Midbrain vlPAG inhibits rVLM cardiovascular sympathoexcitatory responses during electroacupuncture

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Tjen-A-Looi, Stephanie C., Peng Li, and John C. Longhurst. Midbrain vlPAG inhibits rVLM cardiovascular sympathoexcitatory responses during electroacupuncture. Am J Physiol Heart Circ Physiol 290: H2543–H2553, 2006. First published January 27, 2006; doi:10.1152/ajpheart.01329.2005.—The periaqueductal gray (PAG) is an important integrative region in the regulation of autonomic outflow and cardiovascular function and may serve as a regulatory center as part of a long-loop pathway during somatic afferent stimulation with acupuncture. Because the ventrolateral PAG (vlPAG) provides input to the rostral ventrolateral medulla (rVLM), an important area for electroacupuncture (EA) regulation of sympathetic outflow, we hypothesized that the vlPAG plays a role in the EA-related modulation of rVLM premotor sympathetic neurons activated during visceral afferent stimulation and autonomic excitatory reflexes. Cats were anesthetized and ventilated, and heart rate and mean blood pressure were monitored. Stimulation of the splanchnic nerve by a pledget of filter paper soaked in bradykinin (BK, 10 μg/ml) every 10 min on the gallbladder induced consistent cardiovascular reflex responses. Bilateral stimulation with EA at acupoints over the pericardial meridian (P5-6) situated over the median nerve reduced the increases in blood pressure from 34 ± 3 to 18 ± 5 mmHg for a period of time that lasted for 60 min or more. Unilateral inactivation of neuronal activity in the vlPAG with 50–75 nl of kainic acid (KA, 1 mM) restored the blood pressure responses from 18 ± 3 to 36 ± 5 mmHg during BK-induced gallbladder stimulation, an effect that lasted for 30 min. In the absence of EA, unilateral microinjection of the excitatory amino acid dihomocysteic acid (DHL, 4 nM) in the vlPAG mimicked the effect of EA and reduced the reflex blood pressure responses from 35 ± 6 to 14 ± 5 mmHg. Responses of 21 cardiovascular sympathoexcitatory rVLM neurons, including 12 that were identified as premotor neurons, paralleled the cardiovascular responses. Thus splanchnic nerve-evoked neuronal discharge of 32 ± 4 spikes/30 stimuli in six neurons was reduced to 10 ± 2 spikes/30 stimuli by EA, which was restored rapidly to 28 ± 4 spikes/30 stimuli by unilateral injection of 50 nl KA into the vlPAG. Conversely, 50 nl of DHL in the vlPAG reduced the number of action potentials of 5 rVLM neurons from 30 ± 4 to 18 ± 4 spikes/30 stimuli. We conclude that the inhibitory influence of EA involves vlPAG stimulation, which, in turn, inhibits rVLM neurons in the EA-related attenuation of the cardiovascular excitatory response during visceral afferent stimulation.

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Electroacupuncture (EA) is used to treat chronic ailments including cardiovascular disease (32a) and its symptoms, which included hypertension, hypotension, arrhythmias, and angina (2, 3, 13, 34, 38, 45). One well-recognized set of acupoints, Jiangshi-Neiguan (P5-6), positioned directly over the median nerve on the wrist is used frequently to treat symptomatic coronary heart disease (10, 16a, 27). Our work, for instance, has demonstrated that EA stimulation at P5-6 acupoints with low current and low frequency (2–4 mA, 2 Hz) reduces the extent of myocardial ischemia in response to an imbalance between oxygen supply and demand during reflex autonomic stimulation (27). The EA effect is related to activation of group III and IV fibers in the median nerves, which activate μ- and δ-opioid and nociceptin receptors in the rostral ventrolateral medulla (rVLM) to inhibit the sympathetic outflow and the resulting cardiovascular sympathoexcitatory response (9, 11, 27, 29, 40, 41). The rVLM neurons, in particular, premotor cardiovascular sympathoexcitatory cells, contribute greatly to the beneficial effect of EA (41, 42, 46). Thus this medullary region appears to be an important nucleus in the modulation of sympathoexcitatory cardiovascular reflex responses during EA.

We recently observed that cardiovascular excitatory premotor sympathetic rVLM neurons receive convergent input from visceral afferents arising from the gallbladder and somatic afferents in the median nerves, which are activated by EA (41). Premotor neurons in the rVLM exhibit differential degrees of prolonged inhibition during and after stimulation of P5-6, LI4-L7, or LI6-LI7 acupoints for 30 min, thus contributing to point-specific responses during EA (42). In addition, immunohistochemical investigation of the rVLM and the ventrolateral periaqueductal gray (vlPAG) employing c-Fos and enkephalin or β-endorphin labeling demonstrates that both nuclei are activated during EA (14). The presence of projections from the vlPAG to the rVLM (8) further suggests the potential for involvement of the vlPAG in cardiovascular-related EA responses. Thus EA involves bulbospinal neurons in the rVLM that likely receive input from vlPAG that modulates EA-related cardiovascular responses.

We have proposed that the cardiovascular influence of acupuncture occurs through the long-loop neuronal pathway that involves the hypothalamic arcuate nucleus and the midbrain vlPAG (41). A previous study suggested a role for arcuate nucleus during EA (19). Furthermore, we recently demonstrated that the arcuate nucleus provides excitatory projections to the vlPAG that are critical to the EA-cardiovascular response (30). For example, in this study, we observed an increase in the evoked neuronal activity in the arcuate nucleus during 30 min of EA. On the other hand, EA decreases excitatory responses in rVLM premotor cardiovascular sympathetic neurons.

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The midbrain vlPAG modulates neuronal activity of rVLM cells and attendant cardiovascular reflexes. However, the type of interaction between these two nuclei is unclear. For example, several studies report excitatory input to the rVLM during lateral and ventrolateral PAG stimulation, whereas other investigations indicate an inhibitory influence from vlPAG to these medullary neurons (1, 8, 35, 43). Thus either or both excitatory and inhibitory projections from the vlPAG to the rVLM could influence cardiovascular responses. However, evidence supports an inhibitory influence of the vlPAG on the rVLM that could serve as an important pathway in the modulatory influence of EA on cardiovascular excitatory reflexes. Therefore, in the present study we hypothesized that EA exerts its influence on the autonomic cardiovascular and medullary responses through an inhibitory interaction from the vlPAG to the rVLM.

A preliminary report of this work has been presented (39).

MATERIALS AND METHODS

Surgical Procedures

Studies were performed on cats of either sex (2.3–4.6 kg). Experimental preparations and protocols were reviewed and approved by the Animal Care and Use Committee of the University of California, Irvine, CA. Animals were anesthetized initially with ketamine (40 mg/kg im). The left femoral vein was cannulated to enable administration of α-chloralose (50 mg/kg) and other drugs. Supplemental doses of α-chloralose (10 mg/kg) were given as necessary to maintain an adequate depth of anesthesia, as assessed by the animals’ lack of response to noxious toe pinch, a respiratory pattern that followed the ventilator, and a stable blood pressure. Cats were intubated and artificially ventilated (model 661, Harvard Apparatus). Arterial blood gases and pH were measured every hour with a blood gas analyzer (ABL5, Radiometer America) and were maintained within the normal range (Pco2 32–35 mmHg; Po2 > 100 mmHg) by enriching the inspired O2 supply and adjusting the ventilatory rate or volume. Arterial pH was kept between 7.35 and 7.40 and was corrected as necessary by administration of sodium bicarbonate. Body temperature was monitored with a rectal probe (model 44TD) and was maintained between 36°C and 37.5°C by a thermostatically controlled heating pad that was monitored with a rectal probe (model 44TD) and was maintained to expose the spinal cord (T1–T5) and dorsal surfaces of the medulla of the cat was stabilized with a spinal holder and a stereotaxic head (Wallington, CT) was used to isolate the nerve and hold it in place. The splanchnic nerve was placed on a bipolar electrode with an outer diameter of 200 μm (Frederick Haer) and was connected to the stimulator to deliver bipolar stimuli.

A laparotomy enabled exposure of the gallbladder and isolation of the splanchnic nerve. The splanchnic nerve was stimulated with current of 0.1–0.4 mA, 2 Hz, 0.5-ms duration. The IML was stimulated electrically (0.1–0.4 mA, 2 Hz, 0.5-ms duration). The IML was confirmed previously that EA at P5-6 acupoints stimulates the median nerve and modulates sympathoexcitatory cardiovascular responses (9, 29). For collision testing in the IML, the thoracic spinal cord was stimulated electrically (0.1–0.4 mA, 2 Hz, 0.5-ms duration). The IML was identified provisionally through chemical stimulation (DLH, 4 nM, 50 nl) to evoke a small, but consistent, increase in blood pressure of 5–10 mmHg, which along with anatomical evaluation after the experiment, confirmed that the stimulating electrode was situated in the IML.

To investigate neuronal activity, an extracellular recording electrode with inner diameter of 1 μm was advanced slowly through the rVLM. Single-unit extracellular activity in the rVLM was recorded with a platinum electrode inserted in the barrel of a glass pipette filled with 0.5 M sodium acetate containing 2% Chicago sky blue (Sigma Chemical, St. Louis, MO). Action potentials were amplified with a preamplifier (Grass P511) attached to a high-impedance probe (Grass H1 P5) and were filtered (0.3–10 kHz) and monitored with an oscilloscope (Tektronix 2201). Action potentials, blood pressure, and heart rate were digitized and analyzed offline with a Pentium III computer and EGAA software (R. C. Electronics).

Axonal conduction velocities of rVLM neurons were calculated by dividing the distance between the recording and stimulating electrodes in the rVLM and IML by the antidromic latency. To assess the evoked responses to stimulation of splanchnic and median nerves, peristimulus time histograms were constructed for each neuron. Action potentials were analyzed both visually and with the EGAA program for similar wave shapes, heights, and latency from the time of stimulation. As we have described previously (4, 42), the relationship between neuronal activity and blood pressure waves was assessed by both time- and frequency-domain analysis by using arterial pulse-triggered averaging and coherence analysis.

EA was applied bilaterally at 2–4 Hz at the P5-6 acupoints using 4 mA and 0.5-ms stimulus duration (9, 41, 42). Needles were placed at a depth of ~4 mm. An electrical stimulator with an isolation unit provided current to acupuncture needles, one inserted percutaneously at each acupoint (two for the set of two acupuncture points, i.e., P5-6) to deliver bipolar stimulation. Needles at P5 and P6 located 2–3 cm proximal to the flexor crease on the wrist were separated by 5–7 mm and were connected to the stimulator to deliver bipolar stimuli. Prolonged stimulation for 30 min was used during EA to simulate the clinical use of this procedure.

Microinjection, Stimulation, and Recording Methods

Input to the rVLM from the vlPAG was determined by microinjection of dl-homocysteic acid (DLH) and KA. Unilateral delivery of 50–75 nl of saline, kainic acid (KA, 1 nM), or DLH (4 nM) into the vlPAG utilizing a Hamilton syringe of 1 μl occurred 2 min before stimulation of the gallbladder (see Effects of EA on excitatory reflexes) or splanchnic afferents. Low concentrations of KA, as shown by others (16), leads to a transient depolarization blockade of neuronal function that reverses within minutes. To determine the location of the vlPAG, blood pressure was monitored after injection of DLH to assess for the expected decrease. Baseline blood pressure after microinjection of DLH, which typically displayed a transient 2- to 5-mmHg decrease, recovered before the onset of stimulation of the gallbladder or splanchnic nerve. The splanchnic nerve was stimulated with currents of 0.4–0.6 mA and pulses of 0.5 ms duration at 2 Hz. We have confirmed previously that EA at P5-6 acupoints stimulates the median nerve and modulates sympathoexcitatory cardiovascular responses (9, 29). For collision testing in the IML, the thoracic spinal cord was stimulated electrically (0.1–0.4 mA, 2 Hz, 0.5-ms duration). The IML was identified provisionally through chemical stimulation (DLH, 4 nM, 50 nl) to evoke a small, but consistent, increase in blood pressure of 5–10 mmHg, which along with anatomical evaluation after the experiment, confirmed that the stimulating electrode was situated in the IML.
Experimental Protocols

Effects of EA on excitatory reflexes. To induce reflex increases in blood pressure, chemosensitive afferents of the gallbladder were stimulated with a 1-cm² pledget of filter paper soaked with a solution of BK (10 μg/ml). After the maximal blood pressure response was attained, the filter paper was removed, and the gallbladder was washed four times with normal saline to remove BK. The increase in blood pressure was determined as the difference between prestimulus mean blood pressure and pressure at the peak of the reflex response. In five groups of animals, pledges soaked with BK were used to induce increases in blood pressures. In a group of control animals, we evaluated repeatability of 12 sequential blood pressure responses induced by BK applied to the gallbladder every 10 min (to prevent tachyphylaxis) without EA. In two other groups of animals, after observing two consistent increases in blood pressure in response to stimulation with BK, we began bilateral EA at the P5-6 acupoints 7–10 min before the third application of BK; EA was continued for 30 min. During EA, BK was applied three times at 10-min intervals. After completion of EA, BK was reapplied every 10 min for the next 80 min. Thus BK was applied during control (two responses), EA (three responses), and recovery following EA (eight responses) for a total of 13 sequential data points. Unilateral injection of saline (control) or KA into the viPAG occurred 2 min before the seventh gallbladder stimulation following termination of EA at a time when its modulatory influence was still present. Finally, stimulation or inactivation of viPAG neurons with DLH or KA, respectively, in the absence of EA, was conducted in two additional groups of animals to examine the influence of this midbrain region on sympathoexcitatory cardiovascular reflex responses.

Medullary neuronal response to stimulation of viPAG. To examine rVLM responses to input from the viPAG, we identified responsive neurons in the rVLM using one of two paradigms. The first method involved location IML neurons that could be antidromically driven from the IML. With the use of this method in 12 neurons, the IML was stimulated continuously at 2 Hz at 0.1–0.4 mA while the recording electrode was lowered slowly at 1-μm increments through the rVLM. Neurons in the rVLM that responded to stimulation of the IML were evaluated for criteria that indicated antidromic activation. First, they were examined for constant latency, a stable threshold of the evoked all-or-none response and a faithful response to high rates of stimulation (200 Hz). Second, the neurons were evaluated for evidence of collision of triggered antidromic spikes from the IML with either spontaneous or stimulus-induced orthodromic action potentials evoked by stimulating the splanchnic or median nerves. The antidromically driven neurons were examined for faithful responses. The refractory period was determined to ensure that the orthodromic spike occurred in the critical interval (the latency plus the refractory period) required to block the antidromic spike (42). Collision was observed when the sum of the latency and the refractory period was greater than the time interval (42). Conduction velocities of neurons in the rVLM that projected to the IML were determined by measuring the distance between the thoracic IML and the recording site in the rVLM and dividing this number by the latency of conduction of the antidromic response from the IML to the rVLM (42). Neurons were examined for convergent input from the median and splanchnic nerves. Stimuli were applied at 2 Hz. We measured evoked neuronal activity over a 15-s period to construct peristimulus histograms (42). We also recorded baseline activity of these neurons over a 5-min period to provide data for time and frequency domain analysis (42). Second, in nine other rVLM neurons, after spontaneous activity was recorded, the neurons were evaluated during responses evoked by stimulation of the splanchnic nerve and P5-6 acupoints using peristimulus histograms (42).

Once cells were identified, we evaluated their neuronal responses during viPAG stimulation with or without EA-induced prolonged inhibition. The influence of DLH stimulation of the viPAG on splanchnic nerve-evoked rVLM neuronal responses was then examined. We also evaluated the splanchnic nerve-evoked rVLM neuronal discharge following administration of saline or KA into the viPAG following termination of EA stimulation but during its prolonged inhibitory effects. Inactivation of the prolonged inhibitory neuronal response to EA thus was used to evaluate the inhibitory influence of the viPAG in this response.

To characterize the “cardiovascular” responsiveness of all rVLM neurons, the effect of altered baroreceptor input following administration of nitroglycerin or phenylephrine also was evaluated. Cardiovascular rhythmicity likewise was determined to further differentiate the rVLM neurons. The firing pattern of rVLM neurons was subjected to time- and frequency-domain analyses, respectively, using arterial pulse-triggered averaging and coherence analysis of the relationship between arterial pulse and rVLM neuronal activity.

Discharge activity during splanchnic nerve stimulation without EA was recorded every 10 min in six cardiovascular and/or premotor sympathetic neurons serving as a time control group. Of note, our previous investigation (41) demonstrated that most rVLM cells (86%) receiving input from splanchnic and median nerves function as premotor sympathetic neurons.

Histology

At the end of each experiment, the animal was euthanized with α-chloralose and saturated KCl. The brain stem and midbrain were removed and fixed in 10% formalin. Frozen serial 60-μm brain sections were cut with a freezing microtome (Leica CM 1850). Slices were examined with a microscope (Nikon eclipse 6400) to identify the recording and microinjection sites. Microinjection sites in the viPAG were reconstructed with Corel Presentation software and were plotted on coronal sections separated by ~2-mm intervals with respect to the interaural line. Recording sites in the rVLM were plotted on coronal sections likewise were separated by 2-mm intervals with respect to the obex. Stimulating sites of the spinal cord IML also were confirmed with the aid of a microscope.

Data Analysis

Data are presented as means ± SE. The assumption of normal data distribution was analyzed by the Kolmogorov-Smirnov test. All data were distributed normally. Blood pressure responses to BK were analyzed with a one-way repeated measures analysis of variance, followed post hoc with the Student-Newman-Keuls test. These tests represent a pairwise multiple comparison procedure. We utilized
SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) for statistical analysis and graphing. The level of statistical significance was chosen as $P < 0.05$.

We also evaluated time and frequency relationships between rVLM neuronal activity and arterial blood pressure using pulse-triggered spike analysis as well as coherence. Arterial pulse-triggered analysis used a threshold that was set at the systolic phase of the arterial pulse. We used spike height discrimination and wave shape recognition to sort action potentials during the 300-s period of evaluation. Averages of the arterial pulse and histograms of neuronal activity then were constructed as we (41, 42) and others (4) have described.

Frequency-domain analysis was used to assess coherence between rVLM activity and arterial blood pressure using a fast Fourier transform (FFT) algorithm (33, 42). Original data were recorded using a sampling rate of 10,000 Hz. Reconstructed data utilized every tenth sample and included assessment of the mean and peak amplitudes as well as the maximum and minimum slopes of the original spike to be certain that all action potentials were preserved. Action potentials were sorted and identified with a window discriminator to construct a histogram. The histogram was then divided into sections. Representative sections of data (15–20), each lasting 12.8 s, were chosen. Data from all sections were averaged and used to construct an averaged histogram. Thereafter, seven overlapping windows from the averaged histogram were used to calculate the FFT. Autospectra of rVLM discharge and arterial blood pressure were generated with the FFT. Thus coherence was generated with seven overlapping windows, each with a length of 12.8 s, consisting of 256 bins and bin widths of 50 ms. The autospectral analysis was adapted from Shin et al. (36) using contiguous segments of 256 beats with 50% overlap between contiguous segments. The frequency resolution was 1/12 s or 0.08 Hz. The coherence function (normalized cross spectrum) provided a measure of the strength of linear correlation of rVLM neuronal activity and blood pressure at each frequency. Coherence values of $\geq 0.5$ were chosen to reflect a statistically significant relationship between rVLM spikes and arterial blood pressure (4, 21, 36, 41, 42).

RESULTS

Inactivation of vlPAG Neurons

To determine the influence of inactivation of vlPAG on the reflex responses, five animals were stimulated repeatedly with...
BK applied on the gallbladder. After two consistent responses, inactivation of glutamatergic activity in the vIPAG with KA did not alter the consistent increases in blood pressure in response to BK-gallbladder stimulation. Baseline heart rate and mean blood pressure before the onset of each reflex response were unchanged throughout the protocol (Fig. 1).

As a time control, we repeatedly stimulated chemosensitive afferent nerve endings in the gallbladder every 10 min to evaluate for consistency of increases in blood pressure. In seven animals, we observed consistent blood pressure increases ranging between 36 and 42 mmHg, over a 2-h period (Fig. 2A, I). Baseline heart rate and mean blood pressure before the onset of each reflex response were unchanged throughout the protocol.

To examine the role of the vIPAG in the EA influence on the sympathetic cardiovascular responses, we inactivated cell bodies in this midbrain region with KA. Application of EA at P5-6 for 30 min reduced the blood pressure increases for a prolonged period of 80 min in six animals. Microinjection of saline did not alter the EA inhibitory response (Fig. 2B, II). However, inactivation of glutamatergic activity in the vIPAG with KA soon after termination of EA reversed the acupuncture-related inhibition of the reflex increases in blood pressure for at least 20 min in nine other animals. After the action of KA subsided, the long-lasting EA inhibitory effect was again noticeable 40 min after cessation of stimulation of the P5-6 acupoints over the median nerve (Fig. 2B, III). An example of KA-induced inactivation of the EA-blood pressure modulatory response is shown in Fig. 2A. Baseline heart rates and mean blood pressures before the onset of each reflex response were unchanged throughout both protocols.

In addition to the reflex cardiovascular responses, we evaluated neuronal activity in the rVLM to determine the role of the vIPAG in the medullary responses. Repeated stimulation every 10 min of the splanchnic nerve evoked consistent increases in discharge frequency in six rVLM neurons (Fig. 3A). Five medullary rVLM neurons activated by stimulation of the splanchnic nerve following microinjection of the vehicle control (saline) demonstrated decreased activity during and after 30 min of EA at P5-6 (Fig. 3B). Similar to the cardiovascular responses, the EA-related inhibition of six premotor sympathetic cardiovascular rVLM neurons was reversed significantly ($P < 0.05$) by microinjection of KA in the vIPAG (Fig. 3C). An individual neuron demonstrating the modulatory influence of EA and reversal of this inhibition by vIPAG-KA injection is shown in Fig. 4.

**Stimulation of Neurons in vIPAG**

Microinjection of small volumes (50 nl) of the excitatory amino acid DLH into vIPAG mimicked the inhibitory effect of EA on the reflex cardiovascular responses to gallbladder stimulation. Figure 5A, I and II, shows the heart rate and blood pressure responses of an animal subjected to two repeatable

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**Fig. 3. Role of vIPAG in inhibitory effects of EA at acupoints P5-6 overlying median nerves on rVLM neuronal responses.** Inhibition of rVLM neuronal activity with microinjection of KA in vIPAG reversed EA inhibition of rVLM activity. A: consistent responses of rVLM neuronal activity during splanchnic nerve stimulation every 10 min. B: EA inhibition of rVLM responses during and after 30 min of EA. Saline microinjection did not influence EA response. C: displays evidence for temporary inactivation of EA influence of premotor sympathetic rVLM neuronal activity following microinjection of KA unilaterally into the vIPAG. *Decrease of the reflex response after onset of acupuncture. †Significant reversal of EA inhibitory response.
reflex responses followed by microinjection of DLH into the vIPAG 2 min before the onset of three sequential BK-induced reflex responses, repeated every 10 min. Likewise, in a group of six animals, cardiovascular reflexes were reduced in a consistent fashion following each application of DLH (Fig. 5A, III). Baseline heart rate and mean blood pressure before the onset of each reflex response were unchanged throughout this protocol.

In a similar fashion, the responses of five premotor sympathetic cardiovascular neurons in the rVLM that received input from the vIPAG, splanchnic, and median nerves were reduced following microinjection of DLH in the vIPAG in (Fig. 5B). One neuron in this group also was utilized as a time control.

**rVLM Neurons**

Neurons in the rVLM evaluated in this study represent cells that were selected initially because they responded to convergent input from visceral, somatic, and cardiovascular (baroreceptor) afferent stimulation. Baseline activity of the rVLM neurons was $3.7 \pm 0.5$ impulses/s.

All neurons evaluated were found to be premotor sympathetic rVLM cells (12 of 12 neurons). The axonal conduction velocity averaged $5.6 \pm 0.7$ m/s. The average distance of these 12 neurons was $65 \pm 11$ mm. Figure 6 provides an example of collision used to define the bulbospinal nature of these rVLM neurons. The antidromically induced action potential collided with the rVLM orthodromic spike evoked by the stimulation of the splanchnic nerve when the interval between the evoked potential and the triggered antidromic potential before and after the collision was reduced to 4.6 ms. This neuron was inhibited by EA and received input from the vIPAG. It demonstrated abrupt reversal of the EA inhibition response to microinjection of KA (Fig. 7A).

We evaluated the responses of all 21 rVLM neurons to altered baroreceptor input using phenylephrine or nitroglycerin, depending on the baseline activity. Nitroglycerin decreased blood pressure by $55 \pm 12$ mmHg and increased the discharge activity of 17 neurons from $3.5 \pm 0.7$ to $8.4 \pm 2.1$ impulses/s. Phenylephrine consistently increased blood pressure by $75 \pm 17$ mmHg and decreased activity in each of four neurons from $4.8 \pm 0.8$ to $2.1 \pm 1.1$ impulses/s, indicating that the cells were barosensitive. These neurons therefore were classified as sympathoexcitatory. An original response of an rVLM neuron during treatment with phenylephrine is displayed in Fig. 7B.

Each of the 21 medullary neurons studied were examined with respect to their relationship to the cardiac rhythm. Arterial pulse-triggered averaging showed a strong relationship be-
tween the discharge rate of the rVLM neurons studied and arterial blood pressure (Fig. 7C). Likewise, we observed a strong relationship (average coherence, 0.8 ± 0.03) between neuronal activity and arterial blood pressure at a cardiovascular frequency of 3.2 ± 0.2 Hz, which represented an average heart rate of 192 beats/min. An individual example of an rVLM neuron with a coherence of 0.85 at 2.63 Hz (158 beats/min) is provided in Fig. 7D.

Anatomical Location of Recording and Stimulating Sites

All recording sites were confined to an area 1.2–4.5 mm rostral to the obex, 2.5–3.9 mm to the right or left of midline, 0.2–1.4 mm from the ventrolateral surface, lateral to the nucleus inferior olive and pyramidal tracts, as well as ventral and medial to the facial and retrofacial nuclei (Fig. 8). The majority of the cells were located at least 2 mm rostral to the obex. Two cells in this region were located between 1.2 and 2 mm rostral to the obex. Like the other identified cells that received input from the vIPAG, these two more caudally positioned cells could be antidromically driven from the IML of the spinal cord and were suppressed by baroreceptor activation. Thus, except for these two cells, the majority of the premotor sympathoexcitatory neurons receiving input from the midbrain were within the area of the rVLM that has been described by several authors (7, 9, 15, 31, 42, 46). Sites of stimulation in the thoracic spinal cord between T2 and T4 also were identified to be in the IML, located in the central lateral gray area of the spinal cord similar to a previous report by Morrison et al. (32). We located 42 microinjection sites in the vIPAG. Injection sites outside the vIPAG did not inactivate the modulatory response to EA.

DISCUSSION

This study assessed the role of the midbrain in the long-loop pathway serving the beneficial effects of EA, which have been shown to cause prolonged inhibition of autonomic excitatory...
KA-induced depolarization blockade was utilized to examine the influence of the vIPAG on rVLM-evoked neuronal activity and sympathoexcitatory cardiovascular responses. In this regard, microinjection of KA into vIPAG transiently reversed the inhibitory EA effect on the pressor reflexes as well as the rVLM-evoked activity. The EA-related inhibition of the reflex response resumed within 30 min. Reversibility of the cardiovascular responses after KA treatment has been shown previously. For example, Soltis and coworkers (37) observed recovery of blood pressure and heart rate within minutes after microinjection of KA in the dorsomedial hypothalamus, thus demonstrating that the vIPAG neurons were not destroyed. We conclude therefore that the supramedullary vIPAG is integrally involved in the modulatory influence of EA on cardiovascular reflex responses. Thus, the vIPAG-rVLM connection plays an essential role in the prolonged inhibition of sympathetic outflow consequent to EA.

Activation of vIPAG neurons can modulate the sympathetic outflow (1). In this regard, stimulation of the vIPAG can decrease baseline blood pressure and renal sympathetic nerve activity (1). However, responses of the reflex cardiovascular responses during stimulation of vIPAG neurons have not been examined. In the present study, we found that stimulation of the vIPAG modulated the reflex blood pressure responses in a manner similar to EA. We also observed that DLH caused small decreases in baseline blood pressure, whereas KA elicited no alterations. We interpret these data to mean that there is little or no tonic inhibitory activity from the vIPAG to the rVLM. The present study, therefore, supports a role for vIPAG in the EA-associated sympathoexcitation by exerting an effect on presympathetic cardiovascular neurons in the rVLM.

The ventrolateral region of PAG was examined for its influence on sympathetic premotor neurons in the rVLM (9, 28, 29, 41, 42). We observed inhibition of the gallbladder-induced pressor reflex during activation of vIPAG neurons by repeated stimulation with DLH. Furthermore, evoked activity of premotor sympathetic cardiovascular rVLM neurons, through stimulation of visceral afferents in the splanchnic nerve, was inhibited by DLH activation of the neurons in the vIPAG. We also observed that the EA-associated decrease of the visceral afferent-induced cardiovascular sympathoexcitatory reflex response and the inhibition of evoked rVLM neuronal activity were reversed following microinjection of KA into the vIPAG. Taken together, these results provide the first evidence to suggest that neurons in the vIPAG form part of a long-loop pathway involved in the inhibitory influence of EA on autonomic cardiovascular responses during stimulation of chemosensitive visceral afferents.

Future studies should evaluate neurotransmitter system(s) in the vIPAG that participate in the EA-cardiovascular response. Neurons activated by EA within the rVLM contain enkephalin (14), whereas nerve fibers containing enkephalin or β-endorphin are present in both the rVLM and the vIPAG (14). Nitric oxide (NO) in the ventral PAG also may influence the benefi-
cial effects of EA on stress-induced hypertension in rats (26). Other studies demonstrate that neurons activated by muscle contraction within the lateral PAG also contain NO synthase (24, 25). Furthermore, the muscle contraction-cardiovascular reflex response is attenuated by NO in the lateral PAG through its action on the inhibitory neurotransmitter γ-aminobutyric acid (GABA), specifically through a GABA<sub>A</sub> receptor mechanism (23). Moreover, Li et al. (22) reported that whereas

![Image](Fig. 7. A–D: responses of a neuron in rVLM that received input from vIPAG. This premotor rVLM neuron (see Fig. 6) was inhibited by EA (A) and received baroreceptor input (B). Microinjection of KA into vIPAG reversed EA-induced inhibition of neuronal activity (A). Neuron also received convergent input from splanchnic and median nerves and displayed cardiac rhythmicity (C and D) shown by strong relationship to blood pressure in pulse-triggered averaged time domain and coherence of 0.85 frequency domain analyses. BP, blood pressure; AS, autospectra.)

![Image](Fig. 8. Left: histological summary of stimulating sites in the vIPAG microinjected with KA (●), DLH (○), and saline (△). All injection sites were unilateral (side chosen randomly). Right: recording sites (*) in the rVLM. PC, Pedunculus cerebri; 5SP, laminal spinal trigeminal nucleus; RFN, retrofacial nucleus; ION, inferior olive nucleus; X, injection site outside the vIPAG.)
stimulation of muscle and baroreceptor afferents leads to summation in the release of glutamate in the dorsal lateral and lateral PAG, stimulation of convergent input from baroreceptor and muscle afferents decreases the release of the glutamate in the same regions of the PAG. In addition, anandamide and 2-arachidonoylglycerol comprising the endogenous cannabinoid system appear to serve a role in stress-induced analgesia through activation of cannabinoid-1 receptors in the dorsal PAG (17). These endocannabinoids may represent another group of neurotransmitters involved in sympathoinhibition in the vIPAG. Thus opioids, NO, GABA, glutamate, and endocannabinoids may prove to play important roles in the vIPAG.

The neuronal pathway from the splanchnic nerve to the rVLM appears to involve some decussating sympathetic spinal fibers because we recorded increased neuronal activity located in both the right and left rVLM during stimulation of the right splanchnic afferents. Furthermore, unilateral microinjections of KA into the vIPAG showed altered evoked neuronal activity present at the same and opposite sides of the rVLM. These observations suggest that vIPAG neurons project bilaterally.

Projections from the vIPAG to the rVLM may pass through the midline raphe nuclei, including the nucleus raphe obscurus, among others (18, 19, 41). In fact, the vIPAG has been reported to provide inhibitory inputs to the rVLM through a relay in the medullary raphe nuclei (44). Sympathetic renal nerve activity is reduced following stimulation of the vIPAG through the raphe nuclei (12). Stimulation of cells in the raphe nucleus and the rostral half of the raphe obscurus inhibits neuronal activity in the rVLM (44). As such, the raphe nuclei, including the rostral part of the raphe pallidus, the raphe obscurus, and the caudal raphe magnus are additional centers that may process somatic input during EA.

A potential limitation of the current study is that only a subset (12) of the 21 cardiovascular rVLM neurons were demonstrated to be premotor sympathetic in nature. Previously, we showed that 86% of the cells in the rVLM that receive convergent input from the splanchnic and median nerves can be driven antidromically from the IML (41), suggesting that the majority of rVLM neurons that receive visceral and somatic input function as premotor sympathetic cells. Thus it is likely that many of the other nine cardiovascular sympathoexcitatory rVLM neurons, not examined by antidromic stimulation of the spinal IML, function as premotor neurons with convergent input from vIPAG.

In conclusion, the present study demonstrates the importance of neurons in the vIPAG that process input from somatic afferent stimulation during EA. Projections from the vIPAG to the rVLM are an important source of the inhibitory influence of EA on rVLM premotor neurons and ultimately sympathoexcitatory cardiovascular responses. Therefore, this study supports the significance of the midbrain vIPAG as part of a long-loop pathway that also involves the hypothalamic arcuate nucleus in the EA-related cardiovascular response.

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