Periaqueductal Gray Stimulation-Induced Inhibition of Nociceptive Dorsal Horn Neurons in Rats Is Associated with the Release of Norepinephrine, Serotonin, and Amino Acids

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ABSTRACT

The stimulation of the periaqueductal gray (PAG) produces behavioral analgesia in rats, cats, monkeys, and humans. This analgesia is believed to be mediated by several neurotransmitter systems, including the serotonergic, noradrenergic, glycnergic, y-aminobutyric acidergic, and opiateergic systems. The present study was designed to determine whether PAG stimulation produces the release of serotonin (5-HT), norepinephrine (NE), Gly, and y-aminobutyric acid in the spinal cord dorsal horn and whether the release of these neurotransmitters by PAG stimulation is associated with a long-lasting inhibition of the evoked nociceptive responses of dorsal horn neurons. The effect of different frequencies of stimuli on the release of neurotransmitters in the spinal cord was also examined. Microdialysis in combination with HPLC was used to measure the concentrations of neurotransmitters in the lumbar dorsal horn before, during, and after electrical stimulation of the PAG. The PAG was stimulated with electrical pulses at 333 Hz first and then at 67 Hz with the same intensity for 27 min, respectively. Both stimulus frequencies produced a significant increase in the release of 5-HT, NE, Gly, and Asp in the spinal dialysate, but the low-frequency stimulus was more potent in causing the release of neurotransmitters. Low-frequency stimulation also significantly increased the release of Glu. The time course of inhibition of dorsal horn neurons induced by long-lasting PAG stimulation corresponded to the time course of neurotransmitter release. Therefore, the results suggest that the long-lasting inhibition induced by PAG stimulation is mediated in part by the release of 5-HT, NE, and inhibitory amino acids in the spinal cord.

The stimulation at sites throughout the periaqueductal gray matter (PAG) can evoke behavioral analgesia in rats, cats, monkeys, and humans (Reynolds, 1969; Mayer et al., 1971; Liebeskind et al., 1973; Richardson and Akil, 1977; Fardin et al., 1984). The analgesia is mediated, at least in part, by descending pathways that inhibit the responses of dorsal horn neurons to noxious stimuli (Liebeskind et al., 1973; Willis, 1982). Several lines of evidence indicate that bulbo-spinal-projecting noradrenergic and serotonergic neurons mediate at least part of the antinociception produced by the PAG (Aimone et al., 1987; Tseng and Tang, 1990). Both serotonergic neurons, such as those in the nucleus raphe magnus (NRM), and noradrenergic neurons, such as those in the locus ceruleus (LC), A7, and A5 groups, project to the superficial dorsal horn of the spinal cord (Westlund et al., 1982; Fritschy and Grzanna, 1990; Jones and Light, 1990; Proudfit and Clark, 1991; Holden and Proudfit, 1998). However, mesencephalic nuclei, including the PAG, have only a sparse projection to the spinal cord but have significant projections to the caudal brain stem (Castiglioni et al., 1978; Yezierski et al., 1982). Therefore, it has been suggested that spinal actions resulting from PAG stimulation are mediated by excitatory synaptic connections from the PAG to the serotonergic NRM and the noradrenergic LC, A5, and A7 cell groups (Basbaum and Fields, 1979; Willis et al., 1984; Cameron et al., 1995; but see Kwiat and Basbaum, 1990; Ennis et al., 1991). The descending projections inhibit nociceptive dorsal horn neurons through activation of serotonergic receptors and adrenoceptors at the spinal cord level (Yaksh, 1979; Barbaro et al., 1985; Danzebrink and Gebhart, 1990).

Considerable pharmacological evidence supports this hypothesis. The inhibition of behavioral and dorsal horn neuronal responses to noxious stimulation produced by electrical or chemical activation of the PAG is blocked by lesions of or local anesthetic injection into the rostral ventral medulla (Gebhart et al., 1983; Chung et al., 1987). The iontophoretic application of 5-hydroxytryptamine [5-HT (serotonin)] or of a2 agonists to dorsal horn neurons, including STT cells, in-
hhibits their nociceptive-evoked activity (Willcockson et al., 1984), and serotonergic and noradrenergic antagonists block the effects of PAG stimulation (Lin et al., 1996; Peng et al., 1996a,b). Furthermore, the intrathecal injection of α9 adrenoceptor or serotonergic receptor antagonists reduces the antinociception produced through either electrical or chemical stimulation of PAG neurons or of the NRM (Jensen and Yaksh, 1984; Aimone et al., 1987). Finally, systemic depletion of 5-HT with para-chlorophenylalanine diminishes the inhibitory effects of PAG stimulation (Akl and Mayer, 1972).

In addition to the serotonergic and noradrenergic projections from the brain stem, the descending inhibitory pathways may also use amino acid neurotransmitters. Cells in the NRM and the adjacent reticular formation are immunopositive for substances other than 5-HT, including several peptides, γ-aminobutyric acid (GABA), and the excitatory amino acids, Glu and Asp (Reichling and Basbaum, 1990; Nicholas et al., 1992). In many cases, these substances are colocalized with 5-HT (Millhorn et al., 1987; Nicholas et al., 1992). GABA- and Gly-immunoreactive neurons are also concentrated in the spinal cord dorsal horn (Mitchell et al., 1993).

Pharmacological studies show that iontophoresis of GABA or Gly reduces the spontaneous activity of dorsal horn neurons and produces an inhibition of nociceptive evoked responses (Willcockson et al., 1984; Lin et al., 1994; Peng et al., 1996c). The inhibitory action of these two substances is blocked by their receptor antagonists (Lin et al., 1994). Other studies support this by showing that the intrathecal administration of GABA receptor antagonists, such as bicuculline, picrotoxin, and phaclofen, blocks the effect of a 5-HT, agonist in the spinal cord (Alhaider et al., 1991). Behavioral studies in rats also show that the intrathecal application of Gly decreases the scratching and biting behavior induced by intrathecally administered Substance P (Beyer et al., 1985).

If PAG stimulation produces antinociception through the activation of bulbospinal serotonergic, monoaminergic, and amino acid systems, then there should be an increased release of 5-HT, NE, and amino acids into the spinal cord during PAG stimulation. In previous work of a similar nature, it was shown that stimulation of the NRM produced antinociception and increased the release of 5-HT and amino acids in the spinal cord (Hammond et al., 1985; Sorkin et al., 1993). The release of endogenous 5-HT, NE, and amino acids from the spinal cord during electrical stimulation of the PAG, however, has not yet been reported. Therefore, this study was designed to determine 1) whether electrical stimulation of the PAG using stimulus intensities known to produce antinociception in unanesthetized rats increases the release of endogenous 5-HT, NE, and amino acids into superfusates of the rat spinal cord in vivo; 2) whether the frequency of stimulation affects the amount of neurotransmitter release; and 3) whether the time course of release corresponds with the inhibition of nociceptive responses induced by long-lasting PAG stimulation. A preliminary report of this work has been published (Cui et al., 1997).

Materials and Methods

Animal Preparation

Male Sprague-Dawley rats (279–350 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p.). A laminectomy was performed over the lumbar-sacral enlargement to expose the spinal cord for about 3 to 4 cm. The jugular vein and the trachea were cannulated. An initial dose of 0.3 ml of pancuronium was injected i.v. to paralyze the musculature, and then the animal was ventilated artificially. Continuous anesthesia and paralysis were maintained during the experiment through the constant i.v. administration of a mixture of 50 mg of sodium pentobarbital and 2 mg of pancuronium in 30 ml of a 0.9% NaCl solution at a rate of 0.05 ml/min. A craniotomy with a diameter of 2.0 to 2.5 mm was made at 7.0 mm posterior to the bregma and 0.2 mm lateral to the midline to allow insertion of an electrode for PAG stimulation (Paxinos and Watson, 1986). The head and vertebral column were fixed on a stereotaxic frame. Exposed spinal cord and brain were covered with mineral oil. End-tidal CO2 was kept between 3.5 and 4.5%, and the rectal temperature was maintained at 37°C by a servo-controlled heating blanket. All procedures were consistent with the guidelines of the International Association for the Study of Pain and the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and were approved by the local animal care and use committee.

Electrophysiological Recording

A tungsten microelectrode (125-μm shank, 12 MΩ) was used to record extracellular single-unit discharges in the lumbar enlargement. Dorsal horn neurons were identified by their responses to innocuous and noxious mechanical stimulation of the receptive field. A stimulation site in the PAG was located by introducing a monopolar stainless steel electrode stereotaxically 7.50 mm caudal to the bregma, 0.2 mm lateral to the midline, and 4.2 to 5.0 mm deep from the surface of the cerebral cortex and by adjusting the depth of the electrode until an effective inhibition was observed. Inhibition was recognized as a reduction in responses evoked in a nociceptive cell by stimulating its receptive field with mechanical cutaneous stimuli. Innocuous brush stimuli (Brush) were delivered with the use of a camel’s hair brush. Press and pinch stimuli were applications of arterial clips of different sizes, with the pressures applied being 144 g/mm² (Press) and 583 g/mm² (Pinch), respectively. Cells were then categorized as wide dynamic range (WDR), high threshold, or low threshold. High threshold neurons had a maximal response to Pinch and a response to Brush that was <10% of the Pinch response. WDR neurons had responses to all three test stimuli. Low threshold neurons responded best to Brush. The recording started with 10 s of background activity and then activity evoked by the application of Brush, Press, and Pinch stimuli to the receptive field for 10 s each. Unit activity was observed on analog and digital storage oscilloscopes and fed to a window discriminator and finally to a computer data collection system (CED 1401, 586 PC). Short (1-s) duration trains of 0.2-ms square pulses (333 Hz) were used to stimulate the PAG with intensities ranging from 100 to 400 μA.

Microdialysis Preparation

The fiber used for microdialysis was prepared from 30-cm-long hollow cellulose tubing (150-μm inner diameter, 9-μm-thick wall, 9-kDa molecular cutoff; Spectrum). Except for a 2-mm dialysis zone in the middle, the fiber was coated with a thin layer of silicon rubber (3140 RTN coating; Dow Chemical). The fiber was inserted into the spinal cord at the level just above the central canal and was as close as possible to the recorded dorsal horn neuron. The 2-mm collection zone was placed in the spinal cord gray matter. One side of the fiber was connected to a syringe that was controlled with a Harvard infusion pump. The perfusion rate was 2.5 μl/min, and the perfusate was artificial cerebrospinal fluid containing 151.1 mM Na⁺, 2.6 mM K⁺, 0.9 mM Mg²⁺, 1.3 mM Ca²⁺, 122.7 mM Cl⁻, 21.0 mM HCO₃⁻, and 2.5 mM HPO₄²⁻. The artificial cerebrospinal fluid (pH 7.2) was bubbled with 95% oxygen/5% CO₂ before the experiment. After exploratory stimulation of the PAG and the receptive field was finished, a period of 1.5 h was used as the washout and control time. The control period was chosen based on previous studies in our laboratory and the fact that basal concentrations of amino acids were...
stable or close to stable at this time (Sorkin et al., 1993). Samples were collected every 10 min and stored immediately on dry ice. Aliquots (25 μl) were then frozen at −80°C for later HPLC analysis. For NE measurements, 6.25 μl of 0.1 N HClO₄ was added to each sample to prevent the degradation of NE. Concentrations of 5-HT, NE, and amino acids in the dialysate were later measured with HPLC. The PAG was stimulated with trains of 0.2-ms square pulses (333 Hz) for a total of 27 min (9 min on and 1 min off, repeated three times). The intensities of stimulation for release ranged from 200 to 400 μA. To test whether the PAG was still able to cause neurotransmitter release in the spinal cord after the first period of stimulation, a second series of stimuli with 0.5-ms pulses at 67 Hz and the same intensity was applied to the same site 40 min after the first period of stimulation.

**Histology**

At the end of the experiment, the stimulation site in the PAG was marked by injecting D.C. current and later verified histologically. Brain stem and spinal cord tissue were fixed in 10% formaldehyde with added potassium ferrocyanide, sectioned later at 60 μm, and stained with neutral red dye. The stimulation sites in the PAG and the center of the dialysis zone were examined.

**HPLC**

5-HT and NE. Aliquots of dialysate were assayed for 5-HT or NE on a Beckman System Gold HPLC System with an ESA Coulochem II electrochemical detector set to 275 mV. A guard cell set to 400 mV was also used. The column temperature was maintained at 27°C throughout these analyses. An ESA MD-150 particle C₁₈ HPLC column (3-mm inner diameter, 150 mm long, 3-μm particle size) was used in conjunction with different mobile phases for 5-HT or NE analysis. The mobile phase for 5-HT analysis consisted of 10% acetonitrile, 75 mM NaH₂PO₄, 1.7 mM 1-octanesulfonic acid sodium salt, 100 μl of HPLC grade triethylamine, and 25 μM disodium EDTA adjusted to pH 3.0 with phosphoric acid. The HPLC flow rate was 0.6 ml/min. The mobile phase for NE consisted of 1840 ml of distilled water, 160 ml of methanol, 8.2 g of sodium acetate, 8.4 g of citric acid, 1.63 g of 1-octanesulfonic acid, 0.1 g of disodium EDTA, and 125 μl of dibutylamine adjusted to pH 4.3 to 4.6 with acetic acid. Because the loss of 5-HT within 2 months and NE within 1 month are less than 1%, the degradation of 5-HT and NE was negligible under the conditions of our experiments (all the measurements in this study were completed within 2 weeks). Comparisons of sample peak areas with those from standards were used to quantify 5-HT and NE in samples; average detection limits were about 0.01 pg/μl for 5-HT and for NE. The detection limits were determined by making multiple injections of diluted standards into the column. When the peak generated by diluted standards cannot be distinguished from noise, the concentration of the standards is considered to be at the detection limit. Peak areas were a linear function of the amount of substance injected over the concentration range of the samples. An external standard was run every five samples.

**Amino Acids.** Dialysate samples were thawed and injected into an HPLC with fluorescence detection after a precolumn derivatization with naphthalene-2,3-dicarboxyldihydrazide reagent. Samples, 2.55 mM NaCN in 0.01 M sodium borate buffer, and 1.009 mM naphthalene-2,3-dicarboxyldihydrazide in methanol were vortexed and reacted for 50 min at 40°C. The mixture was then chilled to 4°C in the compartment of a Waters automatic sample injector. These samples were assayed for several amino acids, including Asp, Glu, and Gly. Although we tried to measure the level of GABA, the low concentration of GABA and the limitation of the sensitivity of our chromatography technique made reliable measurements of GABA impossible in the majority of experiments (in many of them, GABA concentration was below detection threshold). External standards were run between every five dialysis samples. L-Homocysteic acid was used as an internal standard. The internal standard varied less than 5% across samples on any given day. Peak areas were first normalized to the l-homocysteic acid internal standard. Final quantification was obtained by comparing normalized peak areas with those of the external standards. The peak area was dependent on the amount of amino acid injected and was linear in the range of sample concentrations measured. Identification was based on chromatographic retention times.

The HPLC setup consisted of two pumps and a fluorescence detector. A Bioanalytical Systems 200 A and a Beckman 114 M HPLC system were used. A Waters Spherisorb S5 (ODS2) C18 column (150 × 4.6-mm inner diameter, 3-μm particle size) was used. The mobile phase consisted of a gradient of increasing acetonitrile concentration in a sodium acetate buffer. One compound of the HPLC gradient was 60% acetonitrile/40% water, and the other was 0.14 M sodium acetate (pH 4.84) containing 0.5% triethylamine by volume. The flow rate of the buffer through the chromatograph was 1.0 ml/min. The excitation wavelength of the detector was set at 250 nm, and an emission filter cutoff of 456 nm was used.

**Data Analysis**

Amino acid peak areas were first normalized to the L-homocysteic acid standard and then were quantified based on linear calibration of corresponding external standards. The mean release of each amino acid in the two samples preceding stimulation was designated as the baseline. Each release of amino acids was then expressed as a percentage of baseline level to reduce the variation among animals. All mean concentrations are reported with the standard error for the population. Serotonin and NE were also expressed as a percentage of the mean baseline concentration. A one-way ANOVA was used to determine whether the basal release of 5-HT, NE, and amino acids differed from stimulation groups. Dunnett’s test was used to determine whether the release of each species measured during electrical stimulation differed from that measured under basal conditions. P < .05 for the effect of the sampling period was considered to be significant.

**Results**

Samples were collected from a total of 47 animals; however, data could be used only from 35 animals because of technical difficulties. In each animal, a nociceptive dorsal horn cell was identified by its responses to Brush, Press, and Pinch, and the microelectrode was removed after the recordings were completed. A microdialysis fiber was then inserted as close as possible to the dorsal horn neuron from which the recording had been made. The depth of the dorsal horn neurons ranged from 400 to 900 μm, approximately the level of laminae IV to VI in rats. The histological locations of the PAG-stimulation sites are shown in Fig. 1. Samples collected when stimulation occurred at sites outside the PAG or in animals in which the microdialysis fiber was not within the dorsal horn were excluded from data analysis because they did not show any change in the release of neurotransmitters. In 30 animals (Fig. 1, filled circles), electrical stimulation of the PAG produced an inhibitory effect. In 5 other animals, the stimulation sites were outside the PAG (Fig. 1, open circles), and stimulation at these sites did not produce inhibition of evoked nociceptive responses of dorsal horn neurons. Most of the effective stimulation sites were located in the ventral, ventral lateral, lateral, or dorsal PAG and produced an increase in the release of at least one of the neurotransmitters measured. There was no obvious difference in neurotransmitter release when stimulating the ventral, ventral lateral, lateral, or dorsal PAG.
Inhibitory Effects on Nociceptive Dorsal Horn Neurons Produced by PAG Stimulation

Stimulation in the PAG was effective in inhibiting both the background activity and the activity evoked by innocuous (Brush, Press) and noxious (Pinch) stimuli applied to the cutaneous excitatory receptive field. Figure 2 shows the effect of PAG stimulation on the responses of a WDR cell to cutaneous Brush, Press, and Pinch stimuli. The PAG was stimulated with trains of 0.2-ms square pulses at 333 Hz with an intensity of 250 μA for 10 s. The neuron was characterized as a WDR cell because it responded to graded mechanical stimuli: Brush, Press, and Pinch (Fig. 2A). In Fig. 2B, stimulation of the PAG inhibited the Press and Pinch responses substantially, whereas there was a smaller inhibitory effect on the Brush response.

We also examined the effect of long-lasting PAG stimulation, by using the same time course and stimulation parameters as used in the release experiments (see below), on the evoked nociceptive responses of dorsal horn neurons. In Fig. 3, pinches were applied to the receptive field every 5 min. For the first 30 min, there was no PAG stimulation. After this, there was a stimulation period of 30 min (9 min on and 1 min off, repeated 3 times); then there was a recovery time of about 30 min. Not only did long-lasting PAG stimulation inhibit each individual pinch response (Fig. 3A), but also the background firing rate of the dorsal horn neuron was inhibited (Fig. 3B). At 30 min after PAG stimulation, the nociceptive response showed signs of recovery; however, the recovery was not complete at this time. Similar results were obtained in an additional four animals.

Effect of Stimulation in PAG on Release of 5-HT in Spinal Cord

Basal 5-HT Concentration. In a total of 23 experiments, dialysate was assayed for 5-HT. Basal concentrations of 5-HT were below our limit of detection in five of the experiments. Basal concentrations for the other 18 animals averaged 0.46 ± 0.23 pg/μl (±S.E.M.) with a range from 0.018 to 4.28 pg/μl.

Evoked 5-HT Release. Stimulation of 18/23 PAG sites elicited an increase in the release of 5-HT in the spinal cord dorsal horn. Stimulation outside of the PAG did not produce inhibition of evoked nociceptive responses of dorsal horn neurons or an increase in the release of 5-HT in the lumbar dorsal horn. Of five samples for which the stimulation sites were outside the PAG, three had detectable baseline concentrations of 5-HT. The mean release of 5-HT within the stimulation period in these samples was 69 ± 23% of basal release (n = 3, P < .05). In an additional 18 animals, stimulation in the PAG produced an inhibition of the evoked responses of dorsal horn neurons that was accompanied by detectable 5-HT release. Figure 4 illustrates the time course of changes in the normalized concentrations of 5-HT in the dialysate over time. The PAG was stimulated three times with trains of 0.2-ms pulses at 333 Hz for 9 min, with 1-min intervals when stimulation was turned off, starting at the arrow. Samples were collected during each 10-min period. The mean evoked releases over a 30-min stimulation period were 161 ± 27%, 139 ± 23%, and 163 ± 27%. These mean values were significantly different from control (n = 18, P < .05). Twenty minutes after stimulation, the level of 5-HT had returned almost to the basal level.

The values for the evoked release of 5-HT were lower in Fig. 4 than the mean evoked peak release of 5-HT because the peak of the release appeared at different time points within the 30-min stimulation period in different animals. Therefore, the mean evoked peak release of 5-HT is presented in Fig. 5. The mean evoked peak release of 5-HT was
41% of control for the 18 animals (n = 18, P, .01). The range was from 0.08 to 4.7 pg/ml. Post1 and Post2 were releases at 20 and 30 min after the stimulation, and they were close to basal level. However, the release of 5-HT 60 min after the stimulation period was lower than basal (about 40%). To investigate whether such a low release of 5-HT was due to damage of neurons in the PAG or to depletion of neurotransmitter, the PAG was stimulated for a second period with either high- (333 Hz; n = 2) or low- (67 Hz; n = 4) frequency pulses at the same intensity. This second stimulation period caused either the same amount of neurotransmitter release with a high-frequency stimulus or increased neurotransmitter release with a low-frequency stimulus. The mean evoked release during low-frequency stimulation was 255% of control (n = 18, *P < .01). In the poststimulus period, the concentration of 5-HT was similar to control. The mean evoked peak release of 5-HT is the average of the every individual peak within the stimulation period in each sample. The values of 5-HT concentration were normalized to the average of B1 and B2, which represent two basal measurements preceding stimulation. Post 1 and post 2 represent the release 20 and 30 min after the termination of PAG stimulation.

The PAG was repeatedly stimulated three times for 10-min intervals with a train of 0.2-ms pulses at 333 Hz for 1 s of every 3 s for 9 min. The values of 5-HT concentration were normalized to the average of B1 and B2, which represent two basal release measurements preceding stimulation. Stimulation started at the arrowhead.

255 ± 41% of control for the 18 animals (n = 18, P < .01). The range was from 0.08 to 4.7 pg/μl. Post1 and Post2 were releases at 20 and 30 min after the stimulation, and they were close to basal level. However, the release of 5-HT 60 min after the stimulation period was lower than basal (about 40%). To investigate whether such a low release of 5-HT was due to damage of neurons in the PAG or to depletion of neurotransmitter, the PAG was stimulated for a second period with either high- (333 Hz; n = 2) or low- (67 Hz; n = 4) frequency pulses at the same intensity. This second stimulation period caused either the same amount of neurotransmitter release with a high-frequency stimulus or increased neurotransmitter release with a low-frequency stimulus. The mean evoked release during low-frequency stimulation was 487 ± 282% of control (4.9 ± 3.3 pg/μl) for the 4 animals with detectable basal 5-HT (0.14 ± 0.06 pg/μl). This result suggests that the intensity and frequency of stimulation used in our experiments were not detrimental to neurons in the PAG, that transmitter depletion did not persist, and that low-frequency stimulation produced a greater release of 5-HT.
Release of NE

**Basal Release of NE.** Basal concentrations of NE for the 10 animals before the first stimulation period were 0.10 ± 0.033 pg/μl with a range from 0.022 to 0.27 pg/μl. Forty minutes after the first stimulation period, the basal level became 0.088 ± 0.032 pg/μl with a range from 0.02 to 0.25 pg/μl. There was no significant difference between these two basal levels.

**Evoked NE Release.** Stimulation at both frequencies produced an increase in the release of NE. The time course of normalized NE concentrations before, during, and after PAG stimulation is shown in Fig. 6. The mean evoked NE release over the 30-min stimulation period (0.2 ms at 333 Hz) was 112 ± 41%, 175 ± 42%, and 160 ± 21% of control for high-frequency stimulation (indicated by first arrowhead in Fig. 6) and 278 ± 96%, 250 ± 37%, and 205 ± 61% for low-frequency stimulation (starting at the second arrowhead; n = 10, *P < .05). These values were lower than the mean evoked peak release of NE in Fig. 7 because peaks appeared at different times within the stimulation period in each animal. The mean evoked peak releases of NE (Fig. 7) for high- and low-frequency stimulation were 195 ± 37% and 354 ± 87% of basal concentration, respectively (n = 7, P < .05). The range was from 0.08 to 4.7 pg/μl.

Release of Amino Acids

**Basal Amino Acid Concentrations.** Basal concentrations of Asp and Glu in the dialysate preceding the first collection period were 0.64 ± 0.14 μM (range 0.11–2.74 μM) and 2.34 ± 0.39 μM (range 0.29–7.34 μM), respectively. After a 1.5-h washout time, extracellular amino acid concentrations gradually became stable. The mean Gly basal concentration at the start of collection was 6.29 ± 1.37 μM (range 0.95–17.19 μM).

**Fig. 6.** The time course of the mean evoked release of NE during high- (333 Hz and 0.2 ms) and low- (67 Hz and 0.5 ms) frequency stimulation of the PAG. Both frequencies of stimulation were sufficient to inhibit the evoked nociceptive responses of dorsal horn neurons and to produce a significant increase in the release of NE (n = 7, *P < .05). The values of NE concentration were normalized to the average of B1 and B2. B1, B2, B1’, and B2’ represent the basal release before each stimulus period. Arrowheads, beginning of each stimulus.

**Fig. 7.** The mean evoked peak release of NE by high and low frequency of PAG stimulation. Peak 1 and peak 2 represent the average of NE peak release within stimulation period in each sample induced by stimulation with same intensity at 333 Hz (0.2 ms) and 67 Hz (0.5 ms), respectively. The values of NE concentration were normalized to the average of B1 and B2, B1, B2', B2, and B2' represent the basal release before each stimulus period. Post 1' and post 2' represent the release 20 and 30 min after the termination of PAG stimulation (n = 7 for both stimuli; *P < .05).

Evoked Amino Acid Release.** Samples collected when stimulation sites were outside the PAG did not produce an increase in the release of amino acids. In 5 animals, during stimulation outside the PAG, the mean levels of Gly, Asp, and Glu were 88 ± 12%, 87 ± 10%, and 85 ± 16% of the basal release, respectively (n = 5, P > .05). Because the release of amino acids at 60 min after PAG stimulation was below the basal level (70% of basal), as was the release of 5-HT, we stimulated the PAG again with 0.5-ms pulses at 67 Hz and the same intensity 40 min after the first stimulation to determine whether the stimulation parameters (0.2 ms, 333 Hz) used in our study produced neuronal damage or depletion of neurotransmitters.

Stimulation of the PAG with 0.2-ms pulses at 333 Hz (peak 1, 1.5 h after start of sample collection) resulted in a small increase in the release of Gly and Asp (in 20 of 29 animals); however, stimulation with the same intensity but with 0.5-ms pulses at 67 Hz (peak 2) led to a larger increase in the release of Asp, Gly, and Glu (Fig. 8). The mean evoked releases for Gly and Asp during high-frequency stimulation were 113 ± 3% and 125 ± 7% (n = 21, **P < .01), respectively. Forty minutes after the first period of stimulation, concentrations of amino acids returned almost to the basal level. The application of low-frequency stimulation (67 Hz and 0.5 ms) to the PAG caused bigger releases than high-frequency stimulation. Stimulation with 0.5-ms pulses at 67 Hz produced an increase in the mean evoked peak release of Gly and Asp to 216 ± 49% and 202 ± 42% (n = 10, P < .05) of the basal levels, respectively. Low-frequency stimulation also caused a significant Glu release (136 ± 12%, n = 10, P < .05), which was not seen during high-frequency stimulation with the same intensity (101 ± 3%, P = 0.058). Therefore, stimulus-evoked release was significant with both low- and high-frequency stimuli (except for Glu, which did not increase significantly during high-frequency stimulation). Low-frequency stimulation seems more potent in causing the release of the examined neurotransmitters.

Not all of the amino acids examined showed increased concentrations with high-frequency stimulation. Amino acids released during the first stimulation period varied from ex-
The mean evoked peak release of Gly, Asp, and Glu by high- and low-frequency PAG stimulation. Peak 1 and peak 2 represent mean peak release of amino acids within stimulation period in each sample induced by stimulation with same intensity at 333 Hz (0.2 ms) and 67 Hz (0.5 ms), respectively. B1, B2, B1*, and B2* represent the basal release before each stimulus period. Post 1 and post 2 represent the release 20 and 30 min after the termination of PAG stimulation (n = 21 for the first stimulation period, **P < .01; for the second stimulation period, n = 10, *P < .05).

The mean evoked peak release of Gly, Asp, and Glu by high- and low-frequency PAG stimulation. Peak 1 and peak 2 represent mean peak release of amino acids within stimulation period in each sample induced by stimulation with same intensity at 333 Hz (0.2 ms) and 67 Hz (0.5 ms), respectively. B1, B2, B1*, and B2* represent the basal release before each stimulus period. Post 1 and post 2 represent the release 20 and 30 min after the termination of PAG stimulation (n = 21 for the first stimulation period, **P < .01; for the second stimulation period, n = 10, *P < .05).

Discussion

In this study, we demonstrated that the release of endogenous 5-HT, NE, and amino acids from the spinal cord increased during stimulation of the PAG using stimulus intensities that inhibited nociceptive responses of dorsal horn neurons. The findings provide direct evidence that the antinociception produced by stimulation of PAG is accompanied by an activation of serotonergic, noradrenergic, glutamatergic, aspartatergic, and glycinergic neurons. These results are also supported by a large body of pharmacological and anatomic studies, including recent demonstrations in our laboratory that PAG stimulation-produced inhibition of nociceptive dorsal horn neurons is antagonized by the microdialysis of SNAP-25 receive before each stimulus period. Post 1 and post 2 represent the release 20 and 30 min after the termination of PAG stimulation (n = 21 for the first stimulation period, **P < .01; for the second stimulation period, n = 10, *P < .05).

GABA concentrations remained below our detection limits in most of our experiments; therefore, it is hard to draw any conclusions about the effect of PAG stimulation on the release of GABA in the spinal cord.

Two Stimulation Periods. In this study, we used two different frequencies with the same intensity to stimulate the PAG. High-frequency stimulation was convenient in the search for nociceptive dorsal horn neurons that could be inhibited by PAG stimulation. The reason for using a second low-frequency stimulation was that low-frequency stimulation in the PAG produces antinociception in animals (Barbaro et al., 1985). Our results showed that both frequencies of stimulation caused the release of 5-HT, NE, and amino acids. Low-frequency (n = 10) or high-frequency (n = 4) stimulation applied 40 min after the first stimulation period caused more release or an equal amount of release of neurotransmitters. This indicates that the high-frequency stimulation used in this and previous studies did not damage neurons in the PAG or cause a long-term depletion of neurotransmitters. The levels of 5-HT and amino acids were lower than the basal concentrations 1 h after the first stimulation (40% and 80% of basal, respectively; data not shown). This might indicate that the release of neurotransmitters activated an uptake system. Further investigation is needed to determine whether this is the correct explanation. In this study, we also observed that low-frequency stimulation caused more release of neurotransmitters than high-frequency stimulation, whereas stimulation at both frequencies inhibited the nociceptive responses of dorsal horn cells. This is consistent with previous studies that showed 5-HT release after raphe stimulation or blockade of raphe-induced antinociception by 5-HT antagonists when stimulation pulses of not less than 0.4 ms width (most > 0.5 ms) and with a frequency of 100 Hz or less (usually 25–100 Hz) were used (Hammond et al., 1985; Sorkin et al., 1993). We initially chose 0.2-ms pulses at 333 Hz because most previous work in our laboratory in which the PAG was stimulated showed that these parameters produced strong inhibition of nociceptive responses of STT cells in monkeys and dorsal horn neurons in rats (Lin et al., 1994).

Our present study suggests that low-frequency stimulation was more potent in causing the release of neurotransmitters than high-frequency stimulation because with the same stimulation intensity, low-frequency pulses not only produced a bigger release than did high-frequency pulses but also caused a significant increase in Glu release, whereas high-frequency pulses failed to do so. In a previous study in this laboratory, stimulation of the NRM with high-frequency pulses failed to cause detectable release of 5-HT (Sorkin et al., 1993). Two possible reasons for this are that 1) the PAG is more responsive to high-frequency stimulation than is the NRM or 2) the intensity of stimuli applied in the NRM previously used was not sufficiently high to produce release.

5-HT and NE. The finding of this study that stimulation in the PAG increased the release of 5-HT in spinal cord dialysate provides support for electrophysiological and behavioral studies that PAG-induced antinociception is associated with activation of a serotonergic descending inhibitory pathway. The release of 5-HT in the spinal cord level induced by PAG stimulation in our study is presumably mediated by synaptic connections from the PAG to the NRM or the adjacent reticular formation. (Proudfit and Anderson, 1975; Basbaum and Fields, 1979; Willis et al., 1984; Lakos and Basbaum, 1988; Beitz, 1990; Cameron et al., 1995). Previous studies have demonstrated that electrical or chemical stimulation of the NRM can induce the release of 5-HT in the spinal cord, and this is accompanied by antinociception (Hammond et al., 1985; Sorkin et al., 1993). Therefore, PAG stimulation-induced release of 5-HT in this study likely occurs via excitatory connections from the PAG to NRM and the adjacent reticular formation, which project through the dorsolateral funiculus to the dorsal horn of the spinal cord (Basbaum and Fields, 1979; Lakos and Basbaum, 1988).

5-HT release was not always accompanied by the release of amino acids. In 9 animals, the increase in the release of 5-HT was not associated with detectable amino acid release. This could be explained if high-frequency stimulation did not cause a sufficient increase in the release of amino acids for detection. Low-frequency stimulation-induced 5-HT release
(n = 4, data not shown) was always correlated with an increase in the release of amino acids.

The finding that the release of NE was also increased during stimulation of the PAG is consistent with reports that the antinociception produced by focal electrical stimulation of, or microinjection of drugs in the PAG, is antagonized by intrathecal administration of noradrenergic antagonists (Yaksh, 1979; Jensen and Yaksh, 1984; Aimone et al., 1987). Stimulation of the PAG may cause NE release in the spinal cord by weakly activating the distal extraneural terminal layers of the LC or by exciting non-LC neurons in the rostromedial pericellular region, which in turn modulate the excitability of the LC neurons (Aston-Jones et al., 1991; Ennis et al., 1991). Alternatively, the PAG may excite noradrenergic sparsely projecting neurons in the A7 or A5 groups (Westlund et al., 1980; Clark and Proudfit, 1993). Using anterograde and retrograde tracers combined with immunocytochemistry, noradrenergic neurons located in the LC and A7 have been shown to project to all segments of the spinal cord (Westlund and Coulter, 1980; Westlund et al., 1981). Therefore, activation of the noradrenergic descending pathway by PAG stimulation may be partly via projections from the PAG to the descending catecholamine system. Another part of the action may be through activation of the NRM, which then projects to A7 to activate descending catecholamine pathways (Holden and Proudfit, 1998). Because Hammond et al. (1985) showed that electrical stimulation of the NRM evoked the release of both 5-HT and NE into the spinal cord intrathecal space, it is possible that catecholaminergic neurons might collateralize to the spinal cord, PAG, and rostral ventral medulla (compare with Kwiat and Basbaum, 1990). Stimulation in PAG, therefore, may activate noradrenergic neurons either directly or indirectly and may induce NE release in the spinal cord in part by activating ascending and descending collaterals of the same neurons.

**Amino Acids.** Antinociception produced by PAG or raphe stimulation may also be mediated by neurotransmitter systems other than the serotonergic and noradrenergic systems (Sorkin et al., 1993; Lin et al., 1994). Our results showed that Gly, Glu and Asp were released during inhibition induced by PAG stimulation. This is consistent with a previous demonstration in this laboratory that Gly, Ser, Asp, and/or Glu were released after stimulation in the NRM in cats (Sorkin et al., 1993). The released Asp and Glu could serve as mediators in a fast descending pathway from the brain stem that activates GABAergic and glycinergic neurons in the spinal cord. In the primate, a well myelinated bulbospinal axon could conduct fast enough, even allowing time for synaptic transmission, to cause the initial NRM-induced inhibitory postsynaptic potentials (Giesler et al., 1981; Willis et al., 1984). If serotonergic axons within the rat central nervous system are exclusively unmyelinated (Basbaum et al., 1988), the rapid onset of inhibition produced by PAG stimulation must be due to the activation of a fast, nonserotonergic myelinated pathway (Willis et al., 1977). The release of GABA and Gly could also be from axons of supraspinal origin. Sorkin et al. (1993) provided support for this notion by showing that strychnine totally blocked the raphe stimulation-induced inhibition when no 5-HT release was detected. In our study, Gly was released during PAG stimulation. However, the GABA concentration was often too low for detection by our system. Therefore, our study supports the pharmacological and behavioral studies that purport that Gly is involved in the PAG-induced antinociception but does not contribute to the proposal that GABA is also involved (Lin et al., 1994; Peng et al., 1996c).

**Interaction of Neurotransmitters Released by PAG Stimulation.** The working hypothesis for this study is that stimulation of the PAG may activate neurons in the NRM and LC, A7, and A5, which send descending serotonergic and noradrenergic projections to the spinal cord. NE and 5-HT may act directly on dorsal horn neurons through a1-adrenoceptors and 5-HT1A receptors, respectively. They may also, indirectly, activate inhibitory interneurons in the superficial layers of the dorsal horn through 5-HT3 receptors and possibly a1 adrenoceptors, which in turn inhibit dorsal horn projection neurons in the deeper laminae by releasing inhibitory neurotransmitters, such as Gly and GABA (Lin et al., 1994; Peng et al., 1996b,c). Because GABA of supraspinal origin has been found in bulboospinal axons (Millhorn et al., 1987; Reichling and Basbaum, 1990), stimulation of the PAG could directly activate GABAergic neurons in the NRM and cause a release of GABA in the spinal cord to inhibit nociceptive dorsal neurons.

There is also evidence indicating that NE and 5-HT interact at the spinal cord level to produce a more powerful antinociception (Archer et al., 1986). It was found (Bervoets and Millan, 1994) that spinal populations of a1 adrenoceptors mediate the induction of spontaneous tail flicks in rats by 5-HT1A receptors, yet a2 adrenoceptors inhibit such responses. Thus, cross-interactions among these systems at the spinal cord level are complicated. It is possible that PAG-induced inhibition occurs via a synergistic action of NE and 5-HT at the spinal cord level. The action of NE and 5-HT can be directly on dorsal horn neurons or indirectly via inhibitory interneurons in the spinal cord.

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**References**


Cui et al.


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