21). Thus, studies in primate species (which likely possess ASIC1a channels with similar steady-state desensitization curves) will be invaluable to assess the potential neuroprotective aspects of PcTx1 to human populations.

The APKDL residues cluster within 25 amino acids in a region of the extracellular domain that has not previously been linked to ASIC1a function. How these residues specifically alter proton dependence of ASIC1a steady-state desensitization is unknown. However, the crystal structure of desensitized chicken ASIC1a was recently reported and may provide insight (51). Residues corresponding to proline 285, lysine 291, aspartic acid 298, and leucine 299 are present within a loop on the extracellular surface of the channel. This loop extends directly into the core of the desensitized ASIC1a channel where the residue corresponding to alanine 275 is located (serine 275 in chicken ASIC1a). In the desensitized chicken channel structure, serine 275 is very near glutamate 270 and 291 which are part of a negatively charged pocket thought to be important for proton-dependent gating (51). Based on the location of these residues, we suggest that amino acid substitutions in the APKDL residues alter the structure of the channel and allosterically affect the apparent proton affinity.

Our data suggest that the region containing APKDL is also involved in RF-amide modulation. Mutations in human ASIC1a

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**FIGURE 7. The impact of RF-amide peptides on the pH dose response of ASIC1a.** Representative traces show RF-amide peptide effects on human (A) or mouse (B) ASIC1a pH 6.5-evoked current. Current was evoked by either pH value indicated by **shaded bars above trace** in the presence or absence of peptide (100 μM). FMRF-amide (gray bars) or FRRF-amide (black bars) was applied in the extracellular solution (pH 7.4) for 2 min prior to activation. **Graphs** show the pH dose response of human (A) and mouse (B) ASIC1a in the presence or absence of peptide. $I_{\text{max}}$ is the peak current amplitude evoked by the test activating pH normalized to peak current evoked by pH 5.0 in each experimental condition (no peptide, FMRF-amide, or FRRF-amide) ($n = 2–11$). **Error bars** represent the mean ± S.E.

was evoked following conditioning with pH 7.0 (compared with 3.78 ± 1.2% when peptide was absent ($n = 3$, $p = 0.007$ versus control)) (Fig. 9B). QFRP had no significant effect on steady-state desensitization of APKDL (Fig. 9, A and B). Taken together, the data suggest that the APKDL residues also mediate the ASIC1a response to endogenous peptides. These data also show that endogenous RF-amide-related peptides are capable of modulating ASIC1a steady-state desensitization and suggest that the interaction of ASIC1a with endogenous RF-amide-related peptides may have physiological significance in vivo.
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A

Mouse

B

Human

FIGURE 8. FMRF-amide prevents FRRF-amide-induced inhibition of mouse ASIC1a steady-state desensitization. A, representative trace (top) and quantification (below) showing the impact of co-application of FMRF-amide and FRRF-amide on steady-state desensitization of mouse ASIC1a. Current was evoked by pH 5.0 (as indicated by white bars above trace) from the indicated conditioning pH (shaded bars). A-i, steady-state desensitization was induced by a conditioning pH of 7.0 for 2 min before activation with pH 5.0. A-ii, 100 μM FMRF-amide or 10 μM FRRF-amide (A-iii) was applied to oocytes for 2 min at pH 7.9 and in conditioning pH 7.0 for 2 min before activation with pH 5.0. Co-application of both peptides is shown in A-iv. 100 μM FMRF-amide was applied for 2 min at pH 7.9 (not shown) followed by a co-application of 100 μM FMRF-amide and 10 μM FRRF-amide (black bar with white dots) for 2 min. Steady-state desensitization was then induced by application of pH 7.0 solution containing both peptides at the same concentrations (indicated above trace) for 2 min before activation with pH 5.0 (A-iv). Graph shows quantification of peak current amplitudes evoked by pH 5.0 in each of the experimental conditions described in A-i-iv normalized to pH 5.0-evoked current from conditioning pH 7.9 in the absence of peptide. The response to 50 μM FRRF-amide alone (A-v) as well as co-application of 100 μM FMRF-amide and 50 μM FRRF-amide (A-vi) are shown (n = 6–13). B, representative traces showing the impact of co-application of FMRF-amide and FRRF-amide on human ASIC1a steady-state desensitization are shown at left. Experiments were done as described in A, except conditioning pH 6.8 was used to induce steady-state desensitization and 10 μM FMRF-amide and 10 μM FRRF-amide were used (n = 5). Right, quantification of the peak current amplitudes evoked by pH 5.0 in each of the experimental conditions described in B-i-iv normalized to pH 5.0-evoked current from conditioning pH 7.9 in the absence of peptide. *, p < 0.05; **, p < 0.02 compared with control (no peptide) using Student’s t test with unpaired data.

altered the complement of peptides that affected ASIC1a steady-state desensitization. Specifically, mouse ASIC1a was modulated by FRRF-amide and NPFF, but FMRF-amide and QRFP did not impact steady-state desensitization. Human ASIC1a was affected by FRRF-amide, FMRF-amide, NPFF, and QRFP. Substitution of the APKDL residues in human ASIC1a peptides decrease the pH sensitivity of ASIC1a steady-state desensitization. This action is in opposition to the PcTx1 peptide (20, 21). Both NPFF and QRFP decreased the pH sensitivity of steady-state desensitization of human ASIC1a. However, mouse ASIC1a was only affected by NPFF, suggesting that human ASIC1a may be more susceptible to RF-amide modula-
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Figure 9. Endogenous RF-amide-related peptides affect steady-state desensitization of ASIC1a. A, representative traces showing the impact of neuropeptide FF (NPFF) and QRFP (also known as PS1B and 26RFa) on steady-state desensitization of human ASIC1a, mouse ASIC1a, and APKDL. Current was evoked by pH 5.0 from the indicated conditioning pH solutions. Upper trace shows the impact of 100 nM NPFF (dark gray bars). Lower trace is the effect of 50 nM human QRFP (light gray bars). Peptides were applied at pH 7.9 for 2 min, and in the conditioning pH solutions for 2 min prior to activation with pH 5.0. B, quantification of endogenous peptide effects on steady-state desensitization following a conditioning pH of 6.8 for human or 7.0 for mouse and APKDL. I/I_{max} is the peak current amplitude evoked by pH 5.0 from the test conditioning pH normalized to peak current amplitude evoked by pH 5.0 from conditioning pH 7.9 in the absence of peptide (n = 6–8 for human ASIC1a, n = 6–7 for mouse ASIC1a, and n = 3–4 for APKDL). C, quantification of the impact of endogenous peptides on pH-dependent activation. Current was evoked by the indicated pH solutions from basal pH 7.4 in the presence or absence of NPFF (100 nM) and QRFP (50 nM). I/I_{max} is the peak current amplitude evoked by the test pH normalized to maximal peak current evoked by pH 5.0 (n = 6–12). None of the data in C were statistically different from control. Error bars represent the mean ± S.E. *, p < 0.05; **, indicates p < 0.02 compared with control (no peptide) using Student’s t test with unpaired data.

In vivo mouse ASIC1a, NPFF also limits desensitization of human ASIC1a following activation (24), an action that would further facilitate the consequences of ASIC1a activation. Although the concentration of peptide required for ASIC1a modulation is high (μM), such concentrations may be encountered within microdomains near the site of peptide release. In addition, we predict that other endogenous RF-amide-related peptides will impact steady-state desensitization of ASIC1a, possibly at lower peptide concentrations. Finally, it is known that neuropeptide concentration can change dramatically following inflammation, injury, or seizure (53–56), and RF-amide levels could become pathologically elevated. As such conditions also involve extracellular acidosis, the interaction between RF-amides and ASIC1a may facilitate ASIC1a activity throughout the nervous system in response to multiple pathophysiological conditions.

ASIC1a has recently been shown to contribute to inflammatory pain, and intrathecal administration of PcTx1 reduces inflammatory perception (57). NPFF also contributes to pain and has a pro-nociceptive action (33, 34). Both NPFF and ASIC1a are localized to the dorsal horn (33, 57, 58). In addition, NPFF and ASIC1a levels increase following inflammation (58, 59). NPFF is known to limit ASIC1a desensitization following activation, an action that would potentiate ASIC1a activity (24). Our data show that NPFF also limits steady-state desensitization of ASIC1a and suggest that modulation of ASIC1a steady-state desensitization by NPFF could enhance impact pain perception.

Our results could also provide insight into ASIC1a-mediated neuronal death following cerebral ischemia. The difference in proton sensitivity of steady-state desensitization between human and mouse ASIC1a could have several ramifications relevant to ischemia-induced neuronal death. In particular, the extracellular pH decreases gradually (min) after induction of ischemia (1, 60–62). This amount of time is sufficient to induce steady-state desensitization of ASIC1a, which limits ASIC1a activity and may prevent ASIC1a-induced neuronal death during ischemia and stroke (4, 6, 22). Therefore, the fact that human ASIC1a requires more acidic pH values for steady-state desensitization suggests that human channels may be less likely to desensitize before the extracellular proton concentration reaches the threshold for ASIC1a activation. This could result in a larger contribution of ASIC1a to neuronal death following ischemia in humans compared with rodents. However, other factors such as lactate, glutathione, and intracellular proton concentration may also affect the pH dependence of steady-state desensitization in vivo (18, 45, 63–66). These other modulators could either abrogate or enhance the differences between species. In addition, there are differences in the pH dependence of activation between mouse and human. At the extracellular pH, which is attained following ischemia in mice (pH 6.5), human and mouse ASIC1a produce currents with the same amplitudes. However, human ASIC1a produces less current at pH values greater than 6.5, and this decrease in activation could partially compensate for a loss in steady-state desensitization induction in vivo. Neuropeptide modulation of ASIC1a may also enhance neuronal death following ischemia. Both NPFF and QRFP are expressed in the brain and are found in areas containing high levels of ASIC1a protein (27, 34, 50). By limiting ASIC1a steady-state desensitization, RF-amides may promote ASIC1a activity during prolonged extracellular acidosis caused by ischemia or stroke and enhance ASIC1a-mediated neuronal death. The fact that human ASIC1a channels are affected by a larger complement of RF-amide-related peptides suggests that human ASIC1a chan-
n1els are sensitive to a broader array of neuropeptides in vivo and that peptide modulation may play a significant role in acidosis-induced neuronal death following ischemia in humans.

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