Role of medullary GABA, opioids, and nociceptin in prolonged inhibition of cardiovascular sympathoexcitatory reflexes during electroacupuncture in cats

Stephanie C. Tjen-A-Looi, Peng Li, and John C. Longhurst

Department of Medicine, Susan Samueli Center for Integrative Medicine, School of Medicine, University of California, Irvine, California

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Tjen-A-Looi SC, Li P, Longhurst JC. Role of medullary GABA, opioids, and nociceptin in prolonged inhibition of cardiovascular sympathoexcitatory reflexes during electroacupuncture in cats. Am J Physiol Heart Circ Physiol 293: H3627–H3635, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00842.2007.—Electroacupuncture (EA) causes prolonged suppression of reflex elevations in blood pressure for 1–2 h in anesthetized preparations. A long-loop pathway involving the arcuate nucleus (ARC), ventrolateral periaqueductal gray, and rostral ventrolateral medulla (rVLM) is involved in sympatho inhibitory cardiovascular EA effects. However, the mechanisms and locations of the prolonged EA inhibition are unknown. We hypothesized that this effect is mediated through a long-loop pathway involving opioid, nociceptin, and γ-aminobutyric acid (GABA) receptor activation in the rVLM. In anesthetized, ventilated cats application of bradykinin to the gallbladder every 10 min induced consistent reflex increases in blood pressure. Bilateral EA stimulation at the cardiovascular acupoints P5–6 overlying the median nerves reduced the reflex responses for at least 80 min. Bilateral blockade with kynurenic acid in the ARC 60 min after onset of EA inhibition reversed the cardiovascular response, suggesting a role for the ARC in the long-loop pathway during the prolonged inhibitory response. Unilateral microinjection with either an opioid or a GABAA receptors antagonist in rVLM 50–60 min after the beginning of the EA response reversed EA inhibition of the cardiovascular excitatory reflex. Gaba- zine also reversed EA inhibition of cardiovascular premotor sympathetic rVLM neurons. Conversely, microinjection of a nociceptin/orphanin FQ peptide antagonist did not affect the prolonged inhibitory effect. Thus the ARC, an important component in the long-loop pathway in the EA cardiovascular response, is required for prolonged suppression of reflex cardiovascular excitatory responses by EA. Furthermore, in the rVLM, opioids and GABA, but not nociceptin, participate in the long-term EA-related inhibition of sympathoexcitatory cardiovascular responses. nociceptin; premotor neurons; rostral ventrolateral medulla; arcuate nucleus

ACUPUNCTURE OR ELECTROACUPUNCTURE (EA) is used as an adjuvant treatment for a number of chronic ailments including cardiovascular diseases like hypertension (6). One well-recog- nized set of acupoints, Jianshi-Neiguan (located along the pericardial meridian, P5–6), positioned directly over the median nerve near the wrist, have been used to treat symptomatic coronary heart disease (5, 10, 22). We showed previously (37) that the cardiovascular actions of EA depend on point-specific responses, with some acupoints like P5–6 causing large cardiovascular actions and others exerting little or no cardiovascular modulation. Thus our laboratory has developed a model of partial coronary artery occlusion to study the mechanism of EA’s cardiovascular influence during stimulation of the P5–6 acupoints as well as the median nerve directly. Myocardial ischemia was induced in this model by creating an imbalance between oxygen supply and demand during reflex increases in arterial blood pressure induced by stimulation of chemosensitive sensory nerve endings in the gallbladder (4, 17). These studies demonstrated that low-current, low-frequency EA stimulation of the P5–6 acupoints in cats significantly reduces the extent of myocardial ischemia.

Electrolytic lesions of the arcuate nucleus abolish EA inhibition of defensive excitatory responses (13), suggesting that the arcuate nucleus is essential for EA inhibition. Furthermore, EA stimulation of somatic afferents activates the arcuate nucleus, ventrolateral periaqueductal gray (vIPAG), and nucleus raphe obscurus to inhibit the rostral ventrolateral medulla (rVLM) (15), supporting the existence of a long-loop pathway that facilitates the influence of EA (20). This concept has been refined by our recent studies showing projections between the arcuate nucleus and the vIPAG (20) and between the vIPAG and the rVLM (38). These studies indicate that the arcuate nucleus and the rVLM critically participate in the EA-related modulation of sympathoexcitatory cardiovascular reflexes.

A distinguishing aspect of acupuncture is its ability to cause prolonged modulation of cardiovascular excitatory reflex responses. In this regard, previous studies have suggested that 30 min of EA in unanesthetized animals can inhibit reflex blood pressure and premotor sympathoexcitatory cardiovascular rVLM neuronal responses for as long as 4 h or more, although typically in anesthetized preparations inhibition lasts 60–90 min (7, 18, 19, 36, 38, 45, 49). The pathways and mechanisms contributing to the long-lasting EA effect are unknown.

To identify the underlying mechanisms of acupuncture effect, we have examined the role of neurotransmitters/neuro- modulators in a cardiovascular regulatory region of the brain stem, the rVLM. We showed that during and immediately after termination of EA, nociceptin and μ- and δ-opioid receptors are activated in the rVLM, which in turn inhibit sympathetic outflow and the resulting cardiovascular sympathoexcitatory response (4, 7, 17, 19, 34, 36).

In addition to opioid and opioid-like neurotransmitters, another potential inhibitory neurotransmitter that might play a role in the EA-related cardiovascular inhibitory response is γ-aminobutyric acid (GABA). GABA in the rVLM is responsible for baroreflex-related inhibition of medullospinal neurons during elevations in blood pressure (14, 30, 33, 41–43). GABA receptors in the rVLM also mediate depressor re-
sponses induced by stimulation of the greater splanchnic nerve (28). Participation of this inhibitory amino acid in the hypotensive effect of EA has not been evaluated.

Therefore, in the present study, we hypothesized that the long-loop pathway involving the arcuate nucleus mediates this prolonged inhibition and that opioids, nociceptin, and GABA in the rVLM participate in the long-lasting cardiovascular effect of EA. A preliminary report of this study has been published in abstract form (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. 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 7.42 and was corrected as necessary by administration of sodium bicarbonate. Body temperature was monitored with a rectal thermometer (model 44TD) and was maintained between 36 and 37.5°C by a thermostatically controlled heating pad and a heating lamp. Systemic blood pressure was measured with a cannula inserted into the femoral artery that was connected to a pressure transducer (model 1290, Hewlett-Packard).

A laparotomy was used to expose the gallbladder or to isolate the splanchnic nerve. The splanchnic nerve was placed on a bipolar flexible platinum stimulating electrode connected to an isolation unit and a stimulator (Grass, model S88). Hypoxy dental glue (Pentron, Wallington, CT) was used to isolate the nerve and hold it in place. The abdominal wall was closed with clips to maintain moisture in the abdominal cavity and to prevent heat loss. Thereafter, the neural axis of the cat was stabilized with a spinal holder and a stereotactic head frame (Kopf Instrument, Tujunga, CA). A laminectomy and a craniotomy were performed to expose the spinal cord (T₄–T₅) and dorsal surface of the medulla. The abdominal cavity was reopened only when a pledget soaked with bradykinin (BK) was applied to the serosal surface of the intestinal wall (7), which was connected to the blood pressure. Concentric bipolar electrodes with an outer diameter of 0.8–1.5 mm to reach the intermediolateral column (IML) between T₂ and T₄ to facilitate antidromic stimulation of rVLM neurons. Electrodes were lowered in increments of 100 μm into the spinal cord, searching for the lowest stimulus threshold required to induce an increase in blood pressure (39). At the end of the experiment, the site was marked with an electrolytic lesion for later histological verification. With visual approximation a three-barrel pipette was positioned perpendicularly to the dorsal surface of the medulla, 2.75–3.75 mm lateral to the midline and 2.5–3.0 mm rostral to the obex, and then lowered 5 mm into the medulla to reach the rVLM for subsequent extracellular recordings and iontophoresis of chemicals. The barrels contained either gabazine or saline, a recording electrode, and Chicago blue dye at the end of each experiment for later histological confirmation.

**Methods of Blockade**

The importance of the arcuate nucleus in the prolonged inhibitory EA response was determined by microinjection of the glutamatergic antagonist kynurenic acid (Kyn) into the hypothalamic region. Thus 50 nl of saline or Kyn (100 mM) (29) was injected into the arcuate nucleus 40 min after cessation of EA application (see Effects of EA on excitatory reflexes).

To examine the role of several rVLM neurochemicals in the prolonged EA inhibitory cardiovascular reflex response, 50 nl of the saline vehicle (0.9% NaCl), the nociceptin/orphanin FQ peptide (NOP) antagonist [Nα-Phe¹]-nociceptin(1–3)NH₂ (10 nM) (7), the GABAₐ receptor antagonist gabazine (SR-95531, 27 mM) (23, 32), or the nonspecific opioid antagonist naloxone (100 nM) (4) was microinjected into the rVLM ~40 min after termination of EA (or 50–60 min after the onset of the EA response).

Gabazine or saline was iontophoresed (Neuro Phore BH-2 system, Medical System, Greenwaile, NY) for 2 min (~40 min after EA termination to assess rVLM neuronal activity during the response of EA. A current of 100–120 nA was used for iontophoresis.

**Stimulation Methods**

The splanchnic nerve was stimulated with currents of 0.4–0.6 mA and pulses of 0.5-ms duration at a frequency of 2 Hz to evoke rVLM activity. The median nerves beneath the P₅–₆ acupoints were stimulated during acupuncture and during characterization of rVLM neurons. Electroacupuncture was applied bilaterally at 2–4 Hz at the P₅–₆ acupoints with 2–4 mA and 0.5-ms pulses (38). We confirmed previously (4, 17) that these EA stimulation parameters at P₅–₆ activate Aβ and C fibers in the median nerve to modulate sympathoexcitatory cardiovascular responses. Prolonged stimulation for 30 min was used during EA to simulate the clinical use of this procedure.

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 (2 for each set of 2 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 connected to an isolation unit and stimulator (Grass, model S88) to deliver the bipolar stimuli.

The thoracic spinal cord IML was stimulated electrically (0.1–0.4 mA, 2 Hz, 0.5 ms) for collision testing. Electrical stimulation (10–40 μA) was used to evoke small, reproduce excitatory responses of 5–10 mmHg, which also with anatomic evaluation after the experiment confirmed the location of the stimulating electrodes.

**Methods of Recording**

Single-unit activity in the rVLM was recorded with an extracellular platinum recording electrode inserted in one of the barrels (of a 3-barrel electrode) filled with 0.5 M sodium acetate containing 2% Chicago sky blue (Sigma, St Louis, MO). The pipette was advanced slowly through the rVLM to identify neuronal activity. 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 poten-
tials, blood pressure, and heart rate were digitized for data acquisition on an RC Electronics or a CED micro1401 MK II interface system. Data were analyzed off-line with a Pentium III computer and EGAA software (R. C. Electronics) or a Pentium IV computer and CED Spike 2 Windows software.

Action potentials were analyzed both visually and with the EGAA or Spike 2 programs, using waveshape recognition algorithms to allow detection of similar waveshapes, heights, and latencies of response.

Peristimulus time histograms were constructed for each neuron to assess evoked responses to stimulation of splanchnic and median nerves. The relationship between neuronal activity and blood pressure was assessed by both time and frequency domain analyses using arterial pulse-triggered averaging and coherence analysis, as we and others have described previously (1, 36–38).

Experimental Protocols

Effects of EA on excitatory reflexes. Chemosensitive afferents in the gallbladder were stimulated with a 1-cm² pledget of filter paper soaked with BK (10 μg/ml) to induce reflex increases in blood pressure. The filter paper was removed after the maximal blood pressure response was attained, and the gallbladder was washed four times with normal saline to remove excess BK. The increase in blood pressure was determined as the difference between mean arterial blood pressure before BK application and at the peak of the reflex response. Pledgets soaked with BK were used to induce reflex increases in blood pressure every 10 min in eight groups of animals. We first evaluated repeatability of 12 sequential blood pressure responses induced by BK applied to the gallbladder (to evaluate for tachyphylaxis) in the absence of EA in a control group of six animals. We also examined the influence of EA on cardiovascular sympathoexcitatory reflex response in four animals. We then studied six other groups of animals microinjected with Kyn, naloxone, ORL-1 nociceptin antagonist, gabazine, or their vehicle controls. In each group, we observed two consistent increases in blood pressure in response to stimulation of the gallbladder with BK followed by EA applied bilaterally at the P5–6 acupoints beginning 5–10 min before the third application of BK. Application of EA lasted for 30 min, followed by a recovery period lasting 70–80 min. Thus BK was applied during control (2 responses), EA (3 responses), and recovery after EA (7–9 responses) for a total of 12–14 sequential data points.

A previous study has shown that recovery from EA inhibition occurs between 40 and 50 min after termination of EA (37). Therefore, microinjection to inactivate the arcuate nucleus and to establish blockade in the rVLM occurred ~38 min after termination of EA. Thus saline (vehicle control) or Kyn was microinjected into the arcuate nucleus 40 min (2 min before ninth gallbladder stimulation) after acupuncture, when EA’s modulatory influence was still present, to examine the role of the long-loop pathway in the long-lasting EA inhibition. Also, [N-Phe¹]-nociceptin(1–3)NH₂, gabazine, naloxone, or saline was microinjected into the rVLM 40 min after termination of acupuncture to evaluate the neurotransmitter mechanism underlying EA’s prolonged inhibition. These times of injection occurred 50–60 min after the initial EA-related inhibitory action was observed (i.e., 10–20 min after onset of EA stimulation).

Fig. 1. Role of arcuate nucleus in long-loop pathway was examined with respect to the prolonged inhibitory electroacupuncture (EA) effect. Gallbladder stimulation with bradykinin (BK) every 10 min caused consistent reflex increases in mean arterial blood pressure (MBP) in the absence of acupuncture (A). Responses to EA with saline were similar to those without saline microinjection (37) (see RESULTS). Thus EA reduced the reflex response for a prolonged period and was not influenced by injection of the vehicle control (saline) into the arcuate nucleus (B). On the other hand, glutamatergic ionotropic receptor blockade with kynurenic acid (Kyn) in the arcuate nucleus rapidly reversed the EA-related inhibition of the cardiovascular sympathoexcitatory reflex responses (C), indicating that this hypothalamic nucleus participates in the long-term suppression of visceral excitatory reflexes. Values below each bar indicate baseline blood pressures (means ± SE) before gallbladder stimulation. Bars represent increase in blood pressure responses following BK stimulation. *Decrease of reflex response after onset of EA.

Fig. 2. γ-aminobutyric acid (GABA) antagonist gabazine in the rostral ventrolateral medulla (rVLM) does not influence sympathoexcitatory cardiovascular reflex responses during gallbladder stimulation.
Medullary neuronal activity in response to EA. We identified responsive neurons in the rVLM by examining for medullospinal premotor sympathetic rVLM neuronal responses, input from both splanchnic and median nerves, baroreceptor afferent convergence, and cardiac rhythmicity. We antidromically stimulated rVLM neurons from the IML that was stimulated continuously at a frequency of 2 Hz and 0.5-ms pulses while the recording electrode was lowered slowly at increments of 1–2 μm in the rVLM to locate premotor neurons. Neurons (11 of 17) 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, neurons were evaluated for collision of evoked antidromic spikes from the IML with orthodromic action potentials from the splanchnic or median nerves. The refractory period was measured to determine the critical time interval (the latency plus the refractory period) during which the orthodromic spike cancelled the antidromic spike (38, 48). Axonal conduction velocity of rVLM neurons that projected to the IML was calculated by dividing the distance between the recording and stimulating electrodes in the rVLM and IML by the antidromic latency (37, 38). After testing for collision, all 17 neurons were evaluated for responses during median and splanchnic afferent stimulation. Stimuli to the median and splanchnic nerves were applied at 2 Hz (38). We measured evoked activity over a 15-s period to construct peristimulus histograms (36). In addition to assessing convergence of splanchnic and median nerve input in rVLM neurons, we further characterized their responsiveness to cardiovascular stimulation by altering baroreceptor input with nitroglycerin or phenylephrine to lower or raise blood pressure. Cardiovascular rhythmicity over a period of 5 min also was determined to further differentiate rVLM neurons. The firing pattern of rVLM neurons over this recording period was subjected to time and frequency domain analysis using arterial pulse-triggered averaging and coherence analysis of the relationship between arterial pulse and rVLM neural activity (38). Our previous investigation (36) demonstrated that most rVLM cells (86%) receiving input from the splanchnic and median nerves function as premotor sympathetic neurons.

Once the cells were identified and classified, we evaluated their evoked responses to EA, with the exception of a time control group that was not subjected to EA. Their discharge activity during splanchnic nerve stimulation without EA was recorded every 10 min in six neurons. These neurons were determined to be either sympathoexcitatory cardiovascular or baroreceptor-related sympathetic premotor rVLM neurons by the methods described above.

Finally, we evaluated the splanchnic nerve-evoked rVLM neuronal discharge following iontophoresis of either saline or gabazine into the rVLM of 11 cats 40 min after termination of EA, i.e., during the prolonged period of inhibition, to evaluate electrophysiologically the role of rVLM GABA_A receptors in this response.

Histology

At the end of each experiment, animals were euthanized with α-chloralose followed by saturated KCl. The hypothalamus, brain stem, and spinal cord 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. Recording and microinjection sites in the arcuate nucleus and rVLM were plotted on coronal sections. Additionally, stimulation sites in the spinal cord IML were confirmed microscopically.

Fig. 3. The reflex responses to repeated gallbladder stimulation that were decreased by EA were not immediately influenced by saline but were rapidly reversed with opioid or GABA receptor antagonists microinjected into rVLM (A, C, and D). Reflex responses to EA are similar with and without saline injection (see RESULTS). In contrast, nociceptin receptor blockade did not reverse the EA inhibition of the cardiovascular responses (B). Values below each bar indicate baseline blood pressures (means ± SE). *Decrease of reflex response after onset of EA; †, time of BK application; a–d, individual examples of arterial blood pressure tracings; AP, arterial pressure.
Data Analysis

Data are presented as means ± SE. Evoked activity was measured as the increase in number of spikes above baseline. The assumption of normal data distribution was analyzed with 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 evaluated time and frequency relationships between rVLM neuronal activity and arterial blood pressure with pulse-triggered averaging as well as coherence analysis. For the time domain analysis, arterial pulse-triggered averaging methods used a threshold that was set at the systolic phase of the arterial pulse. We used spike height discrimination and waveform recognition to sort action potentials during the 300-s period of evaluation. Averages of the arterial pulse and histograms of neuronal activity were constructed as we (36–38) and others (1) described previously.

Frequency domain analysis was used to assess coherence between rVLM activity and arterial blood pressure with a fast Fourier transform (FFT) algorithm (27, 37, 38). Original data were recorded with 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 histograms before coherence analysis. The number of data sections (15–20, each lasting for 12.8 s) was chosen to determine the average histogram. 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 with bin widths of 50 ms. The autospectral analysis was adopted from Shin et al. (31), using contiguous segments of 256 beats with 50% overlap between the 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 (1, 16, 31, 38, 49).

RESULTS

Effects of EA on Cardiovascular Excitatory Responses

Long-loop pathway. Consistent sympathoexcitatory responses were observed with repeated gallbladder stimulation in six animals (Fig. 1A) similar to our previous studies (35, 37). Microinjection of saline in the arcuate nucleus did not appear to alter the EA-associated prolonged inhibition of the reflex excitatory response (Fig. 1B) as we previously observed when EA without saline was employed (35, 37). In contrast, glutamate receptor blockade with Kyn in the arcuate nucleus reversed the prolonged influence of EA on the cardiovascular excitatory reflex (Fig. 1C). In the absence of EA, inactivation of this nucleus with kainic acid did not affect the sympathoexcitatory reflex responses (20).

Neurotransmitter mechanisms in rVLM. We showed previously (7) that microinjection of nociceptin antagonist (ORL-1) and naloxone did not affect the BK-induced sympathoexcitatory reflex. Similarly, gabazine did not influence the reflex cardiovascular response to BK on the gallbladder (Fig. 2). Microinjection of saline or the NOP receptor antagonist...
[N-Phe]-nociceptin(1–3)NH₂ into the rVLM 40 min after EA did not disturb the inhibitory effect of EA on the sympathoexcitatory reflex (Fig. 3, A and B). However, both the nonspecific opioid antagonist naloxone and the GABAA receptor blocker gabazine microinjected into rVLM significantly reversed the EA depressor response (88% and 75%, respectively; Fig. 3, C and D).

Medullary neuronal EA-evoked activity. Neurons in the rVLM were selected for their responsiveness to visceral and somatic stimulation of afferent pathways in the splanchnic and median nerves. Neurons also were found to be responsive to baroreceptor afferent stimulation and hence were classified as cardiovascular sympatoexcitatory. In this regard, nitroglycerin increased the discharge rate of 12 neurons from 3.8 ± 1.4 to 9.0 ± 3.9 spikes/s, while phenylephrine decreased neuronal activity of 5 other neurons from 3.8 ± 0.8 to 1.8 ± 1.2 spikes/s. Baseline activity of the group of neurons as a whole averaged 3.8 ± 1.1 spikes/s.

The majority (65%) of the neurons tested (11 of 17) could be antidromically driven from the IML of the thoracic spinal cord. The axonal conduction velocity of this group of rVLM neurons averaged 5.9 ± 0.5 m/s. The average distance between the recording and stimulation sites was 87 ± 4 mm. An example of an individual rVLM neuron is shown in Fig. 4. The axon of this neuron projected to the thoracic IML. In this regard, Fig. 4A displays collision of the antidromic induced spike with an orthodromic median nerve evoked action potential, when the interval between the triggered antidromic and the orthodromic spikes was reduced by 3 ms. It received convergent input from splanchnic, median, and baroreceptor afferents. Thus the discharge rate of this neuron increased after intravenous injection of nitroglycerin (Fig. 4B) and was strongly correlated with the cardiac cycle, as demonstrated by a coherence value of 0.72 at frequency of 3.56 Hz (Fig. 4, C and D). Hence this premotor sympahtoexcitatory rVLM neuron demonstrated cardiac rhythmicity when assessed with time and frequency domain analyses.

All 17 neurons were evaluated with respect to their relationship to the cardiac rhythm. Time domain analysis using arterial pulse-triggered averaging showed a strong relationship between the discharge activity of the neurons examined and arterial blood pressure. Likewise, using frequency domain analysis, we observed a strong relationship (average coherence 0.72 ± 0.05) between neuronal activity and blood pressure at a cardiovascular frequency of 3.4 ± 0.2 Hz.

After classification, we evaluated the responses of neurons to repeated splanchnic nerve stimulation in the absence or presence of EA. Evoked responses were measured before and after iontophoresis of either saline or gabazine after 60–70 min of EA inhibition (Fig. 5). Figure 5A demonstrates consistent neural responses to visceral stimulation every 10 min in the absence of EA. The response to EA with saline iontophoresis (Fig. 5B) was similar that in to our previous studies that did not employ saline (36). In general, we noted that the neurons recovered after an average of 70 min. However, blockade of GABAA receptors 60 min after the onset of inhibition by EA rapidly reversed the modulatory response (Fig. 6). Baseline activity was increased after blockade with gabazine (Fig. 6, peristimulus histogram C). It should be noted that the evoked activity was increased over and above baseline, thus reversing the EA inhibitory effect in the rVLM neuronal response.

Fig. 5. A: repeated splanchnic nerve stimulation evoked consistent responses, as shown by post-stimulus time histogram analysis (imp/30 stim). B: stimulation of acupoints P5–6 reduced this evoked activity for >80 min and was not influenced by injection of the vehicle control (saline) into the rVLM compared with EA without saline microinjection (36) (see RESULTS). *Significant changes from control pre EA period.

Anatomic Location of Recording and Stimulation Sites

We observed that the injection sites for the long-loop inhibition study were located in the arcuate nucleus near the ventral surface of the hypothalamus (Fig. 7A). We found that the neuronal recording sites were located within an area 2.2–4.5 mm rostral to the obex, 2.5–3.9 mm to the right or left of midline, and 0.1–1.2 mm from the ventrolateral surface (Fig. 7B). The sites were lateral to the nucleus inferior olive and pyramidal tracts as well as ventral and medial to the facial and retrofacial nuclei, an area that is within the region previously described by ourselves (4, 36–38) and others (3, 9) to comprise the rVLM. The sites of stimulation in the lateral central gray area of the thoracic spinal cord between T2 and T4 were identified to be in the IML spinal cord as described previously (25, 38).

DISCUSSION

We recently demonstrated (20, 38) an important role for a long-loop pathway involving supramedullary structures including the arcuate nucleus and vPAG, in the early portion of the EA-associated rVLM sympathetic premotor inhibitory response. Our previous studies (8) also have shown that EA activates opioid-containing cells located in the arcuate nucleus. Finally, we have shown (20, 21) that EA stimulation evokes activity in the arcuate nucleus and that inhibition of this nucleus with kainic acid significantly inhibits modulation of...
rVLM activity by EA. In the present study, we have shown that this long-loop pathway participates importantly in the prolonged inhibition of reflex cardiovascular excitation by EA. Furthermore, the present study demonstrates that GABA and opioids, but not nociceptin, underlie the prolonged inhibitory action of EA in the rVLM.

Prolonged acupuncture responses have been reported in clinical and animal studies, including pain and hypertension. For example, chronic pain relief after spinal cord injury can last for 3 mo or more after cessation of acupuncture treatment (26). Also, a long-lasting cardiovascular modulatory response to acupuncture (5–12 h) has been documented in unanesthetized (12, 44) and anesthetized (11) rats. Recent studies of anesthetized rats and cats from our laboratory (19, 36, 37, 49) show that autonomic reflex increases in blood pressure can remain depressed for >1.5 h after 30 min of low-frequency EA. However, these previous studies identified neither the central neural regions nor the mechanisms underlying acupuncture’s long-term inhibition.

In addition to the prolonged influence of EA on reflex blood pressure responses, we have demonstrated prolonged inhibitory rVLM neuronal responses during and after EA, lasting for over an hour (36, 37). Other studies have observed depressed neuronal evoked activity ranging from milliseconds to a few minutes in the rVLM and nucleus tractus solitarii (NTS) in response to afferent stimulation (46, 50). For example, in the rVLM neurons activated by the aortic depressor nerve during brief (0.2 ms) stimulation display increased duration (>200 ms) of inhibitory response with vagal input (46). Five minutes of low-frequency stimulation of primary afferents providing input to the tractus solitarii decreased synaptic strength in the NTS for >30 min, suggesting long-term depression (50). The present study is the first to demonstrate that during and after 30 min of somatic afferent stimulation, sympathoexcitatory cardiovascular premotor rVLM neurons exhibit long-term inhibition lasting for >80 min.

Fig. 6. Role of GABA was evaluated in EA-related long-term suppression of rVLM neuronal activity. Microinjection of the GABA$_A$ receptor antagonist gabacline into the rVLM after >60 min of EA-related suppression (peristimulus histogram B) rapidly reversed the inhibition by acupuncture (peristimulus histogram C). Peristimulus histograms A–D displayed above bar histogram represent evoked activity of 1 neuron. *Significant changes from control (A).

Fig. 7. Sections of the cat medulla and hypothalamus illustrate microinjection and recording sites. Recording sites in rVLM (*) include neurons studied during assessment of convergence of input from splanchnic and median nerve stimulation. Microinjection sites for Kyn (○), gabazine (■), [N-Phe$^1$]-nociceptin(1–3)NH$_2$ (●), and saline (●) in rVLM likewise are indicated. Sections represent combinations of medullary and hypothalamic planes rostral to the obex or interaural line. Inferior olivary nucleus (ION), laminar spinal trigeminal nucleus (5SP), and retrofacial nucleus (RFN) are shown for reference.
The arcuate nucleus, vIPAG, and rVLM are known to facilitate the early modulatory responses of EA. In this regard, kainic acid depolarization blockade in the arcuate nucleus eliminates the immediate EA modulatory response (21). The present study extends these observations by showing that inhibition of ionotropic glutamate receptors in this hypothalamic region 50 min after the onset of EA inhibition reverses acupuncture-related inhibition. Thus glutamate receptor activation in the arcuate nucleus, forming part of a long-loop neural pathway activated by EA, contributes to the prolonged suppression of reflex responses.

We have shown that EA evokes both short- and long-term excitatory neuronal responses in arcuate nucleus and vIPAG (20). On the other hand, while the rVLM responds immediately with excitation during EA, during more prolonged somatic afferent stimulation (lasting longer than 15–15 min) reflex-induced excitation is inhibited by EA (38). Although we know that a long-loop pathway is essential for the initial activation of inhibitory response (20), it is not clear whether the prolonged inhibition is a result of activation of this pathway or is a property of the area where inhibition seems to occur, i.e., within the rVLM where sympathetic activity is modulated by EA (36). The present study demonstrates that the rVLM itself is a key region of EA-associated prolonged sympathoinhibition and that activation of the long loop pathway contributes to rVLM activation.

Endogenous opioids in the rVLM do not contribute to processing visceral-cardiovascular excitatory reflexes (7). However, opioids do play a role in the early inhibitory effect that EA has on the gallbladder-cardiovascular response. In this regard, opioid μ- and δ-receptors both contribute to the immediate acupuncturereponse (19), suggesting that β-endorphin, endomorphin, and enkephalins are important in modulation of sympathetic premotor neurons. In the present study, we observed similar reversal of the cardiovascular responses with late opioid receptor blockade. Thus opioids appear to be important for both the early and late EA responses.

Nociceptin in the rVLM contributes to the early excitatory response (7), but, as we have shown here, this opioid-like neuromodulator does not participate in the long-lasting EA depressor effect. Therefore, there is a dichotomy between opioids and NOP receptor activation in EA’s prolonged inhibition of reflex cardiovascular sympathoexcitation. To confirm that the nociceptin antagonist had no effect during prolonged inhibition, we also examined the response to two repeated microinjections of the antagonist 40 min after the end of EA in a few cats and still observed no effects (data not shown). Thus we believe that nociceptin antagonism does not influence the prolonged inhibitory action of EA.

The action of GABA in EA cardiovascular modulation as mediated by ionotropic GABA_A receptors was examined for the first time in the present study. GABA microinjection in the rVLM induces hypotension (28) and inhibits barosensitive sympathetic premotor rVLM neurons that are influenced by the caudal ventrolateral medulla (cVLM) (30, 41–43). The cVLM will deserve further investigation in the future to determine if it participates in the prolonged cardiovascular inhibition by EA. In addition to tonic GABAergic inhibition in the rVLM, the cVLM also relays somato-sympathetic inhibitory reflexes through a postsynaptic GABA_A receptor mechanism in this region (24, 47). However, previous studies have concentrated on the short-term influence of GABA. In the present investigation, we found that the prolonged inhibitory effect of EA on cardiovascular excitatory reflex responses was reversed rapidly after unilateral GABA_A rVLM receptor blockade without gabazine while the receptor blockade did not influence the primary reflex response. Of note, we used the neutral antagonist gabazine since this inhibitor provides negligible inhibition of spontaneously active GABA_A receptors in the absence of GABA (23). Other antagonists like bicuculline are less reliable because they can inhibit tonic activity (23).

Long-term neural inhibition could result either from prolonged action during short-term release of a neurotransmitter or from prolonged release of a neurotransmitter with a short half-life of action. Although we have shown previously (19) that single application of exogenous μ- and δ-opioid agonists exerts a neural response for up to 30 min, we cannot account for such prolonged inhibition lasting 80 min or longer that would result from a brief EA-related release of opioids and GABA. Thus future studies will need to focus on the possibility that 30 min of EA results in prolonged rVLM modulatory neurotransmitter release that most likely could be related to long-term activation of the long-loop neural circuitry involving the arcuate nucleus.

A potential limitation of the present study is that only one cat was used, and we cannot account for interanimal variability. However, we believe that nociceptin antagonism does not influence the prolonged inhibitory action of EA.

In summary, prolonged inhibitory cardiovascular activity requires an intact long-loop pathway from the arcuate nucleus to the medulla, which, in turn, influences cardiovascular excitatory reflex responses by altering sympathetic outflow. In particular, long-term inhibition of the premotor cardiovascular sympathoexcitatory rVLM neurons by EA involves opioids and GABA but not nociceptin.

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REFERENCES

NEUROTRANSMITTER SYSTEMS IN ACUPUNCTURE MODULATION OF PRESSOR REFLEXES


