Medullary GABAergic mechanisms contribute to electroacupuncture modulation of cardiovascular depressor responses during gastric distention in rats

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Tjen-A-Looi SC, Guo ZL, Li M, Longhurst JC. Medullary GABAergic mechanisms contribute to electroacupuncture modulation of cardiovascular depressor responses during gastric distention in rats. Am J Physiol Regul Integr Comp Physiol 304: R321–R332, 2013. First published January 9, 2013; doi:10.1152/ajpregu.00451.2012.—Electroacupuncture (EA) at P5–P6 acupoints overlying the median nerves typically reduces sympathoexcitatory blood pressure (BP) reflex responses in eucapnic rats. Gastric distention in hypercapnic acidic rats, by activating both vagal and sympathetic afferents, decreases heart rate (HR) and BP through actions in the rostral ventrolateral medulla (rVLM) and nucleus ambiguus (NAmb), leading to sympathetic withdrawal and parasympathetic activation, respectively. A GABA_{\text{A}} mechanism in the rVLM mediates the decrease in sympathetic outflow. The present study investigated the hypothesis that EA modulates gastric distention-induced hemodynamic depressor and bradycardia responses through nuclei that process parasympathetic and sympathetic outflow. Anesthetized hypercapnic acidotic rats and bradycardia responses through nuclei that process parasympathetic and sympathetic outflow. Anesthetized hypercapnic acidotic rats manifested repeatable decreases in BP and HR with gastric distention every 10 min. Bilateral EA at P5–P6 for 30 min reversed the hypotensive response from -26 ± 3 to -6 ± 1 mmHg and the bradycardia from -35 ± 11 to -10 ± 3 beats/min for a period that lasted more than 70 min. Immunohistochemistry and in situ hybridization to detect c-Fos protein and GAD 67 mRNA expression showed that GABAergic caudal ventral lateral medulla (cVLM) neurons were activated by EA. Glutamatergic antagonism of cVLM neurons with kynurenic acid reversed the actions of EA. Gabazine used to block GABA_{\text{A}} receptors microinjected into the rVLM or cVLM reversed EA’s action on both the reflex depressor and bradycardia responses. EA modulation of the decreased HR was inhibited by microinjection of gabazine into the NAmb. Thus, EA through GABA_{\text{A}} receptor mechanisms in the rVLM, cVLM, and NAmb modulates gastric distention-induced reflex sympathoinhibition and vagal excitation.

vagal sympathetic and parasympathetic afferent; somatic afferent; \gamma-aminobutyric acid; nucleus ambiguus; rostral ventrolateral medulla; caudal ventrolateral medulla

ACUPUNCTURE INFLUENCES CARDIOVASCULAR function in about 70–80% of subjects (16). Acupuncture and the more standardized form of stimulation, low-frequency, low-intensity electroacupuncture (EA) affect sympathoexcitatory cardiovascular responses by stimulating somatic afferent nerves (18, 38). EA at different acupoints leads to differential cardiovascular responses with respect to both duration and intensity (43). More specifically, we have observed that EA stimulation at P5–P6 acupoints (overlying the median nerve) reduces sympathoexcitatory related cardiovascular responses in human and experimental animal studies (16, 17, 19, 40). Thirty minutes of EA modifies the activity of cardiovascular neurons in the arcuate hypothalamus, ventrolateral periaqueductal gray (vlPAG) midbrain, medullary raphe pallidus, as well as sympatoexcitatory responses that originate from the rostral ventrolateral medulla (rVLM) in both rats and cats (22, 23, 27, 43, 44, 50). Excitatory and inhibitory neuropeptides in these cardiovascular regions contribute to the inhibition of visceral reflex evoked rVLM neuronal activity during EA (20, 27, 42, 44). In addition to serotonin, nociceptin, endocannabinoids, and opioids (8, 21, 27, 42), GABA, through stimulation of GABA_{\text{A}} receptors in the vlPAG and rVLM, participates in the modulating actions of EA (12, 42, 44).

We have demonstrated that EA at P5–P6 acupoints reduces excitatory cardiovascular responses and lowers sustained hypertension by decreasing sympathetic outflow (17, 40, 42). Although others have suggested that EA at P5–P6 acupoints is able to raise arterial blood pressure when it is low (36, 47), the central neural processing mechanisms underlying this action of EA have not been elucidated.

We have shown that gastric distention typically leads to transient increases in blood pressure. (8, 19, 42). In this regard, we have shown that repeated gastric distention induces consistent increases in arterial pressure in normoxic normocapnic rats and that EA modulates these pressor responses (8, 12, 19, 42, 48, 49). Fewer studies have reported that gastric distention also can reflexly decrease blood pressure (29, 39). In this latter regard, we recently showed that distending the stomach decreases blood pressure specifically in hypercapnic-acidotic rats through sympathoinhibition (39). The sympathoinhibitory reflex response is processed in the caudal ventrolateral medulla (cVLM) and rVLM (39). The current study investigates mechanisms associated with reversing sympathoinhibitory reflex responses during EA.

In addition to its action on sympathoexcitatory reflexes, acupuncture also appears to be capable of influencing conditions associated with lowering of heart rate. We have observed that the bradycardia responses to intravenous phenylbiguanide, which activates cardiopulmonary afferents to reflexly lower heart rate, is reduced by low-frequency low-current EA at P5–P6 through a GABA_{\text{A}} mechanism involving vagal preganglionic nucleus ambiguus (NAmb) neurons (41). More recently, we have shown that distending the stomach decreases heart rate in hypercapnic-acidotic rats through both sympathoinhibition and vagal excitation (39). The action of EA in gastric distention-induced vasodepression and its central neural mechanisms of action have not been investigated. Thus, we postulated that EA reduces gastric distention evoked vasode-
pressor responses through actions in the cVLM and rVLM that are known to regulate sympathetic outflow. We also hypothesized that EA modulates gastrointestinal-induced reflex bradycardia through its actions in the NAmb, as well as the rVLM and cVLM. A preliminary communication of this study has been published (37).

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The GABA\textsubscript{A} antagonist gabazine (27 mM) and the glutamatergic antagonist kynurenic acid (100 nM) were dissolved in normal saline (20, 27, 42). Significant concentrations of kynurenic acid have been used by others investigating cardiovascular and respiratory responses (7, 35). We microinjected 50 nl of gabazine, kynurenic acid, or saline unilaterally into the NAmb, rVLM, or cVLM. Of note, several of our previous studies (8, 19, 42) have demonstrated significant blockade of EA’s actions following unilateral administration.

Surgical Preparations

Experimental preparations and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. The study conformed to the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings” (American Physiological Society, 2002). Studies were performed on adult Sprague-Dawley male rats (400–700 g). After an overnight fast of 18 h, anesthesia was induced with ketamine (80 mg/kg im) and xylazine (12 mg/kg im). Additional doses of ketamine-xylazine (16/2.4 mg/kg iv) were given as necessary to maintain an adequate level of anesthesia, as determined by the lack of response to noxious toe pinch, a respiratory pattern that followed the respirator, as well as a stable blood pressure and heart rate. A femoral vein was cannulated for administration of fluids. The trachea was exposed and intubated to ventilate animals artificially with a respirator (model 663; Harvard Apparatus). A femoral artery was cannulated and attached to a pressure transducer (P23XL; Ohmeda) to monitor blood pressure. Heart rate was derived from the pulsatile blood pressure signal with a biotach (Gould Instrument Systems).

Anatomical Study

Adult male Sprague-Dawley rats (350–500 g) were used for this protocol. Arterial blood gases and pH were monitored with a blood gas analyzer (model ABL-5; Radiometer). They were kept within normal limits (PO\textsubscript{2}, 100–150 mmHg; PCO\textsubscript{2}, 28–35 mmHg; pH, 7.35–7.45) by adjusting the volume and/or the ventilation rate, enriching the inspired O\textsubscript{2} supply and administration of 1 M NaHCO\textsubscript{3}. After the rats were stabilized for 4 h following surgical preparation, EA was conducted at the P5–P6 acupoints for 30 min, as described below in the section on physiological studies. Control rats (sham control at P5–P6) were treated identically with the exception that the acupuncture needles after placement were not stimulated electrically.

Physiological Studies

To establish hypercapnia, 5% CO\textsubscript{2} was added to the oxygen-enriched room air. Arterial blood gases and pH were measured periodically with a blood gas analyzer (ABL-5; Radiometer America). PCO\textsubscript{2} was maintained between 42 and 55 mmHg and PO\textsubscript{2} 200 ± 45 mmHg by adjusting the rate of delivery of 5% CO\textsubscript{2} to the enriched inspired O\textsubscript{2} (39). Arterial pH varied between 7.22 and 7.34. Body temperature, monitored with a rectal thermistor (model 44TD), was kept between 36°C and 38°C with a heating pad and lamp.

An unstrained 2-cm diameter latex balloon (Traub) was attached to a polyurethane tube (3-mm diameter) and inserted into the stomach through the mouth and esophagus. Transmural pressure was determined by measuring the pressure required to inflate the balloon with the various volumes of air before it was inserted into the stomach (19). The balloon was palpated manually transcutaneously during insertion as it was passed through the esophagus into the stomach to confirm positioning of the balloon inside the stomach. A syringe was attached to the cannula to inflate and deflate the balloon with air, while a manometer through a T-connection was used to monitor balloon pressure. Distention pressures were selected to fall within the range that a rat normally experiences during ingestion of food and fluids in a single meal (1, 9). To induce decreases in blood pressure and heart rate, the balloon was inflated inside the stomach. Decreases in blood pressure and heart rate were observed within 30 s of inflation. The balloon was deflated within 30 s after reaching the maximum decrease in blood pressure. We did not include animals in the study when the balloon was verified post mortem to be in the esophagus.

Animals were placed in a stereotaxic head frame to position their heads with the floor of the fourth ventricle in a horizontal position. A partial craniotomy was performed to expose the medulla to allow access to the NAmb, rVLM, and cVLM. A microinjection probe was inserted with visual approximation at a 90° angle relative to the dorsal surface of the medulla. A 1.8 mm lateral either right or left from the midline, 1.5 mm rostral to the obex, at a depth of 2.4 mm to access the NAmb. To reach the rVLM, the probe was positioned 2.3 mm lateral to the midline, 1.5 mm rostral to the obex, at a depth of 3.3 mm. The probe was lowered into the cVLM in a position perpendicular to the dorsal surface, 0.25 mm rostral to 0.5 mm caudal to the obex, 2.0 mm lateral to the midline, and 2.8 to 3.0 mm in depth. Insertion of the probe into the cVLM near the obex was used to deactivate GABAergic-barosensitive neurons (32). A modified CMA microdialysis AB probe that was 14 mm long (CMA, Stockholm, Sweden, tip diameter 0.24 mm) and lacked the microdialysis membrane (39, 42) was advanced toward the ventral surface of the brain stem to reach the NAmb, rVLM, and cVLM for unilateral or bilateral microinjection using coordinates taken from the atlas of Paxinos and Watson (28). Proper positioning of the probes in the NAmb, cVLM, and rVLM was confirmed by noting transient decreases in heart rate, and approximately a 5-mmHg reduction in blood pressure and a 5–10-mmHg elevation in arterial pressure following activation of neurons in the respective nuclei with 25–50 nl of 4 nM d,L-homocysteic acid (DLH) or with probe insertion. The probe was connected to a CMA 402 syringe pump (CMA, Stockholm, Sweden) to deliver 50 nl at a rate of 0.3 μl/min over a 10-s period 2 min prior to the next gastric distention. Furthermore, following administration of kynurenic acid in the cVLM, we observed a small transient increase in blood pressure of ~5 mmHg, while gabazine caused a short-lived decrease averaging 10 mmHg. Conversely, gabazine in the rVLM transiently increased blood pressure by an average of 8 mmHg. Gabazine microinjection into the NAmb briefly decreased heart rate by an average of 10 beats per minute (bpm). These changes are similar to those observed by us and others investigating neurotransmitter action in these nuclei (15, 25, 41).

Acupuncture needles (32-gauge stainless-steel) were placed bilaterally at P5–P6 acupoints overlying the median nerves above the paw or LI6–LI7 located over the superficial radial nerves on the radial side of dorsal surface on the lower one-third of forelimb (43). Acupuncture needles were placed at depths of ~3 mm (P5–P6) or ~1 mm (LI6–LI7) (43, 48). The needles were connected to a constant current stimulator with a stimulus isolation unit and stimulator (model S88; Grass Instruments). Each set of electrodes was stimulated separately, so that current did not flow from one location to the contralateral side. Correct placement of the needles at the P5–P6 acupoints was confirmed by observing slight repetitive paw twitches at or near motor threshold during EA stimulation. The twitches were important observations to confirm stimulation of motor fibers in the median nerve to indicate that we were stimulating the correct nerve (5, 18, 19). Stimulation of EA at LI6–LI7-activated superficial nerves and did not induce muscle movement. Correct placement of acupuncture needles

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positioned close to the superficial radial nerve was confirmed by postmortem dissection of the forelimbs. Gallamine triethiodide (4 mg/kg) was administered intravenously before application of 30 min EA (2–4 Hz, 1–4 mA, 0.5 ms) to avoid muscle movement during stimulation of median nerves. Of note, motor nerve stimulation does not participate in the EA-cardiovascular response, since we have shown EA inhibition of reflex cardiovascular responses following muscle paralysis (22, 41). Application of EA lasted 30 min, while gastric distention during somatic stimulation occurred every 10 min.

Injection sites were marked with 50 nl of Chicago Sky Blue dye (5% in 0.5 M sodium acetate) at the end of each experiment following administration of drugs into the NAcmb, rVLM, or cVLM. Thereafter, rats were euthanized under deep anesthesia with additional ketamine and xylazine, followed by saturated KCl. The stomach was exposed to confirm placement of the balloon. The medulla was removed and submerged in 4% paraformaldehyde for at least 72 h. Frozen 40-μm coronal sections were cut with a CM 1850 cryostat microtome (Leica) to confirm histologically the microinjection sites. Dye spots were identified with a binocular microscope. Using the atlas of Paxinos and Watson as a guide, sites of microinjections in the medulla were plotted with Corel Presentation software on reconstructed coronal sections (28).

Experimental Protocols: Anatomical Study

Tissue preparation. As described previously (14), 90 min after termination of EA or the sham control, deep anesthesia was induced by another larger dose of the ketamine/xylazine (0.5–0.7 ml im). Transcardiac perfusion was performed using 500 ml of 0.9% saline solution followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The medulla oblongata was harvested and sliced coronally (30 μm) using a cryostat microtome (Leica CM1850 Heidelberger Strasse, Nussloch, Germany). Brain sections were placed serially in cold cryoprotectant solution (4) for subsequent in situ hybridization and immunohistochemical labeling, as described below. Only free-floating sections were used for labeling.

c-Fos immunohistochemical fluorescent staining. Brain tissue was washed for 30 min (10 min, 3 times) in PBS containing 0.3% Triton X-100 (PBST; pH 7.4), and placed for 1 h in 1% normal donkey serum (Jackson Immunoresearch Laboratories). Sections were incubated with a primary polyclonal rabbit anti-Fos antibody (1:2,000 dilution, Oncogene research product; Calbiochem) at 4°C for 48 h. Tissue subsequently was rinsed 3 times (10 min for each rinse) in PBST and incubated with a fluorescein-conjugated donkey anti-rabbit antibody (1:200; Jackson Immunoresearch Laboratories) for 24 h at 4°C. Each section was mounted on a slide and air-dried. The slides were coverslipped using mounting medium (Vector Laboratories). All c-Fos staining was abolished in immunohistochemical control studies when 1 ml of the diluted primary antibody was preincubated with 5 μg of the peptide that corresponds to amino acids 4–17 of human c-Fos (SGFNADEYASSSRC, Oncogene Research Product, Calbiochem, no. PP10). No labeling was detected when the primary antibody was omitted.

GAD67 mRNA in situ hybridization and c-Fos immunohistochemical labeling. In situ hybridization fluorescent histochemistry was used to detect glutamic acid decarboxylase isoform 67 (GAD67, cytoplasmic marker for GABAergic neurons) mRNA using antisense digoxigenin-labeled cRNA probes (10, 11, 31). Antisense and sense (control) cRNA probes were generated from full-length cDNA encoding 3.2 kb GAD67 (supplied by Dr. P. Guyenet) (31). Plasmids (pBluescript SK+) were linearized with SalI (New England Biolabs). Riboprobe transcripts were transcribed with digoxigenin-11-UTP (Roche) using T3 RNA polymerase. Template DNA was digested with RQ1 RNase-free DNase (Promega) for 20 min (37°C) and unpurified nucleotide removed with Sephadex G-50 columns (Roche). Efficiency of digoxigenin-11-UTP incorporation was determined by direct detection on dot blots with an antidigoxigenin antibody conjugated to alkaline phosphatase (Roche). The intensities of the dilution series of digoxigenin-labeled RNA and control RNA (Roche) were compared by exposure to X-ray film. Labeled riboprobes were aliquotted and stored at ~80°C.

The methods for in situ hybridization of GAD67 mRNA have been described in detail by others (33, 34). Briefly, brain sections first were placed in preincubation solution of antisense or sense cRNA probes for 1 h. Sense riboprobes were evaluated using the same protocols and induction times as the antisense counterpart that served as a control. After incubating the sections with 50–100 pg/μl of the labeled riboprobe for 16 h at 55°C, they were washed at 37°C with saline-sodium citrate for 40 min and with the RNAse A buffer for 30 min. Thereafter, brain sections were rinsed with 0.1 × SSC solution (15 nM sodium chloride and 1.5 mM trisodium citrate, pH = 7.0) at 55°C for 60 min. Sections were placed in 10% horse serum for 30 min and incubated overnight at 4°C with sheep polyclonal antidigoxigenin antibody conjugated to alkaline phosphatase (Roche) to detect the digoxigenin-labeled riboprobe.

Subsequently, brain tissue was washed for 30 min (10 min, 3 times) in PBST. Similar to labeling c-Fos, as described above, sections were incubated with a primary polyclonal rabbit anti-Fos antibody (1:2,000 dilution) and then with a fluorescein-conjugated donkey anti-rabbit antibody (1:200) after being placed in 1% normal donkey serum for 1 h.

Finally, after rinsing sections in the washing buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, at pH 7.5) for 30 min and with the detecting buffer (100 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2, at pH 8.0) for 10 min, alkaline phosphatase was reacted with a mixture of 2-hydroxy-3-naphthoic acid-2-phenylalanilide phosphate (10 mg/ml in demethylformamide) and fast red (10 μl each in 1 ml of the detecting buffer; Roche) for 30–60 min (2). Sections then were washed with sterile water for 10 min and mounted on slides. The slides were coverslipped using mounting medium (Vector Laboratories).

Data analysis. Brain sections were scanned with a standard fluorescent microscope (Nikon, E400). Two epifluorescence filters (B-2A, G-2A) equipped in a fluorescent microscope were used to identify single stains appearing as green (fluorescein) or red (rhodamine) or dual stains of both colors in brain sections. Sections containing the cVLM were identified according to their best matched standard stereotaxic plane, as shown in Paxinos and Watson’s atlas of the rat brain (28). The cVLM region was identified as the region ventral to nucleus ambiguous, dorsal to the lateral reticular nucleus (LRN) and between the two LRN subdivisions at the most rostral aspect. The dorsal landmarks of medulla oblongata, like the obex or calamus scriptorius (~0.7 mm caudal to the obex), are often used for reference (3, 28, 32). The region of the cVLM investigated in the present study was located 1.0 mm caudal and rostral to the obex, 1.8–2.5 mm lateral to the midline, and 0.5–1.3 mm dorsal to the ventral surface of the medulla oblongata.

c-Fos labeling appeared at ×40 magnification as round dots ~7–12 μm in diameter and was clearly distinguishable from background staining. The numbers of cells singly labeled with c-Fos were counted in sections that represent three different levels from the rostral to caudal extension of the cVLM (obex, +0.72, +0.12, –0.48 mm) (28) in each animal. The average number of labeled neurons derived from these sections was used to represent the average number of neurons per section for statistical analysis. Data are expressed as means ± SE. Statistical analyses were conducted with statistical software (SigmaStat, version 3.0, Jandel Scientific Software). The Kolmogorov-Smirnoff test was used to determine whether data were distributed normally. Comparisons between two groups were analyzed with the Student’s t-test or Mann-Whitney rank sum test. Values were considered significantly different at P < 0.05.

Selected sections that contain two labels were evaluated further with a laser-scanning confocal microscope (Zeiss LSM 710, Meta System) to confirm colocalization. This apparatus was equipped with Argon and HeNe lasers and allowed operation of multiple channels. Lasers of 488- and 543-nm wavelengths were used to excite fluores-
The rats were made hypercapnic and acidotic before any gastric distention reflex responses. Gastric distention was induced by slowly inflating the balloon over a 10-s period with a volume ranging from 5 to 8 ml of air, while elevated PCO2 was held constant. Once the maximal decrease of blood pressure and heart rate was attained (generally within 30 s), the injected air was withdrawn slowly from the balloon. Peak inhibitory blood pressure and heart rate responses were noted typically within 20 to 30 s following inflation. Ten- to fifteen-minute recovery intervals were necessary to prevent attenuation of the cardiovascular reflex responses. Reflex responses were evaluated in five animals. EA at L16–L17 acupoints, shown previously not to alter sympathoexcitatory reflex cardiovascular responses (43, 48), was applied for 30 min during repeated gastric distention as a control and to examine point-specific actions of acupuncture on inhibitory hemodynamic reflex responses.

EA at P5–P6 and brain stem saline microinjection controls during repeated gastric distention. In 17 rats, EA (n = 7) or EA combined with a 50-nl saline microinjection (n = 10) were applied to examine the actions of EA and to serve as a control for the protocols involving gabazine or kynurenic acid microinjection, respectively (see Experimental Protocols: Anatomical Study). After recording two repeatable responses to gastric distention, EA was applied bilaterally for 30 min during three additional distentions. Three subgroups of rats (NAmb, n = 3; rVLM, n = 2; cVLM, n = 5) received microinjections of saline to serve as volume and vehicle controls. Microinjection of saline into the NAmb, rVLM, or cVLM occurred after EA, 2 min prior to the sixth gastric distention. Five or six additional gastric distentions were evoked subsequently to assess the cardiovascular responses after EA alone or EA + microinjection during recovery. Thus, 10 or 11 distentions in total were used to evaluate the actions of EA. Unless indicated, all microinjections were administered unilaterally.

**Role of GABA in EA cardiovascular actions in NAmb, rVLM, and cVLM.** The responses to EA were examined by microinjection of gabazine, a specific GABA\(_A\) receptor antagonist (26, 44), in the NAmb (n = 6), rVLM (n = 6), or cVLM (n = 4) immediately after termination of acupuncture stimulation. Responses following microinjection of gabazine into the caudal aspect of the cVLM during the action of EA were evaluated in five additional rats. We also examined the responses to microinjection of gabazine in the NAmb (n = 4) and cVLM (n = 5) without EA to evaluate their actions on the primary gastric distention reflex. We previously have shown that gabazine injected into the rVLM modulates the primary reflex (39).

**Role of glutamate in cVLM processing EA input.** Glutamate receptor blockade in the cVLM was examined during the action of EA to assess the role of this excitatory neurotransmitter in the depressor hemodynamic reflex responses. Blockade of glutamatergic receptors in cVLM rostral to obex (n = 4) or caudal to obex (n = 5) was applied 2 min prior to the sixth gastric distention. Five additional reflex responses were measured after delivery of the antagonist. Responses of the primary reflex to kynurenic acid injected into the cVLM were examined in the absence of EA in four other rats.

**Statistical analysis.** Reflex responses are expressed as the maximal difference between prestimulus mean arterial blood pressure (steady-state baseline blood pressure) and pressure at peak response. Changes in mean arterial pressure and heart rate are presented as bar histograms. Data are presented as means ± SE. The decreases in blood pressures and heart rates before and after delivery of experimental drug or saline were compared by a one-way repeated measure of ANOVA followed post hoc by the Student-Newman-Keuls test. Additionally, a two-way repeated-measures ANOVA followed post hoc by the Student-Newman-Keuls test was used to compare the inhibitory responses between control and gabazine treatment groups. Data are plotted and analyzed with the Kolmogorov-Smirnov test for normal data distribution and normalized when necessary with Sigma plot (Jandel Scientific). All statistical analyses were performed with Sigma plot/Stat (Jandel Scientific). The 0.05 probability level was used to detect significant differences.

**RESULTS**

**Anatomical Study**

CVM neurons colabel with GAD67 mRNA and c-Fos protein. Cells positive for c-Fos were detected in the rostral-caudal region of the cVLM (obex, +0.72 to −0.60 mm) in...
both control and EA-treated rats. Significantly more (P < 0.01) c-Fos-labeled neurons were observed in the cVLM of the EA treated (n = 6; 26 ± 3 cells per section) compared with the control group (n = 4; 6 ± 1 cells per section).

GAD67 mRNA labeling was detected in the sections treated with antisense digoxigenin-labeled cRNA probes, but not in those treated with the sense riboprobes. Similar to other observations (3, 33), we observed perikarya labeled with GAD67 mRNA throughout the rostral and caudal cVLM in both control and EA-treated rats (n = 2 for each group). Distribution of GAD67 mRNA in these two groups was similar. Neurons labeled with both c-Fos and GAD67 mRNA were identified frequently in both rostral and caudal cVLM (obex, +0.72 to −0.48 mm) in two EA-treated rats, but very rarely in two sham controls. Fig. 1 demonstrates confocal images of neurons double-labeled with c-Fos and GAD67 mRNA in the cVLM of a rat subjected to 30 min of EA at P5–P6 (Fig. 1, D–F) in contrast to the sham control (Fig. 1, A–C).

Physiological Studies

Reflex responses under hypercapnic acidic condition. High PCO_2 and low pH were associated with inhibitory hemodynamic responses evoked by gastric distention (Table 1). Thus, the increase in carbon dioxide from 48 to 54 and decrease in pH from 7.21 to 7.23 were associated with mean reflex decreases in blood pressure of 24 to 29 mmHg and a bradycardia ranging from 29 to 49 beats/min. The magnitudes of the depressor and bradycardia responses observed in the current and previous studies (39) were similar.

Table 1. Influence of hypercapnia and acidosis on MAP and HR reflex responses during gastric distention

<table>
<thead>
<tr>
<th>Protocol</th>
<th>PCO_2</th>
<th>pH</th>
<th>∆MAP, mmHg</th>
<th>∆HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAmb (n = 6)</td>
<td>54 ± 2.4</td>
<td>7.23 ± 0.02</td>
<td>−24 ± 4</td>
<td>−32 ± 8</td>
</tr>
<tr>
<td>rVLM (n = 6)</td>
<td>48 ± 0.8</td>
<td>7.22 ± 0.02</td>
<td>−29 ± 4</td>
<td>−49 ± 12</td>
</tr>
<tr>
<td>cVLM (n = 18)</td>
<td>52 ± 0.8</td>
<td>7.21 ± 0.01</td>
<td>−28 ± 3</td>
<td>−29 ± 4</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; MAP, mean arterial pressure; HR, heart rate; bpm, beats per minute; NAmb, nucleus ambiguous; rVLM, rostral ventrolateral medulla; cVLM, caudal ventral lateral medulla.

Fig. 2. Point-specific EA actions influence inhibitory cardiovascular reflex responses. Mean arterial pressure (MAP) and heart rate (HR) remain unaltered during repeated gastric distention before, during, and after application of EA at LI6–LI7, overlying the superficial radial nerves (A). Conversely, the changes in MAP and HR were reduced during EA at P5–P6 (B). Original recordings of blood pressure (BP) and HR of an individual rat are displayed above the bars (B, a–f). Note: reduced change in MAP and HR during EA at P5–P6 (shown in panels b and e). GD, gastric distention. *Significant differences, P < 0.05. Values above bars represent baseline BP and HR and are expressed as means ± SE.

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Influence of EA on gastric distention reflex response. EA at LI6–7 did not alter the decreases in blood pressure and heart rate during repeated gastric distention applied every 10 min in the presence of hypercapnia-acidosis (Fig. 2A). In contrast, 30 min of EA stimulation at P5–P6 acupoints during repeated gastric distention reduced the inhibitory cardiovascular reflex responses for over 80 min (Fig. 2B).

Role of NAmb and GABA in EA modulation of reflex cardiovascular inhibition. Gabazine microinjection in the NAmb reduced EA modulation of the bradycardia but not the vasodepressor responses to gastric distention (Fig. 3B). In contrast, application of saline did not change either hemodynamic value (Fig. 3A). Steady-state baseline blood pressure and heart rate were unaltered by gabazine. In the absence of EA, gabazine did not affect the inhibitory cardiovascular reflex responses to gastric distention (Table 3).

Actions of EA through GABA system in rVLM and cVLM. Interruption of GABA neurotransmission with unilateral microinjection of gabazine in the rVLM during EA stimulation reversed the acupuncture-related modulation of the distention-induced hypotensive and bradycardia responses (Fig. 4B). Saline control did not influence the actions of EA (Fig. 4A). Also, gabazine did not influence steady-state baseline blood pressure or heart rate.

Microinjection of gabazine into the caudal cVLM reversed EA modulation of the depressor response but not the negative chronotropic response following gastric distention (Table 2). On the other hand, blockade of GABA<sub>A</sub> receptors in cVLM neurons rostral to obex following EA reversed both blood pressure and heart rate reflex responses (Fig. 5B). Microinjection of saline did not alter the action of EA at P5–P6 on either the blood pressure or heart rate reflex responses (Fig. 5A). Gabazine did not influence steady-state baseline blood pressure or heart rate and did not alter the inhibitory reflex blood pressure and heart rate responses to gastric distention (Table 3).

Role of glutamate in cVLM in processing effects of EA. Kynurenic acid microinjected into the rostral cVLM reversed EA modulation of both the depressor and bradycardia responses (Fig. 5C). As mentioned above, saline did not alter the hemodynamic actions of EA (Fig. 5A). Kynurenic acid in the
Fig. 4. GABA in rVLM contributes to the modulatory actions of EA at P5–P6. EA reduced both inhibitory cardiovascular responses induced by gastric distention in the setting of hypercapnia-acidosis. Blockade of GABA<sub>A</sub> receptors reversed the actions of EA. Microinjection of saline (control) did not influence EA modulation of MAP and HR. Individual examples of changes in BP and HR are shown in a–h. *Significant differences, compared with control. †Significant differences compared with preceding response, P < 0.05. Values above bars represent baseline BP and HR and are expressed as means ± SE. R327

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cVLM did not influence steady-state baseline blood pressure and heart rate and did not influence the gastric reflex responses in the absence of EA (Table 3). Inhibition of ionotropic glutamate receptors in the caudal cVLM with kynurenic acid reversed EA modulation of the decreased heart rate but not blood pressure reflex response (Table 2).

A few of the microinjections into cVLM, rostral to obex, were administered bilaterally. The extent of inhibition of the cardiovascular responses following bilateral microinjections were found to be similar to the inhibition observed following unilateral blockade. Hence, the bilateral and unilateral responses (Fig. 5, A–D) were grouped. The numbers of bilateral microinjections were \( n = 1 \) (saline), \( n = 2 \) (gabazine + EA), \( n = 3 \) (kynurenic acid + EA), \( n = 2 \) (gabazine), and \( n = 1 \) (kynurenic acid).

**Confirmation of microinjection sites.** Physiological studies at microinjections sites located within the NAmb, rVLM, and cVLM were included in the data analysis (Fig. 6). Three injection sites found to be medial and ventral to the cVLM and NAmb did not alter the reflex responses and were excluded from analysis.

**DISCUSSION**

We recently have shown that gastric distention in the setting of high \( \text{CO}_2 \) and acidosis leads to reflex decreases in blood pressure and heart rate, responses that result from mechanical activation of both vagal and splanchnic (sympathetic) visceral afferents (39). The current study shows that EA stimulation at P5–P6, in contrast to EA at L16–L17, reduces the gastric distention-induced inhibitory reflex responses through nuclei that process parasympathetic and sympathetic outflow in the hypercapnic condition. Specifically, GABA\(_A\) receptor blockade in the NAmb reverses EA inhibition of the bradycardia, while similar blockade in the rVLM and cVLM reverses EA inhibition of both the hypotension and bradycardia responses. Thus, EA inhibits increased parasympathetic outflow through actions in the NAmb and reverses suppressed sympathetic activity in the rVLM and cVLM through GABAergic mechanisms.

To examine GABAergic neurons activated by EA, we first evaluated immunohistochemical staining of GABA or GAD in the cVLM in normal and colchicine-treated rats. Labeling was observed only in neuronal processes in this region. Hence, we utilized in situ hybridization to detect GAD67 mRNA in the cell body. In situ hybridization detects GAD67 mRNA in neurons with a single stain under light microscopy but does not generate reliable results when combined with fluorescent immunohistochemical imaging using confocal microscopy. Colocalization of c-Fos and a neurotransmitter in a neuron is identified commonly as a c-Fos-labeled neuron surrounded by the immunohistochemical-labeled transmitter in the cytoplasm. However, labeled GAD67 mRNA detected with confocal microscopy typically is found in only a small portion of the perikarya. Thus, in situ hybridization is suitable for qualitative assessment but is not accurate for quantitative assessment of dual-fluorescence-labeled cells. For these reasons no quantitative studies have been published that identify colocalization of c-Fos nuclei and neurotransmitters detected with in situ hybridization using fluorescent labels and laser confocal microscopy to evaluate the anatomical relationship between these labels. As such, in the present study, we qualitatively evaluated neurons containing dual-fluorescence-labeled GAD67 mRNA and c-Fos using laser confocal microscopy and quantitative assessment of c-Fos expression following EA. We found that c-Fos-positive nuclei in the cVLM were significantly increased in EA-treated rats compared with controls. Although we noted a similar distribution of GAD67 mRNA in the cVLM of both EA-treated and control rats, cells colabeled with c-Fos and GAD67 mRNA were identified frequently in EA-treated but rarely in controls. These anatomical data are consistent with the physiological findings presented in the present study, suggesting that EA activates GABAergic neurons in the cVLM.

Kynurenic acid blockade of ionotropic receptors in the cVLM reversed the action of EA on the visceral cardiovascular vasodepressor reflex, indicating that glutamate also contributes to the actions of EA under these conditions. Thus, EA’s actions are complex, involving both inhibitory and excitatory neurotransmitter systems and several brain stem regions that profoundly influence autonomic outflow.

The NAmb is well recognized as an important site for regulation of parasympathetic outflow through its action on preganglionic cardiac vagal efferent fibers (41). In this regard, we recently have shown that this region participates in gastric distention-induced bradycardia associated with hypercapnia and respiratory acidosis (39). EA at P5–P6 acupoints activates NAmb neurons in close proximity to enkephalinergic processes, implying that EA exerts at least part of its cardiovascular actions through this nucleus and that enkephalin participates in EA processing in this region (13). Furthermore, we observed that EA at the P5–P6 acupoints inhibited evoked neuronal discharge in the NAmb during vagal afferent fiber stimulation (41), an action that was attenuated by blockade of GABA\(_A\) receptors with gabazine. GABA\(_A\) receptor blockade in this region also reverses EA-related modulation of bradycardia responses elicited during stimulation of cardiopulmonary vagal afferent endings in cats by intravenous administration of PBG, a 5-HT\(_3\) receptor agonist (41). The current data collected in rats are consistent with our previous observations, since we demonstrated that inhibition of GABA action in the NAmb attenuates EA’s modulatory influence on the negative chronotropic responses elicited during stimulation of cardiopulmonary vagal afferent endings in cats by intravenous administration of PBG, a 5-HT\(_3\) receptor agonist (41). The current data collected in rats are consistent with our previous observations, since we demonstrated that inhibition of GABA action in the NAmb attenuates EA’s modulatory influence on the negative chronotropic responses elicited during stimulation of cardiopulmonary vagal afferent endings in cats by intravenous administration of PBG, a 5-HT\(_3\) receptor agonist (41). The current data collected in rats are consistent with our previous observations, since we demonstrated that inhibition of GABA action in the NAmb attenuates EA’s modulatory influence on the negative chronotropic
Fig. 5. GABA and glutamate in the cVLM facilitates EA modulation of the distention-induced reflex responses in hypercapnic rats. Blockade of GABA<sub>A</sub> receptors in the cVLM immediately following EA reversed EA modulation of BP and HR (B), in contrast to saline (A). Original data above the bars display changes in BP and HR (a–h). Glutamate receptor blockade in the cVLM following EA reversed EA modulation of MAP and HR (C). Microinjection of gabazine or kynurenic acid into the cVLM did not influence the primary reflex in the absence of EA. *Significant differences, compared with control. †Significant differences compared with preceding response, P < 0.05. Values above bars represent baseline BP and HR and are expressed as means ± SE.
response induced by gastric distention in hypercapnic rodents (Fig. 3). Similar to our previous study in cats (41), microinjection of gabazine transiently decreased baseline HR (−10 ± 6 beats/min). However, basal heart rate had returned to normal before subsequent gastric distention. Thus, EA at P5–P6 is capable of inhibiting parasympathetic outflow to the heart through a GABAergic mechanism in the NAmb during abdominal and cardiopulmonary inhibitory reflexes.

Importantly, the rVLM participates in the regulation of sympathetic outflow in general and more specifically, through a GABA\textsubscript{A} mechanism, processes signals leading to the hypotension and bradycardia elicited by gastric distention in hypercapnic rats (39). Hence, GABA is involved in rVLM processing of the primary reflex. The present study documents for the first time that EA likewise modulates the depressor and bradycardia responses through a GABAergic mechanism in this nucleus (Figs. 4 and 7). Thus, GABA\textsubscript{A} blockade with gabazine reduces magnitude of the primary reflex (39), whereas after EA, this antagonist increases the reflex depressor and bradycardia responses (present data). Therefore, although GABA in the rVLM participates in gastric distention-induced sympathoinhibition, EA, also through a GABAergic mechanism, serves to inhibit the magnitude of this reflex. Clearly, the circuitry in the rVLM that is activated during visceral afferent stimulation with gastric distention and somatic afferent stimulation with EA is complex, but in both respects, it appears to involve GABA.

The cVLM also plays a role in processing somatic input during gastric distention-evoked inhibitory cardiovascular reflex responses in hypercapnic-acidotic rats (39). A number of studies have reported that the baroreflex activates a GABAergic cVLM-rVLM projection, leading to rVLM-mediated sympathoinhibition (30, 32, 46). We hypothesized that a similar mechanism might likewise participate in EA-induced modulation of the gastric distention-induced depressor/bradycardia reflexes (39). This hypothesis was confirmed when we demonstrated that 30 min of EA reduced the distention-related sympathoinhibition through a GABA\textsubscript{A} receptor mechanism in the cVLM (Fig. 5). Further support of this conclusion was the demonstration that EA-activated cVLM neurons express GAD67 by visualizing colocalization of in situ hybridized GAD67 mRNA and c-Fos immunoreactivity conjugated with fluorescent labels (2). Thus, the physiological and histological data in combination suggest that EA suppresses sympathoinhibitory cVLM neurons through a GABAergic mechanism (Fig. 7).

Schreihofer and Guyenet (32, 33) have demonstrated that increases in blood pressure activate barosensitive GAD67-labeled cVLM neurons that, in turn, influence both arterial pressure and heart rate. They reported that most of these cells were located rostral to and near the obex (±0.25 mm). We similarly found that the somatic input evoked by EA modulates blood pressure and heart rate changes through the GABA system in the rostral, but not the caudal, cVLM (Table 2).

Our data also show that glutamate participates in the modulatory action of EA in the cVLM (Fig. 5). One explanation for these observations is that, through a glutamatergic mechanism, EA activates GABAergic interneurons in the cVLM to ultimately disinhibit cVLM GABAergic neurons that project to the rVLM (Fig. 7).

Whereas EA modulated the depressor responses when it was applied at P5–P6, known to overlie the median nerve near the paw of the forelimb (43), no effect was observed when EA was applied at L16–L17, overlying the superficial radial nerve, also on the forelimb (43). It is interesting to note that point-specific cardiovascular modulation by EA, involving both sympathetic

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**Table 3. Gastric distention-induced blood pressure and HR responses to microinjection of kynurenic acid and gabazine into cVLM and NAmb, in the absence of EA**

<table>
<thead>
<tr>
<th></th>
<th>cVLM</th>
<th>NAmb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ΔMAP, mmHg</td>
<td>ΔHR, bpm</td>
</tr>
<tr>
<td>Before kynurenic acid</td>
<td>−17 ± 6 (n = 4)</td>
<td>−18 ± 9 (n = 4)</td>
</tr>
<tr>
<td>After kynurenic acid</td>
<td>−18 ± 7 (n = 4)</td>
<td>−18 ± 9 (n = 4)</td>
</tr>
<tr>
<td>Before gabazine</td>
<td>−24 ± 5 (n = 5)</td>
<td>−18 ± 7 (n = 5)</td>
</tr>
<tr>
<td>After gabazine</td>
<td>−26 ± 6 (n = 5)</td>
<td>−20 ± 7 (n = 5)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Kynurenic acid (100 mM, 50 nl); gabazine (27 mM, 50 nl). n, number of animals.
and parasympathetic centers in the brain stem, is similar in its action on reflex excitatory and inhibitory responses. However, further studies are needed to examine the action of other cardiovascular related acupoints that in addition to P5–P6 also may reverse inhibitory hemodynamic reflex responses.

**Limitations.** Concern might be raised that the majority of the experiments utilized unilateral microinjections. Unilateral blockade may have only partially limited the action of a neurotransmitter system, which, in turn, incompletely blocked the reflex responses. However, bilateral glutamatergic or GABAergic antagonism in the cVLM yielded virtually identical responses as unilateral blockade. Furthermore, unilateral microinjections of gabazine in the NAmb and the rVLM clearly show significant reversal of EA’s action and thus demonstrate the role of GABA in EA modulation of the reflex vasodepression and bradycardia. As such, we accomplished our objective to evaluate the role of these inhibitory neuuropeptides.

**Conclusion**

The present study shows that EA at P5–P6 leads to prolonged reduction in gastric distention-induced inhibitory responses in the setting of elevated CO2 and decreased pH. EA actions in this visceral-cardiovascular reflex are processed in the NAmb, rVLM, and cVLM regions of the brain stem. Specifically, activation of NAmb neurons during mechanical stimulation of the stomach is modulated by EA input through a GABAergic mechanism that, in turn, leads to reduction of the vagal excitatory reflex responses. The rVLM and cVLM modulate both the depressor and negative chronotropic reflex responses during EA, also through a GABAergic mechanism that reduces sympathetic withdrawal. As such, both parasympathetic and sympathetic outflows are modulated by 30 min of EA in this model of bradycardia-hypotension.

**Perspectives and Significance**

The current study has utilized a model of hypotension that involves sympathoinhibition and activation of the parasympathetic nervous system to lower heart rate much like vasovagal syncope (39). Postprandial hypotension and vasovagal syncope are well-recognized causes of transient unconsciousness (24, 45) for which EA might serve as a therapeutic option since our data show that point-specific acupuncture treatment substantially modulates the reflex cardiovascular vasodepression and bradycardia. EA has been shown to be capable of increasing low blood pressure associated with hemorrhage (36) and to elevate blood pressure in patients with shock (6). Acupuncture applied at P5–P6 also reduces elevated blood pressure (16, 17). Thus EA at P5–P6 attenuates both inhibitory and excitatory cardiovascular reflex responses, suggesting that this therapy has a unique modulatory action on both excitatory and inhibitory cardiovascular responses that cannot be replicated by pharmacological therapy.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**