Original Article

Monosynaptic Excitatory Connection from the Rostral Ventrolateral Medulla to Sympathetic Preganglionic Neurons Revealed by Simultaneous Recordings

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To directly investigate whether a monosynaptic connection exists between neurons in the rostral ventrolateral medulla (RVLM) and sympathetic preganglionic neurons (SPNs), we used simultaneous extracellular recordings of RVLM neurons and whole-cell patch-clamp recordings of SPNs at the Th2 level and analyzed them by spike-triggered averaging. We averaged 200 sweeps of membrane potentials in SPN triggered by the spikes in the RVLM neuron. No clear postsynaptic potentials were detected in the averaged wave of SPNs before angiotensin II (Ang II) superfusion, whereas during superfusion with Ang II (6 μmol/L) on the medulla oblongata side alone excitatory postsynaptic potentials (EPSPs) were clearly found in the SPN of 3 out of 10 pairs at 40±1 ms after the averaged triggering spike in the RVLM neuron. We consider them to be monosynaptic EPSPs, because 1) the averaged EPSPs exhibited a sharp rise time, 2) the onset latency of the averaged EPSPs in the SPNs after the trigger spike in the RVLM was the same as the latency of the antidromic action potentials in the RVLM neurons in response to electrical stimulation of the SPNs, and 3) the amplitude of the averaged EPSPs was over 2 mV. In summary, combining simultaneous recording and spike-triggered averaging allowed us to demonstrate a monosynaptic excitatory connection between a single RVLM neuron and a single SPN in the thoracic spinal cord. Such connections provide the basis for the maintenance of sympathetic tone and the integrative reflex that relays through the RVLM. The results explain the mechanism by which Ang II in the RVLM area increases peripheral sympathetic activity and blood pressure. (*Hypertens Res* 2008; 31: 1445–1454)

***Key Words*: brainstem–spinal cord preparation, excitatory postsynaptic potential, whole-cell patch-clamp technique, spike-triggered averaging, angiotensin II**

Introduction

The rostral ventrolateral medulla (RVLM) is a critical site for tonic and reflex control of the sympathetic nervous system (*1–5*). Although neurons in the RVLM project to the intermediolateral cell column (IML), where they connect with sympathetic preganglionic neurons (SPNs) (*6–8*), the electrophysiological nature of these connections has not been fully defined. SPNs are a functionally heterogeneous group of neurons that regulate the heart, adrenal medulla, vascular smooth muscle, kidney, and many other organs (*8*), and they form the final common pathway of the central nervous system that regulates peripheral sympathetic function and blood pressure.

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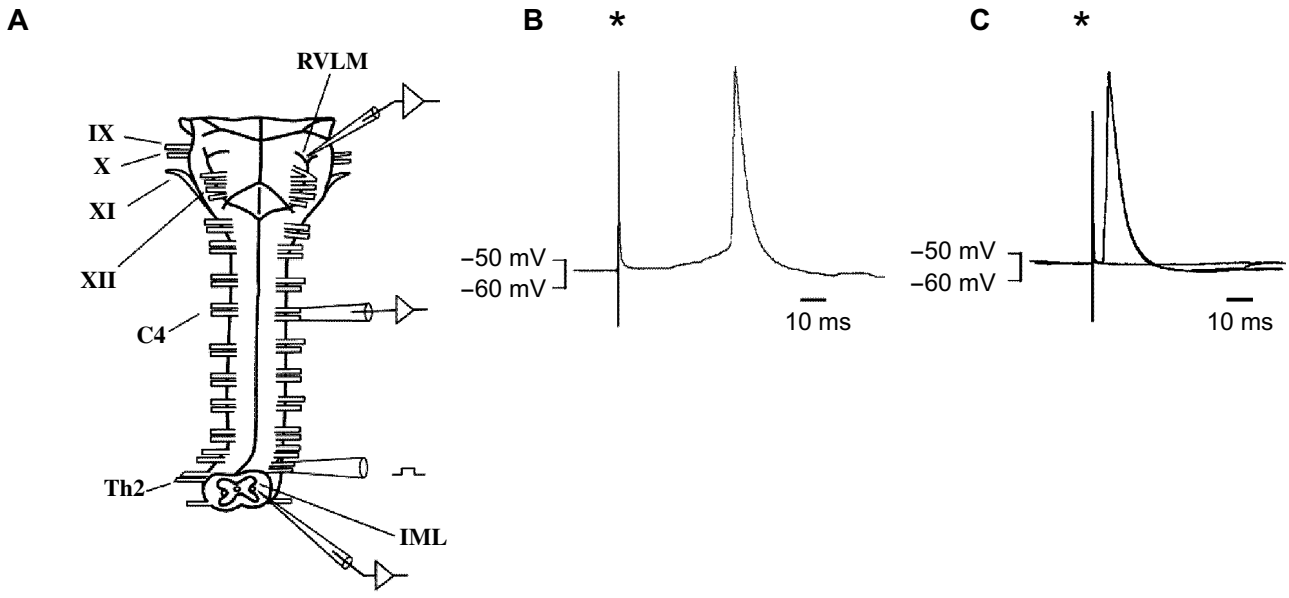

Fig. 1. *A: Brainstem–spinal cord preparation of a neonatal Wistar-Kyoto rat. RVLM and IML indicate sites of recording of rostral ventrolateral medulla (RVLM) units and sympathetic preganglionic neurons (SPNs), respectively. IX, X, XI, and XII are the numbers of cranial nerves. Electrical stimulation was applied to the ventral nerve root at the Th2 level via a suction electrode. B: An orthodromic action potential in an SPN (at the Th2 level) evoked by electrical stimulation (*) of the ipsilateral RVLM area. Note the gradual depolarization of the membrane potential prior to action potential discharge. C: Antidromic action potentials in an SPN evoked by electrical stimulation (*) of the ventral nerve root at the Th2 level to verify that the neuron was an SPN. Three sweeps are superimposed in C. Membrane trajectories containing action potentials evoked by 8-V and 10-V stimuli completely overlap, but no action potentials were evoked by the 3-V stimulus. Note the absence of a preceding excitatory postsynaptic potential (EPSP).*

Factors that affect SPN activity therefore play a crucial role in determining BP and the normal function of the body. However, despite 1) the convincing anatomical evidence that neurons in the RVLM form monosynaptic connections with SPNs, 2) the electrophysiological evidence that baroreceptor-inhibited RVLM neurons project to the spinal cord (*4*, *8–10*), and 3) the evidence that SPNs have the same behavioral properties as vasomotor sympathetic postganglionic neurons and neurons in the RVLM (*11*, *12*), proof is lacking that any identified neurons in the RVLM make a monosynaptic excitatory synapse with an SPN in the spinal cord (*5*).

In a previous study in a brainstem–spinal cord preparation by means of the whole-cell patch-clamp technique we found that RVLM neurons can be classified into 3 types according to their discharge patterns (*13*). Since the brainstem–spinal cord preparation is a useful preparation that preserves the sympathetic neuronal network (*14–17*), the whole-cell patch-clamp technique can be applied to SPNs and other neurons in order to investigate their electrophysiological properties and record postsynaptic potentials (PSPs) in SPNs.

In the present study we tried to make simultaneous recordings of RVLM neurons and SPNs in order to determine whether the connections between neurons in the RVLM and SPNs are excitatory, inhibitory, polysynaptic, or monosynaptic. We also investigated the effect of angiotensin II (Ang II) on the electrophysiological connections between the RVLM neurons and SPNs by using a spike-triggered averaging method (*14*, *18–22*). We tested the relationship between 10 pairs of RVLM neurons and SPNs by averaging 200 sweeps of membrane potentials in the SPN triggered by spikes in the RVLM neuron, before and during Ang II superfusion of the medulla oblongata side alone.

Methods

General Preparations

Experiments were performed on brainstem–spinal cord preparations collected from 1- to 4-d-old Wistar-Kyoto (WKY) rats, as previously described (*13*, *14*, *16*, *17*, *23*). The protocols of the experiments were approved by the Institutional Review Board, and were in accord with the National Guidelines for the Conduct of Animal Experiments. Briefly, with the animals under deep ether anesthesia, the brainstem-spinal cord was isolated at the Th2 level (Fig. 1A), and the brainstem was sectioned between the roots of cranial nerve VI and the lower border of the trapezoid body. The preparation was continuously superfused with a solution containing in (mmol/L)

124 NaCl, 5.0 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, and 30 glucose, and maintained at 25–26°C (control solution). The pH (7.4) and oxygenation were maintained by bubbling the solution with a gas mixture of 90% O_2–5% N_2 –5% CO_2. To avoid recording respiratory neuron activity we recorded phrenic nerve activity throughout the experiments and excluded from further study those neurons that showed a strong phase relationship with the phrenic nerve discharge (*13*, *16*, *17*).

Extracellular Electrodes in the RVLM

Electrodes were pulled in one stage from thin-wall borosilicate filament capillaries (GC120TF-10, outer diameter 1.2 mm; Clark Electromed, Reading, UK) with a vertical puller (PE-2; Narishige, Tokyo, Japan). The tip resistance of the electrodes was 5–20 MΩ. The electrodes were filled with 2% Pontamine Sky Blue in 0.5 mol/L sodium acetate solution.

Patch-Clamp Electrodes in the Spinal Cord

Electrodes were pulled in one stage from thin-wall borosilicate filament capillaries (GC100TF-10, outer diameter 1.0 mm; Clark Electromed) with the vertical puller. The electrodes had a tip diameter of 1.8–2.0 μm, and a resistance of 4–8 MΩ. The electrode solution for whole-cell recording consisted of (in mmol/L): 130 potassium gluconate; 10 HEPES; 10 EGTA; 1 $CaCl_2$; 1 $MgCl_2$; pH 7.2–7.3, adjusted with KOH. The electrode tips were filled with 1% Lucifer-Yellow (Aldrich Chemical, Milwaukee, USA).

Recording Procedure of the Patch-Clamp Method

First, the electrodes were inserted into an intermediolateral cell column (IML) to record SPNs. A patch-clamp amplifier (CEZ 3100; Nihon Kohden, Tokyo, Japan) was used to record the membrane potentials. Prior to obtaining whole-cell recordings, we observed the firing pattern of the target neurons by extracellular recording. After obtaining a gigaOhm seal, a single-shot hyperpolarizing pulse (0.6–0.9 nA; duration, 30 ms) was applied to rupture the neuronal membrane. Membrane potentials were stored on a digital-audio tape (DAT) recorder and corrected for junction potentials at the pipette tip (−11 mV). During the course of the whole-cell recordings, neurons were labeled with Lucifer-Yellow either by spontaneous diffusion or iontophoresis. A chloride ion equilibrium potential of −89 mV was calculated by using the Nernst equation and the intracellular and extracellular chloride concentrations.

Protocols

To confirm that the brainstem–spinal cord preparation was viable, the RVLM was stimulated (5–30 V, 0.1 ms, single pulse) with a tungsten electrode (30 μm tip diameter) to evoke orthodromic action potentials in SPNs.

To determine whether a neuron recorded in the IML area was actually an SPN, we stimulated the ipsilateral Th2 ventral nerve root (5–10 V, 0.1 ms, single pulse) with a suction electrode (resistance: 0.4 MΩ) to evoke an antidromic action potential in the SPN. We used the following criteria, which are similar to those of Lipski (*24*), to identify antidromic action potentials. We considered an action potential to be "antidromic" if 1) a constant latency was obtained between stimulation in the Th2 ventral nerve root and the onset of the action potential, 2) the responses at the threshold voltage were all-or-none, and no underlying potentials were observed when the spike failed, and 3) the action potential had a sharply rising initial slope with no evidence of a preceding expiratory PSP (EPSP).

To examine whether Ang II applied on the medulla side increased the activity of the SPNs, we used a "separate perfusion system." We tried to make sure that the stimulation given by medicines (*e.g.*, Ang II) on the medulla oblongata side alone truly activated SPNs in this preparation. A plastic board was used to divide the preparation at the C1 level, and the solution was changed on the medulla oblongata side alone (separate perfusion system). Whole-cell recordings and extracellular recordings were performed in the SPNs while the medulla side alone was superfused with Ang II (6 μmol/L). In a recent study we found that this dose of Ang II depolarizes RVLM neurons and increases their discharge frequency (*16*). To check the leakage of Ang II to the spinal cord side, we put a few drops of Lucifer-Yellow on the medulla side solution at the end of all experiments and observed that there was no leakage of Lucifer-Yellow to the spinal cord side.

We began performing extracellular recordings of RVLM neurons first, and then performed whole-cell patch-clamp (intracellular) recordings of SPNs. During the recordings the membrane potential of the SPN was slightly hyperpolarized by continuous current injection (−20 pA) to decrease the action potential discharge and thereby improve the likelihood of observing underlying PSPs.

The simultaneous recordings of RVLM neurons and the SPNs were stored on a DAT recorder and later analyzed by spike-triggered averaging with a 0.1 ms sampling clock in a signal processor (7T18A; NEC San-ei, Tokyo, Japan), based on our earlier study (*14*). Two hundred sweeps of membrane potentials in the SPN triggered by spikes in the RVLM neurons were averaged. We analyzed the action potentials in 10 pairs of RVLM neurons and membrane potentials in SPNs. Ang II (6 μmol/L) was superfused on the medulla oblongata side alone (RVLM side) in order to determine whether Ang II increases the occurrence of monosynaptic EPSPs in SPNs.

We defined monosynaptic EPSPs of SPNs according to the following three criteria based on the results of earlier studies (*14*, *19–22*, *25*). 1) Averaged EPSPs have a relatively sharp rise time; 2) the onset latency of the averaged EPSPs in the SPNs after the trigger spike in the RVLM was the same as the latency of the antidromic action potentials in the RVLM neu-

Table 1. Electrophysiological Parameters of the Three Types of SPN*

	Resting membrane potential (mV)	Frequency of action potentials (spikes/s)	Input resistance (MΩ)
Regularly firing neurons (n=5)	57.7±4.2	4.3±1.0	700±350
Irregularly firing neurons (n=20)	54.9±4.3	1.7±1.1	850±140
Silent neurons (n=6)	58.0±3.3		430±390

*Values shown are means±SD. SPN, sympathetic preganglionic neuron; n, number of neurons.

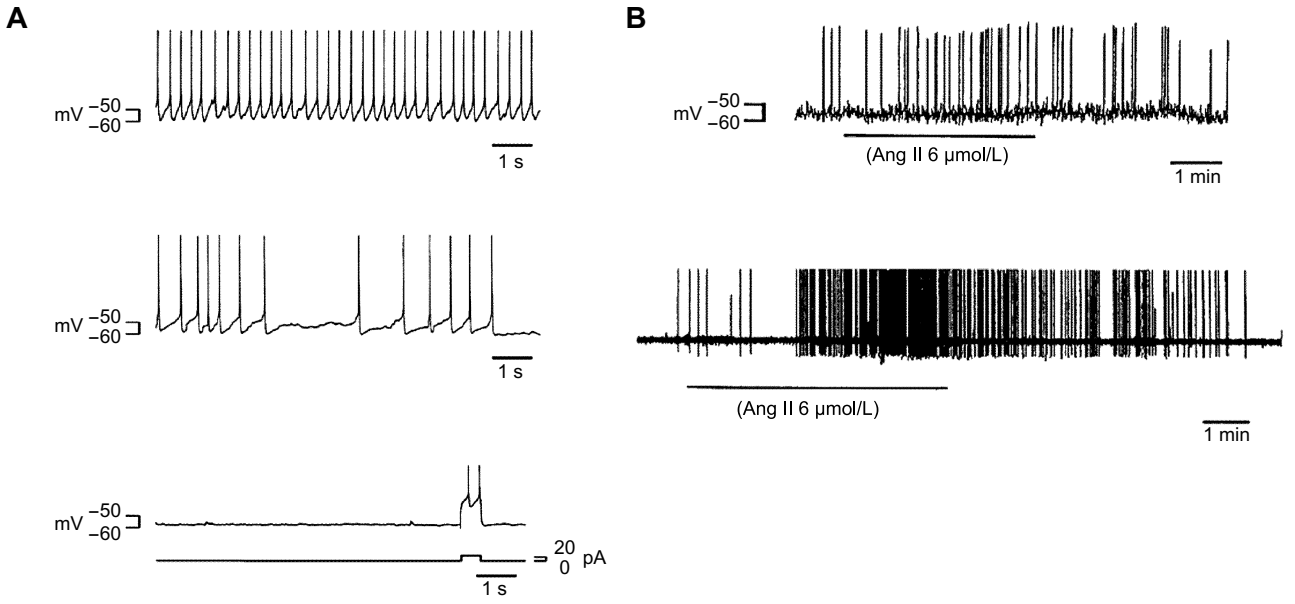

Fig. 2. *A: Three types of SPNs. Top: an example of a regularly firing neuron. Middle: an irregularly firing neuron. Some excitatory postsynaptic potentials (EPSPs) are observed. Bottom: a silent-type neuron. Action potentials are induced only when the membrane potential is depolarized. B: Membrane potentials of an SPN recorded in a separate perfusion system while Ang II (6 μmol/L) was superfused on the medulla oblongata side alone. Membrane depolarization (4.1±0.6 mV) and increased action potential discharge frequency in the SPN were demonstrated by the whole-cell patch-clamp technique (intracellular recording, upper), and an increase in action potential discharge frequency was detected by extracellular recording (lower).*

rons in response to electrical stimulation of the SPNs at the Th2 level; and 3) the average amplitude of the EPSPs is over 2 mV (*14*).

Histology

At the end of the experiments, the preparations were fixed for at least 48 h at 4°C in phosphate-buffered 10% formalin, and transverse 70-μm frozen sections were cut on a cryostat. The recording sites in the RVLM were marked by using Pontamine Sky Blue and photographed through a microscope (Olympus, Tokyo, Japan). Neutral-Red (Aldrich Chemical) staining was performed to verify the position of the neuron within the RVLM area. The sites at which the SPNs were recorded were labeled by Lucifer-Yellow and were photographed through a fluorescence microscope (Olympus). Selected neurons were traced with a *camera lucida* attachment at a magnification of ×400. Neutral-Red staining was performed to verify the position of the neurons within the IML, and the precise location of the marked neurons was identified.

Statistics

The results were expressed as the means±SEM. Comparisons between before and during Ang II superfusion were evaluated by Student's t-test for paired observation. Statistical significance was set at $p<0.05$.

Results

Three Types of SPNs in the Spinal Cord

Orthodromic action potentials were evoked in 31 SPNs after

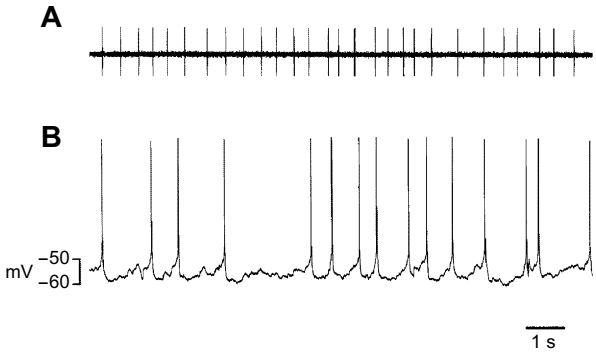

Fig. 3. *One example out of 10 simultaneous recordings of an RVLM unit (A) and an SPN (B). RVLM unit activity was recorded extracellularly, and intracellular events in an SPN were recorded by the whole-cell patch-clamp technique.*

40–60 ms (51.7±3.3 ms) by electrical stimulation of the RVLM area in 7 brainstem–spinal cord preparations (Fig. 1B). All SPNs were antidromically activated by stimulation of the Th2 ventral root, and the latency was 4.8±0.5 ms (Fig. 1C). The axonal conduction velocity was 0.11±0.02 m/s.

Based on their firing patterns, 3 types of SPNs were found in this study: regularly firing SPNs (n=5); irregularly and slowly firing SPNs (n=20); and silent-type SPNs (n=6) (Table 1 and Fig. 2A). PSPs were rarely seen in the regularly firing SPNs and had little effect on the firing pattern. The average spike frequency of the regularly firing neurons was 4.3±1.0 spikes/s (Table 1). PSPs were observed in the irregularly firing SPNs and affected the firing pattern in some neurons.

Effect of Ang II Superfusion of the Medulla Oblongata on PSPs in SPNs

The separate perfusion system was used to superfuse Ang II (6 μmol/L) on the medulla oblongata side alone (n=13). An increase in the action potential firing rate was observed in the SPNs in 11 out of 13 recordings from the brainstem–spinal cord preparations (Fig. 2B, n=13). The 11 recordings consisted of 5 whole-cell recordings of SPNs (1 silent type and 4 irregularly firing neurons) (Fig. 2B, upper trace) and 6 extracellular recordings of SPNs (Fig. 2B, lower trace).

Simultaneous Recording and Spike-Triggered Averaging

We succeeded in making simultaneous recordings of 10 pairs of RVLM neurons and SPNs (Fig. 3). The SPNs exhibited an irregular firing pattern in all 10 preparations. No clear PSPs were detected in the averaged membrane potentials of the SPNs before Ang II superfusion (Fig. 4A).

By contrast, when Ang II was superfused on the medulla oblongata side alone, in 3 out of the 10 pairs of RVLM neurons and SPNs, relatively large amplitude averaged EPSPs (larger than 2 mV) with a sharp rise time appeared in the SPNs at 40±1 ms after an averaged triggering spike in the RVLM neuron (Fig. 4B). In other words, the action potential in the RVLM neuron seemed to elicit the averaged EPSP in the SPN. The latency (40±1 ms) between the spike in the RVLM neuron and the averaged EPSP in the SPN was similar to the latency (45±7 ms) of the antidromic action potentials in an RVLM neuron evoked by electrical stimulation of an SPN (*13*). The calculated conduction velocity was 0.24 m/s. No inhibitory PSPs were observed in the present study. However, since the calculated equilibrium potential for chloride ion was −89 mV, the EPSPs in the SPNs were not reversed inhibitory PSPs (IPSPs).

We measured the electrophysiological properties of the 3

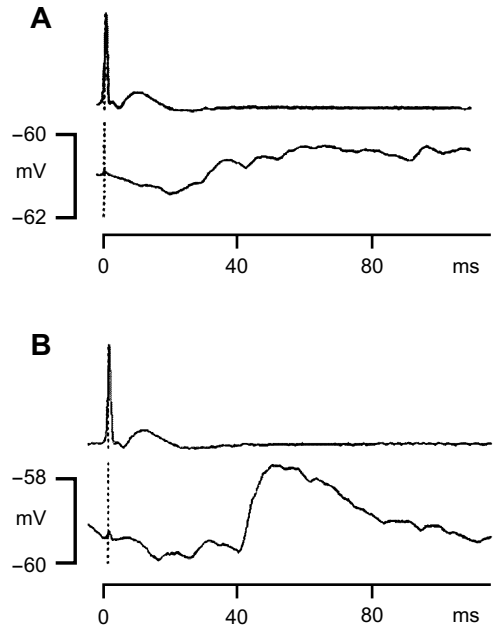

Fig. 4. *Spike-triggered averaging. In each analysis we used 200 consecutive spikes of an RVLM neuron as the trigger and averaged 200 sweeps of membrane potentials in the SPN. We analyzed 10 pairs of RVLM action potentials and PSPs in SPNs. In each simultaneous recording, the top trace shows the averaged triggering spike in the RVLM neuron, and the bottom trace shows the averaged membrane potentials in the SPN. The action potentials of SPNs were inactivated by continuously hyperpolarizing the SPNs in order to observe PSPs. A: Without Ang II superfusion, gradual depolarization and vague elevation of the membrane potential were seen in the SPN, but there was no clear PSP. B: In 3 out of the simultaneous recordings of 10 RVLM neuron-SPN pairs, an averaged EPSP (more than 2 mV) was clearly seen in the SPN during Ang II superfusion on the rostral medulla oblongata alone. It was shown 40±1 ms after the averaged triggering spike in the RVLM neuron.*

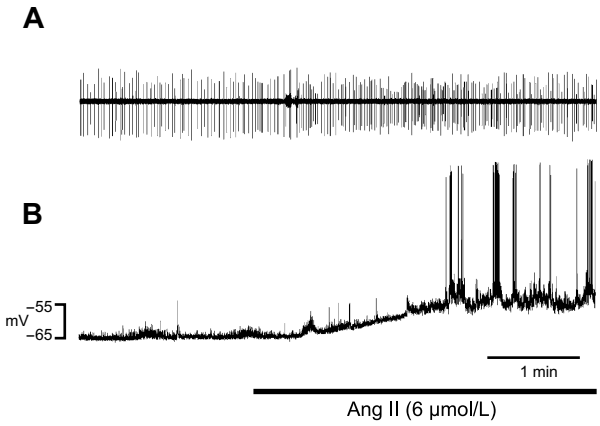

Fig. 5. *Simultaneous recording of the RVLM unit and the membrane potential in the SPN before and during Ang II superfusion. Ang II superfusion caused A: an increase in frequency of action potentials in the RVLM neuron and B: depolarization of the membrane potential in the SPN. During the record the membrane potential of the SPN was slightly hyperpolarized by continuous current injection (−20 pA) to decrease the action potential discharge. In this recording the action potential discharge of the SPN disappeared because of the continuous current injection (−20 pA) before Ang II superfusion.*

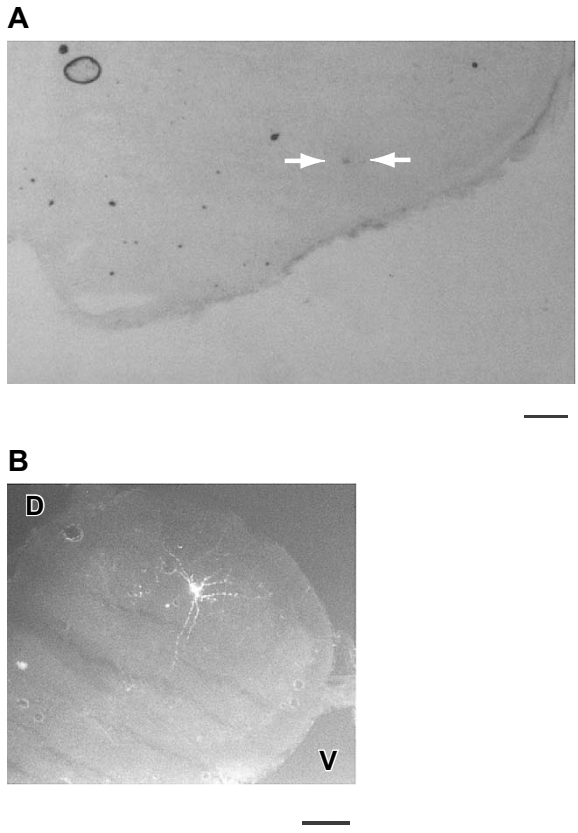

Fig. 6. *A: Pontamine Sky Blue staining of the RVLM indicates the site of extracellular recording. The scale bar is 250 μm. B: Lucifer-Yellow staining of an SPN at the Th2 level. The SPN is located in the intermediolateral cell column. The scale bar is 100 μm. D, dorsal surface; V, ventral surface.*

pairs of RVLM neurons and SPNs in which clear EPSPs were observed. In all three pairs, during Ang II superfusion the spike frequency of the RVLM neurons increased and the membrane potentials in SPNs depolarized (Fig. 5). Ang II superfusion increased the spike frequency of the RVLM neurons from 1.1±0.4 to 1.7±0.2 spikes/s ($p<0.05$), and the number of action potential spikes in SPNs increased significantly, from 0.7±0.4 to 1.3±0.3 spikes/s ($p<0.05$). The membrane potentials of the SPNs tended to depolarize from −56.0±1.6 mV to −53.0±1.2 mV (not significant).

Histology

After extracellular recording of an RVLM neuron, Pontamine Sky Blue staining was performed to localize the recording site in the RVLM (Fig. 6A). Neutral-Red staining demonstrated that its position was within the RVLM area (not shown). Lucifer-Yellow staining was performed after the whole-cell recordings of the SPNs had been completed (Fig. 6B).

Discussion

Technical Considerations and Limitations

In this study we tried to find RVLM neurons which sent axons to SPNs monosynaptically. In this preparation it was technically difficult to examine whether recorded RVLM neurons were baro-sensitive. We thought of RVLM neurons which sent axons monosynaptically to SPNs as putative presympathetic neurons. As we reported in a previous paper (*13*), one method is to examine the antidromic action potentials in the RVLM neurons evoked by electrical stimulation of the SPNs at the Th2 level to prove that those RVLM neurons are projecting axons monosynaptically to the SPNs. However, in this study this was impossible because we needed to put patch-clamp electrodes on SPNs at the Th2 level. So, to show monosynaptic connections between the RVLM neurons and SPNs we used the spike-triggered averaging method, which has been used in previous reports to prove monosynaptic connections between pairs of neurons (*14*, *19–22*).

Only pairs of irregularly firing RVLM neurons and irregularly firing SPNs were examined in the present study, and no other types of neurons were examined. In our previous paper, however, we showed that regularly firing and silent-type RVLM neurons also have antidromic action potentials of the same latency in response to electrical stimulation of SPNs

(*13*). We therefore think that all three types of RVLM neurons might have the same latency.

The latency between the spike in the RVLM neuron and the averaged EPSP in the SPN was relatively long, and its length is ascribed to immature neuronal connections in neonatal rats, which we discussed previously (*13*).

It would have been best if we could have proved that the latency of the averaged EPSP in the SPN after the trigger spike in the RVLM neuron was the same as the latency of antidromic action potentials in the RVLM neuron in response to electrical stimulation of the SPN in each pair of neurons. To this end, we could have examined the latency of antidromic action potentials in the RVLM neuron in response to electrical stimulation of the SPN in each pair by placing the stimulating electrode on the SPN after recording the action potentials of the RVLM neuron and SPN. However, we did not generate the antidromic stimulation in order to avoid destroying the SPNs labeled by Lucifer-Yellow.

Monosynaptic Connection between RVLM Neurons and the SPNs

We have performed simultaneous recordings of RVLM neurons and SPNs and analyzed the data by spike-triggered averaging, a sensitive technique that permits definitive determination of the presence of a connection and its excitatory or inhibitory character. The spike-triggered averaging method was originally developed to document unitary inputs to spinal neurons (*26*, *27*), and it has been used to prove monosynaptic connections between pairs of neurons in many previous studies (*14*, *19–22*). In other words, spike-triggered averaging has been used to evaluate how the PSPs correlate with presynaptic spikes from an identified neuron, and it is especially useful when individual synaptic potentials are buried in noise. In this study we used the spike-triggered averaging method to examine the relation between RVLM neurons and SPNs. This method has two advantages. By using action potentials of RVLM neurons as triggers, it is possible to prove whether action potentials of an RVLM neuron directly stimulate an SPN. Also, the result from the spike-triggered averaging method indicates the change of synaptic strength between an RVLM neuron and an SPN when the circumstances around these neurons are altered. To examine whether the connections between the RVLM neurons and SPNs are monosynaptic, we tested the relationship between 10 pairs of RVLM neurons and SPNs by averaging 200 sweeps of membrane potentials in the SPN triggered by spikes in the RVLM neuron. In the present study we did not find any clear monosynaptic connection between any of the 10 pairs of RVLM neurons and SPNs tested without using Ang II. However, by using Ang II we were able to demonstrate a clear averaged EPSP in SPNs triggered by an action potential in RVLM neurons in 3 out of 10 pairs of RVLM neurons and SPNs (Fig. 4B).

As mentioned in the Methods section, we defined monosynaptic EPSPs based on earlier studies (*14*, *19–21*, *25*). In 3 out of the 10 simultaneous recordings during Ang II superfusion, averaged EPSPs with an amplitude above 2 mV and a sharp rise were constantly observed in SPNs at 40 ± 1 ms after the averaged triggering spike recorded in RVLM neurons. This latency between the action potential in RVLM neurons and averaged EPSPs in SPNs is similar to the latency (45 ± 7 ms) of the antidromic action potentials recorded in RVLM neurons in response to electrical stimulation in the IML (*13*). These results indicate the presence of the monosynaptic connections between RVLM neurons and SPNs in these 3 pairs.

We characterized SPNs by both antidromic activation and dye labeling with Lucifer-Yellow. Previous studies detected connections between RVLM neurons and sympathetic nerves by simultaneous recordings of the activity of RVLM neurons and sympathetic nerves. However, they did not use the intracellular recording method (*28–30*). To our knowledge, there have been few examples of simultaneous recording of RVLM units extracellularly and SPNs by whole-cell recording that have permitted the demonstration of monosynaptic connections electrophysiologically.

The first author of the present study and colleagues have recently shown that monosynaptic connections between RVLM neurons and IML neurons were clarified when arterial blood pressure was decreased (*15*). They concluded that increased action potential frequency in RVLM neurons was needed to demonstrate the monosynaptic connections between RVLM and IML neurons. In the present study, Ang II increased the frequency of firing in RVLM neurons and thus potentiated the synaptic strength between RVLM neurons and SPNs. Although Oshima *et al*. (*15*) did not prove that IML neurons were SPNs, in the present study we were able to demonstrate monosynaptic connections between RVLM neurons and SPNs since we confirmed the antidromic action potentials in the SPNs in response to the stimulation of the ventral root. Therefore, the present study extends the previous study of Oshima *et al*. (*15*) in that we were able to demonstrate monosynaptic connections between RVLM neurons and SPNs during Ang II superfusion.

The detection of connectivity was facilitated by increasing the firing rate of neurons in the RVLM by Ang II superfusion in the rostral section of the chamber and by continuously hyperpolarizing the SPNs during whole-cell patch recording. The most likely mechanism by which Ang II revealed the monosynaptic connection is by somehow increasing the proportion of somatic action potentials that propagate along the axons of the RVLM neurons, presumably by facilitating initial segment depolarization. Previous reports have demonstrated frequency-dependent facilitation of neurotransmitter release from nerve terminals (*31*, *32*), and we speculate that EPSPs may have been present in the SPNs prior to Ang II superfusion in the present study, but that they were not large or frequent enough to be detected easily. Alternatively, they may have been concealed by other PSPs derived from neurons that synapse with SPNs. Ang II superfusion thus

increased the frequency of the action potentials in the RVLM neurons, which in turn increased the amount of neurotransmitter release at the axon terminal onto the SPNs and resulted in larger-amplitude EPSPs that were more easily detected. The finding in the present study that Ang II revealed and potentiated a synaptic connection between an RVLM neuron and an SPN explains the mechanism by which Ang II in the medulla oblongata increases peripheral sympathetic nerve activity and arterial blood pressure. Our data are supported by a previous study showing that Ang II activates the Ang II type 1 (AT_1) receptors on the glutamatergic neurons in the RVLM and that the activation enhances the sympathoexcitatory signals to the IML (*33*). The presence of AT_1 receptors enhanced by Ang II in the RVLM area has been verified in previous studies, including our own (*3*, *16*, *17*, *33*, *34*).

We did not observe "averaged inhibitory PSPs" in SPNs. The possibility that RVLM neurons send the inhibitory signals to SPNs cannot be ruled out. If we had examined many more pairs of RVLM neurons and SPNs, we would have been able to show "averaged inhibitory PSPs" in SPNs that are evoked by the action potentials of RVLM neurons. However, we think that the probability of "EPSPs in SPNs" is higher than that of "IPSPs in SPNs." The fact that RVLM neurons sent excitatory signals to SPNs has been reported in previous papers (*35*, *36*), and we were able to show that RVLM neurons induced EPSPs in SPNs in all 3 pairs in the present study.

There are polysynaptic connections as well as a monosynaptic connection between the RVLM neuron and SPN, and they may be inhibitory or excitatory (*37*, *38*). The physiological significance of the polysynaptic inputs remains to be determined. Furthermore, in addition to synapsing with RVLM neurons, SPNs also receive synaptic input from neurons in the rostral ventromedial medulla, the pontine A5 noradrenergic cell group, the caudal medullary raphe, and the hypothalamic paraventricular nucleus (*39*). Further study is needed to characterize the nature of the connections between the neurons in these higher regions and SPNs.

In our previous paper, we reported that Ang II superfusion induced a greater increase in the activity of RVLM neurons in spontaneously hypertensive rats (SHR) than in WKY rats (*16*). RVLM neurons in SHR might be more sensitive to neuro-transmitters or -modulators which can increase arterial blood pressure (*i.e.*, noradrenaline, Ang II and so on) than those in WKY rats. The more the frequency of action potentials in RVLM neurons increases, the more synaptic strength between RVLM and SPNs would be potentiated. As a result, SPNs would be more activated and arterial blood pressure would increase. Such a mechanism would help to explain why arterial blood pressure is higher in SHR than in WKY rats.

Electrophysiological Characteristics of the SPNs

The 3 types of firing patterns of the SPNs were similar to those of the neurons of the RVLM which we have reported previously (*13*). Few PSPs were recorded in regularly firing SPNs, and more EPSPs and IPSPs were found in the irregularly firing SPNs. The seminal classification of SPNs was reported in *in vivo* studies using high impedance electrodes (*6*). We obtained the same results by using the whole-cell patch-clamp in a brainstem–spinal cord preparation in which the sympathetic neuronal network was preserved. We speculate that irregularly firing neurons receive more synaptic inputs from other neurons or astrocytes, although it is unclear from which neurons the inputs come. The frequency of regularly firing SPNs was 4.3 ± 1.0 spikes/s. Since the frequency was similar to that of regularly firing neurons in the RVLM, all of which discharged spontaneously (*13*), a frequency of around 4 spikes/s may be the basal frequency of individual elements in the sympathetic nervous system of newborn rats studied in the bath at 25–26°C. Many SPNs clearly receive inputs from the presympathetic neurons in the RVLM that play a major role in generating their activity (*8*, *14*, *39*). However, there may also be SPNs that discharge spontaneously to maintain sympathetic activity and rhythmicity at the spinal level (*40*, *41*).

Effect of Ang II Superfusion of the Rostral Medulla Oblongata on the Activity of SPNs

By using the separate perfusion system we were able to demonstrate that Ang II superfusion on the medulla oblongata side activated the SPNs (Fig. 2B). The simplest explanation for this finding is that Ang II directly activated bulbospinal sympathoexcitatory neurons in the RVLM, which in turn activated the SPNs. In previous studies Ang II was found to depolarize neurons in the RVLM and increase their firing rate (*3*, *16*). There are also some reports which showed that Ang II increased the activity of RVLM neurons (*33*, *34*). We hypothesized that Ang II in the RVLM area was one of the important modulators which can cause hypertension. However, other areas besides the RVLM, such as the caudal raphe nucleus and the rostral ventromedial medulla (*39*), can activate SPNs in terms of sympathetic activation.

In summary, we succeeded in simultaneous recording of RVLM neurons and SPNs in the spinal cord of neonatal rats and in demonstrating a monosynaptic sympathoexcitatory connection between the RVLM neuron and SPN by spike-triggered averaging. Ang II superfusion of the medulla side alone unmasked the connection between neurons in the RVLM and SPNs, suggesting that when RVLM neurons are stimulated and generate high frequency action potentials, they can evoke clear EPSPs in SPNs. The combination of simultaneous recording and spike-triggered averaging enabled us to reveal a monosynaptic bulbospinal sympathoexcitatory pathway from a single RVLM neuron to a single SPN, and no inhibitory connections were observed.

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