Processing cardiovascular information in the vlPAG during electroacupuncture in rats: roles of endocannabinoids and GABA

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Tjen-A-Looi SC, Li P, Longhurst JC. Processing cardiovascular information in the vlPAG during electroacupuncture in rats: roles of endocannabinoids and GABA. J Appl Physiol 106: 1793–1799, 2009. First published March 26, 2009; doi:10.1152/japplphysiol.00142.2009.—A long-loop pathway, involving the hypothalamic arcuate nucleus (ARC), ventrolateral periaqueductal gray (vlPAG), and the rostral ventrolateral medulla (rVLM), is essential in electroacupuncture (EA) attenuation of sympathoexcitatory cardiovascular reflex responses. The ARC provides excitatory input to the vlPAG, which, in turn, inhibits neuronal activity in the rVLM. Although previous studies have shown that endocannabinoid CB1 receptor activation modulates γ-aminobutyric acid (GABA)-ergic and glutamatergic neurotransmission in the dorsolateral PAG in stress-induced analgesia, an important role for endocannabinoids in the vlPAG has not yet been observed. We recently have shown (Fu LW, Longhurst JC. J Appl Physiol; doi:10.1152/japplphysiol.91648.2008) that EA reduces the local vlPAG concentration of GABA, but not glutamate, as measured with high-performance liquid chromatography from extracellular samples collected by microdialysis. We, therefore, hypothesized that, during EA, endocannabinoids acting through CB1 receptors, presynaptically inhibit GABA release in the vlPAG. Reduction of GABA release disinhibits the vlPAG and ultimately modulates excitatory reflex blood pressure responses. Rats were anesthetized, ventilated, and instrumented to measure heart rate and blood pressure. Gastric distention-induced blood pressure responses of 18 ± 5 mmHg were reduced to 6 ± 1 mmHg by 30 min of low-current, low-frequency EA applied bilaterally at pericardial P 5–6 acupoints overlying the median nerve. Like EA, microinjection of the fatty acid amide hydrolase inhibitor URB597 (0.1 nmol, 50 nl) into the vlPAG to increase endocannabinoids locally reduced the gastric distention cardiovascular reflex response from 21 ± 5 to 3 ± 4 mmHg. This inhibition was reversed by pretreatment with the GABAa antagonist gabazine (27 mM, 50 nl), suggesting that endocannabinoids exert their action through a GABAergic receptor mechanism in the vlPAG. The EA-related inhibition from 18 ± 3 to 8 ± 2 mmHg was reversed to 14 ± 2 mmHg by microinjection of the CB1 receptor antagonist AM251 (2 nmol, 50 nl) into the vlPAG. Pretreatment with gabazine eliminated reversal following CB1-receptor blockade. Thus EA releases endocannabinoids and activates presynaptic CB1 receptors to inhibit GABA release in the vlPAG. Reduction of GABA release disinhibits EA stimulation of acupoints, located along the pericardial meridian (P 5–6) and positioned directly over the median nerve near the wrist, we have demonstrated that low-current, low-frequency EA stimulation in animals decreases the extent of myocardial ischemia through reduction of myocardial oxygen demand and reduces sympathoexcitatory cardiovascular reflex responses, in part through its action in the rostral ventrolateral medulla (rVLM) (6, 13). Group III and IV fibers are activated during EA application (13, 33, 35). To identify the underlying mechanisms of the acupuncture effect, we have examined the role of several neurotransmitters in several cardiovascular regions of the medulla. For example, we have shown that, during and after EA stimulation, nociceptin, γ-aminobutyric acid (GABA), and opioids, including endorphins, and enkephalins, act on μ- and δ-opioid receptors in the rVLM, inhibit sympathetic outflow and the resulting cardiovascular sympathoexcitatory response (6, 29, 30). Thus EA modulates sympathoexcitatory responses by releasing several neurotransmitters that inhibit the activity of bulbospinal presympathetic neurons.

We also have shown that EA modulates sympathoexcitatory reflex responses through activation of a long-loop pathway extending from the hypothalamus to the midbrain and ultimately to the medulla. Specifically, the hypothalamic arcuate nucleus (ARC), ventrolateral periaqueductal gray (vlPAG), raphe nuclei, and rVLM each appear to play a role during EA (16, 22, 32). Both the ARC and the vlPAG receive convergent input from stimulation of a number of acupoints (16, 32). The ARC provides excitatory projections to the vlPAG, which, in turn, inhibits premotor cardiovascular sympathoexcitatory rVLM neurons to modulate sympathoexcitatory reflexes evoked by visceral afferent stimulation (16, 32). Furthermore, recent examination of pathways involved in the inhibitory EA effect has shown that excitation of the ARC, like EA, inhibits rVLM neuronal activity, while inactivation of neurons in the caudal vlPAG abolishes ARC-related inhibition (Fig. 1, vlPAG panel) (15). Hence, the midbrain vlPAG is an important relay station between the ARC and the rVLM that likely assists in the processing of somatic information during EA. Importantly, this long-loop pathway also appears to contribute to the long-lasting, acupuncture-related attenuation of sympathetic premotor outflow and excitatory cardiovascular reflex responses (29).

In addition to classical neurotransmitters and neuromodulators, recent evidence suggests that endocannabinoids may participate in cardiovascular regulation in the nucleus tractus solitarius (25). The endocannabinoid system includes two endocannabinoids (anandamide and 2-arachidonoylglycerol) that act on cannabinoid receptors (CB1 and CB2), as well as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase that degrade endocannabinoids (9, 27). Inhibition of the FAAH with URB597, which increases local concentrations of endo-
cannabinoids, enhances cannabinoid signaling and has been used experimentally to evaluate the role of the endocannabinoid system (11, 19). The active component of marijuana, cannabis, functions as an agonist for CB1 receptors in the central nervous system (1). Blockade of CB1 receptors with AM251 has been used to examine the relevance of endocannabinoid CB1 receptors in nociception (11). Endocannabinoids, produced in the brain in response to stress, activate CB1 receptors (11) located on presynaptic terminals to inhibit GABAergic transmission (12, 18, 24, 34). For example, low-frequency (1 Hz) stimulation of afferents in the lateral amygdala leads to long-term CB1 receptor-mediated depression or disinhibition of GABAergic inhibitory transmission in the basolateral amygdala (20). Low-frequency stimulation of median nerve inducing the long-lasting EA inhibitory effect on cardiovascular reflex responses similarly may be influenced by the CB1-receptor-mediated disinhibition of GABAergic transmission in the vIPAG. The endocannabinoid-GABA system has been evaluated with respect to its role in cardiovascular regulation (25). We have used the GABA\textsubscript{A} receptor antagonist gabazine in the rVLM to evaluate the role of this receptor in cardiovascular responses to EA (29).

Endocannabinoid-mediated, stress-induced analgesia apparently does not involve the vIPAG (11). However, endocannabinoids acting through CB1 receptors do appear to be involved in regulation of nociception in the vIPAG (19). The significance of the vIPAG in the long-loop pathway underlying EA’s influence on the cardiovascular system thus led us to evaluate more closely the role of endocannabinoids in this region of the mesencephalic central gray. In this regard, using microdialysis to sample neurotransmitter concentrations in the extracellular fluid, our laboratory recently demonstrated that endocannabinoids modulate the release of vIPAG GABA but not glutamate (8). In the present study, we hypothesized that endocannabinoid-related inhibition of GABA release sufficiently disinhibits neurons in the vIPAG to modulate excitatory reflex blood pressure responses evoked by gastric distension (Fig. 1).

MATERIALS AND METHODS

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 (400–600 g). After an 18-h overnight fast, anesthesia was induced initially with ketamine (100 mg/kg im) followed by α-chloralose (5 mg/kg iv). Additional doses of α-chloralose 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 artificially ventilate animals 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. Arterial blood gases and pH were measured periodically with a blood-gas analyzer (ABL5, Radiometer America) and were kept within normal physiological limits (PCO\textsubscript{2} 35–40 Torr, O\textsubscript{2} >100 Torr) by adjusting the ventilatory rate or volume and enriching the inspired O\textsubscript{2}. Arterial pH was maintained between 7.35 and 7.43 by infusion of a solution of 8% sodium bicarbonate. Body temperature, monitored with a rectal thermistor (model 44TD), was kept between 36 and 38°C with a heating pad and lamp. A 2-cm-diameter (unstressed dimension) latex balloon (Traub) was attached to a polyurethane tube (3-mm diameter) that was inserted into the stomach through the mouth and esophagus. The balloon was palpated manually during insertion as it 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. 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 (14). Distention pressures were selected to fall within the range that a rat normally experiences during ingestion of food and fluids in a single meal (2, 7). To induce increases in blood pressure, the balloon was inflated to a 2-cm-diameter (unstressed dimension) latex balloon (Traub) was attached to a polyurethane tube (3-mm diameter) that was inserted into the stomach through the mouth and esophagus. The balloon was palpated manually during insertion as it 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. 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 (14). Distention pressures were selected to fall within the range that a rat normally experiences during ingestion of food and fluids in a single meal (2, 7). To induce increases in blood pressure, the balloon was inflated...
inflated inside the stomach. An increase in pressure was observed within 30 s of inflation. The balloon was deflated within 30 s after reaching the maximum increase in blood pressure. We did not include animals in the study, if the balloon was verified postmortem not to be within the stomach, but rather in the esophagus.

Experimental Procedures

Animals were placed in a stereotaxic head frame, and their heads were positioned such that the floor of the fourth ventricle was horizontal. A partial craniotomy was performed to expose the dorsal cortex to allow access to the vlPAG. A microinjection probe was inserted at a 90° angle relative to the dorsal surface of the cortex, 0.2 mm lateral either right or left from the midline, and within 7 mm caudal to the bregma. A modified CMA microdialysis AB probe of 14 mm long (CMA, Stockholm, Sweden, tip diameter 0.24 mm), lacking the microdialysis membrane, was advanced 6 mm toward the ventral surface to reach the vlPAG for unilateral microinjection, as depicted in the atlas of Paxinos and Watson (23). The probe was connected to the CMA 402 syringe pump (CMA) to deliver 50 nl at a rate of 0.3 μl/min for 10 s. The microdialysis probe enabled delivery of different solutions into the vlPAG without repositioning.

The injection site was 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 vlPAG. Thereafter, rats were euthanized under deep anesthesia with additional α-chloralose and saturated KCl. The stomach was exposed to confirm placement of the balloon. The midbrain was removed and submerged in 4% paraformaldehyde for at least 2 days. Frozen 40-μm coronal sections were cut with a CM 1850 cryostat microtome (Leica) to confirm microinjection sites histologically. The dye spots were identified with a binocular microscope. Microinjection sites in the vlPAG were plotted with Corel Presentation software on coronal sections reconstructed according to the atlas of Paxinos and Watson (23).

Chemicals

The FAAH inhibitor URB597, which limits the metabolism of endocannabinoids, was dissolved in a solution of 5% Tween 80, 5% polyethylene glycol, and 90% normal saline to a final concentration of 0.1 nmol (11). URB597, Tween 80, and polyethylene glycol were obtained from Sigma Aldrich (St. Louis, MO). An inactivator of CB1 receptors, AM251, was dissolved initially in ethanol (Fisher Scientific, Fair Lawn, NJ) to achieve a concentration of 10 mg/ml and, on the day of the experiment, 10 μl were dissolved in 100 μl of Tocrisolve 100 to achieve a final concentration of 2 nmol (26). AM251 and Tocrisolve 100 were obtained from Tocris Bioscience (Ellisville, MO). The GABA<sub>A</sub>-receptor antagonist gabazine (Sigma Aldrich, St. Louis, MO) was dissolved in normal saline to yield an initial concentration of 1 mg/ml. A final concentration of 27 μM was achieved through serial dilutions (21, 29).

Experimental Protocols

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. Once maximal blood pressure was attained (generally within 30 s), the balloon was withdrawn slowly from the balloon. Peak blood pressure reflex responses typically were noted within 20–30 s following peak inflation. Ten-minute recovery intervals were necessary to prevent attenuation of the cardiovascular reflex responses.

Responses to increase in endocannabinoids. Tween 80-polyethylene glycol, the solvent for URB597, was microinjected into vlPAG in a vehicle control group of five animals. In six other animals, the FAAH inhibitor (URB597) was microinjected in the vlPAG after two consistent gastric distention responses were observed to evaluate the reflex increase in blood pressure in response to increased concentrations of endocannabinoids.

Interaction between endocannabinoid and GABA systems. In another group of five rats, gabazine was microinjected 20 min before microinjection of the FAAH inhibitor URB597. Saline, serving as the vehicle control for gabazine, was microinjected 20 min prior to delivery of URB597 in five other rats.

Responses to inactivation of CB<sub>1</sub> receptors. The responses of EA attenuation of the reflex response to gastric distension were evaluated following microinjection of the vehicle control, Tween 100, or Tocrisolve 100 in four rats. In four other rats, action of AM251 on the gastric distention-induced reflex also was examined (Table 1).

Responses to repeated gastric distention, EA, and inactivation of CB<sub>1</sub> and GABA<sub>A</sub> receptors. In a time control group, eight rats were subjected to 10 repeated gastric distentions without EA. In three other groups, EA was applied to examine the role of endocannabinoid-GABA system. After recording two repeatable responses to gastric distention, EA (1–2 mA, 0.5-ms duration, 2 Hz) was performed bilaterally with 32-gauge stainless steel acupuncture needles. The needles were placed at Neiguan-Jianshi acupoints on the pericardial (PC 6–9, overlying the median nerves) located above the paw (31). Acupuncture needles were inserted perpendicularly to a depth of 2–3 mm. Correct placement of the needles at the acupoints was confirmed by observing slight repetitive paw twitches, i.e., motor threshold, during EA stimulation. The paw twitches were important observations to confirm stimulation of motor fibers in the median nerve to indicate that we were stimulating the correct nerve (4, 13, 14). Of note, motor nerve stimulation does not participate in the EA-cardiovascular response, since we have shown that EA inhibition of reflex cardiovascular responses occurs following muscle paralysis (16). Application of EA lasted 30 min, while gastric distention during somatic stimulation occurred every 10 min. Five additional gastric distentions applied every 10 min were evoked to assess cardiovascular responses after EA during recovery. Thus 10 distensions were used in the EA protocol.

We monitored the response to multiple microinjections of saline into the vlPAG during and after EA in five rats. Finally, to examine the role of endocannabinoids in the vlPAG during EA, the CB1 antagonist, AM251, was microinjected immediately after termination of acupuncture in six rats, i.e., during its long-lasting modulatory effect. Then, five additional gastric distentions were performed to evaluate the effects of CB<sub>1</sub>-receptor blockade. In six other animals, the interaction between GABA and the endocannabinoid system was evaluated by blocking GABA<sub>A</sub> receptors with gabazine before microinjection of AM251.

Statistical Analysis

Data are presented as means ± SE. Changes in mean arterial pressure are presented as bar histograms. The increases in blood pressure before, during, and after EA and after delivery of experimental drugs or saline were compared by one-way ANOVA followed with the post hoc Student-Newman Keuls test. Data were analyzed with the Kolmogorov-Smirnov test for normal data distribution and normalized when necessary. All statistical analyses were performed with Sigma Stat (Jandel Scientific). The 0.05 probability level was used to detect significant differences.

Table 1. Gastric distention-induced change in mean arterial pressure before and after microinjection of vehicle Tocris 100 or AM251 CB<sub>1</sub> receptor antagonist

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<td>Tocris 100</td>
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<tr>
<td>AM251</td>
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Values are means ± SE in mmHg; n, no. of rats. GD 1–4, gastric distensions 1–4, respectively.
RESULTS

Response to Inhibition of Endocannabinoid Metabolism

Microinjection of the vehicle for URB597 did not influence cardiovascular reflex responses evoked by gastric distention. Conversely, microinjection of the FAAH inhibitor URB597 to limit the metabolism of endocannabinoids reduced the sympathoexcitatory reflexes (Fig. 2, A and B).

Interaction Between Endocannabinoids and GABA

Pretreatment with the GABA_A-receptor antagonist gabazine prevented the URB597-related vlPAG sympathoinhibition (Fig. 2C). In contrast, saline, the vehicle control for gabazine microinjected before delivery of URB597, did not affect the endocannabinoid-related cardiovascular reflex inhibition in five animals.

Reflex Response to Inactivation of Endocannabinoid CB1 Receptors

Microinjection of the antagonist AM251 or its vehicle did not alter the cardiovascular reflex responses. (Table 1).

Response to Repeated Gastric Distention, EA, and Inactivation of CB1 and GABA_A Receptors

We observed that 10 repeated gastric distentions over a period of 100 min induced consistent sympathoexcitatory cardiovascular responses in the absence of EA. In contrast, 30 min of low-frequency, low-intensity EA reduced reflex blood pressure responses by ~64% for over 60 min, even after multiple vlPAG microinjections of saline (Fig. 3, A and B). Blockade of CB1 receptors in the vlPAG with AM251 during the inhibitory EA response transiently attenuated the influence of acupuncture (Fig. 3C). Microinjection of gabazine into the vlPAG before AM251 administration prevented the reversal of the EA-related inhibition by CB1-receptor blockade (Fig. 3D).

Anatomic Location of Microinjection Sites

All injection sites located within the vlPAG were included in the data analysis. Two microinjection sites found to be too ventral were excluded. The injections of URB597 outside the vlPAG did not modulate the reflex responses (Fig. 4).

DISCUSSION

The vlPAG serves a central role in EA-associated inhibition of rVLM sympathetic premotor responses to visceral afferent stimulation (32). Recently, our laboratory demonstrated that the extracellular concentration of GABA in the vlPAG is decreased by acupuncture stimulation at P 5–6 (8). This observation suggests that disinhibition of GABA in this area of the midbrain may promote EA-related inhibition of rVLM activity, since projections from the vlPAG to the rVLM are mainly inhibitory in nature (32). The present study provides further support for an interaction between endocannabinoids and GABA in the vlPAG during EA inhibition of the cardiovascular responses. Specifically, our data demonstrate that blockade of CB1 receptors reverses the EA inhibitory effect, while pretreatment with gabazine to block the GABA_A receptors prevents this response. Thus, in concert with our laboratory’s previous data showing EA-related decreases in vlPAG GABA release, endocannabinoids do appear to disinhibit neurons in the vlPAG through a GABAergic mechanism.

Application of exogenous cannabinoids in the ARC nucleus influences the release of both GABA and glutamate.
In contrast, manipulation of the endocannabinoid system in the central nervous system has been shown to influence mainly GABA release (10). Our data are consistent with this observation. Furthermore, because we recently observed that acupuncture does not alter the extracellular concentration of glutamate, we concentrated on the role of CB1-receptor blockade with respect to GABA. However, further studies will be required to determine the role of glutamate in the vlPAG with respect to EA attenuation of cardiovascular reflex responses, including assessing the response to excitatory input from the ARC nucleus (17).

Cannabinoid inhibition of GABA transmission also may underlie baroreflex processing in the nucleus tractus solitarius (25). In this regard, while exogenous anandamide in this region does not alter baseline blood pressure or sympathetic nerve activity, it does enhance baroreflex inhibition of renal sympathetic nerve discharge during baroreceptor stimulation through a GABA mechanism (25). The previous study was limited by the use of exogenous anandamide, which, as noted above, may influence both glutamate and GABA. The present study, examining vlPAG mechanisms during EA, confirms that manipulating constitutive endocannabinoids similarly does not alter baseline blood pressure but can mimic EA-related inhibition of sympathoexcitatory reflexes through a GABAergic mecha-
Since endocannabinoids are thought to preferentially inhibit the release of GABA, rather than glutamate, these results are the first to show that endocannabinoids serve an important disinhibitory role in somatic-visceral reflex processing in the mesencephalon.

In addition to the role of endocannabinoids in the EA cardiovascular response, the primary cardiovascular reflex response upon which EA acts could be influenced by the cannabinoid-GABA system. The present study has shown that neither GABA<sub>A</sub> nor cannabinoid receptors in the vlPAG play a role in the excitatory autonomic visceral reflex. We cannot absolutely rule out the possibility that CB<sub>1</sub> and GABA receptors acting in concert influence the reflex responses that are processed in the vlPAG. However, we (15) recently have shown that stimulation at P 5–6 evoked more vlPAG action potentials than splanchnic nerve stimulation, suggesting that this nucleus mainly is important for processing somatic rather than visceral afferent information. The absence of this nucleus mainly is important for processing somatic EA with respect to suppression of sympathoexcitatory reflexes.

Conclusion

The endocannabinoid-GABA system in the vlPAG, operating through CB<sub>1</sub>-receptor activation, is an important mechanism underlying EA-related inhibition of sympathoexcitatory cardiovascular reflexes.

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REFERENCES


