P/Q-Type Calcium-Channel Blockade in the Periaqueductal Gray Facilitates Trigeminal Nociception: A Functional Genetic Link for Migraine?

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The discovery of mis-sense mutations in the α1A subunit of the P/Q-type calcium channel in patients with familial hemiplegic migraine indicates the potential involvement of dysfunctional ion channels in migraine. The periaqueductal gray (PAG) region of the brainstem modulates craniovascular nociception and, through its role in the descending pain modulation system, may contribute to migraine pathophysiology. In this study we sought to investigate the possible link between the genetic mutations found in migraineurs and the PAG as a modulator of craniovascular nociception. We microinjected the P/Q-type calcium-channel blocker ω-agatoxin IVA into the rat ventrolateral PAG (vlPAG). We examined its effect on the nociceptive transmission of second-order neurons recorded in the trigeminal nucleus caudalis and activated by stimulation of the parietal dura mater. After injection of agatoxin into the vlPAG ($n = 20$) responses to dural stimulation were facilitated by 143% ($p < 0.0001$) for Aδ-fiber activity and 180% for C-fiber activity ($p < 0.05$). Similarly, spontaneous background activity increased by 163% ($p < 0.0001$). These results demonstrate that P/Q-type calcium channels in the PAG play a role in modulating trigeminal nociception and suggest a role for dysfunctional P/Q-type calcium channels in migraine pathophysiology.

Key words: migraine; electrophysiology; periaqueductal gray; P/Q-type calcium channel; trigeminal; nociception

It has been suggested that migraine may involve a calcium channelopathy. Although the gene *CACNA1A* has also been implicated in normal migraine, approximately one-half of patients with familial hemiplegic migraine carry mis-sense mutations in this gene, which encodes the α1A subunit of the voltage-gated calcium channel (May et al., 1995; Ophoff et al., 1996; Nyholt et al., 1998; Kors et al., 2001).

CACNA1A encodes the pore-forming protein of P/Q-type calcium channels. These are a heterogeneous class of calcium channel involved in controlling neurotransmitter release throughout the mammalian brain (Dunlap et al., 1995; Craig et al., 1998). They are located on cell bodies, dendrites, and predominantly presynaptic terminals and are widespread in the CNS, including regions involved in nociception such as the trigeminal ganglion, spinal trigeminal nucleus, spinal cord dorsal horn, nucleus raphe magnus, and ventral periaqueductal gray (PAG) (Hillman et al., 1991; Westenbroek et al., 1995; Craig et al., 1998). P/Q-type calcium channels at the spinal level have been shown to affect nocifensive behavior and the hyperexcitability of nociceptive dorsal horn neurons (Malmberg and Yaksh, 1994; Nebe et al., 1999). However, the role of P/Q-type calcium channels in the supraspinal modulation of nociception remains unclear.

It has been postulated that the brainstem plays a pivotal role in migraine (Goadsby et al., 1991; Welch et al., 2001). Direct evidence of a prominent role for the brainstem, particularly PAG, in migraine has been provided by functional imaging studies of patients (Weiller et al., 1995; May et al., 1998; Bahra et al., 2001; Welch et al., 2001). Also, it is well documented that stimulation (Raskin et al., 1987) or lesions in the PAG can produce migraine-like headache in non-migraineurs (Haas et al., 1993; Veloso et al., 1998). These clinical studies indicate a role for the PAG in migraine and particularly the nociceptive processes of headache.

The PAG has been shown to modulate nociception in various experimental animal models of pain (Reynolds, 1969; Behbehani, 1995). Of particular importance to trigeminal nociceptive modulation is the ventrolateral subdivision of the PAG (vlPAG). It modulates trigeminal nociception in the same model of craniovascular pain as used in this study and selectively receives input from trigeminovascular afferents (Oliveras et al., 1974; Keay and Bandler, 1998; Hoskin et al., 2001; Knight and Goadsby, 2001). PAG modulation of trigeminal nociception has been most thoroughly studied for its inhibitory effects and less so for trigeminal pronociceptive effects (Behbehani, 1995). An aim in this study was to further characterize vlPAG modulation of trigeminal nociceptive transmission and, therefore, to investigate a possible mechanism by which the PAG could produce headache.

In an attempt to draw together a genetically driven, central

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attribute for migraine pathophysiology with a focus on nociceptive transmission, we investigated the effect of blockade of ventrolateral PAG P/Q-type calcium channels in a model of trigeminovascular activation in the rat. In our model, we activated trigeminal nociceptive neurons by stimulating the supratentorial parietal dura adjacent to the middle meningeal artery (MMA), recorded the activity of second-order neurons in the trigeminal nucleus caudalis, and studied their response to blockade of P/Q-type calcium channels in the PAG.

MATERIALS AND METHODS

All experiments were performed under a project license issued by the UK Home Office under the Animals (Scientific Procedures) Act of 1986. A total of 20 male Sprague Dawley rats weighing 329 ± 9 gm (mean ± SD) were anesthetized [Sagatal (pentobarbitone sodium), 65 mg/kg, i.p. induction; maintenance with α-chloralose, 15 mg/kg, i.v.] and, during electrophysiological recording, paralyzed [Pavulon (pancuronium bromide) 1 mg/kg initially, then 0.4 mg/kg maintenance].

Surgery. The head of the animal was fixed in a stereotaxic frame. The parietal dura adjacent to the MMA was exposed through a small cranial window and stimulated. For the multibarreled microinjection unit to enter the brain a burr hole was made in the cranium and the superior sagittal sinus was pierced to allow penetration. The muscles of the dorsal neck were separated and the dura was incised to expose the brain stem at the level of the caudal medulla.

MMA stimulation. To activate trigeminal primary afferents, the dura was stimulated with a Grass S88 stimulator (Grass Instrument Co., Quincy, MA). A pair of bipolar electrodes was placed on the dura on either side of the MMA and electrical square-wave stimuli (0.5–0.6 Hz) of 0.5–2 msec duration up to 10 V were applied.

Trigeminal nucleus caudalis recording. Custom made tungsten in glass electrodes with a tip diameter ranging from 10 to 20 μm and impedances ranging from 400 to 800 kΩ were used to record the activity of trigeminal nociceptive neurons in the trigeminal nucleus caudalis. Extracellular recordings of single or multiple neurons were made from MMA stimulation-evoked or spontaneous nerve signals. Signals were fed into a window discriminator connected to an analog-to-digital interface (CED Power 1401plus; Cambridge Electronic Design, Cambridge, UK) and an IBM-compatible computer. Full spikes were discriminated by means of an electronic delay unit. Poststimulus time histograms (PSTHs) and peristimulus time histograms of neural activity were displayed and analyzed using conventional software (Spike 2.01; Cambridge Electronic Design).

PAG microinjection. A multibarreled glass capillary unit was used for microinjection of drugs into the ipsilateral PAG. The stereotaxic positioning of the multibarrel unit was aimed at co-ordinates for the caudal ventrolateral PAG according to the atlas of Paxinos and Watson (1998): 1.36 mm rostral and 4.2 mm dorsal from the interaural point, 0.5–0.7 mm left of the midline. Drugs were injected over a period of 30–120 sec for a volume range of 50–600 nl.

Characterization of neurons. Trigeminal nucleus caudalis (TNC) neurons were characterized for their cutaneous and deep receptive fields. The cutaneous facial receptive field, including the cornea, was assessed in all three trigeminal innervation territories. The receptive field was mapped by applying non-noxious and noxious stimuli. Non-noxious stimuli were applied by gentle brushing and by applying light pressure with a blunt probe. Noxious mechanical stimuli consisted of pinching with forceps or applying heavy pressure deemed painful when applied to humans. Neurons were classified as wide-dynamic range (WDR) if they responded to non-noxious and noxious stimuli and as nociceptive-specific neurons (NS) if they responded only to noxious input (Hu et al., 1981).

Trains of 20 or 30 stimuli were delivered in 5 min intervals to assess the baseline responses to dural stimulation. Stimulation latencies corresponding to Aδ- and C-fiber were 0–30 msec and 30–100 msec, respectively. Spontaneous activity is presented as spikes per second (in Hertz). Spontaneous activity was recorded within 120–180 sec preceding the dural stimulation epoch.

Inhibition of evoked TNC activity after bicuculline injection in the PAG was considered a functional connection between the region of PAG microinjection and the recorded TNC neuron. Three baseline PSTHs were collected to ensure reproducibility over time of responses to MMA stimulation. Because agatoxin is an irreversible P/Q-type calcium-channel blocker (Mintz et al., 1992), only one TNC neuronal response in each animal was tested after the application of agatoxin into the PAG.

Experimental protocol. The sequence of experimental events were as follows: (1) three baseline collections; (2) bicuculline injection into PAG; (3) collections at 1, 5, 10, 15, 20, and 25 min after bicuculline injection; (4) three new baseline collections; (5) agatoxin or saline injection into the PAG; and (6) collections at 5, 10, 15, 20, 25, 30, 40, 50, and 60 min after agatoxin or saline injection.

Drug preparation and histology. Drugs were prepared as follows: 0.4 mM bicuculline methiodide, pH 8.2–9.6 (Sigma, St. Louis, MO), 0.1 μM ω-agatoxin IVA, pH 8.0–8.4 (Scientific Marketing Association, Barnet, Herts, UK), and 2% Pontamine Sky Blue, pH 8.0–8.7 (Sigma) in 100 mM sodium acetate were used. At the completion of the experiment, an electrical lesion was made in the TNC and a deposit of Pontamine Sky Blue was made in the PAG. The brain was stored for 24–36 hr in a staining and fixing solution, frozen, cut into 40 μm sections, and stained with neutral red or cresyl violet.

Statistical analysis. Data for Aδ- and C-fiber responses were normalized to 100% of baseline before statistical analysis. Data for spontaneous activity were analyzed raw (Hertz), except in the saline controls. Two ANOVAs for repeated measures were used to independently determine the time course of significant drug interventions for bicuculline and agatoxin. Statistical significance was set at $p < 0.05$. For ANOVA output, the data are presented as $F_{(df,\ n)}$, where df is degrees of freedom and n is the number of samples. A two-sample Student's t test for *post hoc* analysis was used to evaluate statistical significance compared with baseline of bicuculline or agatoxin at the time of maximal effect. To account for the large scale of firing rates across C-fiber samples (3–810 Hz) postdrug responses were normalized to 100% and pooled; a one-sample t test was then applied. Data are expressed as mean ± SEM for a number (n) of observations. ANOVA, paired sample, and one-sample t tests were performed using SPSS statistical software, version 9.0 (SPSS, Inc., Chicago, IL).

RESULTS

Recordings were made from 20 neurons (18 WDR and 2 NS) responsive to dural stimulation with cutaneous receptive fields restricted to the ophthalmic division of the trigeminal nerve, including the cornea ($n = 20$). A total of 16 neurons showed input from frontalis muscle. Neurons were found in the deep layers of the dorsal horn of the C_1/TNC transition zone at a mean depth of 967 μm (range 470–1495 μm) (Fig. 1). PAG microinjection sites were localized to the vlPAG, and three were in the ventral border of the lateral PAG (Fig. 1).

Bicuculline injection in the vlPAG

To identify functional inhibitory projections from the PAG to the TNC, bicuculline was injected into the vlPAG. Injection of bicuculline (50–300 nl) into the vlPAG produced inhibition of the trigeminal nociceptive response to dural stimulation. In Aδ-fiber responses, maximum inhibition was observed at 5 min after bicuculline injection ($F_{(19,7)} = 8.321$; $p = 0.001$). Maximum inhibition of mean spontaneous activity (MSA) was also observed at 5 min ($F_{(3.1,17)} = 21.4$; $p < 0.0001$) (Fig. 2). Aδ-fiber responses were inhibited by 41 ± 4% of baseline, ranging from 8 to 67% ($n = 18$; $p < 0.0001$). C-fiber inhibition was 50 ± 6% ($n = 11$; $p < 0.0001$). Baseline MSA was 52 ± 4 Hz, ranging from 18 to 102 Hz. Bicuculline significantly inhibited MSA to 24 ± 3 Hz, ranging from 4 to 95 Hz ($n = 17$; $p < 0.0001$).

Agatoxin injection in the vlPAG

Microinjection of 400–600 nl of agatoxin into the vlPAG produced a facilitation of the trigeminal nociceptive response to dural stimulation and to spontaneous activity.

In Aδ-fiber responses, facilitation became significant at 15 min after agatoxin injection and was 122 ± 4% ($F_{(3,13)}=7.375$; $p = 0.041$). Maximal facilitation was observed at 60 min after injec-

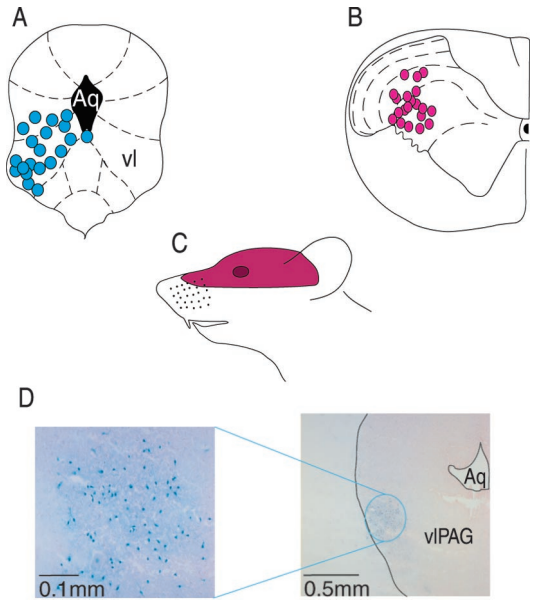

Figure 1. Histological confirmation of PAG microinjection sites in the ventrolateral PAG (*A*), where each *blue dot* represents one injection site. For the recording sites in the deep layers of the TNC (*B*), each *red dot* represents the site of one recording from dura-responsive neurons. A typical example of a cutaneous facial receptive field (*C*) that was restricted to the ophthalmic division of the trigeminal nerve (*red area*) is shown. *D*, Representative example of a Pontamine stain in the vlPAG, showing volume spread of <0.4 mm at low and high magnification. *Aq*, Aqueduct; *vl*, ventrolateral. This illustration was adapted from Molander and Grant (1995) and Paxinos and Watson (1998).

tion and ranged from 116 to 216% (mean, 143 ± 7%) ($n = 14$; $p < 0.0001$) (Fig. 2).

C-fiber facilitation was 180 ± 35% ($n = 12$; $t_{(11)} = -2.277$; $p = 0.044$). Facilitation compared with baseline was significant 5 min after agatoxin injection and was 165 ± 25%. Peak facilitation was at 50 min after injection (Fig. 2).

Baseline spontaneous activity after bicuculline was 55 ± 3 Hz (range, 20–90 Hz). Facilitation became significant at 10 min after agatoxin injection and was 67 ± 7 Hz ($F_{(3,16)}$=16.2; $p = 0.033$). Maximal facilitation was observed at 60 min after injection and was 90 ± 4 Hz (mean), ranging from 19 to 209 Hz ($n = 16$; $p < 0.0001$) (Fig. 3).

In many cases, a notable characteristic of the increased spontaneous activity after agatoxin injection into the vlPAG was that the firing changed to a burst-like pattern after an initial tonic increase of activity; this burst-like pattern lasted throughout the 60 min observation period.

Control injections inside and outside the vlPAG

Five WDR neurons inhibited by bicuculline in the vlPAG were tested for injection of saline at the same site and observed over 60 min. Saline injection in the vlPAG did not significantly affect the nociceptive responses of Aδ-fiber activity ($p = 0.284$), C-fiber activity ($p = 0.113$), or spontaneous activity ($p = 0.574$) compared with baseline. In seven WDR neurons, bicuculline or agatoxin injected outside the PAG, 0.5–1 mm dorsal or lateral to the border of the PAG, did not affect trigeminal nociceptive activity in Aδ-fiber responses ($p = 0.467$), C-fiber responses ($p = 0.512$) or spontaneous responses ($p = 0.418$) compared with baseline.

Blood-pressure effect

Bicuculline in the vlPAG elicited a characteristic transient decrease in blood pressure (Lovick, 1985; Waters and Lumb, 1997) of 15–20 mmHg over 60–100 sec after injection (Fig. 4*B*). This was not correlated with a change in spontaneous activity and this transient blood-pressure decrease returned to baseline before the onset of inhibition, which outlasted the duration of the blood-pressure effect, as reported previously (Sandkuhler et al., 1991; Waters and Lumb, 1997). In some cases, agatoxin also affected blood pressure by inducing fluctuations over a 30–40 mmHg range throughout the observation period. An example is given in Figure 4. We did not observe a correlation between the overall change in spontaneous activity and the blood-pressure fluctuations induced by agatoxin.

DISCUSSION

We have studied the blockade of P/Q-type calcium channels in the caudal ventrolateral periaqueductal gray and observed its effect on spinal trigeminal firing linked with dural stimulation. We report a facilitation of trigeminal nociceptive activity after microinjection of the P/Q-type calcium-channel blocker ω-agatoxin IVA into the vlPAG. These results support a hypoth-

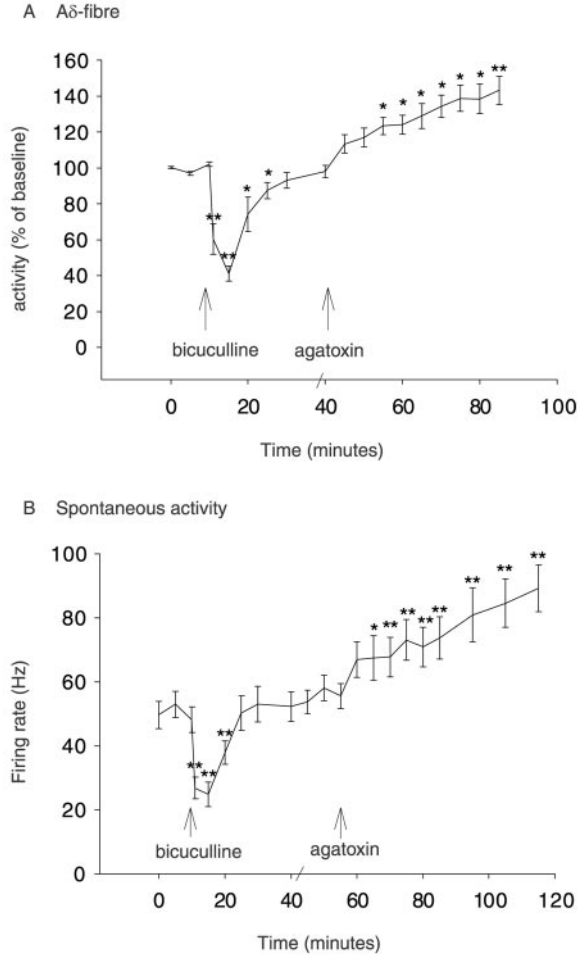

Figure 2. Time course of the group response of TNC neurons to bicuculline or agatoxin injection into the vlPAG, Aδ activity (*A*), and spontaneous activity (*B*). Group data, expressed as mean ± SEM (*$p < 0.05$; **$p < 0.01$; paired *t* test), indicate significant differences compared with baseline.

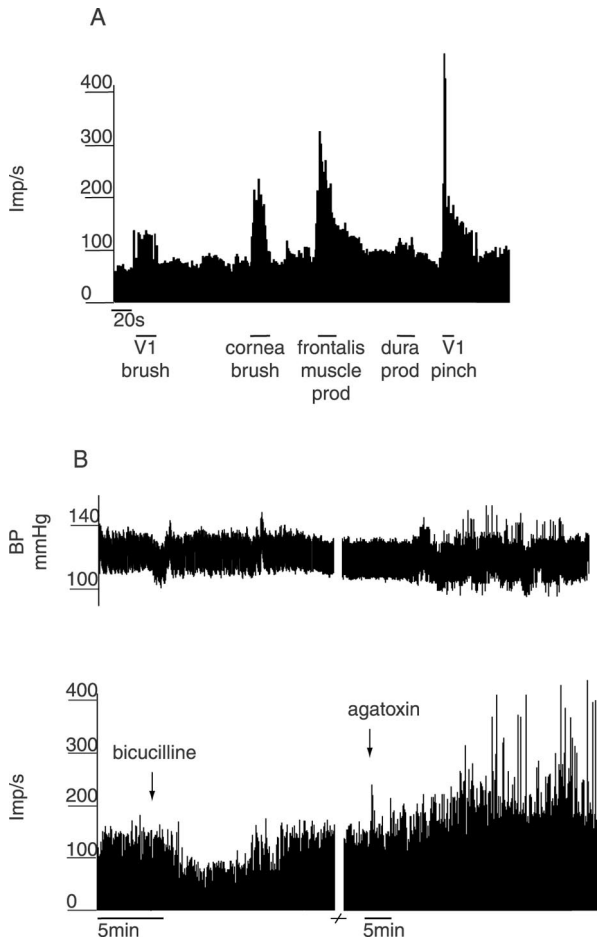

Figure 3. *A*, Receptive field characteristics of a sample TNC recording showing responses to stimulation of superficial and deep receptive fields. *B*, Histograms showing the inhibition of TNC spontaneous activity after bicuculline injection in the vlPAG and facilitation after agatoxin injection. The blood-pressure (*BP*) trace shows a characteristic transient decrease accompanying bicuculline injection in the PAG and sporadic fluctuations observed after agatoxin injection. *Imp/s*, Impulses per second.

esis for the involvement of the PAG in trigeminal pronociception and of P/Q-type calcium channels in trigeminal nociception.

The agatoxin-induced facilitation we observed across the Aδ-fiber, C-fiber, and spontaneous responses occurred within, on average, 10 min of agatoxin injection. This is in accordance with findings of *in vitro* cerebellar and hippocampal slice studies in which agatoxin blockade showed a similar time course to take effect on postsynaptic Purkinje and pyramidal cells (Poncer et al., 1997; Stephens et al., 2001). Because the facilitation we observed was in Aδ-fiber, C-fiber, and spontaneous responses, it suggests that there are no selective actions of the neurons on which the P/Q-type calcium channels were blocked. It is worth noting that the relatively high level of baseline spontaneous activity we recorded is likely to be caused by the type of electrodes we used. Other studies investigating the spontaneous activity of dural-responsive neurons recorded with carbon or stainless-steel electrodes (impedance, 8–12 MΩ) reported considerably lower spontaneous activity (0.05–46 Hz) as a result of the different spatial-recruitment characteristics of the electrodes (Burstein et al., 1998; Schepelmann et al., 1999). ω-agatoxin IVA is an irreversible blocker of P/Q-type calcium channels (Mintz et al., 1992); however, it also has a small effect on T-type channels in some cells (Rusin and Moises, 1995), depending on the concentration of agatoxin at the synapse. Because of difficulty in accurately controlling the toxin concentration at all synapses in complex tissue (Dunlap et al., 1995), we cannot exclude the possibility that non-P/Q-type channels were also blocked by agatoxin. The facilitation induced by agatoxin lasted the duration of the 60 min observation period. That the effect appeared maximal at 60 min may only reflect that our observation period was 60 min; an improvement of the data could be gained by observing the effect of agatoxin for a longer period.

Nociceptive facilitation can arise by anti-analgesic or pronociceptive effects. By injecting agatoxin into the PAG, we irreversibly blocked P/Q-type calcium channels in a population of heterogeneous neurons. At synaptic terminals, P/Q-type channels are required for coupling presynaptic action potentials to the transmitter release process (Dunlap et al., 1995; Sutton et al., 1999). As such, the channels are in predominantly presynaptic locations. Presynaptic locations in the PAG are positioned on GABA inhibitory interneurons and descending projection neurons. After PAG P/Q-type channel blockade, we saw an increase in evoked as

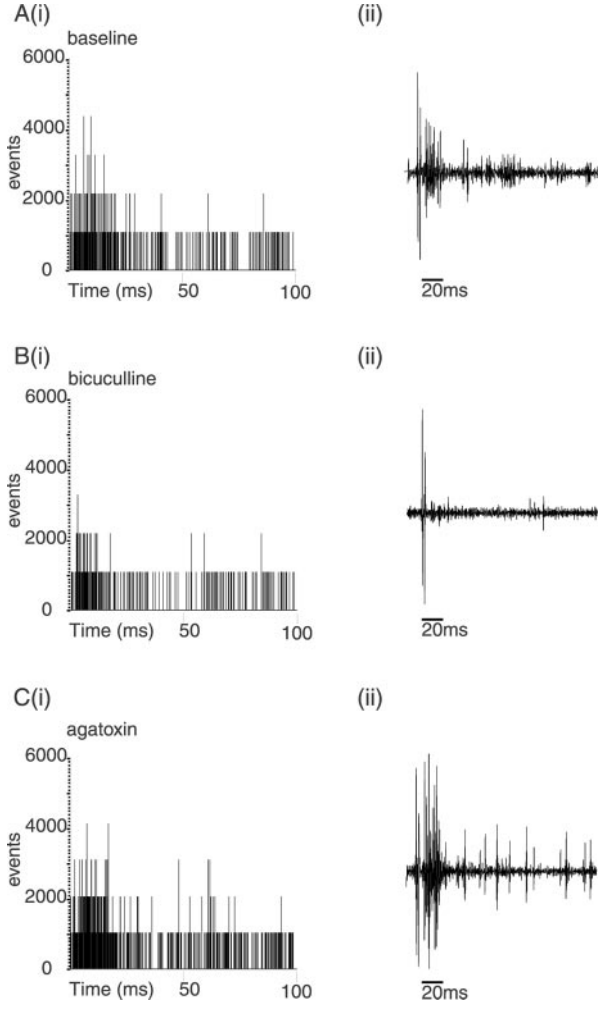

Figure 4. Sample experiment showing an inhibitory or facilitatory effect of bicuculline or agatoxin microinjected into the vlPAG, respectively, on dural stimulation-responsive neurons in the TNC. Poststimulus histograms (*i*) and original oscilloscope traces (*ii*) of the baseline response (*A*), the response 5 min after bicuculline injection in the PAG (*B*) showing inhibition of 42%, and the response 60 min after agatoxin injection in the PAG (*C*) showing facilitation of 144% are shown.

well as spontaneous trigeminal activity. Therefore, the neurons containing the blocked P/Q-type channels were likely to be actively firing before agatoxin injection. This indicates the involvement of neurons involved in tonic inhibitory maintenance of the nociceptive response.

The PAG contains a tonically active antinociceptive GABAergic network that operates through $GABA_A$ receptors (Reichling, 1991). When the $GABA_A$ antagonist bicuculline is injected into the PAG, it excites the majority of cells in the PAG and effectively acts by blocking the inhibition of inhibitory projection neurons (Sandkuhler et al., 1989; Behbehani et al., 1990). This mechanism was the basis for our use of bicuculline as a test search stimulus to identify projections between the PAG and trigeminal neurons. Conversely, a mechanism by which the PAG excites spinal nociceptive neurons is by inhibition of this descending inhibitory input (i.e., disinhibition). It is possible that the increase in trigeminal nociceptive activity that we observed resulted from a blockade of P/Q-type calcium channels negatively coupled to GABA release onto an inhibitory interneuron contacting an inhibitory projection neuron. If so, then blockade of P/Q-type calcium channels would enhance GABA release and disinhibit the dorsal horn neuron. This has been observed in cerebellar neurons, where α1A subunits play a major role in mediating action potential-evoked inhibitory GABA release in mouse Purkinje cells, and in hippocampal pyramidal cells (Poncer et al., 1997; Stephens et al., 2001). Additional studies in our model might confirm that P/Q-type calcium channels mediate such disinhibitory GABA release in the PAG.

Alternatively, rather than by disinhibition, the agatoxin-induced facilitation might have been the result of direct pronociceptive mechanisms. μ-opioid agonists hyperpolarize on-cells projecting to nociceptive dorsal horn neurons (Pan et al., 1990). Patch-clamp studies in dorsal root ganglion and nodose ganglion neurons have shown that P/Q-type channels are negatively coupled with μ-opioid receptors (Rusin and Moises, 1995, 1998). The same may prove to be the case in the PAG. Pronociception might arise from an increase in the basal excitability of dorsal horn neurons. Descending projections from the PAG can enhance the sensitivity of nociceptive dorsal horn neurons (Bederson et al., 1990; Urban et al., 1996). However, in some models the tonic activation of descending facilitation is dependent on afferent input from damaged peripheral nerves (Kovelowski et al., 2000).

How can these experimental findings be applied to the clinical observations of migraine and its pathophysiology? First, we have demonstrated that PAG P/Q-type calcium channels play a role in facilitating trigeminal nociception. In migraine, this places them as possible components of the headache process. Clinical imaging data strongly suggest dysfunction in the region of the PAG in migraineurs. The clinical cases of PAG lesions producing migraine-like headache support this suggestion (Raskin et al., 1987; Haas et al., 1993; Veloso et al., 1998). At the neurotransmitter level, a trigeminal-specific part of the PAG has not been determined; however, it is possible that those PAG-descending inhibitory neurons that target trigeminal regions of the dorsal horn are abnormally regulated by P/Q-type calcium-channel dysfunction in migraineurs. In conclusion, the results of this study suggest a possible site for the dysfunctional P/Q-type calcium channels in migraine and familial hemiplegic migraine.

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