Increased GABA_A inhibition of the RVLM after hindlimb unloading in rats

JULIA A. MOFFITT, CHERYL M. HEESCH, AND EILEEN M. HASSER
Department of Veterinary Biomedical Sciences, Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211
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Moffitt, Julia A., Cheryl M. Heesch, and Eileen M. Hasser. Increased GABA_A inhibition of the RVLM after hindlimb unloading in rats. Am J Physiol Regul Integr Comp Physiol 283: R604–R614, 2002; 10.1152/ajpregu.00341.2001.—Attenuated baroreflex-mediated increases in renal sympathetic nerve activity (RSNA) in hindlimb unloaded (HU) rats apparently are due to changes within the central nervous system. We hypothesized that GABA_A receptor-mediated inhibition of the rostral ventrolateral medulla (RVLM) is increased after hindlimb unloading. Responses to bilateral microinjection of the GABA_A antagonist (-)-bicuculline methiodide (BIC) into the RVLM were examined before and during caudal ventrolateral medulla (CVLM) inhibition in Inactin-anesthetized control and HU rats. Increases in mean arterial pressure (MAP), heart rate (HR), and RSNA in response to BIC in the RVLM were significantly enhanced in HU rats. Responses to bilateral CVLM blockade were not different. When remaining GABA_A inhibition in the RVLM was blocked by BIC during CVLM inhibition, the additional increases in MAP and RSNA were significantly greater in HU rats. These data indicate that GABA_A receptor-mediated inhibition of RVLM neurons is augmented after hindlimb unloading. Effects of input from the CVLM were unaltered. Thus, after cardiovascular deconditioning in rodents, the attenuated increase in sympathetic nerve activity in response to hypotension is associated with greater GABA_A receptor-mediated inhibition of RVLM neurons originating at least in part from sources other than the CVLM.

microgravity; cardiovascular deconditioning; sympathetic nervous system; bedrest; orthostatic intolerance; spaceflight; caudal ventrolateral medulla

HUMANS EXPOSED TO PROLONGED periods of bedrest or microgravity undergo deconditioning of the cardiovascular system. Microgravity and bedrest result in an initial central shift of body fluids and a reduction in plasma volume and blood volume (6, 9, 17, 42, 46). Upon resumption of an upright posture or return to normal gravitational forces, a number of adverse cardiovascular consequences are experienced. These cardiovascular changes include resting tachycardia, decreased exercise capacity, and a marked reduction in orthostatic tolerance (3, 4, 5, 13, 23, 24, 42, 46).

Hindlimb unloading in rodents is an animal model that is used to simulate cardiovascular deconditioning in humans. The effects on the cardiovascular system due to hindlimb unloading in rats are similar to those experienced by humans after bedrest or microgravity (31, 35, 38). These effects include an early central shift in body fluids followed by hypovolemia, tachycardia, reduced exercise capacity, and evidence suggestive of orthostatic intolerance (28, 31, 35, 37, 38, 43). Previous data indicate that hindlimb unloaded (HU) rats exhibit an attenuation in baroreflex-mediated increases in lumbar and renal sympathetic nerve activity in response to hypotensive stimuli (32). In addition, the attenuation in baroreflex control of sympathetic nerve system activity after cardiovascular deconditioning appears to be due to dysfunction in the central nervous system component of the baroreflex arc rather than a change at peripheral baroreceptors (33). Specifically, this dysfunction involves central nervous system processing leading to attenuated sympathoexcitation during baroreceptor unloading (hypotension).

The rostral ventrolateral medulla (RVLM) is a primary site in the central nervous system for control of basal and reflex-mediated changes in sympathetic nerve activity (e.g., cardiovascular reflexes including baroreflex-mediated sympathoexcitation) (12). Sympathetic premotor neurons in the RVLM are tonically active under baseline conditions, and changes in the degree of excitation of these neurons directly influence the activity of sympathetic preganglionic neurons (34). Electrical or chemical stimulation of the RVLM produces an increase in arterial pressure, heart rate (HR), and sympathetic nerve activity, whereas inhibition of the RVLM produces the opposite effects (39, 41).

The level of sympathoexcitation or sympathoinhibition that is mediated by cardiovascular reflexes is primarily achieved by changing the level of RVLM neuronal excitation. Baseline activity in RVLM neurons is influenced tonically by both inhibitory and excitatory inputs (18, 27, 39). The primary neurotransmitter responsible for tonic inhibition in the RVLM is γ-aminobutyric acid (GABA), which tonically activates postsynaptic GABA receptors (25). The majority of these GABAAergic inputs originate from the caudal ventrolateral medulla (CVLM), although sources other
than the CVLM provide additional GABAergic inhibition in the RVLM (7, 25, 44). Increased GABAergic inhibition of the RVLM could account for the attenuated reflex increases in sympathetic nervous system activity after hindlimb unloading. We hypothesized that GABA_\text{A} inhibitor of RVLM neurons is enhanced after hindlimb unloading. In addition, we hypothesized that greater GABAergic input from the CVLM is responsible for increased GABA_\text{A} receptor activation of the RVLM under control conditions and during CVLM inhibition. This allowed us to assess overall GABA_\text{A}-mediated inhibition of RVLM neurons as well as that originating from both the CVLM and sources other than the CVLM. Data indicate a greater GABA_\text{A}-mediated inhibition of the RVLM following hindlimb unloading. In addition, it appears that GABAergic input from sources other than the CVLM contributes to increased GABAergic inhibition of the RVLM in HU rats.

**METHODS**

**Hindlimb Unloading**

All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia. Male Sprague-Dawley rats (n = 29) obtained from Sasco were randomly assigned to HU or control groups. HU rats (n = 13) were acclimated to the unloading procedure by temporary suspension of the hindlimbs for 1–2 h/day for three consecutive days before the hindlimb unloading intervention. The hindlimbs of HU rats were then elevated with a harness attached to the proximal two-thirds of the tail by modification of a technique previously described (20). Briefly, two hooks were attached to the tail with moleskin adhesive material. A curved rigid support made of lightweight plastic (X-lite splint, AOA/Kirschner Medical) was placed beneath the tail to allow adequate blood flow. The hooks were connected by a wire to a swivel apparatus at the top of the cage with the hindlimbs elevated so there was no contact with supportive surfaces. Rats were maintained at a suspension angle of ~30–35°. A small cast made from plaster of Paris was applied to the rat's thorax to reduce lordosis and to help prevent the rat from disturbing the tail apparatus. Control rats (n = 16) had the thoracic cast applied and were maintained in a normal cage environment. Animals remained in the HU or control condition for 14–16 days. This time period was chosen because 1) Spacelab Life Sciences missions have been of similar duration, 2) humans subjected to bedrest exhibit stable changes within this time period (14), 3) this duration of hindlimb unloading provides stable changes in muscle weight and force in rats (47), and 4) this time period allows direct comparison with our previous studies examining arterial baroreflex responses in HU rats (32, 33). Body weights were recorded before and after the caged control or HU period. During the unloading protocol, the rats were closely monitored several times daily for adequate food and water intake, normal grooming behavior, and urination and defecation. All rats were housed individually in a temperature- and humidity-controlled (69–72°F) environment with a 12:12-h light-dark cycle. Rat chow (Purina 5005 rodent chow) and water were provided ad libitum.

**Surgical Procedures**

After the HU or control period, rats were removed from their cages and anesthetized with Inactin (100 mg/kg ip). Supplemental doses (5 mg iv) of anesthetic were administered when necessary. Animals were tracheotimized through a midline cervical incision and ventilated with room air mixed with oxygen. Body temperature was maintained at 37°C by use of a circulating water heating pad. Polyethylene catheters (PE-50 fused to PE-10) were inserted into the aorta and abdominal vena cava via the femoral artery and vein for measurement of arterial pressure and drug administration, respectively.

For recording RSNA, the left kidney was exposed through a retroperitoneal approach and a sympathetic nerve branch was dissected free (32). Two Teflon-insulated silver wire electrodes (0.005-in. diameter, 36 gauge; Medwire) wired through Silastic tubing (0.025 in ID) were placed around the isolated nerve. The nerve and electrode were covered with a polyvinylsiloxane gel (Coltene President), which was allowed to harden before closure. A ground wire was sewn to surrounding tissue, and incision sites were closed. RSNA was amplified 1,000 times (Grass model P511) and filtered using high- and low-pass frequency levels of 30 Hz and 3 kHz, respectively. Action potentials were monitored on an oscilloscope (Tektronix) and an audio monitor (Grass model M8). RSNA was rectified and integrated using a root mean square (RMS) converter with a time constant of 28 ms. The rectified, integrated signal was then electronically averaged, and this mean signal was used as a relative measure of RSNA. Background noise in the sympathetic nerve recording was determined after the rats were euthanized.

**Microinjections**

Rats were placed in a Kopf stereotaxic apparatus and a dorsal midline incision was made to expose and remove the atlanto-occipital membrane. The rat's nose was deflected ventrally so that the calamus scriptorius was positioned 2.2–2.6 mm posterior to the interaural line thus positioning the brain stem in a horizontal plane (21). All subsequent stereotaxic coordinates used calamus scriptorius as a reference with the rat's head in this position. Glass micropipettes (1 or 3 barrels, outside tip diameter ~10–30 μm) were filled with the appropriate drugs and inserted vertically into the brain stem under visual observation. The CVLM was functionally identified bilaterally by observing a depressor response of ~30 mmHg and ~80% inhibition in RSNA in response to microinjection of l-glutamate (10 mM, 30 nl). Similarly, functional identification of the RVLM was made by observing a pressor response of ~15 mmHg and ~100% increase in RSNA in response to microinjection of glutamate (10 mM, 30 nl). In one HU animal, microinjection of GABA (10 mM, 30 nl) was used to functionally identify the CVLM and RVLM by observing a pressor and depressor response, respectively. Stereotaxic coordinates for the CVLM, relative to calamus scriptorius and the dorsal medullary surface, were anterioposterior (AP), −0.2 to −0.25 mm; lateral (Lat), ±1.9 to ±2.1 mm; and ventral (V), −2.2 to −2.4 mm. Stereotaxic coordinates for the RVLM relative to calamus scriptorius and the dorsal medullary surface were AP, +0.7 to +0.8 mm; Lat, ±2.0 to ±2.2 mm; and V, −3.6 to −4.2 mm. In some
control experiments, microinjection into the nucleus tractus solitarius (NTS) was also performed. Stereotaxic coordinates for the NTS relative to calamus scriptorius and the dorsal medullary surface were AP, +0.5 mm; Lat ±0.5 mm; and V, –0.5 mm. Average stereotaxic coordinates for all microinjection sites did not differ between groups. Drugs were ejected from the pipette in volumes of 30–90 nl over a period of <3–9 s (depending on the volume microinjected) by applying pressurized N2 to each barrel using a custom-constructed pressure-ejection system. The volume of drug delivery was controlled by changing the injection pressure and/or duration of the pressure pulse. The volume of the injection was determined by visual inspection of the movement of the fluid meniscus in individual barrels of known internal diameter using a microscope (×150) equipped with a calibrated eyepiece micrometer. Bilateral microinjections were made serially by withdrawing the pipette from the initial microinjection site and then reinserting it into the brain on the contralateral side. Both injections were made within <1 min. All drugs were dissolved in artificial cerebrospinal fluid (aCSF) containing (in mM) 128.7 NaCl, 3.1 KCl, 1.1 MgCl2-6H2O, 21.4 NaHCO3, 2.8 glucose (anhydrous), and 1.2 CaCl2 with the pH adjusted to 7.4. All drugs were obtained from Sigma Chemical (St. Louis, MO). At the end of the experiments, Pontamine sky blue (30 nl) was injected into the CVLM and RVLM, and injection sites were subsequently verified histologically in some animals.

**Blockade of GABA<sub>A</sub> Receptors in RVLM**

Preliminary experiments were conducted to determine the volume of the GABA<sub>A</sub> receptor antagonist BIC (5 mM) (36, 51) sufficient to block GABA<sub>A</sub> receptors within the RVLM. Bilateral microinjections of 90 nl or 120 nl (randomized order) BIC into the RVLM were performed in a separate group of control animals (n = 3). There were no significant differences with respect to the increase in MAP (90 nl, 57 ± 7.2 vs. 120 nl, 60 ± 4.7 mmHg), change in HR (90 nl, –7.2 vs. 120 nl, 5 ± 4.1 beats/min), and increase in RSNA (90 nl, 191 ± 17.5 vs. 120 nl, 157 ± 38% baseline). Thus 90 nl was the volume of BIC chosen to elicit the maximal pressor effect with the minimal volume injected.

To determine the overall effects of tonic GABA<sub>A</sub> inhibition of RVLM neurons, BIC (5 mM, 90 nl) was microinjected bilaterally into the RVLM as described (see Microinjections; Refs. 36, 51). The maximal increase in MAP, HR, and RSNA was recorded. Responses were allowed to fully recover (~1 h) before any further microinjections were performed. To verify that GABA<sub>A</sub> receptors in the RVLM were completely blocked, the effects of endogenous release of GABA in response to CVLM stimulation were evaluated. In some animals (control, n = 7; HU, n = 5), glutamate (10 mM, 30 nl) was microinjected unilaterally into the CVLM before and at the peak pressor and sympathoexcitatory response to BIC microinjection into the RVLM. Activation of the CVLM evokes sympathoinhibition and depressor responses due to release of GABA and activation of GABA<sub>A</sub> receptors in the RVLM. In a separate group of control animals (n = 3), 90 nl of vehicle (aCSF) was bilaterally microinjected into the RVLM before glutamate microinjection into the CVLM to control for nonspecific volume effects of RVLM injection.

**CVLM Blockade**

A large proportion of GABA<sub>A</sub> inhibition in the RVLM is microinjected bilaterally into the CVLM. Kainic acid has been used previously to interrupt neuronal activity, presumably by causing depolarization block (11, 15, 53). This volume was chosen because preliminary studies indicated that stimulation of the CVLM via microinjection of 120 nl of glutamate after the maximal response to 90 nl kainic acid microinjection into the same location within the CVLM did not induce any further change in MAP, HR, or RSNA. These data suggest that 120 nl of glutamate after kainic acid did not diffuse to the RVLM and excite neurons in that region. Similarly, it is unlikely that kainic acid would have spread to RVLM and perhaps influenced RVLM neurons.

The maximal increase in MAP, HR, and RSNA was recorded after bilateral microinjection of kainic acid into the CVLM of control and HU rats. The time course of the peak response to kainic acid (5–9 min) was not different between groups. To verify that the CVLM was completely inhibited, in separate groups of control and HU animals, endogenous release of glutamate in the CVLM was elicited in response to unilateral NTS stimulation by microinjection of glutamate. The depressor response to microinjection of glutamate (10 mM, 30 nl) into the NTS under control conditions was similar in control and HU rats. In addition, the response to NTS activation was blocked during CVLM inhibition in both groups. In these same animals (n = 3), 90 nl of vehicle (aCSF) was bilaterally microinjected into the CVLM before unilateral microinjection of glutamate into the NTS to control for nonspecific volume effects. Vehicle injection into the CVLM had no effect on the response to activation of the NTS.

**GABA<sub>A</sub> Inhibition of RVLM During CVLM Inhibition**

In addition to the CVLM, the influence of other sources of GABA<sub>A</sub>-mediated inhibition of the RVLM was evaluated after hindlimb unloading. At the peak of the pressor and sympathoexcitatory response to CVLM inhibition, remaining GABA<sub>A</sub> inhibition was evaluated by bilateral microinjection of BIC (5 mM, 90 nl) into the RVLM. The maximal increases in MAP, HR, and RSNA were recorded. The additional pressor and sympathoexcitatory response elicited in response to BIC microinjection in the RVLM during CVLM inhibition was used as an indication of the GABA<sub>A</sub> inhibition of RVLM neurons originating from sources other than the CVLM.

Complete recovery from bilateral BIC microinjections occurred in all animals, but complete recovery from bilateral kainic acid microinjection into the CVLM was not always obtained. Therefore, BIC was always microinjected into the RVLM first and was followed by kainic acid microinjection into the CVLM. The subsequent microinjection of BIC into the RVLM was given at the peak response to bilateral CVLM blockade. Several experiments were performed to control for the order of drug microinjections used in this protocol. Bilateral microinjection of kainic acid alone was administered in a separate group of control (n = 6) and HU animals (n = 5 to 7) to verify the time course of the pressor and sympathoexcitatory response to bilateral CVLM inhibition, 2) control for possible effects of prior microinjection of BIC into the RVLM, and 3) confirm a lack of difference in response to CVLM inhibition between the control and HU groups. Also, in a separate group of control animals (n = 6), bilateral microinjection of BIC (5 mM, 90 nl) was performed twice, with complete recovery between bilateral microinjections, to control for the effects of prior microinjection of BIC into the RVLM.

At the end of the experimental protocol, the soleus and plantaris muscles were removed from the rats and weighed. After tissue removal, rats were euthanized with an overdose of Inactin administered through a venous catheter.

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**Table 1. Resting hemodynamic parameters, body weights, and muscle weights**

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<tr>
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<th>n</th>
<th>HR, beats/min</th>
<th>MAP, mmHg</th>
<th>Body Wt, g</th>
<th>Soleus Wt, mg</th>
<th>Plantaris Wt, mg</th>
<th>Soleus Wt/Body Wt (× 10⁶)</th>
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<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>300 ± 8</td>
<td>103 ± 2</td>
<td>316 ± 11</td>
<td>351 ± 9†</td>
<td>149 ± 6</td>
<td>383 ± 16</td>
</tr>
<tr>
<td>Hindlimb unloaded</td>
<td>7</td>
<td>310 ± 9</td>
<td>96 ± 3</td>
<td>328 ± 19</td>
<td>319 ± 14</td>
<td>77 ± 5*</td>
<td>314 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. Body weight was measured before hindlimb unloading on day 1 and after hindlimb unloading on days 14–16. All other variables were measured on days 14–16. *P < 0.01 from control; †P < 0.01 from day 1. HR, heart rate; MAP, mean arterial pressure.

**Results**

Baseline hemodynamic parameters and muscle and body weights for the animals that underwent the entire protocol are presented in Table 1. Baseline MAP and HR values for animals under Inactin anesthesia were similar in control and HU rats. Body weight did not differ significantly between groups in the pre- or postexperimental periods; however, control animals gained weight, whereas there was no significant change in body weight in HU rats over the 14-day period of unloading (control, +11.4 ± 2.4% vs. HU, −2.2 ± 1.7%). Soleus and plantaris muscle weights were significantly reduced (49 and 18%, respectively) by hindlimb unloading. When expressed relative to body weight, the soleus muscle-to-body weight ratio was reduced by 44% and the plantaris muscle-to-body weight ratio was reduced by 11% after hindlimb unloading. Significant atrophy in the soleus and plantaris muscles confirms the effectiveness of the hindlimb unloading intervention in producing a deconditioned state (47).

**Glutamate Responses**

The effects of unilateral microinjection of glutamate (10 mM, 30 nl) into the CVLM and RVLM of control and HU rats are presented in Table 2. Glutamate stimulation of the CVLM significantly reduced MAP, HR, and RSNA. Glutamate activation of the RVLM led to a significant increase in MAP, HR, and RSNA. There were no significant differences between groups in the change in MAP, HR, or RSNA in response to glutamate microinjection into either the CVLM or RVLM.

**GABA<sub>A</sub> Blockade in RVLM**

The effects of bilateral microinjection of the GABA<sub>A</sub> antagonist BIC into the RVLM of control and HU animals are presented in Fig. 1. Before BIC microinjection, baseline levels of MAP, HR, and RSNA were not significantly different between groups. In control and HU animals, there were significant increases in MAP and RSNA in response to BIC microinjection. The time course of this response (3–4 min) was not different between groups. MAP and RSNA were significantly greater after BIC administration in HU animals compared with control animals. In addition, the increases in MAP and RSNA from baseline in response to GABA<sub>A</sub> blockade in the RVLM were significantly enhanced in HU rats (see Fig. 5). There was no significant difference in the change in HR after bilateral microinjection of BIC into the RVLM between control and HU rats.

To verify that GABA<sub>A</sub> receptors were effectively blocked, at the peak BIC response, glutamate (10 mM, 30 nl) was microinjected into the CVLM in control (n = 7) and HU (n = 5) animals to stimulate endogenous release of GABA in the RVLM. These data are presented in Fig. 2. Under control conditions, activation of the RVLM by microinjection of glutamate elicited a decrease in MAP, HR, and RSNA in both groups. This effect has been shown to be due to endogenous release of GABA in the RVLM (25). In the presence of bilateral BIC in the RVLM, the response to CVLM activation was converted to a small pressor and sympathoexcitatory response in control rats and was eliminated in HU rats. After recovery from RVLM BIC, responses to

**Table 2. Hemodynamic responses to microinjection of glutamate**

<table>
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<tr>
<th></th>
<th>CVLM</th>
<th>RVLM</th>
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<tr>
<td></td>
<td>MAP, ΔmmHg</td>
<td>HR, Δbeats/min</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>−36 ± 2.4</td>
</tr>
<tr>
<td>Hindlimb unloaded</td>
<td>6</td>
<td>−34 ± 4.0</td>
</tr>
</tbody>
</table>

Values are mean changes from baseline ± SE; n, no. of rats. All values represent significant differences from baseline. CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla; RSNA, renal sympathetic nerve activity.
CVLM activation of the CVLM completely recovered in both groups. CVLM-evoked responses were not statistically different between groups under any of these conditions. The effect of bilateral microinjection of 90 nl of aCSF into the RVLM on the response to microinjection of 30 nl of glutamate into the CVLM was evaluated in a separate group of animals (n = 3). Prior bilateral application of 90 nl of aCSF into the CVLM had no effect on the depressor and sympathoinhibitory effects produced by glutamatergic stimulation of the NTS, which indicates that this volume of vehicle alone did not induce nonspecific effects. These data indicate that bilateral CVLM inhibition with kainic acid was sufficient to block the depressor response due to endogenously released glutamate from the NTS.

CVLM Inhibition

Microinjection of the depolarization blocking agent kainic acid (5 mM, 90 nl) was utilized to block inputs from the CVLM to the RVLM. To verify that the CVLM was functionally blocked by kainic acid, the depressor response to glutamate (10 mM, 30 nl) microinjected unilaterally into the NTS was evaluated after bilateral kainic acid microinjection into the CVLM. These data are presented in Fig. 3. Under control conditions, glutamate microinjected into the NTS elicited a decrease in MAP, HR, and RSNA. After blockade of the CVLM, this response was eliminated with partial recovery after 1 h. In three control animals, prior bilateral application of 90 nl of aCSF into the CVLM had no effect on the depressor and sympathoinhibitory effects produced by glutamatergic stimulation of the NTS, which indicates that this volume of vehicle alone did not induce nonspecific effects. These data indicate that bilateral CVLM inhibition with kainic acid was sufficient to block the depressor response due to endogenously released glutamate from the NTS.
After microinjection of bicuculline, both MAP and RSNA recovered to within 10% of the original control value. The effects of subsequent bilateral CVLM blockade on MAP, HR, and RSNA in control and HU animals are presented in Fig. 4. Before kainic acid microinjection, baseline levels of MAP, HR, and RSNA were not significantly different between groups. In both groups of animals, bilateral microinjection of 90 nl of kainic acid produced a significant increase in MAP, HR, and RSNA. However, there were no significant differences in the peak levels of MAP, HR, or RSNA between groups (Fig. 4). In addition, the changes in MAP, HR, and RSNA were not different between control and HU animals under any condition.

Non-CVLM GABA<sub>A</sub> Blockade in RVLM

Inhibition of the CVLM by kainic acid removes effects on the RVLM provided by neuronal projections from the CVLM. To evaluate remaining GABA<sub>A</sub>-mediated inhibition originating from sources other than the CVLM, BIC was microinjected bilaterally into the RVLM during CVLM blockade. The effect of GABA<sub>A</sub> blockade in the RVLM in the presence of CVLM inhibition is presented in Fig. 4. At the peak pressor response to bilateral microinjection of kainic acid into the CVLM, bilateral microinjection of BIC into the RVLM elicited a further significant increase in MAP and RSNA in both groups. The peak level of RSNA was significantly greater in HU animals during the combined inhibition of CVLM and GABA<sub>A</sub> blockade in the RVLM. In addition, the total change from baseline in MAP and RSNA due to combined inhibition of CVLM and GABA<sub>A</sub> blockade in the RVLM was significantly greater in HU rats compared to control (Fig. 5). The additional sympathoexcitatory and pressor responses due to BIC microinjection into the RVLM at the peak inhibition of CVLM can be used as an index of GABA<sub>A</sub> inhibition from sources other than the CVLM. The changes in MAP and RSNA due to blockade of GABA<sub>A</sub> receptors in the RVLM during CVLM inactivation are

Fig. 3. Changes in MAP (A), HR (B), and RSNA (C) in response to glutamate microinjection in the nucleus tractus solitarius before, during, and after microinjection of kainic acid in the CVLM in control (n = 3) and HU (n = 3) rats. *P < 0.05, significant effect of KA-CVLM compared with control or recovery. There are no significant differences between control and HU animals under any condition.

Fig. 4. MAP (A), HR (B), and RSNA (C) under baseline conditions and in response to bilateral microinjection of kainic acid into the caudal ventrolateral medulla (KA-CVLM) alone and in combination with BIC in the RVLM (KA-CVLM + BIC-RVLM) of control (n = 8) and HU (n = 7) rats. *P < 0.05, significant effect of KA-CVLM compared with baseline or KA-CVLM + BIC-RVLM for both groups, short line: *P < 0.05, control vs. HU.
shown in Fig. 5 (last set of bars). The additional increases in MAP and RSNA due to the microinjection of BIC into the RVLM during CVLM inhibition were significantly greater in HU rats.

The protocol required the microinjection of BIC into the RVLM before and at the peak response to CVLM inhibition. To verify that a prior microinjection of BIC had no effect on the response to a second injection of BIC, repeated bilateral microinjections of BIC into the RVLM were made in a separate group of control rats (n = 6). There were no significant differences between the first and second injections in the absolute level of MAP (165 ± 5.3 vs. 161 ± 5.5 mmHg), HR (290 ± 15.6 vs. 280 ± 14.5 beats/min), and RSNA (406 ± 41.7 vs. 363 ± 27.2% baseline) due to BIC. Similarly, there were no differences in the change in MAP (+63 ± 3.1 vs. +57 ± 4.2 mmHg), HR (+8.3 ± 3.7 vs. −6.7 ± 12.4 beats/min), and RSNA (+306 ± 41.7 vs. +263 ± 27.2% baseline) between injections. In addition, complete recovery from the initial application of BIC was obtained with no significant differences in the baseline levels of MAP (102 ± 4.5 vs. 104 ± 4.0 mmHg), HR (282 ± 12.3 vs. 286 ± 12.4 beats/min), and RSNA (100 ± 0 vs. 107 ± 18.1% baseline) before the first and second microinjections. These data indicate that prior microinjection of BIC into the RVLM had no effect on the second microinjection of BIC into the RVLM.

The effects of bilateral microinjection of kainic acid (5 mM, 90 nl) alone into the CVLM are shown in Table 3. This control experiment was performed in a separate group of control (n = 6) and HU (n = 5) animals to 1) control for possible effects of prior administration of BIC into the RVLM on the response to CVLM inhibition, 2) confirm that the pressor response to kainic acid had developed to maximal levels before subsequent BIC microinjection into the RVLM, and 3) confirm that the pressor response to CVLM inhibition was not different between control and HU groups. The MAP, HR, and RSNA response to bilateral kainic acid microinjection in these animals (Table 3) was similar to that observed in the previous groups (see Fig. 4). In addition, there were no significant differences between control and HU animals with respect to the absolute level of the changes in MAP, HR, or RSNA in response to CVLM inhibition as shown in the earlier experiment.

**DISCUSSION**

Previous studies indicate that arterial baroreflex-mediated increases in sympathetic nervous system activity are attenuated in HU rats (32). This decrement in baroreflex function appears to be due to changes in the central nervous system component of the reflex (33). In the present study, we tested the hypothesis that the reduced ability to reflexively increase sympathetic nerve activity after hindlimb uploading is associated with increased GABA_A-mediated inhibition of the RVLM. We further hypothesized that greater GABAergic input from the CVLM is responsible for enhanced GABA_A inhibition in the RVLM after hindlimb uploading. Changes in MAP, HR, and RSNA were recorded in response to GABA_A blockade in the RVLM in the presence and absence of CVLM inhibition. The major finding of this study was that GABA_A receptor-mediated inhibition of the RVLM was enhanced after HU. However, contrary to our original hypothesis, this increased inhibition was not dependent on input from the CVLM (see Table 3 and Figs. 4 and 5). These data

| Table 3. Hemodynamic responses to bilateral CVLM blockade |
|-----------------|-----------------|-----------------|
|                 | n   | ΔMAP  | ΔHR  | ΔRSNA |
| Control         | 6   | 75 ± 6.3 | 31 ± 5.6 | 484 ± 87 |
| Hindlimb unloaded | 5   | 86 ± 7.5 | 19 ± 5.7 | 447 ± 70 |

Values are mean changes from baseline ± SE; n, no. of rats.
suggest that after cardiovascular deconditioning in rodents, the attenuated reflex sympathoexcitation in response to hypotension is associated with enhanced GABA<sub>A</sub>-mediated inhibition of RVLM neurons. This augmented GABA<sub>A</sub> tone appears to originate at least in part from sources other than the CVLM.

These conclusions are supported by several lines of evidence. First, bilateral microinjection of the GABA<sub>A</sub> receptor antagonist bicuculline into the RVLM produced a greater increase in MAP and RSNA in HU versus control rats. This indicates that tonic GABA<sub>A</sub> inhibition of the RVLM is enhanced after HU. Bilateral microinjection of kainic acid into the CVLM produced a similar pressor and sympathoexcitatory response in both groups, which indicates that overall tonic effects of the CVLM were not altered after HU. At the peak response to elimination of inputs from the CVLM, BIC was microinjected bilaterally into the RVLM to block remaining GABA<sub>A</sub>-mediated inhibition. When GABA<sub>A</sub> receptors in the RVLM were blocked under these conditions, the increases in MAP and RSNA were significantly greater in HU rats. Thus the additional GABA<sub>A</sub> inhibition of the RVLM after CVLM blockade was augmented in HU animals. Taken together, these data indicate that after hindlimb unloading, GABA<sub>A</sub> inhibition of the RVLM is enhanced, and this inhibition originates at least in part from a site or sites other than the CVLM.

One might expect that increased tonic inhibition of the RVLM in HU rats would result in decreased responsiveness to excitatory agents such as L-glutamate. In this study, we used small volumes of a relatively high dose of glutamate to functionally identify the RVLM and note that the responses to a single dose of glutamate (10 mM, 30 nl) were not different between control and HU rats. To accurately test excitability of the RVLM, larger volumes and a range of concentrations would need to be tested. Therefore, the current experiments do not directly address excitability of the RVLM.

The increased response to BIC is likely due to enhanced GABAergic inhibition in the RVLM in HU rats rather than differences in effectiveness of BIC to block GABA<sub>A</sub> receptors. The volume of bicuculline utilized (90 nl) was sufficient to elicit maximal pressor responses to GABA<sub>A</sub> blockade in the RVLM, as was indicated by the observation that bilateral microinjection of 120 nl of BIC did not produce any greater increase in MAP, HR, or RSNA than 90 nl. In addition, this concentration and volume of BIC was sufficient to block the response to endogenously released GABA in both control and HU animals. This conclusion is supported by data indicating that the depressor and sympathoinhibitory response to GABA release into the RVLM elicited through glutamate microinjection (10 mM, 30 nl) into the CVLM was completely blocked in HU rats and even produced a small pressor response in control rats (see Fig. 2). This blockade was not due to nonspecific pressure or volume effects, because bilateral microinjection of 90 nl of aCSF into the RVLM did not alter the depressor and sympathoinhibitory responses to CVLM stimulation. Thus it appears that the volume and concentration of bicuculline utilized was sufficient to produce maximal blockade of GABA<sub>A</sub> receptors in the RVLM without eliciting any nonspecific effects due to volume or spread of injectate beyond the RVLM.

During bilateral CVLM inhibition, blockade of GABA<sub>A</sub> receptors in the RVLM produced a further significant increase in MAP and RSNA values in both groups. This increase in MAP and RSNA was significantly greater in HU rats (see Fig. 5). This enhanced response suggests that GABA<sub>A</sub> inhibition within the RVLM that originates at least in part from a source other than the CVLM is altered after cardiovascular deconditioning.

An interesting aspect of the response to bicuculline during CVLM blockade is that the overall effect appears to be greater than the response to bicuculline alone. There are several possible explanations for this. The RVLM appears to be under tonic inhibition from inputs mediated by other than just GABA<sub>A</sub> receptors. For example, GABA<sub>B</sub> receptors are present in RVLM, and activation of these receptors produces depressor responses (10). Other investigators (1, 22) have suggested that GABA<sub>B</sub> receptors provide tonic inhibition of the RVLM, because blockade of these receptors within RVLM results in increases in arterial pressure. Recent evidence (22) suggests that the CVLM exerts inhibitory influences on the RVLM that, in addition to GABA<sub>A</sub> receptors, are mediated by both GABA<sub>B</sub> receptors and other inhibitory input from CVLM. Thus the pressor and sympathoexcitatory response to inhibition of the CVLM involves more than removal of only GABA<sub>A</sub>-mediated inhibition of the RVLM. Under these conditions, it appears reasonable that the effects of CVLM blockade and subsequent blockade of GABA<sub>A</sub> receptors within RVLM are greater than the effects of GABA<sub>A</sub> receptor inhibition alone.

The enhanced response to RVLM injection of bicuculline after CVLM blockade is likely due to a difference in remaining GABA<sub>A</sub> inhibition in the RVLM rather than the effectiveness of kainic acid to produce a complete inhibition of cell bodies within the CVLM. The concentration and volume of kainic acid utilized elicited maximal inhibition of the CVLM. First, 120 nl of glutamate was microinjected into the CVLM following 90 nl of kainic acid administration to ensure that the CVLM was functionally inhibited. The microinjection of this large volume of glutamate did not induce any effects after kainic acid, which indicates that CVLM inhibition was complete. Second, this volume and concentration of kainic acid (5 mM, 90 nl) was sufficient to block the depressor and sympathoinhibitory effects produced by glutamate (10 mM, 30 nl) microinjection into the NTS in both groups (see Fig. 3). Microinjection of the same volume of vehicle (90 nl of aCSF) did not block the depressor and sympathoinhibitory responses to NTS stimulation, which indicates that this blockade was not due to any nonspecific volume or pressure effects. These data indicate that CVLM blockade was complete in both groups, and
ineffective blockade cannot account for the enhanced response to further GABA_A blockade in the RVLM in HU rats.

Recovery from kainic acid microinjection into the CVLM was variable. Therefore, we were not able to randomize the order of interventions in this study; the protocol required that microinjection of BIC into the RVLM precede kainic acid microinjection into the CVLM. Several experiments were performed to control for the required sequence of this protocol. We performed two bilateral microinjections of BIC into the RVLM in a separate group of control animals. In this group of animals, responses to the second microinjection of BIC were not significantly different from the first either in terms of the absolute or relative changes in MAP, HR, and RSNA. Recovery of all hemodynamic parameters was complete. In addition, there were no significant differences in the response to either the first or second microinjection of BIC compared to the initial BIC response in the control animals that were subjected to the entire protocol.

It was possible that prior application of BIC into the RVLM had an effect on the subsequent microinjection of kainic acid into the CVLM. Also, because the protocol required that a second microinjection of BIC be applied at the peak pressor and sympathoexcitatory response to CVLM inhibition (kainic acid, CVLM), we wanted to confirm the time course of this peak response. Therefore, in separate groups of control and HU animals, kainic acid was bilaterally microinjected into the CVLM (5 mM, 90 nl) alone. This was done in the absence of any microinjections before or after kainic acid administration with the exception of glutamate (10 mM, 30 nl), which was used to functionally identify the CVLM. The pressor and sympathoexcitatory responses to kainic acid were not significantly different from those in the groups that required microinjection of BIC before bilateral CVLM inhibition. In addition, the increases in MAP, HR, and RSNA as well as the time course were not different between control or HU animals. These data confirm that 1) prior application of BIC in the RVLM had no effect on the response to subsequent CVLM inhibition, and 2) the time course of CVLM inhibition was such that the pressor and sympathoexcitatory response had developed fully before subsequent GABA_A blockade was performed in the RVLM. In addition, these data provide additional evidence that the overall influence of inputs from the CVLM is not different between control and HU animals.

The data indicate that GABAergic inhibition of the RVLM is augmented after cardiovascular deconditioning in rodents. In addition, this increased GABAergic inhibition appears to originate at least in part from a source or sources other than the CVLM. These data are consistent with our previous findings (32, 33), which indicate that the reflex increase in sympathetic nervous system activity in response to hypotension is attenuated in HU rats. When considering that the attenuation in baroreflex control of sympathetic nerve activity occurs during hypotension, when baroreceptor-mediated GABAergic inhibition of the RVLM is withdrawn, it is logical that the increased inhibition would originate from sources other than barosensitive CVLM neurons. However, the CVLM includes both barosensitive and baro-insensitive GABAergic neurons that project to the RVLM (11). Data indicate that when the entire CVLM is blocked by kainic acid, the resulting pressor response is not different between control and HU rats. This effect is the integrated response to removal of both barosensitive and baro-insensitive GABAergic inputs as well as other inhibitory inputs via GABA_B or other mechanisms (11, 22, 36). In addition, any excitatory influences from CVLM also would be eliminated. Thus the overall influence of the CVLM does not appear to be different following hindlimb unloading. However, we cannot eliminate the possibility of specific effects within the CVLM. Additional studies would be required to evaluate potential differences in these inputs due to cardiovascular deconditioning.

Although the data in the present study indicate a greater GABA_A inhibition of the RVLM from sources other than the CVLM, this may not completely account for blunted baroreflex-mediated sympathoexcitation following HU. As indicated above, the current study does not allow us to completely eliminate a potential role for the CVLM. It also is possible that excitatory influences on RVLM neurons may be reduced and further contribute to the attenuated increases in sympathetic nerve activity in response to arterial baroreflex unloading. Among the possible neurotransmitters known to exert excitatory effects in the RVLM are angiotensin II, glutamate, substance P, and acetylcholine (18, 19, 26, 52). Future studies will evaluate a possible attenuation in the excitatory effects of these neurotransmitters on RVLM neurons following HU.

There is increasing evidence that an excitatory projection from CVLM to RVLM exists in the rat (16, 36). This response is unmasked by stimulating the CVLM after removal of tonic GABAergic input into the RVLM and appears to be under tonic glutamatergic and GABAergic control (36). Data in the current study are consistent with these effects as activation of the CVLM after bilateral microinjection of the GABA_A antagonist BIC into the RVLM revealed a small pressor and sympathoexcitatory response in control animals (see Fig. 2). This response was not seen in HU animals, although the difference in this response between groups was not significant. This raises the possibility that there may be an attenuation in the CVLM-mediated excitation of RVLM after hindlimb unloading. Alternatively, the balance between non-GABA_A and excitatory inputs from the CVLM to the RVLM may differ between control and HU rats such that non-GABA_A influences offset the excitatory influence in HU but not control rats.

The source of enhanced GABAergic inhibition after HU is unknown. It is possible that a region other than the CVLM inhibits RVLM neurons via direct inhibitory GABAergic projections. Alternatively, the enhanced GABAergic inhibition may be due partly to enhanced...
activation of local GABAergic interneurons in the RVLM. For example, stimulation of the midline raphe produces both inhibition and excitation of sympathetic nervous system activity and RVLM unit activity (29, 30). The inhibition of RVLM neurons produced from midline raphe stimulation is blocked by BIC, which indicates that this effect is GABA A receptor mediated (29). The time course of these effects is consistent with an excitatory projection from midline raphe activating a local GABAergic interneuron in the RVLM to produce sympathoinhibitory effects. In addition, sympathoinhibition evoked from caudal midline raphe has been reported to be blocked following BIC microinjection in the RVLM (7). Other possible areas of the brain that may act within a similar scenario to alter GABAergic input into the RVLM could include the periaqueductal gray (49) or anterior hypothalamus (50).

Another possible explanation for these results is that a change at the receptor level contributes to enhanced GABA A-mediated inhibition. For example, it is possible that hindlimb unloading results in upregulation in the number of GABA A receptors in the RVLM, an alteration in GABA A receptor responsiveness, or a change in intracellular signaling pathways. A global change in GABA A receptors in the RVLM is unlikely for two reasons. First, the response to endogenous release of GABA into the RVLM evoked through CVLM stimulation was not different between groups. Second, the pressor and sympathoexcitatory response to CVLM blockade was not different between groups. If global changes in GABA A receptor responsiveness or intracellular signaling pathways were present following hindlimb unloading, then endogenous stimulation or removal of tonic GABAergic input into the RVLM through CVLM blockade should elicit different hemodynamic effects between groups. Nevertheless, because the CVLM exerts influences more than simply through GABA A mechanisms, we cannot completely eliminate potential alterations within the CVLM following cardiovascular deconditioning.

In summary, previous work indicates that attenuated baroreflex-mediated increases in RSNA after hindlimb unloading are due to changes in the central nervous system component of the arterial baroreflex (32, 33). Data from the current study indicate greater GABA A receptor-mediated inhibition of RVLM neurons following cardiovascular deconditioning due to hindlimb unloading. The source of this greater GABAergic inhibition is unknown but arises at least in part from sources other than the CVLM. These data suggest that after cardiovascular deconditioning in rodents, the attenuated reflex sympathoexcitation in response to hypotension is associated with augmented GABAergic inhibition of RVLM neurons mediated through sources other than the CVLM.

Perspectives

Orthostatic intolerance is a common problem associated with prolonged bedrest or exposure to microgravity in humans (4, 8). Previous data from our laboratory indicate a significant attenuation in the ability to reflexively increase sympathetic nervous system activity in response to a hypotensive stimulus (32). Baroreflex activation of the sympathetic nervous system mediating increases in peripheral vascular resistance is of primary importance for maintaining arterial pressure during an orthostatic challenge (40, 48). Thus arterial baroreflex dysfunction may be a mechanism responsible for the orthostatic intolerance following cardiovascular deconditioning. Data from the current study suggest that after cardiovascular deconditioning, RVLM-evoked increases in sympathetic nervous system activity are attenuated due at least in part to greater GABA A-mediated inhibition originating from a source other than the CVLM. It is possible that this increased GABA A inhibition prevents sympathetic nervous system activity from increasing total peripheral resistance to a level required to compensate for orthostatic stress. These results provide evidence for dysfunction of a specific neurotransmitter system which may be related to orthostatic intolerance commonly experienced following cardiovascular deconditioning.

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