Abstract—Chronic heart failure (CHF) is characterized by increased sympathetic tone. The glutamatergic input in the rostral ventrolateral medulla (RVLM), which is a key region involved in sympathetic outflow, seems not to be involved in the generation of sympathetic tone in the normal state. The aim of this study was to determine the role of the RVLM glutamate receptors in the generation of sympathetic tone in CHF. CHF was produced by coronary artery ligation. Bilateral microinjection of the glutamate receptor antagonist kynurenic acid, the N-methyl-d-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoate, or the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione into the RVLM dose-dependently reduced resting blood pressure and renal sympathetic nerve activity in CHF but not in sham rats. Picoinjection of kynurenic acid (100 pmol in 5 nL) significantly decreased the basal discharge by 47% in 25 RVLM presympathetic neurons in CHF rats. In contrast, kynurenic acid had no effect on the discharge in all 22 of the RVLM presympathetic neurons tested in sham rats. These data suggest that upregulated glutamate receptors, including NMDA and non-NMDA, in the RVLM are involved in tonic control of elevated sympathetic tone in CHF.

Key Words: sympathoexcitation ■ glutamate receptors ■ micro/picojection ■ extracellular recording ■ presympathetic neuron

It is well known that the rostral ventrolateral medulla (RVLM) is a common pathway for central control of sympathetic outflow and plays a crucial role in tonic and reflex neural control of cardiovascular activity. The RVLM contains presympathetic neurons that have spontaneous activity and directly project to the spinal cord. The basal activity of the RVLM presympathetic neurons is a major mechanism responsible for the generation of resting blood pressure (BP) and sympathetic outflow. In the RVLM, however, glutamatergic input does not appear to be involved in tonic neural control of resting cardiovascular activity, because application of the glutamate receptor antagonist kynurenic acid (KYN) into the RVLM has little effect on resting BP and sympathetic outflow. The excitatory amino acid (EAA) glutamate has been demonstrated to play an important role in cardiovascular regulation. In the RVLM, glutamatergic input does not appear to be involved in tonic neural control of resting cardiovascular activity, because application of the glutamate receptor antagonist kynurenic acid (KYN) into the RVLM has little effect on resting BP and sympathetic outflow. Interestingly, Ito and Sved hypothesized that tonic excitation of EAA inputs may not be obvious because of the balance between excitatory and inhibitory inputs in the RVLM. KYN injected into the RVLM causes a profound fall in resting BP after injection of the γ-aminobutyric acid receptor agonist muscimol into the caudal ventrolateral medulla, which is a major inhibitory input to the RVLM. In some hypertension models, in which there is sympathetic hyperactivity in CHF, there is little evidence supporting the idea that EAA inputs in the RVLM mediate the generation of elevated sympathetic tone in CHF. Therefore, this study was designed to determine the role of RVLM glutamatergic input in the maintenance of resting BP, sympathetic outflow, and basal activity of the RVLM presympathetic neurons in CHF.

Methods

Animals

Male Sprague-Dawley rats weighing between 180 and 200 g were used in these experiments. All of the experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures are similar to those described in our previous studies.

Chronic heart failure (CHF) is characterized by elevated sympathetic outflow, which is closely related to a poor prognosis in patients with CHF. Sympathoexcitation may be an effect intended to compensate for the cardiac pump failure but may ultimately exacerbate the CHF condition. Altered synaptic transmission in cardiovascular regulatory centers may contribute to elevated sympathetic tone. For example, the glutamatergic mediated responses in the paraventricular nucleus are upregulated and γ-aminobutyric acid receptors downregulated in CHF. In the RVLM, sympathetic hyperactivity in CHF may be related to excitatory and inhibitory inputs, such as angiotensin II (Ang II) and nitrergic transmission. However, there is little evidence supporting the idea that EAA inputs in the RVLM mediate the generation of elevated sympathetic tone in CHF. Therefore, this study was designed to determine the role of RVLM glutamatergic input in the maintenance of resting BP, sympathetic outflow, and basal activity of the RVLM presympathetic neurons in CHF.
CHF Model
CHF was produced by left coronary artery ligation; sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation. In this study, cardiac function in all of the experimental animals was measured by echocardiography 6 weeks after coronary ligation or sham operation. Hemodynamic measurements of sham and CHF rats were similar to those described previously.\(^\text{13}\)

General Surgical Procedures
In brief, 6 to 8 weeks after coronary ligation or sham operation, under anesthesia with urethane (800 mg/kg IP) and \(\alpha\)-chlorealose (40 mg/kg IP), the trachea was cannulated to facilitate mechanical respiration. The right common carotid artery was catheterized for BP measurement. Heart rate (HR) was derived from the BP pulse by a PowerLab Chart system (ADInstruments). A femoral vein was cannulated for supplemental anesthesia (\(\alpha\)-chlorealose, 10 mg/kg) and neuromuscular blockade (pancuronium bromide, 1 mg/kg). Body temperature was kept at 37°C.

Recording of Renal Sympathetic Nerve Activity
The renal sympathetic nerves were exposed and placed on a pair of recording electrodes. The distal terminal of the renal nerve was cut to avoid afferent activity. The renal sympathetic nerve activity (RSNA) was amplified and recorded. Baseline RSNA was taken as 100% from the absolute value after the noise level (measured by intravenous hexamethonium) was subtracted.

RVLM Microinjection
The rats were placed in a stereotaxic instrument, and the dorsal surface of the medulla was surgically exposed. Microinjections were made from a triple-barrel micropipette and performed by a 4-channel pressure ejector (PM2000B, WPI). The RVLM (2.0- to 2.5-mm rostral and 1.8- to 2.1-mm lateral to calamus scriptorius and 2.8- to 3.2-mm ventral to the dorsal surface of the medulla) was functionally identified by a prescor response to l-glutamate (1 nmol) ≥30 minutes before subsequent injection of agents. The injections (100 nL) were made over a 10-s period. All of the injection agents (L-glutamate, KYN, the \(N\)-methyl-D-aspartate [NMDA] receptor antagonist D-2-amino-5-phosphonopentanoate [D-AP5], and the non-NMDA antagonist 6-cyano-7-nitroquinoloxaline-2,3-dione [CNQX]) were purchased from Sigma. All of the chemicals were initially dissolved based on previous studies\(^\text{3,6,9,15}\) and with final dilutions made up in artificial cerebrospinal fluid (pH 7.4). The time interval between bilateral injections was within 3 minutes. One barrel of the multibarrel pipette was filled by 2% Pontamine sky blue for marking the injection site.

Extracellular Recording and Picoinjection
For RVLM unit recording, a laminectomy (T1–3) was performed, and a stimulating electrode was placed in the dorsolateral funiculus of spinal segment T2 to allow for antidromic stimulation (pulse width 0.5 to 1.0 ms; current intensity: 0.1 to 0.5 mA). A vascular occluder (Harvard) connected to a syringe (5 mL) was placed around the descending thoracic aorta above the diaphragm for baroreceptor stimulation. An increase in BP (0 to 50 mm Hg) was caused by gradual occlusion of the aorta. One barrel of a 5-barrel micropipette (20- to 30-μm diameter) containing a carbon filament (7 μm in diameter) was connected to a high impedance amplifier (Dagan) for extracellular recording. The spontaneous action potentials were amplified and discriminated for a standard pulse. This pulse output was proportional to the number of spikes per second. As shown in Figure 1, the presympathetic unit was electrophysiologically identified by both barosensitivity and spinal cord projection.\(^\text{16,17}\) The recording site was marked at the end of the experiment by dye injection (5 nL).

Histological Analysis
At the end of the experiments, the brains were removed from the skulls, placed in 10% formalin, and sectioned to verify the microinjection and recording sites. In this study, the centers for microinjection and neuronal recording were demonstrated to be accurately located within the range of the RVLM according to the standard atlas of Paxinos and Watson,\(^\text{21}\) as described previously.\(^\text{15}\)

Data Analysis
All of the values are expressed as means±SEs. Because of the large variability of baseline activity of RSNA and neuronal discharge rate, percentage of change was used for comparison before and after treatments. Student \(t\) test or ANOVA (1- or 2-way) was used for statistical analyses by Sigmasstat software (version 3.5). A value of \(P<0.05\) was considered statistically significant.

Results
Effects of Injections of Glutamate Receptor Antagonists Into the RVLM
The baseline values for mean arterial pressure (MAP) and HR in experimental groups are shown in Table 1. There were no significant differences between groups. In sham rats, bilateral
injection of KYN (0.5 and 5.0 nmol), D-AP5 (0.5 and 5.0 nmol), or CNQX (20 and 200 pmol) into the RVLM did not have a significant effect on baseline MAP, HR, and RSNA compared with vehicle (100 nL of artificial cerebrospinal fluid) injection (Table 2). However, bilateral injection of these glutamate receptor antagonists into the RVLM dose-dependently caused a significant fall in MAP and RSNA in CHF rats (Figure 2). A significant decrease in HR was found only at the 5-nmol KYN injection dose. The peak effects of injection of the antagonists into the RVLM were reached within 5 to 10 minutes and gradually returned to control levels within ~30 minutes.

Effects of Picoinjection of KYN on RVLM Presympathetic Neurons

A total of 22 (3.6 to 22.4 spikes per second) and 25 (4.1 to 29.2 spikes per second) presympathetic neurons in the RVLM were identified (Figure 1) from 9 sham rats and 11 CHF rats, respectively. The averaged baseline discharge of presympathetic neurons was higher in CHF than in sham rats (13.2 ± 1.0 versus 10.4 ± 0.6 spikes per second; *P < 0.05). Figures 3 and 4 show the original tracings and mean data of the discharge of the RVLM presympathetic neurons in response to picoinjected KYN, respectively. In CHF but not in sham rats, picoinjection of KYN produced a profound fall in spontaneous discharge compared with vehicle injection. This decrease began within 10 seconds of KYN application and persisted for 5 to 10 minutes. No significant change in baseline BP was observed after picoinjection of KYN in sham rats (90 ± 3 versus 91 ± 4 mm Hg) and CHF rats (87 ± 4 versus 84 ± 5 mm Hg) rats.

Discussion

The major observation of this study is that blockade of RVLM glutamate receptors produces a significant fall in resting BP, RSNA, and discharge of presympathetic neurons in CHF rats. We conclude that upregulated glutamate receptors in the RVLM contribute to excitatory sympathetic tone in CHF.

Although EAA in the central nervous system has been widely demonstrated to play an important role in control of cardiovascular activity, injection of EAA receptor antagonists into the RVLM has little effect on resting BP in the normal condition.3–5 Interestingly, the current study showed that, in CHF but not in sham rats, KYN microinjected into the RVLM significantly decreased resting BP and RSNA, suggesting that glutamate receptors in the RVLM participate in generating sympathetic tone in CHF rats. We noted that the dose (5.0 nmol) for KYN injection was higher in this study compared with that (2.7 nmol) used in previous studies.3,6,7 We believe that this KYN dose was sufficient to effectively block ionotropic glutamate receptors in the RVLM. The present data show that injection of 5.0 nmol of KYN had no effect on resting BP in sham rats, which is consistent with previous studies.3–5 We confirmed the notion that glutamate receptors in the RVLM are not involved in the maintenance of sympathetic tone in the normal state. In CHF, however, KYN injected into the RVLM significantly reduced resting BP and RSNA. Because this effect is dose dependent, we concluded that the KYN effects may be selectively dependent on

### Table 1. Baseline Levels of MAP and HR in the Different Groups of Rats Before RVLM Microinjections

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham</th>
<th></th>
<th>CHF</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>MAP, mm Hg</td>
<td>HR, bpm</td>
<td>n</td>
</tr>
<tr>
<td>Vehicle, 100 nL</td>
<td>5</td>
<td>93±3</td>
<td>351±15</td>
<td>5</td>
</tr>
<tr>
<td>KYN, 0.5 nmol</td>
<td>6</td>
<td>92±4</td>
<td>362±14</td>
<td>7</td>
</tr>
<tr>
<td>KYN, 5.0 nmol</td>
<td>6</td>
<td>89±3</td>
<td>349±17</td>
<td>7</td>
</tr>
<tr>
<td>D-AP5, 0.5 nmol</td>
<td>6</td>
<td>93±4</td>
<td>337±15</td>
<td>6</td>
</tr>
<tr>
<td>D-AP5, 5.0 nmol</td>
<td>6</td>
<td>91±4</td>
<td>368±17</td>
<td>6</td>
</tr>
<tr>
<td>CNQX, 20 pmol</td>
<td>5</td>
<td>87±5</td>
<td>331±15</td>
<td>6</td>
</tr>
<tr>
<td>CNQX, 200 pmol</td>
<td>5</td>
<td>93±4</td>
<td>354±13</td>
<td>6</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEs. n=5 to 7 for each group.

### Table 2. Peak Changes in MAP, HR, and RSNA Induced by Microinjection of Glutamate Receptor Antagonists Into the RVLM in Sham and CHF Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔMAP, mm Hg</th>
<th>ΔHR, bpm</th>
<th>ΔRSNA, %</th>
<th>ΔMAP, mm Hg</th>
<th>ΔHR, bpm</th>
<th>ΔRSNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle, 100 nL</td>
<td>1.3±0.7</td>
<td>4.8±2.7</td>
<td>3.3±1.9</td>
<td>1.1±0.5</td>
<td>3.4±1.7</td>
<td>2.1±1.4</td>
</tr>
<tr>
<td>KYN, 0.5 nmol</td>
<td>1.1±0.4</td>
<td>3.9±2.1</td>
<td>3.1±2.2</td>
<td>-3.7±1.5</td>
<td>-17.1±0.9†</td>
<td>-10.1±3.1†</td>
</tr>
<tr>
<td>KYN, 5.0 nmol</td>
<td>-2.1±0.5</td>
<td>3.4±1.7</td>
<td>-3.7±1.5</td>
<td>-5.3±0.6*</td>
<td>-5.2±2.1</td>
<td>-14.3±2.3*</td>
</tr>
<tr>
<td>D-AP5, 0.5 nmol</td>
<td>1.7±0.5</td>
<td>6.2±3.1</td>
<td>2.1±1.4</td>
<td>-4.1±2.8</td>
<td>-6.7±0.4*</td>
<td>-4.1±2.8</td>
</tr>
<tr>
<td>D-AP5, 5.0 nmol</td>
<td>-0.9±0.6</td>
<td>-2.7±2.4</td>
<td>-2.2±3.7</td>
<td>-14.1±0.6†</td>
<td>-4.1±3.1</td>
<td>-20.1±3.8†</td>
</tr>
<tr>
<td>CNQX, 20 pmol</td>
<td>-1.5±0.4</td>
<td>2.8±2.6</td>
<td>-4.1±2.8</td>
<td>-6.7±0.4*</td>
<td>3.4±3.3</td>
<td>-10.1±2.7*</td>
</tr>
<tr>
<td>CNQX, 200 pmol</td>
<td>-2.1±0.8</td>
<td>3.4±2.3</td>
<td>-3.9±2.2</td>
<td>-12.1±0.7†</td>
<td>2.4±1.9</td>
<td>-17.1±3.1†</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEs. n=5 to 7 for each group.

*P<0.05 vs vehicle and sham.
†P<0.05 vs low-dose group.
glutamate receptors. Importantly, the role of glutamate receptors on sympathetic tone was further confirmed at the level of the RVLM presympathetic neurons. It is well known that sympathetic outflow in the RVLM is mainly dependent on spontaneous activity of presympathetic neurons. The electrophysiological evidence clearly showed that picoinjection of KYN significantly inhibited resting discharge of presympathetic neurons in CHF but not in sham rats. There may be a limitation that a relatively small number of units (n=22 in sham and n=25 in CHF) were tested in the study. The large variability of activity of neurons may result in, to some extent, the sampling bias of unit recording in the RVLM. However, the present study strongly supports the idea that glutamatergic input in the RVLM plays an important role in the generation of sympathetic tone in the CHF state.

Ionotropic glutamate receptor subtypes include NMDA and non-NMDA ([alpha]-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate) and have been demonstrated to be involved in cardiovascular regulation in the RVLM. In CHF but not in sham rats, we found that bilateral injection of their corresponding antagonists D-AP5 and CNQX into the RVLM had a similar inhibitory effect on resting BP and RSNA, suggesting that NMDA and non-NMDA receptors in the RVLM may have relatively similar importance in the generation of tonic sympathetic outflow in CHF rats. CNQX is a potent non-NMDA receptor antagonist, and at a high dose also blocks the glycine modulatory site on NMDA receptor complex. However, it has been reported that 200 pmol of CNQX selectively blocks non-NMDA receptors without affecting NMDA receptors. Based on the present results, we suggest that both NMDA and non-NMDA receptors in the RVLM are involved in tonic excitatory generation in CHF. It is possible that upregulation in glutamate receptor expression or increase in glutamate release in the RVLM accounts for a significant fall in resting BP, RSNA, and discharge of presympathetic neurons in response to glutamate receptor blockade in the RVLM in CHF.

The present observations of increased glutamatergic input in the RVLM provide new information for our understanding of elevated sympathetic tone in CHF. Sympathetic activity has been widely demonstrated to be increased in CHF rats. Interestingly, we found that spontaneous basal discharge of the RVLM presympathetic neurons was significantly higher in CHF than in sham rats, indicating that sympathetic outflow is increased in CHF. Based on the hypothesis of Ito, Sved, and colleagues it appears that the contribution of glutamatergic inputs to tonic sympathetic outflow depends on the balance between excitatory and inhibitory inputs in the RVLM. Therefore, it is reasonable to speculate that upregulated excitatory inputs and/or downregulated inhibitory inputs would interrupt this balance and trigger tonic effects of glutamatergic inputs. Although this
hypothesis was challenged by a study from Horiuchi et al., in which excitatory inputs are believed to be predominant over inhibitory inputs. Based on our data, glutamatergic input in the RVLM is upregulated, indicating an imbalance between excitatory and inhibitory inputs in CHF. In fact, the contribution of non-EAA excitatory inputs to elevated sympathetic tone should also be considered in CHF. For example, microinjection of Ang II receptor type 1 antagonists into the RVLM significantly reduces resting BP and RSNA in hypertension and CHF but not in normal animals, suggesting that altered Ang II in the RVLM may be involved in elevated sympathetic tone. Because Ang II is increased and Ang II receptors upregulated in the RVLM in CHF, Ang II is a possible mechanism responsible for increased sympathetic tone. On the other hand, it is not clear whether inhibitory inputs in the RVLM are downregulated in CHF. We realize that a lack of observation of γ-aminobutyric acid receptor expression in the RVLM might be a limitation in this study. However, other inhibitory neurotransmitters or modulators such as NO are downregulated in CHF. The evidence from the paraventricular nucleus has shown that NMDA receptors are upregulated and γ-aminobutyric acid receptors reduced in CHF. Therefore, it is possible that the imbalance between excitatory and inhibitory inputs in the RVLM, at least upregulated glutamate input as reported in this work, contributes to elevated sympathetic outflow in CHF.

Perspectives
In CHF, an overall elevation in sympathetic outflow is a common finding. Sympathetic hyperactivity is a compensatory mechanism for cardiac dysfunction but ultimately exacerbates the development of this disease. Understanding the mechanism responsible for altered sympathetic outflow in CHF may provide some useful information for exploring or improving the therapeutic strategies. Taken together with previous evidence, EAA-mediated excitatory inputs were confirmed to have an excitatory effect on tonic sympathetic outflow in sympathoexcitatory diseases, such as hypertension and CHF.

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Disclosures
None.

References
Tonic Glutamatergic Input in the Rostral Ventrolateral Medulla Is Increased in Rats With Chronic Heart Failure

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