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Two Mechanisms Involved in Trigeminal CGRP Release: Implications for Migraine Treatment

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Abstract

Objective—The goal of this study was to better understand the cellular mechanisms involved in proton stimulation of calcitonin gene-related peptide (CGRP) secretion from cultured trigeminal neurons by investigating the effects of two anti-migraine therapies, onabotulinumtoxin A and rizatriptan.

Background—Stimulated CGRP release from peripheral and central terminating processes of trigeminal ganglia neurons is implicated in migraine pathology by promoting inflammation and nociception. Based on models of migraine pathology, several inflammatory molecules including protons are thought to facilitate sensitization and activation of trigeminal nociceptive neurons and stimulate CGRP secretion. Despite the reported efficacy of triptans and onabotulinumtoxinA to treat acute and chronic migraine, respectively, a substantial number of migraineurs do not get adequate relief with these therapies. A possible explanation is that triptans and onabotulinumtoxinA are not able to block proton mediated CGRP secretion.

Methods—CGRP secretion from cultured primary trigeminal ganglia neurons was quantitated by radioimmunoassay while intracellular calcium and sodium levels were measured in neurons via live cell imaging using Fura2-AM and SBFI-AM, respectively. The expression of ASIC3 was determined by immunocytochemistry and western blot analysis. In addition, the involvement of ASICs in mediating proton stimulation of CGRP was investigated using the potent and selective ASIC3 inhibitor APETx2.

Results—While KCl caused a significant increase in CGRP secretion that was significantly repressed by treatment with EGTA, onabotulinumtoxinA, and rizatriptan, the stimulatory effect of protons (pH 5.5) was not suppressed by EGTA, onabotulinumtoxinA, or rizatriptan. In addition, while KCl caused a transient increase in intracellular calcium levels that was blocked by EGTA, no appreciable change in calcium levels was observed with proton treatment. However, protons did significantly increase the intracellular level of sodium ions. Under our culture conditions, ASIC3 was shown to be expressed in most trigeminal ganglion neurons. Importantly, proton

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Category 1

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Category 2

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Category 3

- (a) Final Approval of the Completed Article Paul L. Durham

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stimulation of CGRP secretion was repressed by pretreatment with the ASIC3 inhibitor APETx2, but not the TRPV1 antagonist capsazepine.

Conclusions—Our findings provide evidence that proton regulated release of CGRP from trigeminal neurons utilizes a different mechanism than the calcium and SNAP-25 dependent pathways that are inhibited by the anti-migraine therapies rizatriptan and onabotulinumtoxinA.

Keywords

acid sensitive ion channels; calcitonin gene-related peptide; onabotulinumtoxinA; protons; trigeminal; triptans

Calcitonin gene-related peptide (CGRP) is expressed in trigeminal ganglia neurons that provide sensory innervation to the head and face, and transmit nociceptive signals to the central nervous system (CNS)^{1, 2}. Although the cellular mechanisms involved in the pathogenesis of migraine are still being investigated, several lines of evidence support a central role of CGRP in migraine pathology. CGRP levels in the cranial circulation as well as in saliva are increased during a migraine attack^{3–5}. Results from clinical studies have provided evidence that successful treatment of migraine headache pain with the 5-hydroxytryptamine_{1B/1D} agonist sumatriptan, as well as other triptan drugs, results in the normalization of CGRP levels^{4, 6, 7}. Additionally, while infusion of CGRP was shown to precipitate migraine attacks in susceptible individuals⁸, treatment with non-peptide CGRP receptor antagonists are efficacious as an abortive therapy of migraine^{9, 10}. Following nerve activation, CGRP is proposed to contribute to migraine pathology by promoting neurogenic inflammation following its perivascular release in the meninges, and promoting excitation of second order nociceptive neurons when released in the spinal trigeminal nucleus¹. Importantly, stimulated release of CGRP from the cell body is implicated in peripheral sensitization¹¹ while release from terminals in the spinal cord promotes central sensitization^{2, 12}, which are key physiological features associated with migraine¹³.

Based on models of migraine pathology, several inflammatory molecules including K⁺ ions, bradykinin, 5-HT, histamine, nitric oxide, and protons are thought to facilitate sensitization and activation of trigeminal nociceptive neurons and stimulate CGRP secretion^{14, 15}. Tissue acidosis associated with inflammation, ischemia, or injury leads to activation of primary nociceptive neurons¹⁶. The stimulatory effects of protons that results from a decrease in pH in the extracellular milieu can be mediated via activation of the non-selective cation channels transient receptor potential vanilloid-1 (TRPV1) and the acid-sensing ion channels (ASICs). The six different ASICs include ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4, which belong to the family of proton-gated Na⁺ channels that are formed by homomeric or heteromeric associations between the six ASIC isoforms^{17, 18}. Peripheral sensory neurons are reported to express all the ASICs with the exception of ASIC4^{17, 19–21}. Of the proton-responsive channels, TRPV1 and ASIC3 have been shown to colocalize with CGRP in rat trigeminal ganglia neurons²².

The 37 amino acid neuropeptide CGRP is synthesized in trigeminal ganglia neuronal cell bodies and stored in dense core vesicles to facilitate its secretion from afferent fibers as well as the soma^{23, 24}. Many inflammatory mediators known to cause excitation of trigeminal neurons and the subsequent release of CGRP function via a calcium-dependent process that involves soluble N-ethylamide-sensitive factor attachment protein receptor (SNARE) proteins to promote vesicle docking and fusion to the membrane^{25–27}. Significantly, the inhibitory effects of triptans, drugs commonly used as an abortive migraine therapy²⁸, and onabotulinumtoxinA, which was recently approved as a treatment for chronic migraine^{29–31}, are thought to involve blocking the stimulated release of CGRP from trigeminal neurons. While the inhibitory effect of the specific triptan, sumatriptan, involves blocking elevated

levels of intracellular calcium²⁴, onabotulinumtoxinA prevents vesicle docking by cleaving synaptosomal-associated protein of 25 kDa (SNAP-25)^{25, 27, 32, 33}. SNAP-25 associates with the cell membrane and interacts with the v-SNARE VAMP-2 that is expressed on the vesicle membrane to promote vesicle fusion and the release of neuropeptides and neurotransmitters^{34, 35}. Despite the reported efficacy of triptans and onabotulinumtoxinA to treat acute and chronic migraine, respectively, a substantial number of migraineurs do not get adequate relief with these therapies^{29–31, 36}. A possible explanation is that while triptans and onabotulinumtoxinA can prevent calcium- and SNARE-dependent release of CGRP, these agents are not able to block calcium and SNARE-independent neurotransmitter secretion, which has been reported in sensory neurons^{37, 38}.

Results from a previous study demonstrated that CGRP release from cultured trigeminal neurons could be stimulated via a decrease in extracellular pH with greatest stimulation at pH 5.5³⁹. In this study, the effect of treating trigeminal neurons with a triptan drug as well as onabotulinumtoxinA on CGRP secretion was investigated. In addition, the mechanisms involved in proton-mediated release of CGRP from primary trigeminal neurons were investigated using fluorescent microscopy to monitor changes in intracellular calcium and sodium levels. Results from our study demonstrate the existence of two different mechanisms by which CGRP release can be mediated from stimulated trigeminal neurons. Furthermore, we provide evidence that the stimulatory effect of protons on CGRP secretion is mediated via elevated intracellular levels of sodium and involves activation of ASIC3 ion channels.

METHODS

Animals

All animal experimental procedures were conducted in accordance with institutional and National Institutes of Health guidelines. Every effort was made to minimize animal suffering and reduce the number of animals used. Pregnant female Sprague–Dawley rats (200–250 g; Charles River, Wilmington, MA) were housed in clean plastic cages on a 12-h light/dark cycle with unrestricted access to food and water. Trigeminal ganglia from 3–5 day old neonatal rat pups were isolated and used to establish enriched neuronal cultures.

Materials

L15 medium contained 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 mM glucose, 250 μ M ascorbic acid, 8 μ M glutathione, 2 mM glutamine, and 10 ng/mL mouse 2.5 S nerve growth factor (Alomone Laboratories, Jerusalem, Israel). Penicillin (100 units/mL), streptomycin (100 μ g/mL), and amphotericin B (2.5 μ g/mL) were purchased from Sigma (St. Louis, MO) and added to the supplemented L15 media, which will be referred to as L15 complete medium.

The following chemicals for secretion, calcium, and sodium experiments were purchased from Sigma: potassium chloride (KCl), capsaicin, capsazepine, EGTA, and anti-goat IgG conjugated to horseradish peroxidase. Rizatriptan was kindly provided by Merck Pharmaceuticals (Whitehouse Station, NJ) and onabotulinumtoxinA was obtained from Allergan (Irvine, CA). The ASIC3 inhibitor APETx2 was purchased from Alomone Laboratories. For the calcium and sodium imaging studies, Fura-2 AM, 0.1% pluronic acid, and SBF1 AM were purchased from Invitrogen (Carlsbad, CA). The following antibodies were used in our study for immunohistochemistry and western blotting: primary goat polyclonal antibody against ASIC3 (Cat. No. NBP1-46288, Novus Biologicals, Littleton, CO), and secondary donkey anti-goat Alexafluor antibodies (Invitrogen) and donkey anti-goat IgG conjugated to horseradish peroxidase (Sigma).

Measurement of CGRP Secretion

Primary cultures of trigeminal ganglia were established based on our previously published protocols^{24, 40, 41}. Cells from ganglia isolated from 3–5 day old rats were resuspended in L15 complete medium. For secretion studies, dissociated cells from the equivalent of 24 ganglia were plated on 24-well poly-D-lysine coated tissue culture plates (BD Biosciences, Bedford, MA) and incubated at 37°C at ambient CO₂ for 24 h prior to measurements. To measure CGRP secretion, cells were incubated in 300 µl HBS (22.5 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM MgCl, 2.5 mM CaCl, 3.3 mM glucose, 0.1% BSA; pH 7.4), and the amount of CGRP released from trigeminal neurons into the culture media was determined using a specific CGRP radioimmunoassay as previously described^{24, 40, 41}. CGRP radioimmunoassays were performed according to manufacturer's instructions (Bachem, Torrance, CA). Briefly, standard curve and samples were incubated with a rabbit anti-CGRP primary antibody overnight prior to incubation with a recommended concentration of 125I-radiolabeled CGRP tracer. Standards and samples were immunoprecipitated by centrifugation following addition of goat anti-rabbit IgG and normal rabbit serum. The resultant radioactive pellets were quantitated using a Wizard 2470 gamma counter (Perkin Elmer). A standard curve was plotted on a semi-log scale after subtracting non-specific binding and normalizing to total binding for each standard. This curve was used to determine CGRP concentrations. The detection limit for CGRP was 2 pg/ml of sample. As a control, the basal (unstimulated) rate of CGRP secreted into the media in 1.5 h was determined, and these values were used to normalize for differences between wells. In some experiments, cells were stimulated with potassium chloride (KCl, final concentration of 60 mM diluted from a 3 M stock solution in dH₂O), capsaicin (1 µM final concentration from a 100 mM stock solution in DMSO) or with protons (HBS at pH 5.5) for 30 min. For the inhibitor studies, cultures were pretreated for 60 min with rizatriptan (20 µM in DMSO) or onabotulinumtoxinA (5 units in saline), or for 30 min prior to the addition of stimulatory agents with the selective ASIC3 antagonist APETx2 (300 nM in HBS)^{58,59}, or TRPV1 antagonist capsazepine (10 µM in DMSO). For the experiments designed to test the effect of extracellular calcium, cultures were incubated in standard HBS prior to the addition of the calcium chelator EGTA (10 µM in dH₂O). Media was collected after 1.5 h and assayed for the amount of CGRP. All pretreatment agents were diluted to final concentrations in HBS prior to addition to cultures. As a control, some cultures were treated with DMSO (0.1% final concentration), which was the vehicle used to dissolve capsaicin, capsazepine, and rizatriptan. To determine if any of the various treatments were cytotoxic, neuronal survival was assessed in trigeminal ganglion cultures immediately after treatment and 24 hours later using the trypan blue exclusion method as previously described.^{39, 40} No appreciable change in cytotoxicity (> 5% from control conditions) was observed following any treatment.

Measurement of Intracellular Calcium and Sodium

For calcium and sodium measurements, trigeminal cultures were enriched in neuronal cells (> 90%) by density-gradient centrifugation as previously described⁴¹. Cells were suspended in L15 complete medium and plated on glass coverslips at a density of 2 ganglion per well in a 24-well plate, which corresponds to 50,000–65,000 cells per well, and incubated at 37°C at ambient CO₂ for 24 h prior to the start of the experiment.

Intracellular calcium and sodium levels in neurons were determined essentially as described previously^{24, 41}. Cells were incubated in 300 µl HBS and 5 µM Fura-2 AM with 0.1% pluronic acid for 25–30 min at 37°C for intracellular calcium or perfusion buffer (PB; 136.5 mM NaCl, 3 mM KCl, 1.5 mM NaH₂PO₄, 1.5 mM MgSO₄, 10 mM D-glucose, 2 mM CaCl₂, 10 mM HEPES) and 10 µM SBFI AM with 0.1% pluronic acid for 50–60 min at 37°C for intracellular sodium measurements. Cells were washed twice and incubated for an additional 30 min in HBS or PB prior to analysis. Fluorescence was determined in neuronal

cells, identified by their unique size ($> 20 \mu\text{m}$) and morphology (round cell body) in addition to their measured response to KCl. In all studies, basal intracellular calcium or sodium levels were obtained for a minimum of 2 min before treatment and for as long as 20 min following final treatment. Cultures were initially treated with KCl (60 mM added directly to HBS for calcium imaging) or a media change from HBS pH 7.4 to HBS at pH 5.5 (protons) for calcium imaging or PB pH 7.4 to PB at pH 5.5 for sodium imaging experiments. Some cultures were pretreated with EGTA (10 μM) for 60 min in HBS prior to addition of stimulatory agents. As a control, some cultures were treated with an equal volume of DMSO. Calcium and sodium measurements were obtained at 340 and 380 nm every 2–8 sec on a heated stage at 37°C using a Hamamatsu C4742-95 camera mounted on a Leica DMI 6000 B fluorescent microscope with IP lab software, version 4.0 (North Central Instruments, St Louis, MO).

Immunohistochemistry

Immunohistochemical studies were conducted using untreated primary trigeminal cultures established similar to those used for intracellular calcium and sodium measurements or 14 μm sections of trigeminal ganglia as described previously⁴². For preparation of ganglia tissue, trigeminal ganglia were removed from rats following CO₂ asphyxiation and placed in a solution of 4% paraformaldehyde overnight. Following paraformaldehyde fixation, tissues were incubated in 15% sucrose in distilled water at 4°C for 1 hour and then 30% sucrose overnight at 4°C. Fourteen-micron longitudinal sections of the entire trigeminal ganglion tissue were serially prepared using a cryostat (Microm HM 525, Thermo Scientific, Waltham, MA) set at –20°C. Trigeminal ganglia tissues were then placed dorsal side up on Superfrost Plus microscope slides (FisherScientific, Pittsburg, PA), covered with OCT Compound (Sakura Finetek, Torrance, CA) and quickly frozen. Fixed cells or tissues were incubated for 15 minutes with 0.1% Triton X-100 in PBS to permeabilize cells prior to a 30 min blocking incubation in PBS containing 5% donkey serum. Cells or tissues were stained with donkey anti-goat polyclonal antibodies directed against ASIC3 (1:500 in PBS). Immunoreactive proteins were detected following incubation with anti-goat Alexafluor 488. As a control, some cultures were incubated with only the secondary antibody. All images were taken on a Zeiss Axiocam mRm camera mounted on a Zeiss Imager Z1 fluorescent microscope equipped with an ApoTome (Thornwood, NY). Phase contrast microscopy was used to identify neurons by morphology and size ($> 20 \mu\text{m}$ diameter round cell body) for cell counts. For analysis of primary neurons expressing ASIC3, twelve fields of cells were randomly chosen from three independent experiments done in duplicate. The data are reported as the number of neurons expressing ASIC3 divided by the total number of neurons identified by their unique size and nuclear morphology in DAPI costained images.

Western Blotting

Trigeminal cultures similar to those used for immunohistochemistry were rinsed with PBS and scraped and pelleted for protein extraction in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS). Thirty μg of protein was loaded in reducing Laemmli sample buffer, heated to 95°C for 5 min and loaded onto a pre-cast 4–20% SDS PAGE gel (Tris-glycine, Invitrogen). Protein was transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon, Millipore, Billerica, MA) prior to being blocked in 5% milk for 1 h and incubated overnight at 4°C with a goat polyclonal antibody against ASIC3 (1:1000, diluted in PBS, expected size 60 kDa). The primary antibody was detected with an anti-goat IgG conjugated to horseradish peroxidase (1:70,000, Sigma) and antigens were visualized using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Pittsburgh, PA). Blots were developed on Kodak film.

Data Analysis and Statistics

For all studies, each experimental condition was performed in duplicate and repeated in at least 3 independent experiments. Secretion data are reported as mean $\pm$ SEM of the fold-change when compared to mean control levels that were made equal to one. Intracellular calcium and sodium data are presented as representative plots of the 340/380 nm wavelength values representing bound versus unbound intracellular calcium or sodium ions. In addition, intracellular calcium data are presented as the mean $\pm$ SEM peak amplitude for 6–8 cells for each experiment condition. Statistical analysis was performed using a one-way ANOVA followed by a Scheffe post hoc test. Differences were considered statistically significant at $P < 0.05$. All statistical tests were performed using SPSS Statistical Software, Release 16 (SPSS Inc. Chicago, IL).

RESULTS

To determine whether onabotulinumtoxinA or rizatriptan could repress stimulated CGRP secretion, neuronal-enriched trigeminal ganglion cultures were incubated in HBS, pH 7.4 and 60 mM KCl or with HBS, pH 5.5 (protons, H^+). CGRP secretion was significantly increased more than 4-fold over mean basal control levels of 26 ± 5.4 pg/h/well ($P < 0.01$, $n = 5$) in cultures treated with 60 mM KCl (Fig. 1A). In contrast, secreted CGRP levels in cultures treated with onabotulinumtoxinA prior to KCl stimulation were not different from control levels and were significantly repressed compared to KCl stimulation alone ($P < 0.05$). However, while cultures incubated in acidic media exhibited a significant 3-fold increase in the amount of secreted CGRP when compared to control levels ($P < 0.01$, $n = 5$), the stimulatory effect of protons was not repressed by onabotulinumtoxinA. For the rizatriptan studies (Fig. 1B), CGRP secretion from trigeminal neurons treated with 60 mM KCl was significantly increased over 6-fold when compared to mean basal control levels of 20 ± 6.2 pg/h/well ($P < 0.01$, $n = 5$). Similarly, cultures incubated in acidic media had an approximate 4-fold increase in the amount of secreted CGRP ($P < 0.01$). Although KCl-mediated increases in CGRP levels were significantly repressed by pretreatment with rizatriptan ($P < 0.05$), the stimulatory effect of protons on CGRP release was not suppressed by this anti-migraine drug. The basal amount of CGRP secreted from trigeminal neurons was not significantly changed by treatment with onabotulinumtoxinA or rizatriptan alone when compared to control levels (data not shown). Data from these studies provide evidence that while the anti-migraine therapies onabotulinumtoxinA and rizatriptan can block the stimulatory effect of KCl on CGRP secretion; these agents are not able to suppress proton mediated CGRP release from trigeminal neurons.

To investigate whether the stimulatory effects of KCl and proton-mediated CGRP release were dependent on extracellular calcium, the amount of CGRP secreted from cultured trigeminal neurons was determined in HBS containing EGTA as previously described²⁴. While KCl-stimulated levels of CGRP secretion were increased about 7-fold over mean basal control levels of 23 ± 4.5 pg/h/well ($P < 0.01$, $n = 4$), the amount of CGRP released in media supplemented with EGTA was greatly suppressed ($P < 0.05$) to near basal control levels (Fig. 2). In contrast, there was no significant reduction in the amount of CGRP released from cultured neurons stimulated with protons in the absence or presence of EGTA in the media. CGRP secretion was stimulated 5-fold when compared to control levels ($P < 0.01$, $n = 3$) in normal HBS, and was also significantly increased > 4 -fold ($P < 0.01$, $n = 3$) in the absence of extracellular calcium. The basal, unstimulated amount of CGRP released from trigeminal neurons did not significantly change when cultures were incubated in EGTA containing media when compared to control levels (data not shown). These data demonstrate that trigeminal neurons utilize different mechanisms to facilitate CGRP release from trigeminal neurons in response to KCl and protons (pH 5.5).

To investigate the cellular basis for the difference in KCl and proton stimulation of CGRP secretion, the effect of each agent on levels of intracellular calcium were determined in trigeminal neurons using fluorescent microscopy and the fluorescent calcium dye Fura-2. Intracellular calcium levels were only determined in neurons which were in the size range of nociceptive neurons (20–40 μm). The mean calcium response in cells treated with 60 mM KCl alone resulted in a transient increase in the F340/380 ratio from a basal ratio of 0.48 ± 0.02 to 3.22 ± 0.16 (Fig. 3A). However, chelation of extracellular calcium with EGTA prior to stimulation with KCl blocked the elevation in intracellular calcium (Fig. 3B). The mean $\pm$ SEM from at least 4 independent experiments was calculated and is displayed as a graph to illustrate average calcium changes between conditions (Fig. 3C). The presence of EGTA in the media significantly suppressed the transient increase of intracellular calcium in response to KCl treatment ($P < 0.01$, $n = 45$ neurons) when compared to cultures treated with KCl alone ($n = 44$ neurons). In contrast to KCl stimulation, incubation of cells in acidic media (pH 5.5) did not induce a change in intracellular calcium levels in neurons when compared to basal levels (0.52 ± 0.05 , $n = 36$ neurons, representative plot shown in Fig. 3D). These results provide further evidence of distinct cellular mechanisms by which KCl and protons facilitate CGRP release.

Since protons are known to cause excitation of sensory neurons via activation of ASICs, changes in the level of intracellular sodium levels was investigated using microscopy and the fluorescent sodium dye SBFI. Intracellular sodium levels were only determined in neurons which were in the size range of nociceptive neurons (20–40 μm). Treatment of cultures with acidic media caused a transient sodium increase from a basal level of 1.63 ± 0.08 to a peak ratio value of 3.22 ± 0.21 (Fig. 4). The average change in the peak amplitude of the maximum F340/380 ratio in response to protons was 1.56 ± 0.07 when compared to unstimulated basal levels ($n = 3$ independent experiments). Results from these experiments suggest that the stimulatory effect of protons on CGRP release from trigeminal neurons is mediated by a mechanism involving elevated levels of intracellular sodium.

To determine whether proton-mediated CGRP secretion involved activation of TRPV1 channels, CGRP secretion was stimulated with capsaicin or acidic media in the presence or absence of the TRPV1 inhibitor capsazepine. As seen in Figure 5, the amount of CGRP released into the culture media was significantly stimulated > 5 -fold or > 4 -fold over mean basal control levels of 37 ± 3.8 pg/h/well in response to capsaicin or protons, respectively ($P < 0.05$; $n = 4$). While capsazepine suppressed the stimulatory effect of capsaicin on the level of CGRP secretion ($P < 0.05$), pretreatment with this inhibitor did not significantly repress proton stimulation of CGRP secretion. Capsazepine did not inhibit basal secretion of CGRP. These data demonstrate that proton (pH 5.5) stimulation of CGRP secretion occurs via a mechanism independent of TRPV1 channel activation.

To determine if ASIC3 ion channels are expressed by trigeminal ganglion neurons, the presence of ASIC3 was investigated by immunohistochemistry in tissue sections and primary cultures of trigeminal neurons. As seen in Figure 6A, ASIC3 immunoreactivity was detected in neurons in the V1 and V2 regions of trigeminal ganglia sections. In addition, ASIC3 was expressed in most small to medium size trigeminal neurons, as identified by their unique nuclear morphology and size, under our culture conditions (87.5%, 98/112; Fig 6B). No specific cellular staining was detected in cultures incubated with only secondary antibodies (data not shown). In agreement with ASIC3 staining data, a single 60 kDa immunoreactive band was detected by western blot analysis in trigeminal ganglia (Fig. 6C). Having established the presence of ASIC3 in trigeminal neurons, trigeminal ganglia cultures were stimulated with acidic media in the absence or presence of the selective ASIC3 inhibitor APETx2 (Fig. 6D). The amount of CGRP released from trigeminal neurons increased 3-fold over control levels (42 ± 2.9 pg/h/well) in response to protons. Pretreatment

with 300 nM APETx2 was shown to significantly repress proton-mediated secretion of CGRP when compared to proton stimulated levels ($P < 0.01$, $n = 3$). However, pretreatment with APETx2 alone did not suppress basal CGRP secretion levels (data not shown). Our findings provide evidence of the presence of ASIC3 receptors in trigeminal neurons and that the stimulatory effects of protons are mediated, at least in part, via ASIC3 ion channels.

DISCUSSION

Results from our study provide pharmacological and functional evidence of two distinct mechanisms involved in mediating stimulated release of CGRP from trigeminal ganglion neurons. In agreement with our previously published studies^{24,27}, we found that K⁺ stimulation of CGRP secretion occurs via a calcium and SNAP-25 dependent mechanism that is blocked by the anti-migraine therapeutic agents rizatriptan and onabotulinumtoxinA. However, incubation of trigeminal neurons in acidic media (pH 5.5) stimulated CGRP release via a calcium and SNAP-25 independent mechanism that was mediated via increases in intracellular sodium and was not repressed by either therapeutic agent. Our data are in agreement with findings from an earlier published study by Purkiss et al. (2000) that showed release of the neuropeptide substance P in response to noxious stimuli occurred via two different mechanisms. In their study, capsaicin was found to stimulate release of the neuropeptide substance P from cultured dorsal root ganglia nociceptive neurons via a mechanism requiring extracellular calcium and intact SNAP-25 and another mechanism independent of extracellular calcium and not involving SNAP-25. Taken together, these data provide evidence of two distinct secretory mechanisms by which neuropeptides can be released from sensory neurons in response to inflammatory or noxious stimuli.

At this time we can only speculate on possible physiological functions for the presence of two cellular pathways that facilitate neuropeptide release from sensory neurons. However, a plausible explanation is that multiple vesicle pools provide a mechanism for regulating neuropeptide secretion in accord with the type of inflammatory stimuli and severity of the pathology. In this way, the amount of CGRP released from trigeminal neurons would be greatest if both calcium dependent as well as calcium independent mechanisms were induced as might occur during a migraine attack. Based on models of migraine pathology, elevated levels of several inflammatory agents including bradykinin, nerve growth factor, serotonin, and protons, are thought to cause sensitization and activation of trigeminal nociceptive neurons that provide sensory innervation to the meninges during an attack^{14,43–45}. While many of these inflammatory agents are known to stimulate neuropeptide release via mechanisms involving calcium and SNAP-25, our data provide evidence that protons (pH 5.5) stimulate CGRP release by an alternative mechanism that is independent of extracellular calcium and SNAP-25. However it should be noted, that pH 5.5 used as a stimulus in our study has not been reported in migraine patients but similar pH levels been reported under pathological conditions involving ischemia and tissue hypoxia^{46,47}. In our study, we found that protons promote CGRP secretion via a pathway that involves ASIC3 and elevated intracellular levels of sodium ions. Interestingly, several inflammatory molecules implicated in migraine pathology, including bradykinin, serotonin, nerve growth factor, nitric oxide, and arachidonic acid, are known to increase ASIC3 expression and activity^{48–52}. In this way, the release of other inflammatory mediators within the meninges would lead to heightened sensitivity and expression of ASIC3 channels and an enhanced response to protons during a migraine attack. Thus, we propose the significantly elevated levels of CGRP reported during a migraine attack that correlate with pain intensity^{4,5}, are likely the result of CGRP release from two distinct vesicle pools mediated via different signaling pathways.

The existence of two vesicle pools also has therapeutic implications since we found that while KCl-induced stimulation of CGRP release was inhibited by rizatriptan and onabotulinumtoxinA, proton stimulation was not repressed by these drugs. Thus, our findings provide evidence for a novel mechanism of calcium-independent CGRP release in response to protons that is not blocked by commonly used anti-migraine medications. The triptan class of anti-migraine drugs, which are the standard of care, is thought to function as an abortive therapy by blocking peripheral release of neuropeptides within the meninges and central release of glutamate and CGRP in the spinal trigeminal nucleus^{6, 28, 53}. Inhibiting the secretion of CGRP and other inflammatory mediators in the meninges would repress neurogenic inflammation, while suppressing the central release of these compounds prevents activation of second order neurons and spinal glial cells, and hence the development of central sensitization. Similarly, onabotulinumtoxinA, which is now approved for treatment of chronic migraine^{30, 31}, is thought to function by blocking neurotransmitter release from sensory neurons. The mechanism involves cleavage of SNAP-25 and thus, inhibiting docking of vesicles containing neurotransmitters such as glutamate and CGRP to the cell membrane and consequently their release^{25–27, 54, 55}. Our identification of another mechanism for CGRP secretion provides a plausible reason to explain why some migraine sufferers do not respond as well to triptans and onabotulinumtoxinA treatment since these therapies can only block one mechanism of stimulated CGRP release. Based on our findings, we propose that in migraineurs whose headaches are initiated or sustained by elevated extracellular levels of protons (pH 5.5) would not respond as well to either therapy. Unfortunately, there are currently no drugs approved for the treatment of migraine that selectively target ASICs. However, NSAIDs, which are commonly used to effectively treat migraine, have been reported to inhibit ASIC channel activity at therapeutic doses that produce analgesic effects⁵⁶. Interestingly, findings from recent clinical studies have shown that the combination of a triptan (sumatriptan) with an NSAID (naproxen) was more effective than treatment with either drug alone, possibly, because they inhibit different molecular targets⁵⁷. In addition, it is likely that CGRP antagonists might offer an alternative therapeutic option since these agents would function to block the physiological effects of CGRP rather than its release from trigeminal neurons.

Based on our findings, the stimulatory effects of protons (pH 5.5) are likely to involve activation of ASIC3 channels. We found that under our culture conditions ASIC3 is expressed in most trigeminal neurons and proton stimulation of CGRP secretion was significantly repressed by pretreatment with the selective ASIC3 inhibitory agent APETx2^{58, 59} but not by the selective TRPV1 antagonist capsazepine⁶⁰. Our findings are in agreement with results reported by Yan et al. in which activation of dural trigeminal afferents by acidic pH was shown to involve ASICs but not TRPV1 and was likely to be mediated by ASIC3 channels⁶¹. In future studies, it would be of interest to further confirm the involvement of ASIC3 channels in mediating CGRP release using the recently identified nonproton ASIC3 ligand 2-guanidine-4-methylquinazoline (GMQ) that reportedly activates sensory neurons and causes pain-related behaviors⁶². The reason for the high percentage of neurons expressing ASIC3 may be due to the inclusion of NGF as a growth factor supplement in our media since NGF was shown to maintain basal expression of ASIC3⁶³. Peripheral ASIC3 channels function as essential sensors of acidic pain during inflammation and reported to contribute to primary hyperalgesia in rats^{59, 64}. Our findings are in agreement with data from a previous study in which ASIC3 was reported to colocalize with CGRP in rat trigeminal ganglia neurons and its expression was upregulated with inflammatory mediators leading to altered firing properties of neurons²². Although our results with respect to APETx2 provide evidence of the participation of ASIC3 in mediating proton stimulation of CGRP, we cannot rule out the possible contribution of other ASICs. ASIC3, which forms homomeric channels with selectivity towards sodium ions, has also been reported to form non-selective cation heteromeric channels with ASIC2a^{65, 66} or

ASIC1⁶⁷, or other ASIC subunits^{68, 69} in the dorsal root ganglion and nodose ganglia sensory neurons. In the seminal study by Diochot and colleagues⁵⁸, APETx2 was reported to selectively inhibit homomeric ASIC3 channel activity as well as heteromeric ASIC3 channels formed with ASIC1a, ASIC1b, and ASIC2b. In addition, APETx2 did not significantly inhibit ASIC1a, 1b, or 2a homomeric currents at pH 6 or pH 5. Thus, at this time, our findings demonstrate that activation of ASIC3 channels promotes proton stimulation of CGRP release from trigeminal neurons and as such represent a viable therapeutic target for inflammatory diseases involving tissue acidosis as implicated in migraine.

In summary, we have provided evidence that CGRP release from trigeminal neurons, a physiological event prominently implicated in migraine pathology, is mediated via two distinct mechanisms and is dependent on the type of stimulus. Results from our study support the notion that proton stimulation of CGRP secretion involves ASIC3 channels since cultured trigeminal neurons express ASIC3, proton stimulation increased intracellular sodium ions levels, and the potent peptide ASIC3 inhibitor, APETx2, repressed proton stimulated CGRP release. In addition, based on our work and that of Purkiss and colleagues³⁷, it appears that sensory neurons have the ability to modulate the release of neuropeptides to effectively regulate the cellular response to the amount and type of inflammatory stimuli. Finally, our findings may provide a plausible explanation for the lack of therapeutic benefit reported by some migraine patients in response to rizatriptan and onabotulinumtoxinA.

Acknowledgments

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Abbreviations used

CGRP	calcitonin gene-related peptide
TRPV1	transient receptor potential cation channel subfamily V member 1
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble N-ethylamide-sensitive factor attachment protein receptor
ASIC	acid-sensing ion channel
PBS	phosphate buffered saline
HBS	HEPES buffered saline
BSA	bovine serum albumin
KCl	potassium chloride

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Figure 1.

Effects of onabotulinumtoxinA and rizatriptan on KCl and proton induced CGRP secretion from trigeminal ganglion neurons. A. The relative amount of CGRP secreted in 1.5 h from untreated control cultures (CON), cultures treated with 60 mM KCl or protons (HBS, pH 5.5; H⁺) alone, or pretreated with onabotulinumtoxinA (O) prior to addition of stimulatory agents. B. The amount of CGRP released from control cultures (CON) or cultures following stimulation with KCl or acidic media, or pretreatment with rizatriptan (R). The amount of CGRP secreted into the culture medium in each condition was first normalized to the basal rate for each well. Values are reported as fold-change relative to control levels whose mean was made equal to one. The means $\pm$ SEM from 3 independent experiments are shown. * $P < 0.01$ when compared to control; # $P < 0.01$ when compared to KCl stimulation.

Figure 2.

Proton stimulation of CGRP release does not require extracellular calcium. CGRP secreted in 1.5 h from untreated control cultures (CON), cultures treated with KCl or acidic media alone, or pretreated with EGTA prior to KCl or proton stimulation. The amount of CGRP secreted into the culture medium in each condition was normalized to the basal rate for each well and values reported as fold-change relative to control levels whose mean was made equal to one. The means $\pm$ SEM from 3 independent experiments are shown. * $P < 0.01$ when compared to control; # $P < 0.01$ when compared to KCl stimulation.

Figure 3.

Proton stimulation of trigeminal neurons does not cause an increase in intracellular calcium. Representative plots of F340/380 calcium ratio changes recorded from trigeminal neurons incubated in HBS in response to 60 mM KCl (A) or pretreatment with EGTA prior to KCl stimulation (B). Panel C shows the average change in the peak amplitude of the maximum F340/380 ratio in response to each treatment $\pm$ SEM from 3 independent experiments. Panel D is a representative plot of F340/380 calcium ratio changes in response to proton (H⁺) stimulation (HBS, pH 5.5). * $P < 0.01$ when compared to KCl treatment alone. Solid bar indicates time of treatment.

Figure 4.

Proton stimulation of trigeminal neurons involves an elevation in intracellular sodium levels. A representative plot of F340/380 sodium ratio changes recorded from trigeminal neurons treated with protons, (H^+ , HBS, pH 5.5) is shown. Solid bar indicates time cells exposed to acidic media after change from HBS 7.4 media.

Figure 5.

Capsazepine does not block proton-mediated stimulation of CGRP secretion. The amount of CGRP secreted into the culture medium from untreated control cultures (CON), capsazepine (CZP), capsaicin (CAP), or HBS, pH 5.5 (H⁺) alone, or pretreated with capsazepine prior to capsaicin or proton stimulation was normalized to the basal rate for each well (88 ± 4.1 pg/h/well). Values are reported as fold-change relative to control levels whose mean was made equal to one. * $P < 0.01$ when compared to control. # $P < 0.05$ when compared to capsaicin alone.

Figure 6.

Proton stimulation of CGRP secretion involves ASIC3. (A) Expression of ASIC3 in the V1/V2 region of the trigeminal ganglion. The yellow box shows a region enlarged in the right two panels that are stained only with ASIC3 (green) or stained for ASIC3 and the nuclear dye DAPI (blue). ASIC3 staining is observed in neuronal cells (arrows). (B) The same image of trigeminal cultures viewed using phase microscopy and fluorescent microscopy. ASIC3 expression was detected in most trigeminal neurons (solid arrows). (C) Detection of ASIC3 (60 kDa) in trigeminal ganglia by western blot analysis. (D) The amount of CGRP secreted into the culture medium from untreated control cultures (CON), cultures treated with HBS, pH5.5 (H⁺), or pretreated with APETx2 prior to proton stimulation. Values were normalized to the basal rate for each well (42 ± 2.9 pg/h/well) and are reported as fold-change relative to control levels whose mean was made equal to one. The means $\pm$ SEM from 4 independent experiments are shown. # $P < 0.05$ when compared to pH 5.5.