Electrophysiological responses of sympathetic preganglionic neurons to ANG II and aldosterone

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The sympathetic preganglionic neurons (SPNs) in the intermediolateral cell column (IML) of the spinal cord are an important area where sympathetic impulses propagate to peripheral sympathetic organs. ANG II and aldosterone are important components of the renin-angiotensin-aldosterone system (RAAS), which activate the sympathetic nervous system. Each is partly synthesized in the brain and plays a paracrine role in the regulation of blood pressure independently of RAAS in the periphery. Our purpose in the present study was to clarify the contributions of sympathetic preganglionic neurons in the IML (IML neurons) and the effects of ANG II and aldosterone on the sympathetic nervous system. To examine responses to ANG II and aldosterone, we intracellularly recorded 104 IML neurons using a whole cell patch-clamp technique in spinal cord slice preparations. IML neurons were classified into two types: silent and firing. Both neuron types were significantly depolarized by ANG II, and candesartan inhibited this depolarization. After pretreatment with TTX, firing neurons (but not silent neurons) were significantly depolarized by ANG II. Aldosterone significantly increased the number of excitatory postsynaptic potentials (EPSPs) in both neuron types, but this response disappeared after pretreatment with TTX. ANG II and aldosterone had no synergistic effects on the IML neurons. The silent neurons had large cell soma, and many more dendrites than the firing neurons. These results suggest that ANG II acts presynaptically and postsynaptically in IML neurons, while aldosterone acts mainly presynaptically. Thus, the physiological effects of these substances are likely to be transmitted via specific membrane receptors of IML and/or presynaptic neurons.

The objective of the present study was to clarify the direct and synergistic contributions of IML neurons to the sympathetic nerve activity in response to ANG II and aldosterone. We intracellularly recorded IML neurons using a whole cell patch-clamp technique in spinal cord slice preparations. Through these recordings, we found that the IML neurons responded to ANG II and aldosterone in different ways.

METHODS

Intracellular recordings. Wistar rats of either sex (0–4 days old) were used for the experiments. The animals were anesthetized with ether, and the spinal cord was excised. Transverse sections from segments Th1 to Th2 were resected with scissors and placed in artificial cerebrospinal fluid (ACSF) (25°C). The ACSF was composed of (in mM) 124 NaCl, 26 NaHCO₃, 5.0 KCl, 1.3 MgCl₂, 2.4 CaCl₂, and 30 n-glucose (pH 7.4), and was gassed with 95% O₂-5% CO₂. The ACSF was continuously superfused at a rate of 3 ml/min in a recording chamber. All experimental protocols were approved by the Institutional Review Board of our facility.

Intracellular recordings of IML neurons were taken by the whole cell patch-clamp technique. The patch micropipettes were filled with (in mM) 130 K-glucronate, 1 MgCl₂, 10 HEPES, 10 EGTA, 1 CaCl₂, and 2 Na₂-ATP, adjusted to a pH of 7.3 with KOH. Lucifer yellow (0.5%) (Sigma) was included in the pipette solution of the electrode tip for faster visualization and morphological identification of the recorded cells. IML neurons were identified by their all-or-none depolarization.
antidromic responses to mainly Th2 ventral root stimulation applied with a suction electrode (1–10 V, duration 100 μs) before and after intracellular recording.

**Drugs and protocols.** All drugs were dissolved in standard ACSF. The drugs and solutions were applied by superfusion to the preparation at a rate of 3 ml/min. The ANG II concentration was selected based on data from previous studies (24, 28). To test the direct effect (postsynaptic effect) of ANG II on the IML neurons, superfusion with 0.5 μM TTX, a blocker of the action potential-dependent transmitter release, was commenced 10 min before the start of the ANG II superfusion. After this pretreatment with TTX, the IML neurons were superfused with 6 μM ANG II (human; Sigma) for 5 min.

The contribution of the AT1 receptor to the IML-neuron response was examined by superfusion of 0.12 μM candesartan (Takeda Chemical Industries), an AT1 receptor antagonist (29), for 10 min. Candesartan was dissolved in a 1 M NaOH solution and adjusted to a pH of 7.3 by adding HCl. As soon as the superfusion with candesartan ended, 6 μM of ANG II was superfused for 5 min by a similar method.

The effects of aldosterone on the IML neurons were examined by superfusing 0.01 μM aldosterone (human; Sigma) for 10 min. To test the direct effect (i.e., postsynaptic effect) of aldosterone on the IML neurons, superfusion with 0.5 μM TTX was commenced 10 min before the start of the aldosterone superfusion (0.01 μM, 10 min).

The contribution of the mineralocorticoid receptor to the IML-neuron response was examined by pretreating the neurons for 10 min with 0.1–1 μM eplerenone (Sigma) (4, 46), a mineralocorticoid receptor antagonist, dissolved in ACSF. After this pretreatment, 0.01 μM aldosterone was superfused for 5 min.

The synergic effects of aldosterone and ANG II on IML neurons were examined by superfusing 6 μM ANG II for 5 min, starting from 20 min after the 10-min superfusion of 0.01 μM aldosterone.

**Microscopic examination.** After whole cell recording, preparations were fixed for >48 h at 4°C in Lillie solution (10% formalin in phosphate buffer, pH 7.0), and transverse 100-μm slices were then cut with a custom-made vibratome. Labeled neurons were reconstructed via a camera lucida attached to a fluorescence microscope (BH-2; Olympus, Tokyo, Japan). Most of the recorded neurons were successfully labeled with Lucifer yellow. The locations of the cell bodies of the recorded neurons in the intermediolateral nuclei were confirmed after 1% neutral-red staining.

**Statistical analysis.** All data are expressed as the means ± SD. Statistical significance of the differences (P < 0.05) was determined by the Student’s t-test.

**RESULTS**

**General electrophysiological properties of IML neurons.** We intracellularly recorded 104 IML neurons. The neurons were classified into two types: silent neurons, which exhibited no spontaneous action potential, and firing neurons, which exhibited continuous spontaneous activity (Fig. 1). The firing rate of the firing neurons was 4.9 ± 1.1 Hz, at a membrane potential of −50 mV. The membrane potential of the silent neurons was significantly deeper than that of the firing neurons (−53.0 ± 6.0 mV in silent neurons vs. −47.6 ± 5.2 mV in firing neurons, P < 0.01). The membrane resistance of the
silent neurons was significantly lower than that of the firing neurons (203.1 ± 1.45.5 MΩ in silent neurons vs. 473.3 ± 170.0 MΩ in firing neurons, P < 0.0001).

Effects of ANG II on IML neurons. We examined the effects of ANG II on 20 IML neurons, 7 of which were silent and 13 of which were firing. The IML neurons were gradually depolarized from 1 to 2 min after the start of superfusion with 6 μM of ANG II, and the responses plateaued within 5 min. The membrane depolarization recovered in about 10 min after washout (Fig. 2A). The changes in membrane potential vs. baseline were significant in both silent and firing neurons: silent neurons, -53.6 ± 6.4 mV in control vs. -50.8 ± 7.2 mV in ANG II (P < 0.05); firing neurons, -47.4 ± 5.8 mV in control vs. -43.8 ± 7.1 mV in ANG II (P < 0.0001). Correspondingly, the input resistances tended to increase during ANG II: silent neurons, 194.0 ± 140.9 MΩ in control vs. 227.8 ± 144.3 MΩ in ANG II (P < 0.05); firing neurons, 511.0 ± 173.2 MΩ in control vs. 533.6 ± 229.7 MΩ in ANG II (not significant). After superfusion with 6 μM, ANG II, the number of excitatory postsynaptic potentials (EPSPs) of the silent neurons increased: silent neurons, 0.80 ± 0.27 Hz in control vs. 1.68 ± 0.61 Hz in ANG II (P < 0.05); firing neurons, 0.71 ± 0.17 Hz in control vs. 1.02 ± 0.53 Hz in ANG II (not significant).

Effects of ANG II on IML neurons after candesartan. We examined the effects of ANG II after candesartan on 17 IML neurons (5 silent and 12 firing), none of which exhibited noticeable changes in membrane properties in direct response to a pretreatment with candesartan. ANG II did not significantly depolarize the membrane potentials after candesartan in either neuron type, compared with the membrane potentials at baseline (<0.5 mV). There were no increases in input resistance during ANG II after candesartan (not significant): silent neurons, 253.5 ± 144.7 MΩ in control vs. 277.8 ± 150.8 MΩ in ANG II; firing neurons, 520.1 ± 160.2 MΩ in control vs. 548.9 ± 165.6 MΩ in ANG II after candesartan (not significant). ANG II did not increase the number of EPSPs after candesartan in either neuron type: 0.82 ± 0.35 Hz in control vs. 0.90 ± 0.55 Hz in ANG II (not significant); firing neurons, 0.80 ± 0.44 Hz in control vs. 0.83 ± 0.53 Hz in ANG II (not significant).

Effects of ANG II after TTX on IML neurons. We examined how ANG II affected 20 IML neurons (5 silent and 15 firing) in the blockade of the action potential-dependent synaptic transmission. Spontaneous and current-induced action potentials in the IML neurons disappeared within 10 min after the TTX superfusion was commenced. There were clear differences in the responses of both neuron types to ANG II after the pretreatment with TTX. The firing neurons were depolarized by ANG II in the presence of TTX (Fig. 2B), whereas the membrane potential of silent neurons did not change with ANG II: silent neurons, -49.3 ± 7.9 mV in control vs. -48.8 ± 7.6 mV in ANG II (not significant); firing neurons, -43.9 ± 3.8 mV in control vs. -41.9 ± 3.3 mV in ANG II (P < 0.001). The input resistances tended to increase during ANG II: silent neurons, 311.1 ± 251.7 MΩ in control vs. 389.9 ± 297.8 MΩ in ANG II (P < 0.05); firing neurons 468.8 ± 155.0 MΩ in control vs. 506.6 ± 220.7 MΩ in ANG II (not significant). The number of TTX-resistant EPSPs due to action potential-independent transmitter release tended to increase after superfusion with 6 μM ANG II, but the change was not significant in either neuron type: silent neurons, 0.43 ± 0.19 Hz in control vs. 0.58 ± 0.45 Hz in ANG II; firing neurons, 0.39 ± 0.12 Hz in control vs. 0.56 ± 0.50 Hz in ANG II.
Effects of aldosterone on IML neurons. We examined the effects of aldosterone on 30 IML neurons, 9 silent and 21 firing. Two to three minutes after the superfusion with 0.01 μM aldosterone was commenced, the number of EPSPs of the IML neurons began to increase, and a slight depolarization of the membrane potential was observed (<2 mV). The increase in the number of EPSPs was significant vs. baseline in both silent and firing neurons (Fig. 3): silent neurons, 0.84 ± 0.44 Hz in control vs. 2.11 ± 0.91 Hz in aldosterone (P <0.05); firing neurons, 0.83 ± 0.63 Hz in control vs. 2.20 ± 1.60 Hz in aldosterone (P <0.05). The input resistances did not change in response to aldosterone in either neuron type: silent neurons, 372.1 ± 81.3 MΩ in control vs. 311.4 ± 54.3 MΩ in aldosterone; firing neurons 566.7 ± 161.5 MΩ in control vs. 495.1 ± 136.6 MΩ in aldosterone.

Effects of aldosterone on IML neurons after TTX. We examined the effects of aldosterone in the presence of TTX on seven IML neurons, three silent and four firing. The membrane potentials and input resistances of both neuron types remained unchanged during the application of aldosterone with TTX. Having observed earlier that aldosterone increased the frequency of EPSPs in the absence of TTX, we decided to analyze the effects of aldosterone on TTX-insensitive EPSPs, as well. Aldosterone brought about no significant changes in the frequency of EPSPs in either neuron type after the pretreatment with TTX (Fig. 4): silent neurons, 0.45 ± 0.26 Hz in control vs. 0.65 ± 0.37 Hz in aldosterone; firing neurons, 0.60 ± 0.24 Hz in control vs. 0.70 ± 0.28 Hz in aldosterone.

Effects of aldosterone on IML neurons after eplerenone. We examined the effects of aldosterone on 10 IML neurons, four silent and six firing, after pretreatment of eplerenone. The membrane potentials were not significantly changed by aldosterone in either neuron type, compared with those at baseline (<0.5 mV). The number of EPSPs tended to increase in both silent and firing neurons: silent neurons, 0.88 ± 0.30 Hz in control vs. 1.45 ± 0.62 Hz in aldosterone (P <0.05); firing neurons, 0.47 ± 0.54 Hz in control vs. 0.74 ± 0.23 Hz in aldosterone (not significant).

Synergistic effects of aldosterone and ANG II on IML neurons. We examined the effects of ANG II after aldosterone on 20 IML neurons, 4 silent and 16 firing. The neurons were superfused with 6 μM ANG II for 5 min, starting from 20 min after the end of the superfusion with 0.01 μM aldosterone. A gradual depolarization of the IML neurons was observed from 1 to 2 min after the commencement of the ANG II superfusion, as had been observed in the neurons superfused with ANG II in the absence of aldosterone. The level of membrane potential depolarization by ANG II after aldosterone superfusion was similar to that induced by the single application of ANG II without aldosterone: 3.3 ± 4.1 mV in ANG II vs. 2.5 ± 1.5 mV in ANG II after aldosterone (not significant).

Microscopic examination. The axons of the labeled neurons projected toward the ipsilateral ventral root and the dendritic trees were confirmed within two or three sections. The cell bodies of the identified IML neurons took two forms: large multipolar cells and small oval (or fusiform) -shaped cells (Fig. 5). The longitudinal and transverse diameters of the large cells were 22.9 ± 3.8 × 12.3 ± 4.0 μm, respectively, and those of the small cells were 12.9 ± 1.6 × 10.3 ± 3.2 μm. The large multipolar cells had 5–8 primary dendrites extending by 100–400 μm in the transverse plane, and the small oval cells had 2–4 primary dendrites extending by 50–200 μm in the same orientation. Clear relations between the shapes and electrophysiological properties of the IML neurons were also observed. All of the silent neurons were large, multipolar cells, while all of the firing neurons were small, oval (or fusiform) -shaped cells. The axon projected most often ventrally along the border of the gray matter and turned at the ventrolateral corner in both neuron types.

DISCUSSION

The main findings in this study were as follows: 1) IML neurons were classifiable into two types based on the electrophysiological properties: silent neurons and firing neurons. Both neuron types were significantly depolarized by superfusion of ANG II. Candesartan inhibited this ANG II-induced response in both neuron types. 2) ANG II increased the number of EPSPs in silent neurons, but not in firing neurons. 3) The silent neurons did not exhibit significant depolarization in response to ANG II after pretreatment with TTX, but the firing neurons did. 4) The superfusion of aldosterone significantly increased the number of EPSPs in silent neurons and firing neurons, but this response disappeared in both neuron types after the pretreatment with TTX. 5) The silent neurons had large cell soma and many more dendrites than the firing neurons.

Effects of ANG II on IML neurons. The application of ANG II is into the RVLM known to increase sympathetic nerve activity and blood pressure (6, 7, 16). The administration of ANG II into the ventricles brought about similar results (2, 17). ANG II excites the RVLM presynaptic neurons via angio-
tensin type 1 (AT1) receptors. The AT1 receptor, a major receptor subtype, has been characterized pharmacologically and histologically in the RVLM. The precise electrophysiological responses of RVLM neurons to ANG II have been elucidated in previous studies (18, 29). Recent studies have also revealed the molecular mechanisms and genetic factors (19, 21, 40). Much less is known about the electrophysiological characteristics of IML neurons in response to ANG II (35), though

Fig. 4. IML neuron responses to aldosterone in the presence of TTX. A: postsynaptic activity of a silent neuron before aldosterone with TTX treatment. Five EPSPs were detected over a period of 10 s. B: postsynaptic activity of a silent neuron after aldosterone with TTX treatment. Excitatory postsynaptic potentials (EPSPs) are not increased. Arrows denote examples of EPSPs.

Fig. 5. Microscopic examination of IML neurons in a transverse section. A: an example of a silent neuron. A, a: photograph of the neuron stained with Lucifer yellow. A, b: drawings of a Lucifer yellow-stained neuron. A, c: location of this cell in the IML (indicated by the arrow). B: firing neuron. B, a: location of this cell in the IML (indicated by the arrow). B, b: drawings of a Lucifer yellow-stained neuron. The silent neuron has large multipolar cell body and many more dendrites than the firing neuron. The firing neuron has a small oval cell body and only a few dendrites. The arrowheads (A, b and B, b) denote axons.
IML neurons have been confirmed to express AT1 receptors (1). Only one previous study has been published on the effects of ANG II on IML neurons in spinal cord slice preparations. In 1993, Lewis and Coote (23) used intracellular recordings with fine glass microelectrodes to examine the effects of ANG II on neurons located in the lateral horn of the spinal cord of adult Sprague-Dawley rats. According to their results, ANG II acts on IML neurons both presynaptically and postsynaptically. Although our findings are consistent with their results, a few clear differences between their study and ours are worthy of note. Lewis and Coote administered ANG II at 0.1 mM to 1.0 mM, whereas we applied 6 μM ANG II (24, 28) and recorded the neurons by a whole cell patch-clamp technique. They drew no distinction between silent and firing neurons, whereas the neurons that we recorded had a resting membrane potential in a wide range from −48 mV to −68 mV.

Numerous studies have established that neurons can produce angiotensinogen and that ANG II synthesized within the brain may play a specific role in the regulation of blood pressure and sympathetic nerve activity (20, 37). Yet the precise mechanisms underlying these behaviors are not well understood. AT1 receptors were detected in the lamina X, II, and VIII of the spinal cord, as well as in the IML (1, 12, 26). On this basis, we can speculate that the spinal cord neurons outside the IML are sensitive to ANG II. Indeed, Oz et al. (36) reported that the ANG II depolarized the responses of motor neurons in lamina VIII and IX of spinal cord slices. The IML neurons may receive synaptic inputs from other neurons outside the IML and undergo a presynaptic activation in response to ANG II. Deuchars et al. (8, 10) reported that GABAAergic interneurons in the central region of the spinal cord participate in the adjustment of SNA via inhibitory synaptic inputs (IPSPs) to the IML neurons. Monosynaptic GABAAergic IPSPs to the IML neurons from the RVLM have also been reported (10). Clearly, detectable IPSPs were absent in most of the IML neurons in our experimental condition, although some of the neurons exhibited small hyperpolarizing potential changes, which resembled IPSPs. We cannot assume, on this basis, that most of the IML neurons received no IPSPs, as the clear detection of IPSPs might have been hindered by a reversal potential close to the resting membrane potential. We believe that detailed analyses of IPSPs for the future will require a different method for whole cell recording, such as a method with high Cl− electrode solution.

In the present study, we compared the electrophysiological properties of the IML neurons with the cell morphology of the neurons by microscopic examination. In an investigation of the shape and morphology of SPNs in spinal cord slices of neonatal rats, Shen and Dun (41) classified the IML neurons into two types, one fusiform and one oval or round, each with a resting membrane potential of about −50 mV. Subgroups of the two neuron types in their report may correspond to the silent neurons and firing neurons in our study. We definitively detected a cell cluster consisting of large, multipolar neurons in the IML area. According to our electrophysiological characterization, the neurons in this cell cluster were the silent type. In the report by Pilowsky et al. (38), most of the SPNs had extensive dendritic arborizations in the rostrocaudal direction. Having examined the cell morphology only in cross sections in our study, we have no clear information on the expanse of the dendrites in the horizontal plane or rostrocaudal axis. The features and developmental changes of the dendritic arbors remain to be examined in future studies.

Silent neurons have many more dendrites than firing neurons. Dendrites constitute afferent pathways of impulses from presynaptic neurons. Silent neurons may thus be active in more intact preparations, such as in vivo preparations or in vitro brain stem-spinal cord preparations (9). In our study, firing neurons (but not silent neurons) were significantly depolarized by ANG II in the presence of TTX. This tells us that ANG II acts on firing neurons both presynaptically and postsynaptically. We also recognized moderate (but not significant) increases in the number of EPSPs in response to ANG II after the pretreatment with TTX in IML neurons. This is consistent with the result of a previous study (36), in which we proposed that AT1 receptors are located in presynaptic terminals of IML neurons.

**Effects of aldosterone on IML neurons.** Aldosterone is an important RAAS component with key pathophysiological roles in hypertension and cardiovascular disease (27). Though previously thought to be confined to the adrenal cortex, aldosterone synthesis has recently been detected in extra-adrenal tissues of organs such as the heart, blood vessels, and brain in animal studies (33, 42, 43, 44). Gomez-Sanchez et al. (13) reported that the rat brain is equipped with enzymatic machinery for the synthesis of adrenal corticosteroids and can synthesize aldosterone. Thus, aldosterone synthesized in the brain might play a paracrine role.

Aldosterone, a mineralocorticoid hormone, classically acts via intracellular mineralocorticoid receptors (MRs), but its activities in the brain are still poorly understood. The effects of aldosterone via MRs in the brain have been observed in previous animal studies, many of which were performed over long time frames of at least a few days (47). Aldosterone-induced genomic effects via intracellular receptors are characterized by a delay corresponding to a long series of subcellular events. Very rapid steroidial effects have also been widely recognized, raising questions about the genomic model. The rapid actions of aldosterone vary among renal tubule cells, vascular smooth muscle cells, and vascular endothelial cells (11). The fast-acting, nongenomic steroidial actions are likely to be transmitted via specific membrane receptors. Much remains to be clarified with regard to the rapid effects of aldosterone on target tissues, and the identity of the aldosterone nongenomic receptor remains controversial, as well (5, 22, 25).

Our study is the first to show the responses to aldosterone on IML neurons. We noted, with great interest, that aldosterone induced a rapid response in the IML neurons. The EPSPs of the IML neurons were significantly increased, and slight depolarization of the cells was observed within a few minutes, probably as a consequence the accumulation of EPSPs. After the pretreatment with TTX, the phenomenon disappeared. These results might support the notion that aldosterone acts on cell surface receptors in presynaptic neurons for IML neurons. Although further studies to address the membrane receptors for aldosterone in neurons will be required, our present results demonstrate at least one phenomenon underlying the rapid aldosterone responses. The effects of aldosterone may, therefore, differ from those of ANG II, although the detailed mechanisms remain to be elucidated. Aldosterone has been found to upregulate angiotensin-converting enzyme messenger RNA expression in several studies (15, 45, 47). These reports
suggest that aldosterone acts in the positive feedback loop in the RAAS. Each component could have a mutually synergistic effect. Synergistic actions of aldosterone and ANG II in vascular smooth muscle cells have also been reported (32, 34). Our study failed to corroborate this finding, as no differences were observed between the response of IML neurons to ANG II alone and to ANG II after aldosterone. This issue remains open to question, however, as their studies and ours employed very different materials and methods (they performed immunoblotting studies in vascular smooth muscle cells, for example, while we used the patch-clamp in IML neurons). Although we failed to identify any late responses of the IML neurons, we were able to conclude that the rapid responses of the IML neurons to ANG II were not enhanced by aldosterone. Eplerenone blocks the nongenomic effect of aldosterone, although the mechanism by which it does so remains obscure (3, 31). Eplerenone was not found to block the rapid responses of IML neurons induced by aldosterone in the present study.

Perspectives and Significance

In summary, ANG II induced presynaptic and postsynaptic effects in IML neurons, while aldosterone induced mainly presynaptic effects. These effects could be responses via membrane receptors for IML and/or presynaptic neurons. Although we found no evidence of functional differences between the large silent neurons and small firing neurons, we speculate that the silent neurons are more important for the integration of information from structures of the upper nervous system, such as the RVLM. It would be interesting to ascertain whether similar neurons are found in adult. Although no apparent synergistic effects of ANG II and aldosterone were observed in this study, it would also be interesting to determine whether these effects are absent in an in vivo condition or appear in association with longer-term treatment with these drugs.

REFERENCES


