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DHE Repression of ATP-Mediated Sensitization of Trigeminal Ganglion Neurons

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Abstract

Objective—To investigate the mechanism by which adenosine triphosphate (ATP) causes sensitization of trigeminal neurons and how dihydroergotamine (DHE) represses this modulatory effect.

Background—Dihydroergotamine is an effective treatment of migraine. The cellular mechanisms of action of DHE in treating migraine attacks remain unclear.

Methods—In this study, neonatal rat trigeminal ganglia cultures were used to investigate effects of ATP, alpha, beta-methyl ATP (α,β -meATP), and DHE on intracellular calcium levels and calcitonin gene-related peptide (CGRP) secretion.

Results—Pretreatment with ATP or α,β -meATP caused sensitization of neurons, via P2X₃ receptors, such that a subthreshold amount of potassium chloride (KCl) significantly increased intracellular calcium levels and CGRP secretion. Pre-treatment with DHE repressed increases in calcium and CGRP secretion in response to ATP-KCl or α,β -meATP-KCl treatment. Importantly, these inhibitory effects of DHE were blocked with an α_2 -adrenoceptor antagonist and unaffected by a 5HT_{1B/D} receptor antagonist. DHE also decreased neuronal membrane expression of the P2X₃ receptor.

Conclusions—Our findings provide evidence for a novel mechanism of action for DHE that involves blocking ATP-mediated sensitization of trigeminal neurons, repressing stimulated CGRP release, and decreasing P2X₃ membrane expression via activation of α_2 -adrenoceptors.

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Keywords

dihydroergotamine; P2X₃; trigeminal ganglion; adrenergic; migraine

Migraine is a prevalent painful headache disorder that can be divided into 2 major subtypes, migraine without aura and migraine with aura. In migraine with aura, the headache phase is preceded or accompanied by transient neurological symptoms, most typically visual.^{1,2} It has been proposed that cortical spreading depression (CSD), which is implicated in migraine visual aura, leads to release of protons, potassium ions (K⁺), nitric oxide, prostaglandins, and adenosine 5'-triphosphate (ATP) in cerebrospinal fluid.^{3,4} The increased level of these molecules can cause sensitization and activation of sensory primary afferent fibers of trigeminal ganglion (TG) neurons that transmit nociceptive information from the meninges to second order neurons in the trigeminal nucleus caudalis (TNC). Thus, CSD and the subsequent release of pro-inflammatory molecules are thought to play an important role in the initiation of headache in migraine with aura.

In response to hyperexcitable cortical activity that precedes CSD, ATP levels are known to increase greatly in the extracellular fluid.^{4,5} Elevated levels of ATP are implicated in acute, inflammatory, as well as neuropathic pain mechanisms by mediating either sensitization or activation of nociceptive neurons depending on its concentration. The inflammatory and nociceptive effects of ATP are known to involve activation of P2X and P2Y receptors, which are expressed by primary sensory afferents.⁶⁻⁹ P2X receptors are a member of a family of ligand gated ion channels, expressed in peripheral sensory afferents, and preferentially on nociceptive C-fibers. Importantly, many trigeminal nociceptive neurons that provide sensory innervation of the dura and release calcitonin gene-related peptide (CGRP) are reported to express P2X₃ receptors.¹⁰⁻¹² Thus, activation of P2X₃ receptors is likely to play an important role in the underlying pathology of migraine with aura by facilitating release of CGRP within the dura to promote an inflammatory response as well as CGRP release in the TNC that activates second order neurons involved in pain transmission.

Dihydroergotamine (DHE) is a semisynthetic ergot alkaloid derivative that has been used extensively in the acute treatment of migraine and has been reported to be superior to sumatriptan in preventing migraine recurrence.¹³ DHE exhibits high affinity for several serotonergic, adrenergic, and dopaminergic receptors.¹⁴ It is thought that the therapeutic benefit of DHE in treating migraine involves 5-HT_{1B} and 5-HT_{1D} receptors and leads to the repression of trigeminal neuron activation^{15,16} and constriction of meningeal blood vessels via the 5-HT_{1B} receptor.^{17,18} However, results from more recent studies provide evidence that activation of the α_2 -adrenoreceptors, which are expressed on TG neurons, had an inhibitory effect on nociceptive transmission in the TG system at the level of primary afferent terminals.¹⁹ α_2 -adrenoreceptors have been shown to be involved in inhibition of calcium influx through voltage-gated calcium channels via a G-protein dependent pathway in neuronal cells.^{17,20-22} α_2 -adrenoreceptors also couple to the inhibitory G_o/G_i-protein to inhibit adenylyl cyclase or to interact directly with calcium channels.²³⁻²⁶ Thus, DHE activation of α_2 -adrenoreceptors may also be involved in repressing stimulated CGRP release from trigeminal neurons in response to inflammatory stimuli.

In this study, we used primary cultures of TG neurons to investigate the mechanism by which ATP causes sensitization of trigeminal neurons and DHE represses this modulatory effect. Data from our study provide evidence that ATP, via P2X₃ receptors, lowers the threshold of activation of trigeminal neurons to K⁺ and that DHE activation of α_2 -adrenoreceptors represses ATP-mediated sensitization of trigeminal neurons involves down regulating membrane expression of P2X₃ receptors.

METHODS

Animals

The animal studies were approved by the Institutional Animal Care and Use Committee at Missouri State University in accordance with the guidelines established in the Animal Welfare Act and National Institutes of Health. Every effort was made to minimize animal suffering and reduce the number of animals used in our study. Adult female Sprague–Dawley rats (250–300 g) were housed in structurally sound, clean plastic cages on a 12 hours light/dark cycle and with unrestricted access to food and water for the duration of the experiment.

Cell Culture

Primary cultures of trigeminal ganglia were established based on our previously published protocols.^{27–29} Briefly, cells from ganglia isolated from 3 to 5-day-old rats were resuspended in L15 medium containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 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, Sigma, St. Louis, MO, USA) were also added to the supplemented L15 media, which will be referred to as 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, USA) and incubated at 37°C at ambient CO₂ for 24 hours prior to measurements. For calcium measurements, trigeminal cultures were enriched in neuronal cells (>90%) by density-gradient centrifugation as previously described.²⁹ Briefly, after dissociation, the cell pellet was resuspended in 3 mL of L-15 medium containing 1 mg/mL bovine serum albumin (BSA). Cells were carefully layered onto 6 mL of L-15 medium containing 10 mg/mL BSA in a 15-mL conical tube and centrifuged at 100 $\times$ g for 3 minutes. The pellet was resuspended in L15 complete medium and plated as described above at a density of 2 ganglia 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 hours prior to the start of the experiment. For use in immunocytochemistry studies, TG cells were plated at a density of half a ganglion on 11 mm plastic coverslips coated with poly-D-lysine and incubated at 37°C at ambient CO₂ for 24 hours.

CGRP Assay

For the CGRP secretion studies, cells were incubated in HBS (22.5 mM HEPES, 135 mM NaCl, 3.5 mM potassium chloride [KCl], 1 mM MgCl, 2.5 mM CaCl, 3.3 mM glucose, and 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 (Peninsula Laboratories, Belmont, CA, USA) as previously described.^{27–29} As a control, the basal (unstimulated) rate of CGRP secreted into the media in 1.5 hours was determined, and these values were used to normalize for differences between wells. In some experiments, cells were treated with ATP (100 μ M, Sigma) or the selective P2X₃ agonist α,β -meATP (10 μ M, Sigma), 30 mM KCl alone, or pre-treated with ATP or α,β -meATP 15 minutes prior to the addition of KCl. α,β -meATP is reported to have a K_i of 1 μ M for P2X₃ and >100 μ M for P2X₂ receptors, which are the only two P2X receptors expressed on trigeminal neurons.^{30,31} For antagonist studies, cultures were pretreated with A-317491 (10 nM, Sigma), which has a reported K_i value of 10 nM for the P2X₃ receptor,³² for 15 minutes prior to addition of ATP and KCl. In other studies, DHE (10 μ M, in DMSO; kindly provided by MAP Pharmaceuticals, Inc., Mountain View, CA, USA), which has a reported K_i for 5-HT₁, adrenergic, and dopamine receptors all in the nanomolar range, was added alone to the cultures or was added 30 minutes prior to the addition of ATP and KCl or α,β -meATP and

KCl. Media were collected after 1.5 hours and assayed for the amount of CGRP. The α_2 -adrennergic receptor antagonist, yohimbine (3 nM, Sigma), which has reported K_i values of 3 nM, 10 nM, and 0.68 nM for α_{2A} , α_{2B} , and α_{2C} , respectively, and affinity for serotonergic and dopaminergic sites at greater than 100 nM,³³ was added 15 minutes prior to the addition of DHE. In addition, the 5HT_{1B/1D} receptor antagonist, GR 127935 (3 μ M, TOCRIS, Ellisville, MO, USA), which has a reported K_i of 1 μ M for 5HT_{1B/1D} receptors,³⁴ was also added 15 minutes prior to DHE. As a control, some cultures were treated with DMSO (0.1% final concentration). Following removal of basal media, all pretreatment agents were first diluted to the appropriate concentrations in HBS prior to addition to the cultures. Stimulatory agents (ATP, α,β -meATP, and KCl) were added directly to the culture media. Each experimental condition was performed in duplicate and repeated in at least 3 independent experiments. The data are reported as mean $\pm$ SE of the fold-change when compared with control levels, whose mean was made equal to 1. Statistical analysis was performed using a one-way ANOVA followed by a Scheffe post hoc test. Differences were considered statistically significant at $P < .05$. All statistical tests were performed using SPSS Statistical Software, Release 16 (SPSS Inc., Chicago, IL, USA).

Calcium Measurements

Intracellular calcium levels in cultured trigeminal neurons were measured as described previously.^{28,29} Cells were incubated in HBS and 5 μ M fura-2 AM (Invitrogen, Carlsbad, CA, USA) for 25–30 minutes at 37°C. After the cells were washed twice with HBS, they were incubated in the same buffer for 30 minutes before analysis. Absorbance was determined in neuronal cells, identified by their unique size and morphology in addition to their measured response to 100 μ M ATP or 10 μ M α,β -meATP to confirm purinergic activity, at 340 and 380 nm every 2–8 seconds for a minimum of 10 minutes on a heated stage at 37°C. In all studies, basal intracellular calcium levels were obtained for a minimum of 2 minutes before treatment and for as long as 20 minutes following final treatment. Initially for the sensitization studies, ATP or α,β -meATP were added to cultures for 0, 5, 10, 15, and 20 minutes before the addition of 30 mM KCl to determine the minimal preincubation time required to lower the response threshold to KCl. Based on the results of the time course study, cultures were treated with ATP, α,β -meATP, or KCl alone or treated with ATP or α,β -meATP for 15 minutes prior to addition of KCl. For purinergic antagonist studies, cultures were pre-treated with 10 nM A-317491 for 15 minutes prior to addition of ATP and KCl. In the studies on DHE, cultures were initially treated with 10 μ M DHE alone or cultures were pretreated with DHE for 30 minutes prior to addition of ATP or α,β -meATP alone or ATP and KCl or α,β -meATP and KCl. For receptor antagonist studies, 3 nM of the α_2 -adrennergic antagonist yohimbine or 3 μ M of the 5HT_{1B/1D} antagonist GR127395 was added 15 minutes prior to the addition of DHE. As a control, some cultures were treated with an equal volume of DMSO, which was the vehicle used to dissolve DHE. Representative plots of the ratio of F340/380 nm wavelength values that correspond to bound versus unbound intracellular calcium are shown. Calcium measurements were performed 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, USA). In addition, results are presented as the mean $\pm$ SE peak amplitude for 6–8 cells being analyzed for each experiment condition, which was repeated in a minimum of 3 independent experiments performed in duplicate. Statistical analysis was performed using the one-way ANOVA followed by a Scheffe post hoc test. Differences were considered statistically significant at $P < .05$. All statistical tests were performed using SPSS Statistical Software, Release 16.

Immunocytochemistry

For the immunocytochemistry experiments, cultures were treated with DMSO (0.1% final concentration in HBS), 10 μ M DHE for 30 minutes in HBS, 3 nM yohimbine, or 3 μ M GR127395 for 15 minutes prior to DHE. Cultures were fixed using 4% paraformaldehyde for 15 minutes then permeabilized and blocked with 0.1% Triton-X100 and 5% donkey serum in PBS (pH 7.4) for 15 minutes. Cultures were then incubated overnight at 4°C with rabbit primary antibodies against the purinergic receptor P2X₃, which targeted the C-terminus domain of the transmembrane protein (1:1000 in PBS; Abcam, ab10269, Cambridge, MA, USA). Immunoreactive proteins were visualized following incubation with Alexa-Fluor 594 donkey anti-rabbit IgG for 1 hour at room temperature. After staining, tissues were covered in Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA, USA). Images (630 $\times$) were collected using a Zeiss 60N camera mounted on an Axio Imager Z1 epi-fluorescent microscope fixed with an ApoTome Grid M (Carl Zeiss Inc., Oberkochen, Germany). Typically, the image field analyzed contained on average 2–4 neuronal cells with a diameter of 20–40 μ m. The fluorescence intensity of P2X₃ staining across each cell was determined using Zeiss Axio Vision v4.7 software. For each experimental condition, the number of neurons exhibiting membrane staining for P2X₃ was determined by measuring intensity across 3 lines over each cell from 3 independent experiments performed in duplicate ($N \geq 200$ cells per condition). Cells with at least a twofold increase in staining intensity on the membrane over the cytosol were considered positive for P2X₃ membrane localization. Statistical analysis was performed using the one-way ANOVA followed by a Scheffe post hoc test. Differences were considered statistically significant at $P < .05$. All statistical tests were performed using SPSS Statistical Software, Release 16.

RESULTS

ATP-Mediated Sensitization of Trigeminal Neurons Involves P2X₃ Receptor Activation

To determine whether ATP could sensitize neurons to a concentration of KCl that normally would not stimulate CGRP secretion (subthreshold), neuronal-enriched TG cultures were incubated in HBS with 100 μ M ATP for 15 minutes prior to the addition of 30 mM KCl. The basal, unstimulated amount of CGRP released was measured prior to treatment and used to normalize for differences between wells. The amount of CGRP released from trigeminal neurons did not significantly change when cultures were exposed to 100 μ M ATP or 30 mM KCl when compared with control levels (Fig. 1A). However, cultures exposed to ATP prior to the addition of KCl had an approximately fourfold increase in CGRP levels when compared with unstimulated control levels ($P < .01$, $N = 3$). Interestingly, the P2X₃ purinergic receptor antagonist, A-317491, when added 15 minutes prior to the addition of ATP significantly inhibited CGRP release in response to ATP and KCl treatment ($P < .01$, $N = 3$) (Fig. 1A). Additionally, the P2X₃ agonist, α,β -meATP, was able to induce a similar response to that observed with ATP (Fig. 1B). When cultures were exposed to 10 μ M α,β -meATP or 30 mM KCl alone, CGRP release was not significantly changed. However, cultures exposed to α,β -meATP 15 minutes prior to the addition of KCl had a similar fourfold increase in CGRP levels when compared with control levels ($P < .01$, $N = 3$). Taken together, these results provide evidence that the sensitizing effect of ATP on trigeminal neurons is mediated primarily by activation of P2X₃ receptors.

P2X₃ Receptor Activation Leads to Sensitization of Trigeminal Neurons

To assess the functional responses to ATP, we tested the ability of ATP as well as a P2X₃ agonist, α,β -meATP, to induce intracellular calcium increases in trigeminal neurons loaded with the fluorescent calcium dye Fura-2. Basal calcium levels were determined at 2- to 8-second intervals for a minimum of 2 minutes prior to addition of experimental agents.

Initially, experiments were performed to determine the time in which 100 μ M ATP or 10 μ M α,β -meATP exposure leads to the sensitization of cultured trigeminal neurons. Cultures were exposed to ATP or α,β -meATP for 0, 5, 10, 15, or 20 minutes and the change in intracellular calcium measured in response to a subthreshold concentration of KCl (30 mM). While simultaneous cotreatment with ATP and KCl or α,β -meATP and KCl did not cause a significant rise in intracellular calcium compared with each agent alone, pretreatment with each agonist did cause a significant time-dependent sensitization of trigeminal neurons that was greatest at the 15 and 20 minute time points (Table, N = 33 and 28 for ATP and α,β -meATP, respectively). Based on these results, cultures were incubated with 100 μ M ATP or 10 μ M α,β -meATP for 15 minutes prior to addition of KCl in all subsequent studies involving ATP regulation of trigeminal neurons. Intracellular calcium levels were only determined in neurons that initially responded to either form of ATP and were in the size range of nociceptive neurons (20–40 μ m). The mean calcium response in cells treated with ATP alone resulted in an increase in the F340/380 ratio from a baseline value of 0.23 ± 0.02 to 1.28 ± 0.16 (Fig. 2A). Similarly, α,β -meATP increased the F340/380 ratio from a baseline of 0.24 ± 0.07 to 1.33 ± 0.18 , while 30 mM KCl induced a transient rise in intracellular calcium levels in neurons to 1.31 ± 0.18 compared with basal levels of 0.16 ± 0.04 (Fig. 2B). As seen in Figure 2C, exposure of cultures to ATP or α,β -meATP for 15 minutes prior to treatment with KCl caused a much greater calcium increase from basal ratios of 0.24 ± 0.02 and 0.21 ± 0.04 to mean F340/380 ratios of 2.08 ± 0.06 ($P < .01$, N = 48) and 1.93 ± 0.08 ($P < .01$, N = 45), respectively. The elevated values were significantly greater than those observed with ATP (N = 56), α,β -meATP (N = 28), or KCl (N = 44) alone. However, pretreatment with the P2X₃ receptor antagonist A-317491 repressed the sensitizing effect of ATP such that the amplitude of the calcium increase was similar to KCl control levels (Fig. 2D). Specifically, the calcium ratio was repressed to 1.27 ± 0.05 ($P < .01$, N = 32), with a basal level of 0.23 ± 0.03 , in response to A-317491 pretreatment. The mean and standard error from at least 3 independent experiments was calculated and is displayed as a graph to illustrate calcium changes between conditions (Fig. 2E). Based on our findings, we conclude that the increased amplitude in KCl-mediated calcium response from neurons pretreated with ATP is mediated primarily by P2X₃ as pretreatment with α,β -meATP was sufficient to induce neuronal sensitization to KCl and that the effect of ATP was blocked by the P2X₃ receptor antagonist A-317491.

DHE Repression of ATP and KCl-Induced CGRP Release

To establish whether DHE could inhibit stimulated CGRP secretion by KCl in combination with ATP, trigeminal cultures were treated with DHE for 30 minutes prior to the addition of ATP and KCl. Treatment with 10 μ M DHE alone did not repress the basal level of CGRP secretion (Fig. 3A). Pretreatment with 100 μ M ATP 15 minutes prior to addition of 30 mM KCl stimulated CGRP levels almost 4-fold above basal levels ($P < .01$, N = 3). However, in cultures preincubated with DHE for 30 minutes, the stimulatory effect of ATP and KCl treatment on CGRP secretion was significantly repressed ($P < .01$), when compared with ATP and KCl treatment alone and as well the control ($P < .05$, N = 3) (Fig. 3A). Somewhat surprisingly, the inhibitory effect of DHE on ATP and KCl stimulated CGRP secretion was blocked by pretreatment of cultures with 3 nM yohimbine ($P < .01$, N = 3), yet uninhibited by 3 μ M GR 127935, a 5-HT_{1B/1D} antagonist. As seen in Figure 2B, pretreatment with 10 μ M α,β -meATP 15 minutes prior to addition of 30 mM KCl induced a similar significant 4-fold increase in CGRP secretion levels when compared with control or DHE alone values ($P < .01$, N = 3). However, DHE repressed the stimulatory effect of α,β -meATP and KCl treatment. Importantly, pretreatment of cultures with yohimbine but not with GR 127935 significantly prevented DHE-mediated repression of α,β -meATP and KCl stimulation ($P < .01$, N = 3). Additionally, pretreatment with yohimbine or GR 127935 alone did not repress stimulated CGRP release (data not shown). Similarly, as a control, treatment of cultures with

vehicle alone (DMSO; 1% final concentration) did not change CGRP secretion levels (data not shown). Thus, DHE can repress CGRP release from ATP-sensitized trigeminal neurons in response to a subthreshold concentration of KCl. Furthermore, DHE repression of ATP and KCl stimulated secretion is primarily mediated by α_2 a and/or α_2 c-adrenergic receptors, and not 5HT_{1B/1D} receptors.

DHE Repression of ATP and KCl-Induced Calcium Response

Calcium imaging was used to investigate whether the inhibitory effect of DHE on both ATP compounds and KCl stimulation of CGRP secretion was due to inhibition of the rise in intracellular calcium in response to ATP and KCl treatment. DHE treatment alone did not alter basal intracellular calcium levels (Fig. 4A). However, when added prior to addition of ATP or α,β -meATP, DHE greatly inhibited the intracellular calcium increase with values reaching only 0.56 ± 0.08 and 0.31 ± 0.06 from baseline levels of 0.20 ± 0.02 and 0.21 ± 0.58 , respectively (Fig. 4B). In contrast, pretreatment with DHE caused only a slight reduction in the amplitude of the KCl response (0.96 ± 0.18 over a baseline of 0.19 ± 0.06 , Fig. 4B). ATP and KCl or α,β -meATP and KCl treatment caused a rapid transient increase in intracellular calcium to 2.08 ± 0.06 and 1.93 ± 0.08 , values much greater than the basal levels of 0.24 ± 0.02 and 0.21 ± 0.04 (Fig. 4C). Pretreatment of cultures with 10 μ M DHE repressed the magnitude of the intracellular calcium rise in response to ATP and KCl treatment to a F340/380 ratio of 1.02 ± 0.13 from a basal level of 0.21 ± 0.07 and α,β -meATP and KCl treatment to a F340/380 ratio of 0.95 ± 0.19 from a basal level of 0.24 ± 0.10 (Fig. 4D). Similar to our secretion data, DHE repression of ATP and KCl or α,β -meATP and KCl-mediated increases in intracellular calcium was blocked by pretreatment with 3 nM yohimbine and returned to 1.99 ± 0.06 and 1.75 ± 0.16 , over basal levels of 0.32 ± 0.09 and 0.28 ± 0.04 (Fig. 4E). In contrast, pretreatment with the 5HT_{1B/1D} antagonist GR127395 did not block DHE-mediated repression of P2X₃-mediated sensitization by ATP or α,β -meATP, 0.98 ± 0.14 and 0.71 ± 0.20 over baselines of 0.23 ± 0.08 and 0.28 ± 0.06 (Fig. 4F). Finally, pretreatment with yohimbine or GR 127935 alone did not repress stimulated calcium increases (data not shown).

The mean and standard error for each experimental condition was calculated and graphically represented to demonstrate changes in intracellular calcium in response to the different treatments. While DHE (N = 27) did not significantly repress treatment with 30 mM KCl (N = 44), DHE significantly ($P < .01$, N = 36) repressed the ATP and KCl-stimulated calcium increase (N = 48) as well as significantly repressed ($P < .01$, N = 30) the α,β -meATP and KCl-stimulated calcium increase (N = 45) in trigeminal neurons (Fig. 4G). As seen in Figure 4H, treatment of cultures with ATP and KCl (N = 48) or α,β -meATP and KCl (N = 45) causes a marked increase in intracellular calcium levels that was significantly repressed by treatment with DHE ($P < .01$, N = 36 for ATP; $P < .01$, N = 30 for α,β -meATP). The inhibitory effect of DHE was blocked by pretreatment with yohimbine (N = 41 for ATP and N = 37 for α,β -meATP) but not by GR 127935 ($P < .01$, N = 34 for ATP and $P < .01$, N = 25 for α,β -meATP). In summary, DHE significantly represses ATP or α,β -meATP-mediated increases in intracellular calcium (Fig. 4G) and this inhibitory effect of DHE was blocked by pretreatment with yohimbine but not GR 127935 (Fig. 4H).

DHE Repression of Basal P2X₃ Protein Levels in Trigeminal Neurons

Based on our results of a P2X₃ antagonist repressing the sensitizing effect of ATP on trigeminal neurons, we examined whether DHE could function by decreasing the membrane expression of the P2X₃ receptor protein. To test this notion, we stained trigeminal cultures for expression of this receptor using P2X₃ antibodies, and utilized immunocytochemistry and fluorescent microscopy to visualize the cells. Trigeminal ganglia cultures were treated for 30 minutes with vehicle (DMSO), 10 μ M DHE for 30 minutes, or 3 nM yohimbine for

15 minutes prior to DHE addition (Fig. 5). In the vehicle control cultures (“CON”), P2X₃ staining was primarily detected in the cell membrane of trigeminal neurons as illustrated by the intensity measurements across the neuronal cell body as seen in representative images. However, treatment of cultures with DHE (“DHE”) caused a large decrease in the level of P2X₃ staining in the neuronal cell membranes but an increase in the level of cytosolic staining. Importantly, treatment of cultures with yohimbine prior to the addition of DHE (“YO + DHE”) blocked the effect of DHE to cause internalization of P2X₃ receptors as staining was observed primarily associated with the cell membrane. In contrast, pretreatment of cultures with GR 127935 (“GR + DHE”) did not prevent DHE-mediated internalization of P2X₃ receptors. Furthermore, both yohimbine and GR 127935 treatment alone did not alter membrane P2X₃ levels (data not shown).

Cell counts based on the intensity of P2X₃ immunostaining primarily localized in the neuronal cell membrane versus cytosol was determined for each of the experimental conditions. Under basal untreated conditions, P2X₃ staining was primarily localized to the cell membrane in almost all positively stained neuronal cells ($100 \pm 4.95\%$). In contrast, the number of cells with P2X₃ localized to the membrane was greatly reduced ($4.91 \pm 1.30\%$ of control; $P < .01$, $N = 6$) when compared with basal levels. The percentage of neuronal cells expressing P2X₃ primarily in the membrane treated with yohimbine prior to DHE was $97.87 \pm 6.01\%$, a value not significantly different from control levels. However, the effect of DHE on P2X₃ cellular localization was not blocked by pre-treatment with GR 127935 ($4.12 \pm 0.72\%$; $P < .01$, $N = 6$). Taken together, these data provide evidence that DHE treatment leads to decreased levels of P2X₃ receptor protein in the cell membrane and an accumulation in the cytosol of TG neurons and this inhibitory effect involves activation of α_2 -adrenergic receptors and is independent of 5HT_{1B/1D} receptors.

DISCUSSION

In this study, we used primary cultures of trigeminal ganglia as an *in vitro* model to study the intracellular effects of ATP and potassium (K⁺) ions on trigeminal neurons as levels of these molecules are elevated in CSF in response to CSD and hypercortical activity and thus, could directly modulate the excitability state of trigeminal afferents within the dura. In support of this notion, we found that ATP, in a time-dependent manner, lowered the activation threshold of trigeminal neurons such that a subthreshold amount of K⁺ ions was required to cause neuronal activation as demonstrated by increased release of CGRP. In other studies, ATP has been reported to be involved in inflammatory, allodynic, and nociceptive responses by modulating the expression of key signaling pathways in both neuronal and glial cells leading to increased production and release of stimulatory molecules.^{35,36}

The regulatory effects of ATP have been shown to be mediated via activation of ATP receptors that function as ion channels or coupling to G-proteins that regulate cellular responses through stimulation of intracellular signaling pathways.^{36–38} In our study, we showed that 100 μ M ATP could stimulate a small transient increase in intracellular calcium that was not sufficient to stimulate CGRP release. However, pretreatment of ATP lowered the activation threshold such that a subthreshold concentration of KCl (30 mM) caused a large enough increase in intracellular calcium to mediate CGRP release. Based on our inhibitor studies with the high-affinity non-nucleotide antagonist A-317491,^{32,39} the stimulatory effect of ATP was thought to involve, at least in part, activation of heteromeric P2X_{2/3} or homomeric P2X₃ receptors, which are abundantly expressed by TG neurons projecting to the dura¹¹ and upon activation mediate acute and chronic pain.^{8,12,36,40,41} Importantly, results from another study using A-317491 have provided evidence that blocking P2X₃ receptors was effective in repressing nociception in inflammatory and

neuropathic pain models.³⁹ Our findings are also in agreement with a recently published study that reported ATP activation of P2X₃ and P2X_{2/3} receptors could cause sensitization of primary afferent nociceptors that was involved in development of hyperalgesia.⁴² Further evidence that the sensitizing effect of ATP was being mediated primarily through activation of P2X₃ was provided by our studies using the selective P2X₃ agonist α,β -meATP. We found that treatment with α,β -meATP caused a similar increase in the amount of CGRP release in response to a subthreshold KCl concentration as that observed with ATP. Taken together, we propose that ATP released in response to CSD or cortical hyperexcitability causes sensitization of trigeminal nociceptors via activation of P2X₃ receptors that facilitates CGRP release. CGRP is thought to play an important role in migraine pathology as activation of peripheral trigeminovascular V1 afferents in the meninges releases CGRP, which mediates neurogenic vasodilation and mast cell degranulation, while central efferent release of CGRP contributes to pain, central sensitization, and allodynia associated with migraine.^{43,44}

A novel finding of our study was that the inhibitory effects of DHE on ATP-sensitized trigeminal neurons were primarily mediated via activation of α_2 -adrenoceptors. DHE has been reported to bind with high affinity to α_2 -adrenergic receptors as well as 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} serotonergic receptors, and D1 and D2 dopaminergic receptors.¹⁴ We found that pretreatment of trigeminal neurons with yohimbine blocked the inhibitory effects of DHE on ATP and KCl stimulation of CGRP secretion and elevated intracellular calcium levels, in addition to preventing downregulation of P2X₃ receptor membrane expression. Yohimbine, which functions as an antagonist, has marked affinity for the α_{2A} -adrenergic, α_{2B} -adrenergic, and α_{2C} -adrenergic receptors.⁴⁵ Somewhat surprisingly, the inhibitory effects of DHE on CGRP secretion and intracellular calcium levels were not blocked by pretreatment with the selective 5-HT_{1B/1D} antagonist GR 127935. Our findings differ from results reported in earlier studies that provided evidence that DHE repression of trigeminal neuron excitation by electrical stimulation occurred via mechanisms that involved 5-HT receptor activation.^{15,16} A major difference between our study and those from previous studies is that we were investigating DHE repression of KCl stimulation of ATP-sensitized trigeminal neurons. Thus, DHE repression of trigeminal neuron excitation and neuropeptide release may be mediated via activation of different receptor types, serotonergic or adrenergic, based in part on the response to different inflammatory stimuli. Based on our findings with GR 127935, we speculate that triptans, which have affinity and selectivity primarily for 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors^{46,47} would not repress the stimulatory effects of ATP on trigeminal neurons. Rather, our results implicate a primary role of α_2 -adrenoceptors in mediating the inhibitory effects of DHE on ATP sensitization of trigeminal neurons via P2X₃ receptors. In agreement, activation of α_2 -adrenoceptors that are expressed on TG neurons was found to exert an inhibitory effect on nociceptive transmission within the trigeminal system at the level of the primary afferent terminals as well as neuronal cell bodies.¹⁹ Interestingly, selective activation of α_2 -adrenoceptors has previously been proposed as a useful target for the development of potential anti-migraine drugs because of their ability to cause vasoconstriction.^{48–50} Based on findings from our study, we would agree that selective activation of α_2 -adrenoceptors would be a viable target, but rather because of their inhibitory effect on ATP-mediated sensitization and P2X₃ membrane expression in trigeminal neurons.

Based on our results, we propose that the ability of DHE, via activation of α_2 -adrenoceptors, to treat a migraine attack is likely to involve repression of ATP sensitization of trigeminal neurons and augmentation of CGRP release by inhibiting ATP-mediated increase in intracellular calcium. Our finding in trigeminal neurons is in agreement with other neuronal studies that have shown that α_2 -adrenoceptors couple to inhibition of voltage-gated Ca²⁺ channels via pertussis toxin sensitive G-proteins. Specifically, α_2 -adrenoceptors are reported

to couple to the G_o/G_i -protein to inhibit adenylyl cyclase or to interact directly with calcium channels.^{24,25} Results from our study provide additional evidence that DHE functions in a somewhat different manner than the triptans as we found that DHE treatment of trigeminal neurons did not change intracellular calcium levels as previously reported for the triptans.^{28,29} In those studies, sumatriptan was shown to cause a prolonged low amplitude increase in intracellular calcium that was mediated via activation of 5-HT₁ receptors. Furthermore, the prolonged calcium increase was shown to be sufficient to cause repression of stimulated CGRP synthesis as well as its release. In contrast, we found that DHE repression of KCl-stimulated release of CGRP from ATP-sensitized trigeminal neurons involves inhibition of intracellular calcium levels by decreasing membrane expression of the P2X₃ receptor. Thus, a sufficient number of membrane P2X₃ receptors would not be accessible to facilitate ATP-mediated sensitization of trigeminal neurons. It is likely that DHE-mediated inhibition of membrane P2X₃ expression involves repression of the cAMP/PKA signaling pathway that has been reported to lead to an enhancement of P2X₃ homomeric receptor-mediated ATP responses in dorsal root neurons.⁵¹

Adenosine triphosphate-mediated sensitization of trigeminal neurons leading to increased release of CGRP is likely to have important implications for sustaining a migraine attack as CGRP has been shown to increase the amplitude of P2X₃ receptor currents and accelerated recovery from desensitization as well as stimulated trafficking of the P2X₃ receptors to the cell membrane.¹⁰ In addition, CGRP stimulates transcription of the P2X₃ receptors via activation of protein kinase A and C, thus leading to increased numbers of receptors in trigeminal neurons. It is interesting to speculate that prolonged elevated levels of CGRP as has been reported between migraine attacks^{52,53} may play a role in the transition from acute episodic migraine attacks to more chronic migraine by facilitating increased P2X₃ expression that leads to long lasting neuronal sensitization. Toward this end, a primed state of nociceptive neurons has recently been described in which changes in the expression of ion channels and receptors leads to long term changes in the excitability state of these neurons.^{54,55} In a primed state, which involves increased expression of protein kinases A and C, changes in nociceptors significantly lower the concentrations of inflammatory mediators required to elicit a heightened state of pain (hyperalgesia) that can persist for several weeks. Thus, nociceptors in a primed state would respond to lower concentrations of stimuli, such as ATP, which under normal conditions might only lead to a sensitized state. In essence, sensitizing agents now become activating agents of nociceptive neurons. Importantly, inhibition of P2X₃ expression was recently shown to block chronic pain.⁵⁶ Furthermore, it is thought that sensitization of sensory neurons is a critical step in the development of a constant pain state, which is characteristic of chronic migraine that is presently relatively refractory to standard anti-migraine treatments. Thus, based on findings from our study as well as other studies, we propose that ATP and CGRP interactions are likely to play an important role in the development and maintenance of peripheral sensitization in acute episodic migraine while development of a primed state in trigeminal nociceptors is involved in the transition to chronic migraine.

To our knowledge, our study provides the first evidence that ATP can lower the activation threshold of trigeminal neurons to K⁺ ions and, hence, can function to promote peripheral sensitization of nociceptive afferent fibers that provide innervation of the meningeal blood vessels and are involved in the pain of migraine. Based on our findings, we propose that DHE, as has been reported for the triptans, may be most beneficial therapeutically if taken early in a migraine attack during the initial stages characterized by peripheral sensitization and when headache pain is mild.^{57–59} In addition, our results provide evidence that DHE should be most effective in treating migraine with aura as these types of attacks are characterized by CSD, an event that triggers increased release of ATP and K⁺ ions that can lead to activation of trigeminal nociceptive neurons. It will be of interest to determine in

clinical trials whether more rapid dosing of DHE, as reported for LEVADEX™, will be more beneficial in treating migraine with aura than migraine without aura and whether it will be more effective than triptans. Finally, our finding that DHE can block KCl stimulation of ATP-sensitized trigeminal neurons and decrease membrane expression of P2X₃ receptors may provide a possible explanation at the cellular level for why DHE has a lower recurrence rate than triptans^{13,60} and is used successfully to treat chronic daily headache.^{61,62}

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Abbreviations

ATP	adenosine triphosphate
CGRP	calcitonin gene-related peptide
CSD	cortical spreading depression
DHE	dihydroergotamine
K+	potassium ion
KCl	potassium chloride
TG	trigeminal ganglion
TNC	trigeminal nucleus caudalis

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Fig 1.

Effect of ATP and KCl on CGRP secretion from trigeminal ganglion neurons. (A) The relative amount of CGRP secreted in 1.5 hours from untreated control cultures (CON), cultures treated with ATP or KCl alone, treatment with ATP and KCl, or pretreated with A-317491 (A-317) prior to addition of ATP and KCl. The amount of CGRP secreted into the culture medium in each condition was first normalized to the basal rate for each well. The mean basal rate of CGRP release was 60 ± 7 pg/hour/well. (B) Effect of α, β -meATP and KCl on CGRP secreted in 1.5 hours from untreated control cultures (CON), cultures treated with α, β -meATP ($\alpha\beta$) or KCl alone, and treatment with α, β -meATP and KCl. The amount of CGRP secreted into the culture medium in each condition was normalized to the basal rate for each well (49 ± 5 pg/hour/well). Values are reported as fold-change relative to control levels whose mean was made equal to 1. The means $\pm$ SE from 3 independent experiments are shown. * $P < .01$ when compared with control and # $P < .01$ when compared with ATP and KCl treatment. (C) Temporal sequence of treatments to the cultures. ATP = adenosine triphosphate; CGRP = calcitonin gene-related peptide; KCl = potassium chloride.

Fig 2.

Adenosine triphosphate (ATP) potentiation of KCl-mediated elevation of intracellular calcium levels. Representative plots of F340/380 ratio changes recorded from trigeminal neurons incubated in HBS in response to ATP or α,β -meATP ($\alpha\beta$) (A), KCl treatment (B), pretreatment with ATP or α,β -meATP for 15 minutes before addition of KCl (C), or pretreatment with A-317491 (A-317) prior to treatment with ATP and KCl (D). The average change in the F340/380 ratio $\pm$ SE in response to each treatment is shown in panel E. * $P < .01$ when compared with KCl or ATP treatment alone and # $P < .01$ when compared with α,β -meATP treatment alone. (F) Temporal sequence of treatments to the cultures. KCl = potassium chloride.

Fig 3.

Dihydroergotamine (DHE) repression of ATP-mediated sensitization and inhibition of KCl stimulation of CGRP release. (A) The relative amounts of CGRP secreted in 1.5 hours from trigeminal neurons incubated in HBS from untreated control cells (CON), cells treated with DHE alone, treatment with ATP and KCl in the absence or presence of DHE, yohimbine (YO), or GR 127935 (GR) are shown. The amount of CGRP secreted into the culture medium in each condition was first normalized to the basal rate for each well (68 ± 5 pg/hour/well). (B) The relative amounts of CGRP secreted in 1.5 hours from trigeminal neurons incubated in HBS from untreated control cells (CON), cells treated with DHE alone, treatment with α, β -meATP ($\alpha\beta$) and KCl in the absence or presence of DHE, yohimbine (YO), or GR 127935 (GR) are shown. The mean basal rate of CGRP release was 54 ± 4 pg/hour/well. Values are reported as fold-change relative to control levels whose mean was made equal to 1. The means $\pm$ SE from 3 independent experiments are shown. * $P < 0.01$ when compared with controls. # $P < .05$ when compared with ATP and KCl treatment. (C) Temporal sequence of treatments to the cultures. ATP = adenosine triphosphate; CGRP = calcitonin gene-related peptide; KCl = potassium chloride.

Fig 4.

Dihydroergotamine (DHE) inhibition of ATP potentiation of KCl-mediated elevation of intracellular calcium levels. Representative plots of F340/380 ratio changes recorded from trigeminal neurons incubated in HBS in response to DHE (A), DHE prior to addition of ATP, α,β -meATP ($\alpha\beta$ ATP), or KCl (B). Shown also are plots representing pretreatment with ATP or α,β -meATP for 15 minutes before KCl addition (C), addition of DHE prior to treatment with ATP and KCl or α,β -meATP and KCl (D), pretreatment with yohimbine and DHE prior to the addition of ATP and KCl or α,β -meATP and KCl (E), or pretreatment with GR 127935 (GR) and DHE prior to addition of ATP and KCl or α,β -meATP and KCl (F). The average change in the F340/380 ratio $\pm$ SE in response to various treatments is shown in panels G and H. * $P < .01$ when compared with ATP alone (G) or ATP and KCl treatment (H). # $P < .01$ when compared with α,β -meATP ($\alpha\beta$) alone (G) or α,β -meATP ($\alpha\beta$) and KCl

(H). (I) Temporal sequence of treatments to the cultures. ATP = adenosine triphosphate; KCl = potassium chloride.

Fig 5.

Membrane expression of P2X₃ in trigeminal ganglion neurons is decreased in response to DHE. Fluorescent P2X₃ images and images merged with a differential interference contrast (DIC) image of untreated cultured trigeminal ganglion neurons (CON) or cultures treated with DHE, yohimbine prior to DHE (YO + DHE), or GR 127935 before DHE treatment (GR + DHE) were stained for P2X₃ receptors and images were taken at 630× magnification. Scale bars = 10 μm. Right panels. The relative fluorescence intensity of P2X₃ staining of neurons along the white lines (left to right), as determined using Zeiss Axio Vision software, are shown in the right hand panels. Lower graph. A change in number of cells expressing P2X₃ immunostaining primarily in the neuronal cell membrane of untreated cultured trigeminal ganglion neurons (CON) or cultures treated with DHE, yohimbine prior to DHE (YO + DHE), or GR 127935 before DHE treatment (GR + DHE). Values are reported as percent change relative to control levels whose mean was made equal to 100%. **P* < .01 compared with control levels. DHE = dihydroergotamine.

Table

Temporal Change in Intracellular Calcium Levels

Time of addition of 30 mM KCl (Min)	0	5	10	15	20
F340/380 average for ATP 100 μ M	1.556 $\pm$ 0.087	1.395 $\pm$ 0.124	1.366 $\pm$ 0.468	2.0391 $\pm$ 0.047*	2.139 $\pm$ 0.045*
F340/380 average for α , β -meATP 10 μ M	1.337 $\pm$ 0.130	1.339 $\pm$ 0.162	1.627 $\pm$ 0.169	2.167 $\pm$ 0.160*	2.606 $\pm$ 0.236*

* $P < .01$.

ATP = adenosine triphosphate; KCl = potassium chloride.