Electrophysiological Properties of Rostral Ventrolateral Medulla Neurons in Angiotensin II 1a Receptor Knockout Mice

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Abstract—We compared the electrophysiological properties of neurons in the rostral ventrolateral medulla of neonatal angiotensin II type 1a receptor knockout mice and wild-type mice with responses to angiotensin II, its type-1 receptor blocker candesartan, and its type-2 receptor blocker PD123319. Using the whole-cell patch-clamp technique, we examined the characteristics of rostral ventrolateral medulla neurons in brain stem–spinal cord preparations in which the sympathetic neuronal network is preserved. Baseline membrane potential and firing rate were almost similar between angiotensin II type 1a receptor knockout mice and wild-type mice. Superfusion with angiotensin II depolarized rostral ventrolateral medulla bulbospinal neurons in wild-type mice, whereas it hyperpolarized those in angiotensin II type 1a receptor knockout mice. Because pretreatment with candesartan significantly prevented the angiotensin II–induced depolarization in wild-type mice, the angiotensin II type 1 receptor is crucial for this depolarization. Superfusion with PD123319 depolarized rostral ventrolateral medulla bulbospinal neurons in angiotensin II type 1a receptor knockout mice. PD123319 prevented the angiotensin II–induced hyperpolarization in angiotensin II type 1a receptor knockout mice, and, rather, it induced depolarization. These results suggest that the angiotensin II type 2 receptor in rostral ventrolateral medulla plays an antagonistic role against the angiotensin II type 1a receptor in controlling the neuronal activity of rostral ventrolateral medulla. (***Hypertension*****. 2005;46:349-354.)**

Key Words: angiotensin antagonist ■ angiotensin II ■ brain ■ central nervous system ■ mice ■ receptors, angiotensin II ■ sympathetic nervous system

Rostral ventrolateral medulla (RVLM) neurons are located at an essential site involved in the baroreflex pathway and play a key role in controlling peripheral sympathetic nerve activity (SNA) and blood pressure (BP).[1,2] Previous studies examined the responses of BP and SNA to angiotensin II (Ang II) and Ang II antagonists microinjected into the RVLM of normotensive and hypertensive animals[3,4] because the RVLM area contains Ang II–immunoreactive nerve terminals and a moderately high density of Ang II type-1 (AT_1) receptors.[5,6] Microinjection of Ang II into the RVLM area increased BP and SNA,[3] whereas the AT_1 receptor blocker candesartan reduced BP, renal SNA, and heart rate.[4] To elucidate the precise electrophysiological changes within RVLM neurons elicited by Ang II and candesartan, we previously performed intracellular recordings (whole-cell patch-clamp technique) of RVLM neurons in neonatal Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR).[7] We demonstrated that the electrophysiological properties of RVLM neurons and their responses to Ang II and candesartan differ between neonatal WKY and SHR. These results suggest that endogenously generated Ang II binds to AT_1 receptors on RVLM bulbospinal neurons, thus tonically generating a higher membrane potential and a faster firing rate in SHR.

Ang II has 2 major receptor subtypes: the AT_1 and AT_2 receptors.[8] A single gene on chromosome 3 encodes the AT_1 receptor in humans,[9] whereas 2 AT_1 receptor subtypes, AT_{1a} and AT_{1b}, encoded by distinct genes on different chromosomes, have been identified in rodents.[10] AT_1a receptors are predominant in most tissues (vascular smooth muscle, liver, lung, and kidney), whereas AT_1b receptor expression is restricted to the adrenal gland and anterior pituitary.[10,11] AT_2 receptors are known to be expressed in high levels in several tissues of developing and young animals and then to decrease in density with age.[12] Many studies in animals have provided new insights into the roles of AT_1 and AT_2 receptors using gene transfer or transgenic techniques or antisense gene transfer technology.[13–18]

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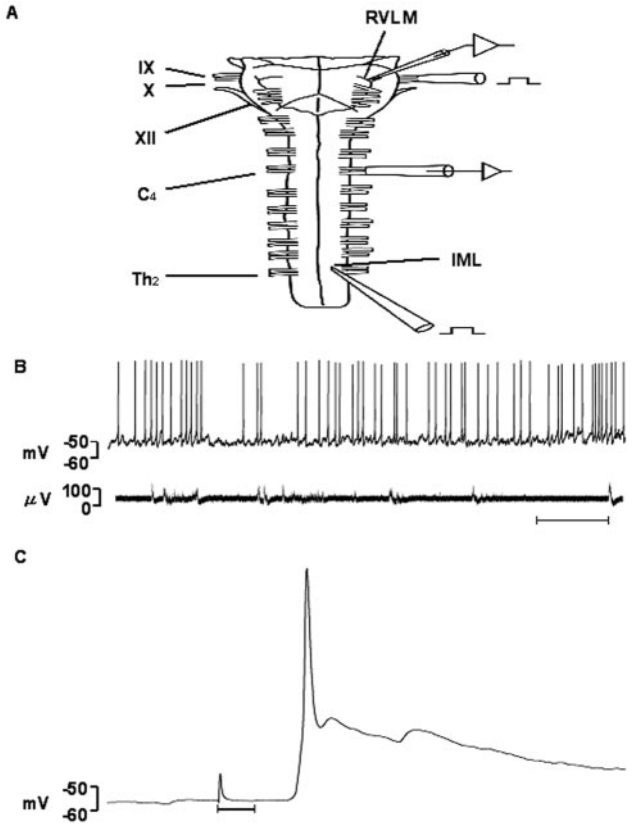

Figure 1. A, Brain stem–spinal cord preparation of an AT1a KO mouse. Using the whole-cell patch-clamp technique, intracellular recordings of RVLM neurons were made. Phrenic nerve activities were recorded from the ventral root of C4. Electrical stimulation was applied to Th2 segment, including the intermediolateral cell column (IML). IX indicates glossopharyngeal nerve; X, vagus nerve; XI, hypoglossal nerve. B, Simultaneous recordings of RVLM neuron activity and phrenic nerve discharge. Bar=10 s. C, An antidromic action potential shown in an RVLM neuron. Bar=10 ms. The latency from stimulation of the IML area to antidromically activation of RVLM neuron was ≈20 to 30 ms. It was shorter than in rats.[19]

There are no receptor binding or immunostaining data that indicate that the RVLM contains AT_{1b} and AT_2 receptors, either in adult or neonatal mice. Nonetheless, we performed intracellular recordings (whole-cell patch-clamp technique) of RVLM neurons in neonatal AT_{1a} receptor knockout (AT_{1a} KO) mice and wild-type (WT; C57BL/6J) mice during superfusion with Ang II and its AT_1 and AT_2 receptor blockers to clarify the role of each receptor in the RVLM electrophysiologically. Our data provide physiological evidence for the existence of AT_{1b} and AT_2 receptors in the RVLM of neonatal mice.

Methods

Intracellular Recordings of RVLM Bulbospinal Neurons

Experiments were performed on brain stem–spinal cord preparations of AT_{1a} KO mice[13] and WT (C57BL/6J) mice pups (1 to 4 days old; Figure 1A).[7,19,20] The preparation was superfused continuously with artificial cerebrospinal fluid (aCSF). All experimental protocols were approved by our facility institutional review board.

Using the whole-cell patch-clamp technique, we observed intracellular recordings of RVLM bulbospinal neurons, which met the following criteria. (1) The RVLM neurons with discharges that were synchronized with phrenic activity were assumed to be respiratory neurons[20] and were excluded from this study. Figure 1B shows representative data of simultaneous recordings of RVLM neuron and phrenic nerve activity. (2) The electrode tip was filled with a solution containing 1% Lucifer-yellow (Aldrich Chemical), which spontaneously diffused into the neurons during the intracellular recordings to verify the location of the neurons examined.[21] (3) To determine 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 with a tungsten electrode and selected only the RVLM neuron that exhibited an antidromic action potential (Figure 1C).[7,19]

The RVLM neurons of both strains of mice were classified into 3 types: regularly firing neurons, irregularly firing neurons (Figure 2), and silent-type neurons (data not shown). Regularly firing and irregularly firing neurons showed spontaneous firing. Silent type neurons did not show spontaneous firing, and they were activated only during current-induced depolarization. Irregularly firing neurons exhibited many excitatory postsynaptic potentials, whereas regularly firing neurons rarely showed excitatory postsynaptic potentials.

The electrode solution consisted of (in mmol/L) 130 potassium gluconate, 10 HEPES, 10 EGTA, 1 CaCl2, and 1 MgCl2, adjusted to a pH of 7.2 to 7.4 with potassium hydroxide.

Drugs and Protocols

All drugs were dissolved in standard aCSF. 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 superfusion with Ang II. We selected 3 and 6 μmol/L of Ang II, the same concentrations that we had used previously in rats.[7] To

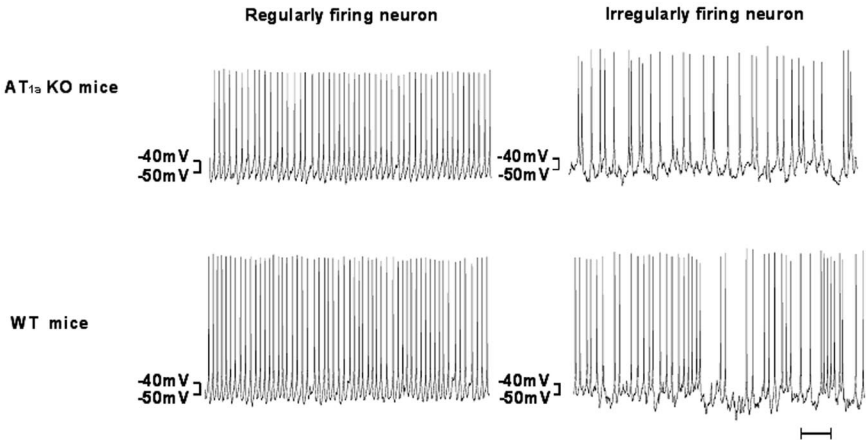

Figure 2. Intracellular recording of RVLM bulbospinal neurons from neonatal AT1a KO mice and WT mice. Bar=1 s.

Basal Electrophysiological Characteristics of RVLM Neurons

	Regularly Firing Neuron		Irregularly Firing Neuron	
	AT_{1a} KO Mice (n=10)	WT Mice (n=5)	AT_{1a} KO Mice (n=31)	WT Mice (n=18)
Membrane potential (mV)	−51.3±2.2	−49.4±1.0	−52.4±1.0	−49.8±1.1
Firing rate (Hz)	6.0±0.9*	6.4±1.5	3.8±0.4	4.0±0.5
Input resistance (MΩ)	526±109	545±104	616±55	671±114

Mean±SEM.
*P=0.016 vs irregularly firing neurons in the same strain.

examine the AT_1 receptor–mediated effects of Ang II, we performed superfusions with the AT_1 receptor blocker candesartan. Candesartan (Takeda Chemical Industries) was dissolved in 1 mol/L NaOH solution, and the pH was adjusted to 7.3 by the addition of HCl. We used 0.06 and 0.12 μmol/L of candesartan, the same concentration as used previously in rats.[7] We applied 6 μmol/L of Ang II for 20 minutes, immediately after the end of 20-minute superfusion with 0.12 μmol/L of candesartan. To assess the AT_2 receptor–mediated effects of Ang II, we performed superfusions with the AT_2 receptor blocker PD123319 (Sigma). Because the effect of 100 nmol/L Ang II on neurons was prevented by 1 μmol/L PD123319,[22] we used 60 and 120 μmol/L of PD123319, doses presumably capable of antagonizing the effect of 6 μmol/L of Ang II. We applied 6 μmol/L of Ang II plus PD123319 (60 and 120 μmol/L) for 20 minutes, immediately after the end of 20-minute superfusion with the same concentration of PD123319.

Statistics

Statistical analyses were performed using SPSS 11.5. Baseline values between AT_{1a} KO mice and WT mice and between regularly and irregularly firing neurons were compared with an unpaired t test (2-tailed). Membrane potentials before and after superfusion with drugs were compared with a paired t test (2-tailed). All data were expressed as mean±SEM. $P<0.05$ was considered statistically significant.

Results

Basal Electrophysiological Properties of RVLM Bulbospinal Neurons

We performed intracellular recordings of bulbospinal RVLM neurons of AT_1a KO mice (n=41) and WT mice (n=23). The RVLM neurons of both strains of mice were classified into 3 types: regularly firing neurons, irregularly firing neurons (Figure 2), and silent-type neurons (data not shown). These characteristics were similar in RVLM neurons of rats, according to our previous studies in WKY and SHR.[7,19] In this study, we examined regularly and irregularly firing neurons and did not examine the properties of silent-type neurons. The basal membrane potential, firing rate, or input resistance did not differ between AT_{1a} KO and WT mice (Table). The firing rate of regularly firing neurons was significantly faster than that of irregularly firing neurons in the AT_{1a} KO mice.

Effects of Ang II on RVLM Bulbospinal Neurons

One to 2 minutes after the start of superfusion with 6 μmol/L Ang II, RVLM bulbospinal neurons of WT mice depolarized, and the firing rate increased (Figure 3A, right panel). This response was similar when comparing regularly and irregularly firing neurons. After that, a tendency to repolarize was observed for 2 to 3 minutes, although the membrane potential remained less negative than that at the presuperfusion level. This represents the accommodation of the neurons to the Ang II stimulus. Thus, the neuronal response of Ang II was biphasic. We quantified the membrane depolarization of the first phase. The neurons for WT mice depolarized with 3 μmol/L Ang II (2.5±0.6 mV; n=6) and with 6 μmol/L Ang II (4.6±1.0 mV; n=12).

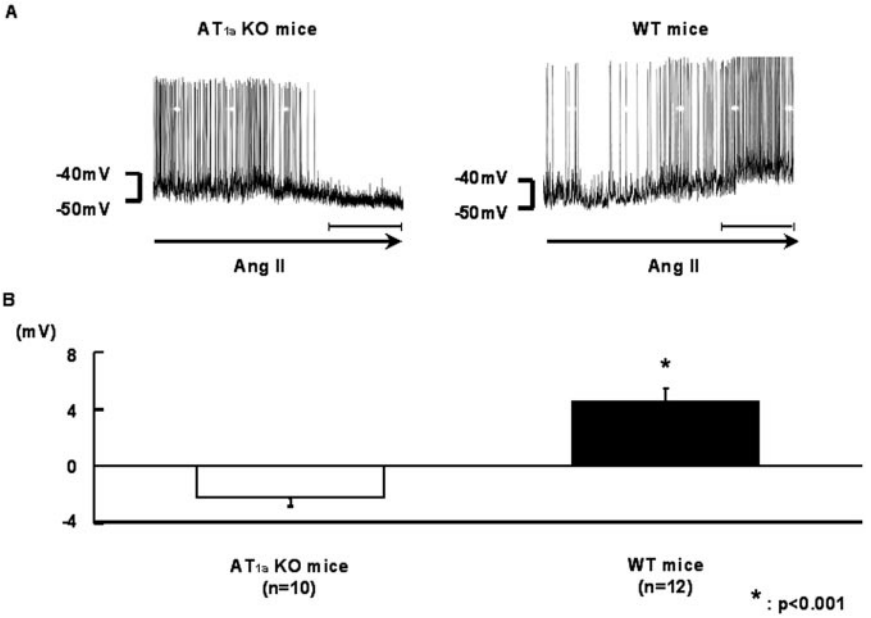

Figure 3. A, Left, Intracellular recording of membrane hyperpolarization and the decrease in the firing rate of an RVLM bulbospinal neuron during Ang II (6 μmol/L) superfusion in an AT1a KO mouse. Right, Intracellular recording of membrane depolarization and the increase in the firing rate of an RVLM bulbospinal neuron during Ang II (6 μmol/L) superfusion in a WT mouse. Bars=1 minute. B, Average change in membrane potential of RVLM bulbospinal neurons during superfusion with 6 μmol/L of Ang II. Values are mean±SEM. *$P<0.001$ vs AT1a KO mice. Changes in membrane potential during Ang II superfusion are significant compared with those before the superfusion in AT1a KO mice ($P<0.001$) and WT mice ($P<0.001$).

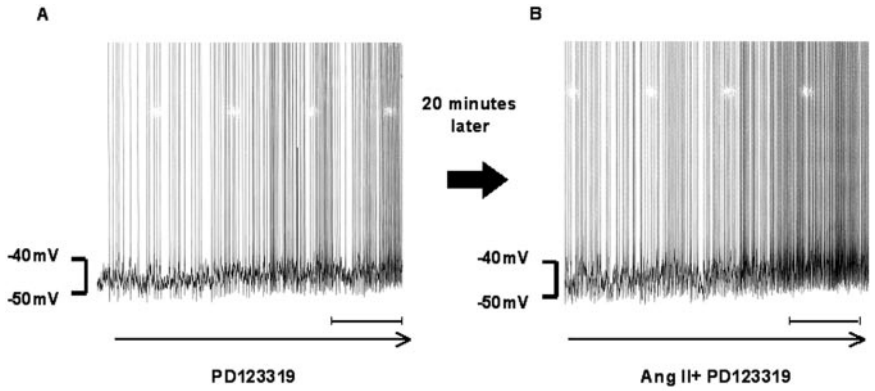

Figure 4. A, Recording of membrane depolarization and the increase in the firing rate of an RVLM bulbospinal neuron during PD123319 (60 μmol/L) superfusion in an AT1a KO mouse. Average change in membrane potential was 1.3±0.5 mV (n=9). Changes in membrane potential during PD123319 are significant compared with those before the superfusion (P=0.022). B, Recording of membrane depolarization and the increase in the firing rate of an RVLM bulbospinal neuron during superfusion with Ang II (6 μmol/L) and PD123319 (60 μmol/L) simultaneously 20 minutes after start of application of PD123319 in an AT1a KO mouse. Average change in membrane potential was 1.8±0.6 mV (n=9). Changes in membrane potential during superfusion with Ang II and PD123319 are significant compared with those before the superfusion with them (after superfusion with only PD123319; P=0.014). Bars=1 minute.

In contrast, 1 to 2 minutes after the start of superfusion with 6 μmol/L Ang II, RVLM bulbospinal neurons of AT_{1a} KO mice hyperpolarized, and the firing rate decreased (Figure 3A, left panel). This time course was almost the same in regularly and irregularly firing neurons. This response to Ang II was also biphasic. The membrane potential depolarized within 2 to 3 minutes after the hyperpolarization, but it remained below the presuperfusion level. We quantified the membrane hyperpolarization during the first phase. The neurons for AT_{1a} KO mice hyperpolarized with 6 μmol/L Ang II (−2.3±0.5 mV; n=10). In this case, 8 of 10 neurons hyperpolarized, and in 2 neurons, there was no response. We also examined the effect of 3 μmol/L Ang II on the RVLM neurons of AT_{1a} KO mice, but it elicited no changes (0.0±0.5 mV; n=6). The difference between the change in membrane potential elicited by Ang II (6 μmol/L) in WT mice and AT_{1a} KO mice was statistically significant. Figure 3B clearly shows that superfusion with Ang II (6 μmol/L) hyperpolarized RVLM bulbospinal neurons of AT_{1a} KO mice, whereas it depolarized those of WT mice.

Effects of Candesartan on RVLM Neurons

Candesartan (0.06 and 0.12 μmol/L) did not significantly change the membrane potential of either WT mice or AT_1a KO mice. However, pretreatment with 0.12 μmol/L of candesartan prevented the Ang II (6 μmol/L)–induced depolarization in the RVLM of WT mice (from 4.6±1.0 mV [n=12] to 0.5±0.4 mV [n=6]). This effect was similar between regularly and irregularly firing neurons. In contrast, pretreatment with 0.12 μmol/L of candesartan did not significantly affect the Ang II (6 μmol/L)–induced hyperpolarization in the RVLM of AT_{1a} KO mice.

Effects of PD123319 on RVLM Neurons in AT_{1a} KO Mice

PD123319 depolarized the RVLM bulbospinal neurons of AT_{1a} KO mice and increased the firing rate (Figure 4A). This effect was similar in regularly and irregularly firing neurons. The average depolarization of RVLM bulbospinal neurons in AT_{1a} KO mice was 1.3±0.5 mV (n=9; 60 μmol/L of PD123319) and 2.2±0.6 mV (n=6; 120 μmol/L of PD123319), significant values (before versus after superfusion with PD123319). The effect of PD123319 persisted for >40 minutes after its washout.

Effect of PD123319 on Ang II–Induced Hyperpolarization in AT_{1a} KO Mice

Pretreatment with either 60 or 120 μmol/L of PD123319 completely prevented the Ang II (6 μmol/L)–induced hyperpolarization of AT_1a KO mice. During superfusion with PD123319, Ang II depolarized the RVLM bulbospinal neurons of AT_1a KO mice (Figure 4B). This effect was similar in regularly and irregularly firing neurons. In this case, the average depolarization induced by Ang II (6 μmol/L) was 1.8±0.6 mV (n=9; 60 μmol/L of PD123319) and 3.8±1.3 mV (n=5; 120 μmol/L of PD123319), significant values (before versus after superfusion with Ang II).

Discussion

In this study, we compared the electrophysiological characteristics of the RVLM bulbospinal neurons in neonatal AT_{1a} KO mice and WT mice, via intracellular recordings using the whole-cell patch-clamp technique. The baseline membrane potential tended to be more negative, and the firing rate tended to be slower in the RVLM neurons of neonatal AT_{1a} KO mice than those of WT mice. However, these differences were not statistically significant. Regularly and irregularly firing neurons showed the same results. Sugaya et al[13] and Ito et al[14] reported that the adult AT_{1a} receptor–deficient mice show hypotension. We did not find any differences in baseline electrophysiological properties between the AT_{1a} KO mice and WT mice, so other mechanisms may play a key role in the difference in BP.

After superfusion with 6 μmol/L Ang II, RVLM bulbospinal neurons of WT mice depolarized, and the firing rate increased. This is the same reaction as RVLM neurons of the SHR in our previous study.[7] The reason that RVLM neurons of normotensive WT mice were activated by Ang II in a

similar fashion to RVLM neurons in the hypertensive rat model SHR is not clear. It may be because of the difference between mice and rats. In any case, the effect of Ang II is one of the important factors for the activation of RVLM neurons in mice and rats.

In contrast, superfusion of RVLM bulbospinal neurons of the AT_1a KO mice with 6 μmol/L Ang II produced a hyperpolarization, and the firing rate decreased. Thus, with a lack of AT_{1a} receptors, there was a lack of Ang II–induced depolarization. Therefore, we postulate that AT_{1a} receptors are essential for the depolarization produced by Ang II. Pretreatment with 0.12 μmol/L of candesartan prevented the Ang II (6 μmol/L)–induced depolarization in the RVLM of WT mice. This result also supports the importance of AT_1 receptors for depolarization induced by Ang II. In another in vivo study, Ito et al[14] reported that the pressor responses to intravenously infused Ang II were virtually absent in AT_{1a} KO mice.

Candesartan by itself did not significantly change the membrane potential of either WT mice or AT_{1a} KO mice. In our previous study using rats,[7] candesartan induced membrane hyperpolarization and a decrease in the firing rate of RVLM bulbospinal neurons in SHR but not in WKY. The response of RVLM neurons elicited by candesartan in WT mice was similar to that in WKY.

A novel finding of this study is that after superfusion with 6 μmol/L Ang II, RVLM bulbospinal neurons of AT_{1a} KO mice hyperpolarized, and the firing rate decreased. To assess the underlying mechanism of this hyperpolarization, we used the AT2 receptor blocker PD123319. PD123319 depolarized the RVLM bulbospinal neurons of AT_{1a} KO mice and increased the firing rate. These results suggested that, at least in AT_{1a} KO mice, AT_2 receptors are crucial for the hyperpolarization produced by Ang II.

Although the role of AT_1 receptors in the brain is essential for regulating BP, basal SNA, baroreceptor reflexes, and fluid balance,[23–26] the role of AT_2 receptors is still not fully understood. The AT_2 receptors play a role in a modulation of apoptosis, neurite development, and exploratory behavior,[17,27,28] and some reports relate AT_2 receptors to BP. Ichiki et al[16] reported that AT_2 KO mice showed a significantly increased BP and increased sensitivity to the pressor action of intravenously infused Ang II. Hein et al[17] also reported an increased vasopressor response of AT_2 KO mice to intravenous injection of Ang II, but the baseline BP was almost the same between AT_2 KO mice and WT mice in their study. A recent study by Li et al[29] suggested an antagonistic action of AT_2 receptors in the brain against AT_1 receptors in the regulation of BP. They reported that the increase in BP elicited by intracerebroventricular injection of Ang II was greater in AT_2 KO mice than in WT mice, and the pressor response to a central injection of Ang II in WT mice was exaggerated by PD123319. These results are consistent with our data in that PD123319 depolarized the RVLM bulbospinal neurons of AT_{1a} KO mice and increased the firing rate. We speculate that Ang II hyperpolarizes RVLM neurons through AT_2 receptors, and this effect antagonizes that of Ang II through AT_1 (especially AT_{1a}) receptors.

The RVLM contains a high density of AT_{1a} receptors,[5,6] but the existence of AT_2 receptors has not been reported as far as we know. The distribution of brain AT_1 receptors is highly conserved across species from rodents to primates and humans, whereas that of AT_2 receptors is highly variable.[30] In the mouse brain, the presence of mixed populations of AT_1 and AT_2 receptors is a widespread occurrence.[31] For example, the nucleus of the solitary tract expresses only AT_1 receptors in rats, whereas mice express AT_1 and AT_2 receptors in that nucleus.[31] We speculate that, at least in neonatal mice, AT_2 receptors can exist in the RVLM, but their density is not so high. In mice, Ang II depolarizes RVLM bulbospinal neurons through AT_1 (mainly AT_{1a}) receptors and hyperpolarizes those neurons through AT_2 receptors. The roles of AT_1 and AT_2 receptors are naturally antagonistic in the RVLM. Superfusion with Ang II depolarized RVLM bulbospinal neurons of WT mice because the density of AT_1 receptors is much higher than that of AT_2 receptors. When we applied Ang II to AT_{1a} KO mice, the membrane potential of RVLM bulbospinal neurons was hyperpolarized. We speculate that it is because AT_2 receptors became relatively predominant.

During superfusion with PD123319, Ang II depolarized the RVLM bulbospinal neurons of AT_1a KO mice. Without the effect of AT_{1a} and AT_2 receptors, it is hard to clarify what type of receptor is important for this depolarization. Oliverio et al[15] reported AT_{1b} receptors contribute to the regulation of resting BP when AT_1a receptors are absent. Therefore, we speculate that depolarization shown in our study may be attributable to AT_{1b} receptors. However, we have not done a histological work in this study, so the existence of AT_{1a}, AT_{1b}, and AT_2 receptors should be elucidated in future.

We recognize the limitations of this study. Because the brain stem–spinal cord preparation removes a lot of the inputs from other brain regions, it only retains the network within the medulla oblongata and spinal cord. However, the major reason why we used this preparation here is that we can choose RVLM neurons that monosynaptically project to the spinal cord. In addition, we should explain why we used neonatal mice. This brain stem–spinal cord preparation is well established in the research field of respiratory neurons. Brockhaus et al[32] showed that the microenvironment (eg, oxygenation) of neurons in this preparation is satisfactorily maintained in rats until 4 days after birth. Although we cannot extrapolate data obtained in the present study to adult rats, we believe that this study that demonstrated the role of AT2 receptor is important.

Another major question that arises from our study is whether superfusion with aCSF alters the distribution of the AT_1/AT_2 ratio. We have not measured AT_1/AT_2 receptor levels in this study. However, we believe that superfusion with aCSF does not change the AT_1/AT_2 ratio because in our previous study, the depolarization of RVLM bulbospinal neurons induced by Ang II via AT_1 receptor was higher in SHR than in WKY.[7] This result is consistent with the fact that the expression of AT_1 receptor, as determined by immunocytochemical studies, was higher in SHR than in WKY.[6] If the ratio of AT_1 receptors in SHR/AT_1 receptors in WKY was maintained in our previous study, then the AT_1/AT_2 ratio in RVLM is likely maintained in the current study.

To clarify the mechanisms that underlie the effects of AT_2 receptors on AT_1-mediated depolarization is also a difficult question. We assume that antagonistic effects of AT_2 receptor activation on AT_1-mediated depolarization are attributable to opposite effects on potassium channels in the RVLM neurons (ie, the increases and decreases in conductance after AT_2 and AT_1 receptor activation, respectively). Indeed, Sumners et al[33,34] demonstrated that Ang II suppresses neuronal delayed rectifier K+ current (IKv) via AT_1 receptors, whereas Ang II stimulates IKv via AT_2 receptors. Detailed analyses of the K+ currents in the RVLM neurons remain for future studies.

Perspectives

We demonstrated that superfusion with Ang II depolarized RVLM bulbospinal neurons in WT mice, whereas it hyperpolarized those in AT_1a KO mice. AT_1 receptors are essential for the Ang II–induced depolarization in WT mice because pretreatment of AT_1 receptor blocker candesartan significantly prevented this effect. Because superfusion with the AT_2 receptor blocker PD123319 depolarized RVLM neurons in AT_{1a} KO mice, Ang II hyperpolarized RVLM bulbospinal neurons through AT_2 receptors.

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