Rostral Ventrolateral Medulla Neurons of Neonatal Wistar-Kyoto and Spontaneously Hypertensive Rats

Tomokazu Matsuura, Hiroo Kumagai, Akira Kawai, Hiroshi Onimaru, Masaki Imai, Naoki Oshima, Katsufumi Sakata, Takao Saruta

Abstract—We compared the electrophysiological properties of neurons in the rostral ventrolateral medulla (RVLM) of neonatal Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR), and responses to angiotensin II and its type 1 receptor antagonist candesartan. Using the whole-cell patch-clamp technique, we examined the characteristics of RVLM neurons in brainstem–spinal cord preparations with a preserved sympathetic neuronal network. The baseline membrane potential of irregularly firing neurons was less negative (−50.1±0.6 versus −52.0±0.6 mV) and the firing rate was faster (3.0±0.2 versus 2.0±0.2 Hz) in SHR (n=56) than in WKY (n=38). Superfusion with angiotensin II (6 μmol/L) significantly depolarized the RVLM bulbospinal neurons in SHR (5.4±1.1 mV, n=15) but not in WKY. In contrast, candesartan (0.12 μmol/L) induced a significant membrane hyperpolarization (−3.7±0.4 mV; n=14) and a decrease in the firing rate in RVLM bulbospinal neurons of SHR but not of WKY. These results suggest that endogenously generated angiotensin II binds to type 1 receptors on RVLM bulbospinal neurons, thus tonically contributing to a higher membrane potential and a faster firing rate in SHR. The electrophysiological properties of RVLM neurons and their responses to angiotensin II and candesartan differ between neonatal WKY and SHR. These differences in RVLM neurons suggest a mechanism that possibly leads to elevation in blood pressure. (Hypertension. 2002;40:560-565.)

Key Words: brain ■ rostral ventrolateral medulla ■ angiotensin II ■ receptors, angiotensin ■ candesartan ■ hyperpolarization

Rostral ventrolateral medulla (RVLM) neurons are located at a pivotal site involved in the baroreflex pathway and play a key role in controlling peripheral sympathetic nerve activity (SNA) and blood pressure (BP).1-3 We have reported impairment of the baroreflex function in hypertension based on recordings of renal SNA (RSNA) in conscious rabbits and rats and on vagal afferent nerve activity.4,5 We have also confirmed that the angiotensin (Ang) II type 1 (AT₁) receptor antagonist candesartan improves the impaired baroreflex in conscious rats with congestive heart failure.6 Earlier studies examined the responses of BP and SNA to Ang II and Ang II antagonists microinjected into the RVLM of normotensive and hypertensive animals,7-11 because the RVLM area contains Ang II–immunoreactive nerve terminals and a moderately high density of AT₁ receptors.12 Microinjection of Ang II increased BP and SNA,7-10 whereas candesartan reduced BP, RSNA, and heart rate (HR).11 However, the precise neuronal mechanisms by which RVLM neurons regulate SNA and BP and how these neurons are involved in the development of hypertension have not been fully elucidated. The firing rate of extracellular units in RVLM neurons was faster in adult spontaneously hypertensive rats (SHR) than in Wistar-Kyoto rats (WKY) in vivo.13 Iontophoretic application of Ang II increased the extracellular activity of 30% of RVLM neurons in both strains, and the increase was greater in SHR.14 However, very few studies have compared the intracellular properties of RVLM neurons and responses to Ang II and its antagonist between WKY and SHR. Recently, we determined the intracellular electrophysiological characteristics of RVLM bulbospinal neurons in neonatal WKY using brainstem–spinal cord preparation, in which the neuronal network is preserved from the vagal afferents to the sympathetic efferents exiting the intermediolateral cell column.15

In the present study, we performed intracellular recordings (whole-cell patch-clamp technique) of RVLM neurons in WKY and SHR during superfusion rather than microinjection of Ang II or candesartan to precisely understand the role of the RVLM neurons. Although BP of SHR increases above that of WKY sometime between 3 and 4 weeks of age,16 RVLM neuron activity cannot be assumed to be identical in neonatal WKY and SHR because the difference between the
Materials and Methods

Intracellular Recordings
The experiments were performed on brainstem–spinal cord prepara-
tions. All experimental protocols were approved by our facil-
ity’s institutional review board. WKY and SHR pups (1 to 4 days old) were deeply anesthetized with ether, and the brainstem and spinal cord were isolated at the Th10 level. The preparation was continuously superfused with artificial cerebrospinal fluid. The solution was maintained at 25°C and equilibrated with 90% O₂, 5% N₂, and 5% CO₂ (pH, 7.3 to 7.4). The patch pipettes had a tip diameter of 1.8 to 2.0 μm and a resistance of 4 to 8 Mohm. The electrode solution consisted of (in mmol/L) potassium gluconate 130, HEPES 10, EGTA 10, CaCl₂ 1, and MgCl₂ 1, adjusted to a pH of 7.2 to 7.4 with potassium hydroxide (KOH). The electrode tip was m and a resistance of 4 to 8 Mohm. The electrode solution consisted of (in mmol/L) potassium gluconate 130, HEPES 10, EGTA 10, CaCl₂ 1, and MgCl₂ 1, adjusted to a pH of 7.2 to 7.4 with potassium hydroxide (KOH). The electrode tip was filled with a solution containing 1% Lucifer-yellow (Aldrich Chemical) and adenosine 5’-triphosphate (Sigma).

Before obtaining intracellular recordings, we observed the firing pattern of the target neurons by extracellular recording. After forming a gigahm seal with gentle suction, a single-shot hyperpolarizing pulse (0.6 to 0.9 nA; 30 ms) was applied to rupture the membrane. Phrenic nerve activity was recorded simultaneously (Figure 1a). The RVLM neurons with discharges that were synchronized with phrenic activity were assumed to be respiratory neu-
rons and were recognized with phrenic activity were assumed to be respiratory neu-
rons and were excluded from this study. Membrane potentials were corrected for junctional potentials at the tip of the pipette (−11 mV). We recorded 1 RVLM neuron from 1 preparation. After obtaining the intracellular recordings, membrane potentials were recorded by the current-clamp technique (from −100 to 20 pA, by 20-pA increments; 500 ms), and input resistance was calculated from the current-voltage curve. Lucifer-yellow spontaneously or ionoto-

Identification of RVLM Bulbospinal Neurons
To determine whether each RVLM neuron monosynaptically proj-
ects to the spinal cord, ie, whether the RVLM neuron recorded is a bulbospinal neuron, we routinely stimulated (5 to 30 V, 0.1 ms, single pulse) the ipsilateral Th2 spinal segment, including the intermediolateral cell column, with a tungsten electrode (30-μm tip diameter) for induction of antidromic action potential in that RVLM neuron²⁵ (Figure 1b).

Drugs and Protocols
All drugs were dissolved in standard artificial cerebrospinal fluid. Drugs and solutions were applied at a speed of 2 to 3 mL/min to the preparation. To examine the effects of Ang II on RVLM bulbospinal neurons, we performed the superfusion with Ang II. Li and Guyenetaved used a bath application of 0.3 to 1.0 μmol/L of Ang II in the slice preparation. We selected a dosage of 6 μmol/L Ang II (human, Sigma), because the distance from the surface of the preparation to the RVLM neurons makes it difficult for Ang II to reach the target neurons. Further, we used 1, 3, and 12 μmol/L of Ang II to see dose responsiveness. We applied Ang II for 20 minutes and observed the response for >40 minutes.

To test whether the effects of Ang II on the RVLM neurons are pre-
or postsynaptic, superfusion with 0.5 μmol/L of tetrodotoxin was started 20 minutes before start of the Ang II superfusion (6 μmol/L). To examine the effects of endogenous Ang II, we performed superfusion with an AT₁ receptor antagonist, candesartan. Candesartan (Takeda Chemical Industries) was dissolved in 1 N NaOH solution, and the pH was adjusted to 7.3 by the addition of HCl. This solution was then dissolved in standard artificial cerebro-
spinal fluid. Few studies have reported the appropriate dose of candesartan to reduce the activity of RVLM bulbospinal neurons. However, because constriction of rabbit aortic preparations elicited by 10 nmol/L Ang II was prevented by 0.1 to 1.0 nmol/L candesar-
tan, we used 0.06 and 0.12 μmol/L of candesartan, dosages presumably capable of antagonizing the effects of 6 μmol/L Ang II. We also used 0.24 μmol/L of candesartan. We applied candesartan for 20 minutes and observed the response for >40 minutes. Finally, to examine whether candesartan prevents the changes produced by Ang II, we applied 6 μmol/L Ang II for 20 minutes, immediately after the end of 20-minute superfusion with 0.12 μmol/L candesar-
tan. In this case, we observed the effect for >40 minutes after the start of Ang II.

To accurately quantify the changes in the firing rate produced by superfusion with Ang II or candesartan, we applied a current to hyperpolarize the membrane of RVLM neurons so that the basal firing rates were kept at ~0.1 to 0.8 Hz before the superfusion.

Statistics
Baseline values of membrane potential, firing rate, and input resistance, and responses to Ang II and candesartan between WKY and SHR and between regularly and irregularly firing neuron were compared with ANCOVA followed by Fisher’s post hoc compar-
sion. Membrane potentials before and after superfusion with Ang II and candesartan were compared with the paired Student t test. All data were expressed as mean±SEM. P<0.05 was considered to be statistically significant.

Results
Electrophysiological Properties of RVLM Neurons
We performed intracellular recordings of RVLM neurons of WKY (n=54) and SHR (n=89). The RVLM neurons of both strains were classified into 3 types: regularly firing neurons, irregularly firing neurons (Figure 2), and silent type neurons (data not shown). Irregularly firing neurons exhibited many
excitatory postsynaptic potentials, whereas regularly firing neurons rarely showed excitatory postsynaptic potentials.

The membrane potential of irregularly firing neurons in SHR was significantly less negative than that in WKY \((P<0.05)\), whereas that of regularly firing neurons was not different (Table). The firing rate of irregularly firing neurons was significantly faster in SHR than in WKY \((P<0.01)\), whereas that of regularly firing neurons was not different. Input resistance between WKY and SHR or between regularly and irregularly firing neurons was not different. The proportion of RVLM bulbospinal neurons, of which we examined the input resistance, was 6 of 20 regularly and 6 of 23 irregularly firing neurons in WKY, and was 10 of 37 regularly and 9 of 38 irregularly firing neurons in SHR. Therefore, this proportion was not different between WKY and SHR, or between regularly and irregularly firing neurons.

**Effect of Ang II on RVLM Neurons**

In protocols using Ang II and candesartan, we report the results of RVLM bulbospinal neuron that showed antidromic action potential. One to 2 minutes after the start of superfusion with 6 \(\mu\)mol/L Ang II, RVLM bulbospinal neuron of SHR depolarized, and the firing rate increased (Figure 3, top). After that, a tendency of repolarization was observed for 2 to 3 minutes, although the membrane potential remained less negative than that at the presuperfusion level. This is accommodation of the neurons to the stimulus of Ang II. Thus, the neuronal response to Ang II was biphasic. We quantified the membrane depolarization of the first phase. The average depolarization of RVLM bulbospinal neurons in SHR during

### Basal Electrophysiological Characteristics of RVLM Neurons and Responses to Ang II and Candesartan

<table>
<thead>
<tr>
<th>Electrophysiological Parameters</th>
<th>Regularly Firing Neurons</th>
<th>Irregularly Firing Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
</tr>
<tr>
<td>Membrane potential, mV</td>
<td>-50.9±1.4</td>
<td>-49.6±0.7</td>
</tr>
<tr>
<td>Firing rate, Hz</td>
<td>4.2±0.3</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>668±70</td>
<td>633±32</td>
</tr>
<tr>
<td>Responses to Ang II (6.0 (\mu)mol/L)</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>Change in membrane potential, mV</td>
<td>1.1±1.0</td>
<td>5.0±0.9*</td>
</tr>
<tr>
<td>Input resistance, % of the control</td>
<td>97.5±5.2</td>
<td>84.5±6.4</td>
</tr>
<tr>
<td>Responses to candesartan (0.12 (\mu)mol/L)</td>
<td>n=4</td>
<td>n=5</td>
</tr>
<tr>
<td>Change in membrane potential, mV</td>
<td>-0.1±0.6</td>
<td>-3.6±0.4*</td>
</tr>
<tr>
<td>Input resistance, % of the control</td>
<td>98.0±6.9</td>
<td>104.6±8.1</td>
</tr>
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</table>

Mean±SEM. Responses to Ang II and candesartan were examined in RVLM bulbospinal neurons that show antidromic action potential.

*\(P<0.05\); †\(P<0.01\) vs WKY in the same type of neuron; ‡\(P<0.01\) vs regularly firing neurons in the same strain.
superfusion with 6 μmol/L Ang II was 5.4±1.1 mV, a significant value compared with that before superfusion (n=15) (Figure 3, bottom). These neurons also depolarized with 3 μmol/L Ang II (2.8±1.1 mV; n=4) and with 12 μmol/L Ang II (7.6±1.3 mV; n=6), whereas they did not depolarize significantly with 1 μmol/L (0.9±1.4 mV; n=5). Therefore, a weak dose-dependency was observed. The magnitude of depolarization of regularly firing neurons did not differ from that of irregularly firing neurons of SHR (Table).

During tetrodotoxin superfusion (n=11) in SHR preparation, the depolarization to 6 μmol/L Ang II was 3.9±1.2 mV, which was significantly smaller than the depolarization without tetrodotoxin. The result suggests that the Ang II–induced depolarization without tetrodotoxin was both pre- and postsynaptic, and at least some of the Ang II action was a direct effect on the RVLM bulbospinal neurons. RVLM nonbulbospinal neurons of SHR (n=15) were significantly depolarized by 5.0±0.8 mV during superfusion with 6 μmol/L Ang II.

Ang II superfusion (6 μmol/L) depolarized the RVLM bulbospinal neurons in 12 WKY preparations and hyperpolarized them in 4 WKY preparations. So, the average change (1.1±0.8 mV; n=16) was not significant. Further, RVLM neurons of WKY did not depolarize significantly with 3 μmol/L (0.4±0.5 mV; n=5) or 12 μmol/L (1.0±1.3 mV; n=6) of Ang II.

The firing rates of irregularly firing neurons of WKY (from 0.5±0.2 to 1.3±0.4 Hz) and SHR (from 0.3±0.1 to 1.7±0.6 Hz) were significantly increased by superfusion with 6 μmol/L of Ang II. The magnitude of the increase in the firing rate was greater in SHR than in WKY on the basis of ANCOVA. On the other hand, because almost all of the regularly firing neurons lost their spontaneous firings when we hyperpolarized the membrane, we did not analyze the changes in the firing rate of the regularly firing neurons.

Effect of Candesartan on RVLM Neurons
Candesartan (0.12 μmol/L) hyperpolarized the RVLM bulbospinal neuron of SHR and reduced the firing rate (Figure 4, top). Average hyperpolarization of RVLM bulbospinal neurons in SHR was −3.7±0.4 mV, a significant value (n=14) (Figure 4, bottom). The effect of candesartan persisted for >40 minutes after its washout. These neurons hyperpolarized with 0.24 μmol/L candesartan by −4.2±0.8 mV (n=6), whereas they did not hyperpolarize with 0.06 μmol/L (n=8). The magnitude of hyperpolarization of regularly firing neurons did not differ from that of irregularly firing neurons of SHR (Table).

Membrane potentials of WKY bulbospinal neurons did not change with any dose of candesartan (0.06 μmol/L, 0.0±0.3 mV [n=6]; 0.12 μmol/L, −0.1±1.3 mV [n=12]; and 0.24 μmol/L, −0.1±1.0 mV [n=6]).

The firing rate of irregularly firing neurons in SHR (from 0.7±0.3 to 0.1±0.3 Hz) was significantly decreased by superfusion with 0.12 μmol/L of candesartan. By contrast, the firing rate in WKY did not change (from 0.3±0.3 to 0.3±0.2 Hz).

Figure 4. Top, Recording of membrane hyperpolarization and the decrease in the firing rate of an RVLM bulbospinal neuron during candesartan (0.12 μmol/L). Bottom, Average changes in membrane potential of RVLM bulbospinal neurons during superfusion with 0.12 μmol/L of candesartan. Values are mean±SEM. *P<0.05 vs before superfusion. Changes in membrane potential during candesartan superfusion are significant as compared with those before the superfusion in SHR (n=14) but not in WKY (n=12).

Effect of Candesartan on Ang II–Induced Depolarization
Pretreatment with 0.12 μmol/L of candesartan significantly prevented the Ang II (6 μmol/L)–induced depolarization in RVLM bulbospinal neurons of SHR (1.0±0.5 mV; n=7, P<0.05). The depolarization in the WKY preparations was not significantly different with (0.9±0.4 mV; n=10) versus without candesartan.

Discussion
From intracellular recordings using the whole-cell patch-clamp technique, we found that the baseline membrane potential was less negative and the firing rate was faster in RVLM irregularly firing neurons of neonatal SHR than those of WKY, whereas the difference was not significant in regularly firing neurons. This is probably owing to the larger number of irregularly firing neurons tested than that of regularly firing neurons. These results are consistent with an earlier in vivo study by Chan et al,13 who reported that the firing rate of extracellular units in RVLM neurons of adult SHR was faster than that of WKY. Dickhout et al16 showed the BPs and HRs of WKY and SHR to be nearly the same until the age of 2 weeks. Thereafter, the HR of SHR became faster from the age of 3 weeks, and the BP of SHR became higher from the age of 4 weeks. Therefore, we identified a change in the electrophysiological properties of RVLM neurons around the time when the BP and HR begin to differ between SHR and WKY.

Superfusion with 6 μmol/L Ang II significantly depolarized all RVLM bulbospinal neurons in SHR in the present...
study. Although 12 of the 16 WKY preparations depolarized, the average change was not significant. The magnitude of the depolarization of regularly firing neurons did not differ from that of the irregularly firing neurons of SHR. AT1 receptors occur in several sites in the medulla oblongata—of which at least two, the nucleus tractus solitarius and the caudal VLM, may influence the activity of RVLM neurons. However, Ang II–induced depolarization was 3.9±1.2 mV during tetrodotoxin superfusion, which blocks the sodium channels, resulting in the inhibition of neurotransmitter release from the presynaptic region. These results suggest that part of the Ang II–induced depolarization without tetrodotoxin (5.4±1.1 mV) was postsynaptic, and at least some of the Ang II action directly affected the RVLM neurons.

Earlier in vivo studies showed that microinjection of Ang II into the RVLM area increases BP and SNA,7–10 and the pressor effect was compared in normotensive and hypertensive rats. Muratani et al8 showed that the pressor effects were similar in adult WKY and SHR. The different results between the studies by Muratani et al8 and our own are explained by differences in the methods and parameters examined. They compared pressor effects in vivo when Ang II was microinjected into the RVLM of adult rats, whereas we compared changes in the membrane potential of RVLM bulbospinal neurons in vitro when the brainstem–spinal cord preparation of neonatal rats was superfused with Ang II. Data showing the presence of more AT1 receptors in the brainstem/hypothalamus of SHR than of WKY12 support our results. Iontophoretic application of Ang II to the RVLM increased the activity of 30% of RVLM neurons tested by extracellular recordings, and the increase was greater in SHR than in WKY.14 However, very few studies have compared intracellular recordings of the response to Ang II between WKY and SHR. Li and Guyenet20 demonstrated that Ang II (1 µmol/L) significantly depolarized the RVLM neurons (6.8±0.6 mV) of 3- to 10-day-old normotensive Sprague-Dawley rats in the slice preparation. Reasons for lack of depolarization in our WKY should be mentioned. (1) RVLM neurons in Sprague-Dawley rats may be more sensitive to Ang II than those in WKY. (2) RVLM neurons may be more sensitive to Ang II when examined in the slice preparation than in the brainstem–spinal cord preparation. If so, the depolarization values in both WKY and SHR would be larger in the slice preparation, and the depolarization in SHR would be larger than in WKY when examined in the slice preparation.

We have demonstrated that candesartan induced membrane hyperpolarization and a decrease in the firing rate of RVLM bulbospinal neurons in SHR but not in WKY. These results suggest that endogenous Ang II binds to the AT1 receptors on RVLM bulbospinal neurons, tonically contributing to the less negative membrane potential and the faster firing rate in SHR. Further, candesartan prevented the Ang II–induced membrane depolarization in SHR, suggesting Ang II–induced depolarization to be mediated by the AT1 receptors. DiBona and Jones11 showed that candesartan microinjected into the RVLM reduced BP, HR, and RSNA in Sprague-Dawley rats, whereas Allen23 demonstrated that microinjected candesartan reduced BP and RSNA in SHR but not in WKY. Zhu et al10 reported the depressor effect of candesartan microinjection to be significantly larger in SHR than in Sprague-Dawley rats. Using intracellular recordings, we showed that the activity of RVLM bulbospinal neurons was significantly suppressed with candesartan in SHR.

Earlier studies showed that candesartan can suppress the electrophysiological properties of neurons situated behind the blood-brain barrier, even when it was administered peripherally.24–26 Therefore, when orally administered in a clinical setting, candesartan can suppress the activities of RVLM bulbospinal neurons, and this mechanism may be involved in the antihypertensive effect of candesartan. We had demonstrated that intravenous injection of candesartan did not induce the reflex-activation of RSNA and that 14-day oral treatment decreased the value of RSNA in conscious SHR, despite significant reduction of BP.27 The candesartan-induced hyperpolarization of RVLM neurons we report here may be involved in the reduction of peripheral sympathetic nerve activity.

Because the RVLM area contains a moderate density of AT1 receptors,12,22,26 endogenous Ang II can stimulate RVLM neurons through AT1 receptors. A quantitative autoradiographic study found that the nucleus tractus solitarius and the dorsal motor nucleus of the vagus possess a higher density of AT1 receptors in adult SHR than in WKY,28 suggesting the number of AT1 receptors per neuron being increased in SHR compared with WKY. Although the study28 did not describe the RVLM neurons, we speculate from our results that the number of AT1 receptors expressed on RVLM neurons is larger in SHR than in WKY. We also presume that upregulation of AT1 receptors on RVLM neurons and/or increased local concentration of Ang II changes the open probabilities of potassium and calcium channels of the neurons,20 thus modulating the membrane potential and firing rate of the neurons.

The brainstem–spinal cord preparation is well established in the research field of respiratory neurons.17,18 Brockhaus et al29 show that the oxygenation of those neurons that exist 300 to 600 µm deep from the ventral surface is sufficient. RVLM bulbospinal neurons that we examined were found 100 µm deep from the surface. The reasons we have chosen the neonate rats are as follows: (1) we aimed to compare the properties of RVLM neurons between WKY and SHR before BP differs, and (2) the microenvironment of RVLM neurons in this preparation is satisfactorily maintained in rats until 4 days after birth.29

Perspectives

It is possible that sympathetic activation, although a component in SHR, is not prevalent in human essential hypertension, and mechanisms of BP elevation in SHR are not necessarily equal to those in human. Although sympathetic nerve activity is not always activated in patients with established essential hypertension, muscle sympathetic nerve activity is potentiated in young hypertensive patients at the initial stage.30 Julius et al31 demonstrated in their longitudinal studies that sympathetic activity was elevated in young hyperkinetic hypertension and in children who were normotensive at 6 years of age and became hypertensive in their fourth decade. Thus, the pathogenesis of essential hyperten-
sion shows a transition from a sympathetic-activated to a sympathetic-independent state. Therefore, our results demonstrating a faster firing rate of RVLM neurons in neonatal SHR have some relevance to the pathogenesis of essential hypertension.

In summary, the electrophysiological properties of RVLM neurons and the responses to Ang II differ in neonatal WKY and SHR. Moreover, the significant hyperpolarization and decrease in the firing rate evoked by candesartan suggest that endogenously generated Ang II, which binds to AT₁ receptors on the RVLM bulbospinal neurons, contributes to a less negative membrane potential and faster firing rate in SHR. These characteristics of RVLM neurons in SHR may account for the BP elevation that occurs in this model.

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