Inhibition of baroreflex vagal bradycardia by activation of the rostral ventrolateral medulla in rats

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Arterial baroreflexes, especially the vagal heart rate component, are known to be suppressed in stressful conditions (see Ref. 22 for review). For examples, baroreflex vagal bradycardia (BVB) is reflexly inhibited by somatic (24) as well as visceral nociceptive inputs (23). Similar effect is produced by activation of some central nervous structures, such as hypothalamic defense area (2) or dorsolateral part of the periaqueductal gray matter (PAG) (9, 25). Since inhibition of BVB induced by these perturbations always accompanies an increase in blood pressure, it is a natural question whether activation of the rostral ventrolateral medulla (RVL), a pivotal source of sympathetic vasomotor tone (4), has an inhibitory influence on BVB as well. This question may involve potential occurrence of sympathetic vagal interaction at brain stem level.

There have been a few reports regarding the RVL influence on BVB. Zhang et al. (37) described that microinjection of glutamate into the RVL region produced pressor and bradycardiac responses; it augmented sensitivity of BVB induced by intravenous phentolamine. According to Lin et al. (18), however, microinjection of angiotensin II into the RVL, which is known to excite presympathetic neurons there (17) by decreasing potassium conductance (16), thereby increasing blood pressure (18), suppressed baroreflex bradycardia induced by intravenous phentolamine. The suppression was reversed to facilitation following administration of angiotensin receptor antagonists (18). Collectively, the role of the RVL in modification of BVB remains unsettled.

The present study was designed to address the important but yet controversial issue of whether the RVL, a region that contains presympathetic neurons, affects BVB or, in a broader sense, the vagal cardioinhibitory mechanism at the medullary level. Using totally baroreceptor-denervated Wistar rats, we chemically stimulated the RVL by microinjection of DL-homocysteic acid (DLH), a broad-spectrum excitatory amino acid agonist. Under β-adrenergic receptor blockade, the aortic depressor nerve (ADN) was stimulated to produce BVB, and the effect of RVL activation on the BVB was studied. With this method, the test baroreceptor input could be held constant no matter how much blood pressure was changed during the conditioning challenge. The result was supplemented by another series of experiments, in which bicuculline methiodide, a GABA A receptor antagonist, was microinjected into the RVL of rats with the carotid sinus nerve uncut, and the effect was examined on BVB. Bicuculline microinjection into the RVL was expected to activate the presympathetic neurons, thereby removing the tonic GABA-mediated inhibition, including the baroreceptor-dependent one (4).
MATERIALS AND METHODS

General procedures. Experiments were performed in male Wistar rats, weighing 350–400 g, anesthetized with intraperitoneal injection of α-chloralose (60 mg/kg) and urethane (600 mg/kg). Polyethylene tubes were introduced into the left femoral artery and vein for blood pressure monitoring and drug administration, respectively. One-sixth of the initial dose was intravenously supplemented every 3 h. The adequacy of this anesthetic recipe had been ascertained by the lack of an avoidance reaction to hind paw pinching under conditions of spontaneous breathing. Animals were immobilized with intravenous succinylcholine (initially 10 mg/kg, supplemented with one-half the dose as needed) and artificially ventilated with room air through a tracheal cannula. After the immobilization, adequate anesthetic conditions were assured according to the stability of blood pressure and heart rate. Throughout the experiment, α-adrenergic receptors were blocked with intravenous injection of propranolol (initially 0.5 mg/kg, supplemented with one-half the initial dose on appreciable increase in resting heart rate). With this blockade, a heart rate change could be attributed to a change in the vagal activity to the heart.

In all series of experiments, the aortic depressor nerves (ADNs) of both sides were dissected and cut at the level of the clavicle. The cervical sympathetic trunk was cut at the same level and crushed at the periphery to block the conduction. To avoid extension and desiccation, the ADN and the sympathetic trunk were collectively handled for electrical stimulation (24). In some rats, the phrenic nerves of both sides were prepared and cut at the entry to the thorax for recording to monitor the central inspiratory activity. The animal was placed on a stereotaxic apparatus. The scalp was incised, the common carotid arteries; the brain was removed and fixed in 7% Formalin. Sections of 70 μm in thickness were prepared on a freezing microtome, and ferritin iron that had been deposited in the brain tissue was histochemically visualized according to the Prussian blue method. All the sections were counterstained according to the method of Tolivia and Tolivia (35). Locations of the injection sites were plotted on a histology-based stereotaxic atlas (29).

Data analysis. Magnitude of BVB was expressed as the decrease in the heartbeat decrement of the test BVB to that of control BVB (24).

All the numerical data obtained in the experiments were given as means ± SE. Data from multiple groups were first analyzed according to the Kruskal-Wallis test. Intergroup differences were then analyzed by the Wilcoxon signed rank or Mann-Whitney U test, depending on paired or unpaired comparison, respectively; differences were regarded as significant when P was <0.05.

RESULTS

DLH stimulation of the RVL. After DLH microinjection to the RVL region, blood pressure elevated promptly, reached the peak level within 20–40 s, and gradually declined to the original level (Fig. 1). Magnitude of the blood pressure increase was 50 ± 2 mmHg (initial level, 81 ± 4 mmHg; peak values, 131 ± 4 mmHg, n = 13).

The ADN was electrically stimulated to provoke BVB. In control state, the stimulation lowered heart rate from 290 ± 8 to 72 ± 5 beats/min (n = 13). Magnitude of the fall was 218 ± 8 beats/min. After DLH injection to the RVL ipsilateral to the ADN stimulated, BVB due to test ADN stimulation was remarkably suppressed (Fig. 1). The stimulation decreased heart rate from 295 ± 9 to 215 ± 15 beats/min, with the
magnitude of the fall being reduced to $77 \pm 10$ beats/min. Percentage of inhibition of BVB was $69 \pm 4\%$ ($n = 13$). The inhibition of BVB was moderately correlated to blood pressure increase ($r = 0.58, P = 0.039$).

The increase in blood pressure was a manifestation of overall sympathoexcitation. It was possible that the cardiac sympathetic nerve, thus excited, inhibits acetylcholine release from the vagus terminals (15). To evaluate contribution of this prejunctional mechanism to the observed inhibition of BVB, the spinal cord was cut, and the magnitude of the inhibition was compared before and after the transection. It was found that even after spinal cord transection, the RVL-induced inhibition of BVB persisted, whereas RVL-induced hypertension was abolished. Percentage of inhibition of BVB was $65 \pm 6\%$ before and $76 \pm 11\%$ after the transection ($n = 4$, Fig. 2). Furthermore, subsequent suprapontine decerebration did not affect the DLH-induced inhibition of BVB (% inhibition, $70 \pm 8\%$, $n = 4$; Fig. 2), ruling out appreciable participation of the forebrain to the inhibition.

Changes in central inspiratory drive. To address whether a change in central inspiratory drive was involved in the inhibition of BVB, the phrenic nerve discharges were recorded in four rats on the side contralateral to the RVL injected with DLH. We found that phrenic nerve discharges were suppressed immediately on DLH injection. The suppression lasted 20–40 s, gradually turning to facilitation. Even during the inspiratory suppression, inhibition of BVB was still observed (Fig. 3A). After bilateral vagotomy, it was evident that DLH given to the RVL inhibited central inspiratory drive (Fig. 3B).

DLH stimulation of sites other than the RVL. Besides the RVL, we microinjected DLH to other sites in the rostral medulla at the level of 2,500 μm rostral to the calamus scriptorius. A number of the nuclei produced inhibition of BVB as well as blood pressure increase. The sites included the following: the lateral paragangiocellular reticular nucleus, the gigantocellular reticular nucleus, the raphe nuclei obscurus/pallidus, the lateral tegmental reticular formation (parvocellular reticular nucleus plus intermediate reticular nucleus), and the spinal trigeminal nucleus. The results are collectively provided in Table 1. The injection sites with their responsiveness are shown in Fig. 4.

Bicuculline disinhibition of the RVL. If the DLH inhibition of BVB involves presympathetic neurons in the RVL, then application of a GABA antagonist, which
removes baroreceptor-dependent, GABA-mediated inhibition of these neurons (4), should inhibit BVB as well. To address this issue, we used the rats with the carotid sinus nerve intentionally preserved. As shown in Fig. 5, bicuculline microinjection to the RVL gradually increased blood pressure from basal level of 94 ± 4 mmHg to peak level of 132 ± 4 mmHg (n = 27), with net increase being 37 ± 2 mmHg.

When blood pressure response to bicuculline reached a near-plateau level, the ADN ipsilateral to bicuculline microinjection was electrically stimulated. The test BVB was clearly suppressed following the bicuculline application as compared with control BVB (Fig. 5). In the control state, ADN stimulation lowered heart rate from 266 ± 6 to 68 ± 4 beats/min (n = 27). Magnitude of the fall was 198 ± 6 beats/min. After bicuculline microinjection, test ADN stimulation at the maximal increase in blood pressure decreased heart rate from 267 ± 6 to 171 ± 10 beats/min; the magnitude of the fall was diminished to 96 ± 8 beats/min. Percentage of inhibition of BVB was 51 ± 3% (n = 27). BVB inhibition was significantly, albeit slightly, correlated with blood pressure increase (r = 0.430, P < 0.025, n = 27). Because of gradual increase in blood pressure, the stimulation could be repeated more than twice at different blood pressure levels. As noted in Fig. 5, the suppression was related to the blood pressure level. So far as the cases subjected to such repetitive measurements were concerned, correlation coefficients (r) of percentage of inhibition of BVB vs. blood pressure increment were increased to 0.66 with P = 0.004 (n = 17). The injection sites and their effectiveness are diagrammatically shown in Fig. 6.

In addition to inhibition of BVB, another feature of cardiovascular responses to ADN stimulation following bicuculline microinjection into the RVL was progressive diminution of reflex hypotension (Fig. 5). This phenomenon was attributed to interrupted signal transmission in the sympathetic baroreflex arc due to blockade of baroreceptor-dependent, GABA-mediated input to the RVL.

To evaluate contribution of a peripheral, sympathetic nerve-dependent mechanism to the inhibition of BVB, the spinal cord was cut at C2 level, and the bicuculline effect on BVB was compared before and after the section. Even following C2 transection, the inhibition of BVB was still preserved (Fig. 7), indicating that a central mechanism plays a major role in it. Percentage of inhibition of BVB was 58 ± 6% before and 50 ± 10% after the transection (n = 6).

**Responses of other sites to bicuculline injection.** In addition to the RVL, the raphe nuclei were another structure in the rostral medulla that produced inhibition of BVB on bicuculline microinjection. After bicuculline injection, the raphe nuclei showed slight to moderate hypertension (13 ± 4 mmHg, n = 10) and inhibition of BVB of variable degrees (percentage of inhibition, 37 ± 10%; Fig. 8). Unlike the RVL, however, bicuculline injection into the nuclei did not appear to affect baroreflex hypotension.

**DISCUSSION**

In the present study, BVB was induced by electrical stimulation of the ADN under β-adrenergic receptor blockade, and the magnitude of the bradycardia was taken as not only the index of heart rate component of baroreflexes but also the index of cardiovagal outflow. According to our experience, BVB thus induced in rats is pronounced, sometimes leading to cardiac arrest. We admit that the best approach of evaluating a given effect on cardiovagal outflow would be determination of the effect on unitary responses or field potentials antidromically activated by stimulation of identified cardiac branches of the vagus. In practice, however, this is subject to technical limitation, since the cardiovagal
preganglionic neurons in rats are dispersed without forming a distinct nuclear organization (26). Instead, the BVB produced by ADN stimulation is considered to provide a currently most convenient, reproducible measure of cardiovagal activity.

The present study has shown that the RVL, when activated either directly by excitatory amino acid or through disinhibition by a GABA antagonist, inhibits BVB. The implications of this finding are twofold, one regarding central modification of BVB and the other...
regarding sympathetic-vagal interaction. In terms of the former issue, there have been a number of reports delineating sites in the central nervous system that suppress or facilitate BVB (see Ref. 22 for review). The sites inhibiting BVB include the following: the cerebral motor cortex (1), the hypothalamus (2), the amygdala (32), dorsolateral part of the PAG (9, 25), the parabrachial nucleus (14, 21, 30), and cerebellar posterior vermis (13, 28). The sites facilitating BVB include the following: the medial prefrontal and insular cortices (27, 31, 36), the preoptic area (8), lateral hypothalamic area (8, 27), ventrolateral part of the PAG (7), and the nucleus raphe magnus (7). In addition to these sites, the present study showed that a number of brain stem structures, especially the RVL, are involved in inhibition of BVB. This accords with a recent observation that the RVL mediates the inhibition of BVB provoked by a more rostral portion of the brain (14).

As the second implication, the finding that excitation of the RVL is accompanied by reduction of vagal outflow is considered to suggest the presence of sympathetic-vagal interaction within the brain stem. Although the mechanism is so far unknown, circadian or occasional reversal of reciprocal tones of sympathetic and parasympathetic divisions is a fundamental property of the autonomic nervous system. Our observation that the RVL inhibits vagal activity within the brain stem seems to offer an important clue for understanding the mechanism underlying the creation of an ergotropic phase in which sympathetic activity dominates over vagus activity. This view is at present a hypothetical one, because the RVL is not a homogenous structure but contains neurons other than presympathetic neurons. Above all, the presence of respiratory neurons in the RVL calls for special caution since the central inspiratory drive is known to suppress BVB (5). In the present study, however, DLH microinjection in the RVL immediately suppressed inspiratory activity as measured by phrenic nerve activity. During the phase of inspiratory suppression, BVB was still inhibited. The inspiratory suppression was likely due to activation of those expiratory neurons in the Bötzingher complex, which are known to suppress the activities of overall inspiratory neurons (3). The location of Bötzingher expiratory neurons overlaps that of presym- pathetic neurons in the RVL (11). Occurrence of inhibition of BVB even during this inspiratory suppression implies that the inhibition does not necessarily depend on the facilitated inspiratory drive.

Another important problem is that the inhibition of BVB could only be produced by those neurons that are
inhibitory in nature. They could be those inhibitory, expiratory neurons in the Boëtzinger complex as mentioned above. However, this view apparently contradicts with the fact that BVB is inhibited in inspiratory phase, not in expiratory phase (5), although their possible involvement in DLH or bicuculline inhibition of BVB is not entirely ruled out. Alternatively, presympathetic neurons in the RVL could indirectly inhibit vagal cardioinhibitory neurons provided that inhibitory neurons intervene between them. Such excitatory-inhibitory conversion may be achieved by GABAergic neurons extensively populated in the medullary reticular formation (10).

Morphological background for the possible intramedullary interaction of the RVL neurons on the vagal activity has been provided by a recent electrophysiology-based anatomical study. Lipski et al. (19) identified “barosensitive” neurons in the RVL that generated inhibitory postsynaptic potentials on electrical stimulation of the ADN and intracellularly labeled them with a tracer dye to examine their morphology. The labeled RVL neurons issued axons that directed dorsomedially and then rostrally or caudally, or coursed directly caudally. Some of them issued thin and short axon collaterals in their medullary course, in the areas dorsal to the RVL, in the dorsal vagal complex, or in the CVL. Projection of these axon collaterals to certain inhibitory neurons might be responsible for the inhibition of BVB by the RVL as revealed in the present study.

The findings of our present study contradict those reported by Zhang et al. (37). These authors microinjected glutamate into the RVL in Sprague-Dawley rats and found that resultant profound hypertension was accompanied by a brisk vagal bradycardia. This bradycardia was considered to be baroreceptor-mediated because it was eliminated by sinoaortic denervation, but the magnitude was greater than baroreflex bradycardia evoked by comparable hypertension induced by intravenous phenylephrine. Also, phenylephrine-induced bradycardia was potentiated by glutamate microinjection into the RVL. The controversy between their studies and ours may be partly but not satisfactorily be explained by differences in rat strains (Sprague-Dawley vs. Wistar), in anesthesia (chloralose vs. chloralose-urethan), in conditions of baroreceptor afferent nerves (preserved vs. transected), or in excitatory amino acids injected (glutamate vs. DLH). Instead, a plausible explanation may be made based on differences of injection sites in rostrocaudal direction. We injected DLH along the track at the level 2,500–3,000 μm rostral to the calamus scriptorius, whereas Zhang et al. (37) injected glutamate at the level 2,000 μm rostral to the calamus. Considering that the vagal cardioinhibitory neurons in rats are distributed at levels from 500 to 1,500 μm rostral to the calamus (26), glutamate injected in the coordinates of Zhang et al. (37) could reach by diffusion the rostral population of cardiovagal preganglionic neurons in addition to activating the RVL neurons. Elimination of the hypertension-associated bradycardia following sinoaortic denervation is accounted for by withdrawal of converging effects of excitatory baroreceptor influence and direct glutamate action on the cardiovagal preganglionic neurons. Sensitization of phenylephrine-induced BVB by glutamate injection is likewise explained in terms of convergence.

Fig. 8. Effects of bicuculline microinjection into the raphe nuclei on BVB. A1: control response to electrical stimulation of the aortic depressor nerve (ADN). A2: blood pressure increase and inhibition of BVB following bicuculline microinjection; A3: control response after recovery (15 min later). Periods of ADN stimulation and bicuculline microinjection are indicated by horizontal bars. B: injection site is indicated by dot. Abbreviations are as in Fig. 1.
Later, it was reported from the same laboratory that a glutamate uptake inhibitor, which increases extracellular concentration of endogenously released glutamate, enhances phenylephrine-induced BVB (20). This effect, however, cannot be attributed to activation of the presympathetic neurons in the RVL, since basal blood pressure was not increased by this procedure. This was probably because the RVL neurons do not receive appreciable amount of tonic glutamatergic input in anesthetized condition (4). In contrast, cardio-vagal preganglionic neurons are proposed to receive tonic glutamatergic input derived from ongoing baroreceptor activity (6). Therefore, the enhancement of BVB by the glutamate uptake inhibitor is likely caused by its diffusion to, and action on, the vagal preganglionic neurons or, alternatively, by activation of a nearby yet unknown baroreflex-sensitizing mechanism.

In the present study, we found that a pressor response and inhibition of BVB were produced not only from the RVL but also from a variety of regions on DLH microinjection. There was a tendency for the ventral-to-lateral portion to provoke these responses relatively more frequently and markedly. Suppose that the RVL is the site of prime importance for these responses, then the rather diffuse distribution of the responsive sites is partly explained by the widespread expansion of dendrites of the RVL neurons as morphological studies have shown (19, 33, 34). Alternatively, the pressor response and inhibition of BVB obtained from part of the reticular formation may be related to those as observed following nociceptive stimulation. The medullary reticular formation is the site of integration of a variety of ascending and descending information; especially, the somatic nociceptive input, which ascends along complex pathways (24), may in part terminate there to cause a variety of autonomic responses on the way to the forebrain. Inhibition of BVB produced by DLH stimulation of the spinal tract nucleus of the trigeminal nerve is possibly related to the inhibition responding to noxious input along the trigeminal nerve (12).

Inhibition of BVB produced by bicuculline microinjection deserves a special mention. The bicuculline-induced GABA A receptor blockade in the RVL increased blood pressure and inhibited BVB. These responses could be attributed to disinhibition of the RVL neurons, including removal of baroreceptor-dependent inhibitory input (4). This notion was supported by the finding that following bicuculline, baroreflex hypotension became progressively attenuated as blood pressure elevated with time, whereas the inhibition of BVB became augmented. In contrast, inhibition of BVB produced by microinjection of bicuculline into the raphe nuclei was apparently unrelated to sympathoexcitation due to impairment of baroreflex circuitry, because it was not accompanied by attenuation of baroreflex hypotension. It is suggested that the raphe neurons have a potential inhibitory action on the cardio-vagal outflow system, but this action may be tonically masked because of a certain GABA-mediated inhibition.

In conclusion, the RVL inhibits BVB when activated directly by excitatory amino acid or through removal of GABA-mediated inhibitory input, including the baroreceptor-dependent one. The inhibition does not necessarily depend on central inspiratory drive. Future studies are needed to identify the neurons of the RVL responsible for the inhibition and to determine the exact nature of the interaction between the RVL and cardiovagal mechanism.

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