Role of glutamate in the rostral ventrolateral medulla in acupuncture-related modulation of visceral reflex sympathoexcitation

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Zhou W, Fu LW, Guo ZL, Longhurst JC. Role of glutamate in the rostral ventrolateral medulla in acupuncture-related modulation of visceral reflex sympathoexcitation. Am J Physiol Heart Circ Physiol 292: H1868–H1875, 2007. First published December 8, 2006; doi:10.1152/ajpheart.00875.2006.—Visceral sympathoexcitatory reflexes induced by stimulation of the gallbladder with bradykinin (BK) are attenuated by electroacupuncture (EA) at Neiguan-Jianshi (P5-6) acupoints located over the median nerve. Previous studies have shown that neurons in the rostral ventrolateral medulla (rVLM) receive convergent input from visceral organs and somatic nerves (activated by EA), Glutamate (Glu), an important excitatory neurotransmitter in the rVLM, processes visceral sympathoexcitatory cardiovascular reflexes. In the present study, we determined the relationship between EA-mediated opioidergic modulation of visceral cardiovascular responses and Glu. Reflex cardiovascular responses were evoked by application of BK to the gallbladder before and after EA in anesthetized cats. Glu concentrations ([Glu]) were measured by HPLC from samples collected by microdialysis probe(s) inserted unilaterally or bilaterally into the rVLM. BK-induced reflex responses and [Glu] were attenuated by 45% and 70%, respectively, after 30 min of EA (n = 6). EA alone did not change [Glu] in the rVLM (n = 6, P > 0.05). However, microdialysis of naloxone (100 mM) into the rVLM reversed EA-related inhibition of blood pressure and [Glu] (n = 5). Immunohistochemical visualization showed that δ-opioid receptors colocalized with, and were in close apposition to, vesicular Glu transporter 3- and c-Fos-double-labeled perikarya and processes of rVLM neurons after gallbladder stimulation with BK. These data suggest that EA attenuates BK-induced visceral sympathoexcitatory reflexes through opioid-mediated inhibition of Glu’s action in the rVLM.

Clinical and experimental studies have suggested that acupuncture at certain acupoints may have therapeutic effects in hypertension, hypotension, arrhythmias, and myocardial ischemia (3, 4, 36, 49, 52, 54). The Neiguan-Jianshi pericardial meridian (P5-6) acupoints overlying the median nerve on the wrist are commonly used for treatment of heart disease in Eastern and, more recently, Western countries (30, 33, 43, 48, 49). We have established a feline model to study the mechanisms of the influence of electroacupuncture (EA) on visceral sympathoexcitatory reflexes evoked by activation of sympathetic visceral chemosensitive afferents. In this respect, EA at P5-6 acupoints overlying median nerves in cats significantly attenuates reflex increases in blood pressure (BP) evoked by stimulation of sensory nerve endings in the gallbladder (10, 36).

The rostral ventrolateral medulla (rVLM) organizes fundamental tonic and reflex sympathetic control of circulatory function (16, 26). The rVLM is involved in EA-related modulation of visceral sympathoexcitatory reflexes (10, 37, 57). In this regard, we have demonstrated that sympathoexcitatory premotor rVLM neurons receive convergent input during EA at P5-6 and stimulation of the splanchnic nerve (56, 57, 63) and that EA reduces bradykinin (BK)-evoked activity of these rVLM neurons for up to 1 h after termination of the stimulus (57, 63). Thus the initial and predominant response of these premotor sympathetic neurons to visceral spinal afferent stimulation is excitation, a response that is inhibited over time by application of EA. The inhibitory response from EA is related to activation of a long-loop pathway involving the arcuate nucleus in the ventral hypothalamus and the ventrolateral periaqueductal gray in the midbrain (39, 58).

Bulbospinal neurons arising from the rVLM contain a number of excitatory neurotransmitters, including glutamate (Glu), serotonin, and catecholamines, that contribute to the regulation of cardiovascular function (13, 46, 51). Our recent study employing microdialysis showed that Glu acting through N-methyl-d-aspartic acid and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in the rVLM serves as an important excitatory neurotransmitter in processing visceral sympathoexcitatory reflex responses (59). Opioid peptides are present in nuclei that process autonomic signals, including the rVLM, caudal VLM, periaqueductal gray, hypothalamus, and dorsal horn of the spinal cord (19, 28). Stimulation of opioid receptors in the rVLM inhibits sympathetic outflow (47), as well as the release of Glu in the brain stem (29, 31). We have shown that activation of the opioid system during EA at P5-6 inhibits sympathetic outflow and visceral excitatory reflex responses induced by application of BK on the gallbladder (10, 57). In addition, using c-Fos immunohistochemical labeling, our group demonstrated that EA at the same pericardial acupoints activates neurons in the rVLM, some of which contain enkephalin (24). Also, many rVLM neurons activated during EA are closely associated with nerve fibers containing enkephalin or β-endorphin that originate from interneurons in the rVLM or supramedullary centers that project to the rVLM (24). However, although Glu appears to be an important excitatory neurotransmitter in the rVLM, whether EA inhibits visceral excitatory reflexes by limiting Glu release in this brain stem region is unclear. Furthermore, the mechanism of EA-related alternations in Glu release in the rVLM, which may be related to activation of the opioid system, is uncertain.

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The purpose of the present study was to determine the anatomic relation between Glu and the opioid system in rVLM neurons responsive to EA stimulation, as well as the influence of acupuncture on visceral reflex-induced rVLM Glu release and the underlying role of the opioid system in this response. We hypothesized that there is a close anatomic relation between Glu and opioids in EA-responsive rVLM cells. We further hypothesized that acupuncture-related inhibition of visceral excitatory reflexes is mediated through opioid-mediated inhibition of Glu release in the rVLM. We therefore predicted that EA would inhibit the increase in extracellular Glu concentration ([Glu]) and that blockade of opioid receptors with naloxone would abolish this inhibitory effect of EA.

MATERIALS AND METHODS

General Surgical Preparation

The experimental preparations and protocols were reviewed and approved by the Animal Care and Use Committee of the University of California, Irvine. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Studies were performed in cats of either sex (2.5–4.5 kg). Anesthesia was induced with ketamine (40–50 mg/kg im) and subsequently maintained with α-chloralose (50–60 mg/kg iv). Supplemental α-chloralose (5–10 mg/kg iv) was administered to maintain an adequate depth of anesthesia as judged by stability of BP and respiration and lack of a withdrawal response to toe pinch. A femoral artery and vein were cannulated for recording BP and heart rate (HR) and for administration of fluids and drugs, as described previously. Body temperature was monitored continuously with a rectal probe and was maintained at ~37.0°C with a water-perfused heating pad and an external heat lamp.

Microdialysis and HPLC Measurement of Glu

The cat’s head was fixed in a stereotaxic apparatus (Kopf Instruments). A craniotomy was performed to expose the ventral surface of the medulla for microdialysis. For placement of probes into the rVLM, the basioccipital bone was removed from the atlanto-occipital membrane with rongeurs, and the craniotomy was extended for ~4 mm on each side of the midline over the medullary surface, as described previously (10, 59). The dura was cut, and the cerebrospinal fluid (CSF) was removed to expose the medullary surface. Gel foam was used to minimize bleeding.

After surgery, a 1-mm-long, 0.5-mm-diameter microdialysis probe (model CMA/12, CMA, Acton, MA) was placed in the rVLM with the use of a stereotaxic carrier (Kopf Instruments). We unilaterally inserted a microdialysis probe in our early experiments and observed a success rate of probe placement into the rVLM of ~67% (4 of 6 cats). To increase the rate of success, we designed a custom-made probe holder for bilateral insertion based on the distance (~6.0 mm) between left and right regions of the rVLM (Barman's atlas, plane 10.0, 10.8). Bilateral insertion improved our success rate from 67% to 86% (12 of 14 cats) in our later experiments. With respect to bilateral insertion, we found that one probe was correctly placed in the rVLM in eight cats and both probes were placed in the rVLM in four cats, as confirmed by subsequent histological examination. When both probes were located in the rVLM, we averaged [Glu] sampled from both sides of the rVLM.

All insertion sites were confined to an area 2.4 ± 0.2 mm caudal to the most caudal portion of the trapezoid body, 3.2 ± 0.3 mm to the right or left of midline, and 0.7 ± 0.1 mm below the ventral surface of the medulla. This area includes the rVLM, as described previously (18, 41, 42, 57). Extracellular fluid was collected during sequential 10-min periods (1.5 μl/min, total 15 μl) using a refrigerated fraction collector, and samples were stored at ~80°C until assay with artificial CSF (0.2% BSA, 0.1% bacitracin, 6.2 mM K+, 134 mM Cl–, 2.4 mM Ca2+ , 150 mM Na+, 1.3 mM P–, 13 mM HCO3–, and 1.3 mM Mg2+ , pH 7.4). Placement of microdialysis probes into a pressor region of the brain stem was verified by notation of the change in BP after local perfusion of 30 nl of 1 mM Glu (RBI, Natick, MA) into the area. An immediate increase in MAP (average 35 ± 4 mmHg) indicated proper probe placement.

Fifteen microliters of microdialysate were mixed for 10 min at 25°C with 15 μl of β-phenylalanic dioxycarboxylic acid solution (20 mg of β-phenylalanic dioxycarboxylic acid, 0.5 ml of 1 M sodium sulfate, and 10 ml of 4 M sodium borate solution) for precolumn derivation (32, 59). [Glu] was immediately analyzed in the mixed sample. The mobile phase for isocratic elution of Glu consisting of 100 mM NaH2PO4 (pH 3.5), 10% methanol, and 0.5 mM Na2EDTA was run through a Spherisorb PS Phase Separation ODS2, 5-μm HPLC analytic column at a flow rate of 1.0 ml/min (510 Pump, Waters, Milford, MA). Glu was detected coulometrically by a Coulomb II detector (ESA, Chelmsford, MA) with a 5020 guard cell and a 5011 analytic cell (ESA). The applied working potentials were 910 mV for the guard cell and 1100 mV (E1) and 860 mV (E2) for the analytic cell. The detection limit for this Glu assay was 50 pg. For confirmation of peak identity, samples were run concurrently with known standards.

Triple-Fluorescent Immunohistochemical Labeling

Cardiovascular hemodynamic changes can cause secondary baroreceptor cardiopulmonary reflex activation, which can lead to c-Fos expression in the brain (24, 25). To control the input from this secondary activation of the neural pathways consequent to stimulation of the gallbladder, bilateral sinoaortic denervation and cervical vagotomy were accomplished. Thus the carotid sinus nerves and cervical vagi were isolated and transected from the internal and common carotid arteries, respectively. Barodenervation was verified by the absence of the normal decrease of HR in response to an increase in arterial BP induced by administration of phenylephrine (10 μg/kg iv; Genedia/Scor Pharmaceuticals, Irvine, CA). Cats were allowed to stabilize for 4 h after surgical preparation.

We examined a relation between δ-opioid receptors and glutamatergic neurons in the rVLM by observing the anatomic relations between labeling of δ-opioid receptors on neurons or neural processes and neurons colabeled with vesicular Glu transporter 3 (vGLUT3) and c-Fos in the rVLM after stimulation of the gallbladder with BK. vGLUT3 is a Glu transporter that transports Glu into vesicles of neuronal cell bodies and, as such, is considered to be a specific marker for neurons that use Glu as a neurotransmitter (53).

Animals were perfused transcardially with 0.9% normal saline and cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4) (22, 23) 90 min after completion of experimental procedures described below. The medulla oblongata was harvested and stored in 4% paraformaldehyde that use Glu as a neurotransmitter (54).

The brain sections were rinsed in PBS (0.1 M) and then placed in 1% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h. The tissues were incubated in PBS solution containing primary antibodies at 4°C for 48 h. Coronal sections of the brain (30 μm) were made with a cryostat microtome (model CM 185, Leica, Nussloch, Germany) and collected serially in cold cryoprotectant solution (9).

The brain sections were rinsed in PBS (0.1 M) and then placed in 1% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h. The tissues were incubated in PBS solution containing primary antibodies at 4°C for 48 h. The antibodies used for double labeling were guinea pig polyclonal anti-vGlut3 (1:500 dilution; Chemicon International, Temecula, CA) and rabbit polyclonal anti-δ-opioid receptor (1:400 dilution; Abcam, Cambridge, MA). For triple staining, guinea pig anti-vGLUT3, rabbit anti-δ-opioid receptor, and monoclonal mouse anti-c-Fos (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were used. Sections then were treated with secondary antibodies raised in the donkey, including rhodamine-conjugated anti-rabbit, fluorescein-conjugated anti-guinea pig, and coumarin-conjugated anti-mouse, at 4°C for 24 h (all 1:100 dilution; Jackson Immunoresearch Laboratories). Each of the second-
ary antibodies has been produced for multiple labeling and has minimal cross-reactivity to other nonspecific species. Sections were mounted on slides and air-dried. Mounting medium (Vector Laboratories, Burlingame, CA) was used to apply coverslips to the slides. Immunohistochemical control studies were performed by omission of a primary or a secondary antibody. No labeling was detected when a primary or a secondary antibody, responsible for specific staining, was omitted.

Brain sections were scanned and examined with a standard fluorescence microscope (model E400, Nikon, Melville, NY). Selected rVLM sections that display the rVLM and demonstrated fluorescent activity from three antibodies were evaluated with a laser scanning confocal microscope (model LSM 510, Meta system, Zeiss, Thornwood, NY) equipped with Ar and He-Ne lasers, as well as a Ti-sapphire femtosecond pulse laser, allowing operation of multiple channels. A 488- and a 543-nm laser were used to excite fluorescein (green) and rhodamine (red), respectively, and a 790-nm laser was applied for two-photon excitation of coumarin (blue). Digital images of the immunoreactive structures were captured and analyzed with software (Zeiss LSM) provided with the confocal microscope. Each confocal section was limited to 0.5 μm thickness in the z plane. Images of three colors in the same plane were merged to define the relation among the three immunoreactive elements (see Fig. 3).

Brain Histology

A modified microdialysis probe was used for microinjection of 50 nl of 0.5% pontamine blue dye (Chicago Sky Blue) into the rVLM; the membrane of the probe was removed at the end of each experiment. The modified probe was placed in the same position as the original membrane-covered dialysis probe to mark the location of the probe in the rVLM. The coordinates of the insertion points were measured from the micromanipulator settings and were plotted for each animal on a diagram of the ventral surface of the brain. The reference points for probe insertion were the midline, the caudal border of the trapezoid body, the most rostral hypoglossal rootlets, and the lateral longitudinal sulcus (see Fig. 2A). The medulla was fixed postmortem by immersion in 4% paraformaldehyde and 20% sucrose for ≥24 h. Dye spots were localized histologically in 60-μm frozen sections that had been counterstained with neutral red. This procedure allowed the injection sites to be mapped in relation to nuclei closely related to the rVLM, particularly the nuclei of the inferior olive and the retrofacial nucleus.

Experimental Protocols

EA influence on visceral reflex increases in extracellular rVLM [Glu]: effect of naloxone. After insertion of the microdialysis probes, the animals were allowed to stabilize for 1 h. During this period, CSF was continuously dialyzed at 1.5 μl/min while 10-min collections of dialysates from each probe (total volume, 15 μl) for each collection period were sampled to establish the baseline (control) extracellular [Glu]. Then BK (10 μl/ml) was applied to the serosal surface of the gallbladder with a 1-cm² pledget of BK-soaked filter paper. The filter paper was removed after the cardiovascular responses were recorded (2–3 min), and the gallbladder was washed at least three times with saline to remove excess BK. We bilaterally inserted 32-gauge stainless steel acupuncture needles into P5-6 acupoints and connected the needles to a stimulator (Grass Instruments, Quincy, MA). In six animals, after initial application of BK to the gallbladder, we performed EA (2 Hz, 2–4 mA, 0.5-ms duration) for 30 min and then applied BK again, 10 and 60 min after termination of EA. In the same six animals, we recorded BP and collected Glu samples before EA, 10, 20, and 30 min during EA, and 40 min after EA without BK stimulation of the gallbladder. In five other cats, we performed the procedure described above, except the acupuncture needles at P5-6 were not stimulated, since our previous study suggests that needle insertion without stimulation at active acupoints serves as an adequate control for EA (60). Five other cats were subjected to a protocol identical to that described for the EA group, with the addition of naloxone (100 mM) (31) perfused with microdialysate for 40 min, initiated 10 min before EA.

Immunohistochemical assessment of vGLUT3, δ-opioid receptors, and c-Fos in the rVLM. vGLUT3 and δ-opioid receptors in the rVLM were visualized in two untreated control cats. We evaluated triple labeling of vGLUT3 + δ-opioid receptors + c-Fos in the rVLM after inducing sympathoexcitatory pressor reflex responses by gallbladder stimulation in three cats. Thus, at 4 h after bilateral barodenervation and vagotomy, BK was applied to the gallbladder six times over a 100-min period with 20 min between each application (1st through 6th stimulation). We used the smallest dose of BK that induced a ≥30-mmHg increase in MAP. The gallbladder was rinsed with saline after the peak BP response, ∼1 min after application of BK (5–10 μg). BP and HR were monitored throughout the experiment and were recorded continuously during application of BK. The medulla oblongata of each cat was harvested for immunohistochemical labeling.

Statistical Analysis

Values are means ± SE. We used the Kolmogorov-Smirnoff test from peak response to determine whether the data were distributed normally. Baseline and peak values of BP and [Glu] were analyzed using a one-way repeated-measures ANOVA followed by comparison for individual differences using Tukey’s test. Values were considered to be significantly different when P < 0.05. All statistical calculations were performed with a statistical software package (SigmaStat for Windows version 3.0, Jandel Scientific Software, San Rafael, CA).

RESULTS

Influence of EA on rVLM [Glu] During Visceral Reflex Stimulation: Effect of Naloxone

We assessed the prestimulation [Glu] in microdialysates collected 60–70 min after probe insertion. Needle insertion at P5-6 without stimulation in six cats did not affect the increases in BP or extracellular [Glu] evoked by gallbladder stimulation (Fig. 1, A and B). Thus BP and extracellular [Glu] in the rVLM were significantly increased during stimulation compared with prestimulation. These increases were attenuated by ∼45% and 70%, respectively, by 30 min of EA at P5-6 (Fig. 1, C and D; n = 6, P < 0.05). Microdialysis of naloxone in the rVLM reversed the EA-related inhibition of BK-evoked BP and Glu responses in five other cats (Fig. 1, E and F). EA alone at P5-6 did not alter baseline BP or extracellular [Glu] (Table 1; n = 6).

In Fig. 2A, 20 probe insertion sites in the rVLM are plotted on the ventral surface of the medulla to show their position relative to surface landmarks. The positions of probe insertion in the rVLM were confirmed histologically. They were located 2.7–4.5 mm rostral to the obex, 2.8–4.0 mm to the midline, and 0.5–1.0 mm from the ventral surface. A frontal section depicting the location of unilateral insertion of a probe in the rVLM of one cat is shown in Fig. 2B.

Relation Between δ-Opioid Receptors, vGLUT3, and c-Fos in the rVLM

In the rVLM of two cats, the labeling of vGLUT3 was located bilaterally in rVLM cell bodies and in neuronal processes (Fig. 3A). δ-Opioid receptors also appeared bilaterally in fibers and perikarya as clusters and/or dots (Fig. 3B). More than half of the neurons containing δ-opioid receptors were in close apposition to or colocalized with vGLUT3-containing
neuronal structures (perikarya and processes of neurons) in the rVLM. Also, the majority (~60%) of neuronal structures labeled with vGLUT3 were in close proximity to and/or colocalized with δ-opioid receptors. Neurons labeled with c-Fos and/or vGLUT3 were found bilaterally in the rVLM in three cats after chemical stimulation of the gallbladder. The pattern of vGLUT3 and δ-opioid receptor labeling in the rVLM of these three cats was similar to that in the two untreated cats. We observed that δ-opioid receptor labeling was very closely related to neuronal processes or colocalized in cell bodies of most (~70%) neurons containing vGLUT3 and c-Fos in the rVLM. Merged confocal micrographs of an rVLM neuron, including triple labeling with c-Fos, vGLUT3, and δ-opioid receptors in a cat treated with BK, are shown in Fig. 3D.

DISCUSSION

Stimulation of the Neiguan-Jianshi (P5-6) acupoints significantly attenuates visceral sympathoexcitatory reflex responses through activation of opioid receptors in the rVLM (10, 37, 60). Visceral reflexes providing input to the rVLM increase the extracellular [Glu], which acts as an important excitatory neurotransmitter in this brain stem region (59). The present study supports and extends previous results by evaluating the interaction between Glu and the opioid system during acupuncture-related modulation of these visceral reflexes with use of a combination of microdialysis, anatomic, and whole animal neural reflex approaches. The results provide the first evidence to suggest that EA at P5-6 located over the median nerve attenuates the visceral sympathoexcitatory reflex by inhibiting increases in rVLM extracellular [Glu] in response to BK stimulation of the gallbladder. Since naloxone reversed this inhibitory effect of EA, opioids, at least in part, underlie this EA effect. When combined with our observation that the majority of glutamatergic rVLM cells activated by BK stimulation (c-Fos positive) colocalizes with or exists in close apposition to cells or cellular process that express δ-opioid receptors, these data suggest that EA-mediated opioid inhibition of Glu release in this medullary region serves as an important mechanism in EA regulation of cardiovascular function.

Acupuncture has been used for centuries to treat a variety of heart diseases and disorders (5, 11, 12, 49, 54, 62). The effects of EA on the cardiovascular system are the result of excitation

Table 1. Effect of low-frequency, low-current EA alone at P5-6 on baseline blood pressure and rVLM Glu (n = 6)

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<th>Basal</th>
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<td>0 min</td>
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<tr>
<td>MAP, mmHg</td>
<td>121.4±8.5</td>
<td>123.0±6.5</td>
<td>118.2±7.7</td>
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<td>[Glu] μM</td>
<td>0.28±0.05</td>
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Values are means ± SE (n = 6). MAP, mean arterial pressure; [Glu], extracellular glutamate concentrations in rostral ventrolateral medulla (rVLM); EA, electroacupuncture.
of group III and IV somatic afferent fibers, which underlie many acupoints (36, 55, 60). Such afferent input activates sympathetic inhibitory systems in the brain, involving endogenous opioids, nociceptin, $\gamma$-aminobutyric acid, and serotonin (10, 15, 34, 38).

Opioid neurotransmitters in the rVLM are capable of regulating sympathetic outflow and, ultimately, cardiovascular function (8, 44, 47). For example, microinjection of opioid agonists into the rVLM produces profound hypotension (47) and decreases in BP and HR in normotensive, chronic stress-induced hypertensive and spontaneously hypertensive rats, whereas naloxone injected into the rVLM blocks these responses. Furthermore, injection of opioid agonists into the rVLM inhibits BP responses to carotid occlusion and muscular exercise (8, 31, 47). These studies suggest that opioids in the rVLM have the potential to inhibit premotor sympathetic cardiovascular outflow. In this regard, we have shown that many premotor sympathetic rVLM neurons receive convergent input from visceral and somatic afferents (57, 63) and that, through an opioid mechanism, EA at P5-6 attenuates excitatory responses of those neurons during splanchnic nerve stimulation (57). Endorphins and enkephalins, but not dynorphin, mediate the inhibitory rVLM effects of EA on reflex-induced elevations in BP (38). Nerve terminals immunoreactive for enkephalin innervate rVLM premotor sympathetic neurons, which regulate BP (40). An earlier anatomic study from our laboratory demonstrates that neurons responsive to EA at P5-6 contain enkephalin and are closely associated with neuronal processes containing enkephalins or endorphins (24). The present obser-

Fig. 2. A: ventral view of brain stem of the cat in which all injection sites in the rVLM are plotted. ○, location of both probes placed in the rVLM during bilateral insertions. TB, trapezoid body; PT, pyramidal tract; XII, hypoglossal nerve. B: probe placement (arrow) in the rVLM.

Fig. 3. Confocal microscopic images showing triple labeling in rVLM (3.5 mm rostral to obex) of a cat stimulated with BK on the gallbladder. Top: merged low-magnification image from 3 (1 each red, green and blue) fluorescently labeled histological sections. □, site from which confocal images (bottom) were obtained. Middle: frontal cross section showing relative position of nucleus of inferior olive (ION) and retrofacial nucleus (RFN). Bottom: confocal images. A, B, and C: neurons containing vesicular glutamate transporter 3 (vGLUT3, green), $\delta$-opioid receptors (red), and c-Fos (blue), respectively; arrow 3, colocalization of labels of $\delta$-opioid receptor and vGLUT3, identified by yellow color (overlapping red and green); arrow 4, staining of $\delta$-opioid receptors in very close proximity to vGLUT3.
vation that local administration of naloxone into the rVLM can inhibit the EA-cardiovascular response confirms previous studies suggesting that opioid peptides are physiological modulators of rVLM premotor neurons activated by EA.

Many premotor neurons in the rVLM contain Glu (45). This excitatory amino acid plays a significant role in processing exercise-related cardiovascular reflexes (1) as well as in visceral reflex activation (59). The excitatory response to Glu may be modulated by opioids through presynaptic or postsynaptic mechanisms (2, 29, 50). For example, opioid receptor stimulation by exogenous agonists in the rVLM lowers extracellular [Glu] and attenuates cardiovascular pressor responses to static muscle contraction (31). However, exogenous opioids may cause nonspecific pharmacological responses. Our observation that administration of naloxone into the rVLM reverses EA-related inhibition of [Glu] and BP responses proves that endogenous opioids are involved in low-frequency, low-intensity somatic afferent (EA) modulation of visceral reflex-induced changes in cardiovascular function. Furthermore, the observation that glutamatergic rVLM neurons (vGLUT3) during visceral reflex-induced muscle contraction (31). However, exogenous opioids may cause nonspecific pharmacological responses. Our observation that administration of naloxone into the rVLM reverses EA-related inhibition of [Glu] and BP responses proves that endogenous opioids are involved in low-frequency, low-intensity somatic afferent (EA) modulation of visceral reflex-induced changes in cardiovascular function. Furthermore, the observation that glutamatergic rVLM neurons (vGLUT3) during visceral reflex contain δ-opioid receptors supports the notion that EA can inhibit the visceral reflex pressor response by modulating rVLM Glu release through an opioid mechanism.

The rVLM region contains bulbospinal and nonbulbospinal neurons, including, for example, interneurons (17, 27). BK stimulation of the gallbladder induces Glu release in the rVLM from neurons containing δ-opioid receptors (Fig. 3). These, in turn, activate bulbospinal neurons, eventually leading to a pressor response. Thus, our data suggest that EA inhibits BK-induced Glu release from nonbulbospinal glutamatergic neurons, possibly through a δ-opioid receptor mechanism in the rVLM, to modulate excitatory neurotransmission of the bulbospinal neurons. We cannot exclude the additional possibility that EA may also directly inhibit neuronal activity of rVLM bulbospinal neurons through an opioid mechanism to modulate sympathoexcitatory reflex responses. Since our previous studies have demonstrated that EA inhibits the neuronal activity of nonbulbospinal (i.e., interneurons) and sympathetic premotor neurons in the rVLM (56, 57, 63), we suggest that EA attenuates visceral reflex responses through modulation of nonbulbospinal and bulbospinal neurons in the rVLM.

Although we did not examine colocalization of μ-opioid receptors and glutamatergic neurons in the present study, we would expect that μ- and δ-opioid receptors are also involved in the regulation of Glu release, since EA at P5-6 reduces the sympathoexcitatory response through an opioid mechanism involving δ- and μ-opioid receptors (38). In total, these observations support our conclusion that rVLM Glu release associated with visceral afferent stimulation is modulated by opioid peptides during EA (Fig. 4).

Investigation is required to identify the sources of each of the opioid peptides in the rVLM.

Interestingly, EA did not alter baseline BP or the basal [Glu] in the rVLM. This finding is consistent with our observation that low-frequency, low-intensity EA does not alter the baseline BP in human subjects and animals (35, 37). In addition, insertion of acupuncture needles into P5-6 did not influence visceral afferent-induced changes in [Glu], supporting our previous work (60) showing no neural input during simple insertion of an acupuncture needle without stimulation. This observation has profound implications for acupuncture needle insertion without manual or electrical stimulation, since it suggests that any clinical response would have to be a placebo response.

Although it seems clear that Glu modulates excitatory responses of rVLM premotor sympathetic neurons, other excitatory neurotransmitters (e.g., catecholamines and serotonin) also may interact with opioid peptides and other inhibitory neurotransmitters (e.g., γ-aminobutyric acid and nociceptin) in processing neuronal activity in this brain stem region. Further studies are required to evaluate the individual roles and interactions between each of these neurotransmitter systems to fully define the processing mechanisms in this sympathoregulatory region.

Previously, we demonstrated that EA at St36 overlying the peroneal nerve also inhibits visceral sympathoexcitatory reflex responses, suggesting that supraspinal mechanisms are involved in EA modulation of the cardiovascular system. Spinal mechanisms also participate in EA-cardiovascular responses. For example, intrathecal naloxone reverses inhibition of visceral sympathoexcitatory reflex responses during somatic afferent stimulation induced by magnetic stimulation at P5-6 as a surrogate to EA (61). Thus supraspinal and spinal mechanisms underlie the EA effects on sympathetic outflow. The current study focuses on the supraspinal aspect of the EA response.

![Diagram](image-url)
The present study provides novel evidence that EA attenuates visceral sympathoexcitatory reflex responses to BK stimulation of visceral afferents by inhibiting the increase in extracellular [Glu] in the rVLM through an opioid-related modulation. These results suggest that EA modulates sympathetic outflow and, ultimately, cardiovascular function through a presynaptic mechanism that involves opioid-induced inhibition of Glu release. This study adds further insight into the inter-action of excitatory and inhibitory neurotransmitters in the medullary neural control of circulatory function during stimulation of visceral sympathetic (spinal) afferent pathways.

ACKNOWLEDGMENTS

A preliminary report of part of this study has been published in abstract form (21).

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