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ORIGINAL ARTICLE

Expression and functions of β_1- and β_2-adrenergic receptors on the bulbospinal neurons in the rostral ventrolateral medulla

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The expression and effects of β-adrenergic receptors (β-ARs) on the neurons of the bulbospinal rostral ventrolateral medulla (RVLM) have been limitedly examined to date. The objective of this study was to examine the expression of β_1- and β_2-ARs on the bulbospinal RVLM neurons electrophysiologically and histologically. To directly investigate whether RVLM neurons display sensitivity to metoprolol (a β_1-AR antagonist), dobutamine (a β_1-AR agonist), butoxamine (a β_2-AR antagonist), and salbutamol (a β_2-AR agonist), we examined changes in the membrane potentials of the bulbospinal RVLM neurons using the whole-cell patch-clamp technique during superfusion of these drugs. During metoprolol superfusion, 16 of the 20 RVLM neurons were hyperpolarized, and 5 of the 6 RVLM neurons were depolarized during dobutamine superfusion. During butoxamine superfusion, 11 of the 16 RVLM neurons were depolarized, and all of the 8 RVLM neurons were hyperpolarized during salbutamol superfusion. These results suggest the expression of β_1- and β_2-ARs on the RVLM neurons. To determine the presence of β_1- and β_2-ARs histologically, immunofluorescence examination was performed. Five metoprolol-hyperpolarized neurons were examined for β_1-AR and tyrosine hydroxylase (TH) immunoreactivity. All of the neurons displayed β_1-AR immunoreactivity, whereas three of the neurons displayed TH immunoreactivity. All of the five RVLM neurons that became depolarized during metoprolol superfusion and hyperpolarized during butoxamine superfusion displayed β_1- and β_2-AR immunoreactivity. Our findings suggest that β_1-AR antagonists or β_2-AR agonists may decrease blood pressure through decreasing the activity of the bulbospinal RVLM neurons.

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INTRODUCTION

The actions of catecholamines include vasoconstriction, endothelial dysfunction and sodium reabsorption and retention,[1,2] all of which increased the blood pressure (BP). The rostral ventrolateral medulla (RVLM), which contains presympathetic neurons, contains C1 neurons.[3,4] C1 neurons express catecholamine-synthesizing enzymes, such as tyrosine hydroxylase (TH), dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase,[5] and have the potential to utilize adrenaline, noradrenaline or dopamine as neurotransmitters. Therefore, catecholamines are considered to participate in the control of the BP through adrenergic receptors (ARs) expressed on the presympathetic neurons.[6] In fact, the neurons in the RVLM area express α_2-ARs,[7,8] and α_2-AR agonists have been used clinically as antihypertensive drugs.[9]

Some previous studies demonstrated that the effects of catecholamines are mediated by β-ARs expressed on the neurons that affect sympathetic nerve activities. Koepke *et al.*[10,11] examined the roles of β-ARs in the central nervous system and reported that β_2-ARs in the posterior hypothalamus mediate the increased renal sympathetic nerve activity and antinatriuresis. The clear interaction between the posterior hypothalamus and RVLM is not well known, but these studies suggest that β-ARs are present on the neurons that affect sympathetic nerve activities. Sun *et al.*[12] reported the presence of β-ARs in the rostral medullary pacemaker neurons by applying propranolol, and Privitera *et al.*[13] demonstrated that microinjection of propranolol (a non-selective β-AR antagonist) into the bilateral C1 areas suppresses the neural activity of neurons in the C1 area. However, few reports have described the presence of β_1- and β_2-ARs on the bulbospinal RVLM neurons and the physiological roles of these receptors expressed on RVLM neurons.

In this study, we examined the effects of β-AR antagonists or agonists on the bulbospinal RVLM neurons using brainstem–spinal cord preparations, which possess a preserved sympathetic nervous system.[14–22] While recording the membrane potentials (MPs) of the

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bulbospinal RVLM neurons using the patch-clamp technique, the neurons were superfused with metoprolol (β_1-AR antagonist), dobutamine (β_1-AR agonist), butoxamine (β_2-AR antagonist) or salbutamol (β_2-AR agonist). Furthermore, to examine the direct effects of metoprolol or butoxamine on the RVLM neurons, the superfusion was conducted with each drug dissolved in a low-Ca^{2+}, high-Mg^{2+} or tetrodotoxin (TTX) solution. After recording the MPs of the RVLM neurons, the β_1- and β_2-AR immunoreactivity of the neurons were examined by immunofluorescence staining.

EXPERIMENTAL PROCEDURES

General preparations

Experiments were performed on brainstem–spinal cord preparations collected from 1- to 5-day-old Wistar rats as previously described.[15–22] The experimental protocols were approved by the Institutional Review Board of National Defense Medical College in accordance 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 T helper type 2 (Th_2) level, and the brainstem was sectioned between the roots of cranial nerve VI and the lower border of the trapezoid body. The preparations were continuously superfused with a solution containing (in mmol l^{-1}) 124 NaCl, 5.0 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$ and 30 glucose. The preparations were maintained at 25–26 °C (artificial cerebrospinal fluid). The pH (7.4) and oxygenation were maintained by bubbling 90% O_2–5% N_2–5% CO_2 through the solution. To avoid recording respiratory neuronal activities, we examined the phrenic nerve activity from the ventral root of C_4 and excluded neurons that displayed a strong phasic relationship with phrenic nerve discharges from the subsequent experiments.[17,18,22]

Patch-clamp electrodes

Electrodes were pulled in one stage from thin-wall borosilicate filament capillaries (GC100TF-10, outer diameter 1.0 mm, Clark Electromed, Reading, UK) with a vertical puller. The electrodes had a tip diameter of 1.8–2.0 μm and a resistance of 4–6 MΩ. The electrode solution for the whole-cell recordings consisted of (in mmol l^{-1}): 130 potassium gluconate, 10 HEPES, 10 EGTA, 1 $CaCl_2$ and 1 $MgCl_2$, and the pH was adjusted to 7.2–7.3 using KOH. The electrode tips were filled with 1% Lucifer Yellow (Sigma, St Louis, MO, USA).

Recording procedure

Electrodes were inserted into the RVLM. A patch-clamp amplifier (AxoPatch, ID; Axon Instruments, Sunnyvale, CA, USA) was used to record the MPs. The RVLM neurons were obtained from the ventral side of the medulla. Before the intracellular whole-cell recordings, we observed the firing pattern of the target neurons using extracellular recordings. After obtaining a GΩ seal, a single-shot hyperpolarizing pulse (0.6–0.9 nA; duration, 30 ms) was applied to rupture the neuronal membrane. To determine whether each of the recorded RVLM neurons was indeed a bulbospinal neuron, the existence of antidromic action potentials (APs) in the RVLM neurons was examined by delivering electrical stimulation in the intermediolateral cell column at the Th_2 level with a tungsten electrode (30 μm tip diameter, Unique Medical, Tokyo, Japan).[17,18,22] The RVLM neurons that displayed antidromic APs with electrical stimulation in the intermediolateral cell column at the Th_2 level were considered to be bulbospinal RVLM neurons. The MPs were recorded using the current-clamp technique (20 pA increments from −100 to 20 pA, 500 ms duration). All data were recorded and analyzed using PowerLab (AD Instruments, Colorado Springs, CO, USA). Membrane resistances of the RVLM neurons were calculated from the current–voltage curves. During the course of the whole-cell recordings, neurons were labeled with 0.2% Lucifer Yellow (lithium salt; Sigma-Aldrich, St Louis, MO, USA) either by spontaneous diffusion or iontophoresis. A chloride ion equilibrium potential of −89 mV was calculated using the Nernst equation and the intracellular and extracellular chloride concentrations.

Experimental protocols

Protocol (1): During the MP recordings of the bulbospinal RVLM neurons, the preparations were superfused with metoprolol (20 μmol l^{-1},[23] Sigma) and/or butoxamine (20 μmol l^{-1},[24] Sigma) dissolved in artificial cerebrospinal fluid. To confirm the effects opposite to those of metoprolol and butoxamine, the preparations were superfused with dobutamine (5 μmol l^{-1},[25] Sigma) and salbutamol (5 μmol l^{-1},[25] Sigma). The duration of each drug superfusion was 4–10 min. During superfusion with each drug, we defined depolarization and hyperpolarization as an increase or decrease of the MP by >2 mV, respectively. The changes in the MPs were determined 4 min after the start of superfusion with each drug.

Protocol (2): The bulbospinal RVLM neurons were superfused with a low-Ca^{2+}, high-Mg^{2+} solution[26] for 30–40 min or TTX (0.5 mmol l^{-1},[27] Na^+ channel blocker, Sigma) for 10 min to block synaptic transmissions from other neurons to the recorded bulbospinal RVLM neurons. Thereafter, the neurons were superfused with metoprolol or butoxamine dissolved in a low-Ca^{2+}, high-Mg^{2+} solution or TTX, and the MPs were recorded.

Immunofluorescence staining

To confirm the presence of β_1- and β_2-ARs histologically, immunofluorescence staining was performed. After the aforementioned experiments, the preparations were fixed for 1 h at 4 °C in 4% paraformaldehyde in 0.1 m phosphate-buffered saline, immersed in 18% sucrose–phosphate-buffered saline overnight, embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), frozen on dry ice and cut into 20-μm-thick transverse sections followed by immunofluorescence staining.

Immunofluorescence staining protocols

Protocol (1): To confirm the existence of neurons expressing both β_1-AR and TH in the RVLM, the following primary antibodies were used: rabbit anti-β_1-AR antibody (1:400 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-TH antibody (1:400 dilution, Sigma-Aldrich). The secondary antibodies used (1:1000 dilution) for the immunofluorescence staining were Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes/Invitrogen, Carsbad, CA, USA) and Alexa Fluor 633 goat anti-mouse IgG. The Lucifer Yellow-stained RVLM neurons that responded to metoprolol were examined for both β_1-AR and TH immunoreactivity using the triple-merged images.

Protocol (2): To confirm the existence of neurons expressing both β_2-AR and TH in the RVLM, rabbit anti-β_2-AR antibody (1:400 dilution, Santa Cruz Biotechnology) and mouse anti-TH were used as the primary antibodies. The secondary antibodies used for the fluorescence staining were Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG. The Lucifer Yellow-stained RVLM neurons that responded to butoxamine were examined for both β_2-AR and TH immunoreactivity using the triple-merged images.

Protocol (3): To confirm the co-existence of β_1- and β_2-ARs on the RVLM neurons, goat anti-β_1-AR antibody and rabbit anti-β_2-AR (1:400 dilution, Sigma-Aldrich) were used as the primary antibodies. The secondary antibodies for fluorescence staining were Alexa Fluor 633 donkey anti-goat IgG (Molecular Probes/Invitrogen) and Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes/Invitrogen). The Lucifer Yellow-stained RVLM neurons that responded to both metoprolol and butoxamine were examined for β_1- and β_2-AR immunoreactivity using the triple-merged images.

Images of the immunofluorescence samples and Lucifer Yellow-stained RVLM neurons were obtained with $\times 20$ or $\times 40$ objectives on an Olympus FV1000 confocal microscope (Olympus Optical) or conventional fluorescence microscope (BX60, Olympus Optical, Tokyo, Japan).[16]

Statistics

The results were expressed as means ± s.e.m. Comparisons of the MPs recorded before and during the superfusion with the drugs were performed using the Student's *t*-test for paired observations. Statistical significance was set at $P<0.05$.

RESULTS

The recorded bulbospinal RVLM neurons ($n=93$) were classified into three types based on their firing patterns:[17] regularly firing neurons ($n=18$; resting MP, -41.0 ± 0.7 mV; frequency of APs (FAPs), 3.6 ± 0.2 spikes s^{-1}), irregularly firing neurons ($n=65$; resting MP,

-44.4 ± 1.1 mV[#]; FAPs, 1.1 ± 0.2 spikes s^{-1}) ([#]$P<0.05$ *vs.* regularly firing neurons), and silent-type neurons ($n=10$; resting MP, -46.0 ± 0.9 mV*) (*$P<0.01$ *vs.* regularly firing neurons).

Of the 20 recorded neurons (regularly firing, 3; irregularly firing, 15; silent-type, 2), 16 (regularly firing, 3; irregularly firing, 12; silent-type, 1) displayed hyperpolarization in response to metoprolol superfusion. Of the 16 recorded neurons (regularly firing, 2; irregularly firing, 12; silent-type, 2), butoxamine superfusion induced depolarization in 11 (regularly firing, 2; irregularly firing, 8; silent-type, 1) of the neurons. No significant changes in the membrane resistance of the recorded RVLM neurons were observed in response to superfusion with any drug (except salbutamol) (Tables 1a and b).

The effects of metoprolol and dobutamine on the bulbospinal RVLM neurons

Of the 20 recorded bulbospinal RVLM neurons, 16 showed hyperpolarization and decreased FAPs during metoprolol superfusion (Figure 1a, Table 1a). Furthermore, when 4 of these 16 neurons were subjected to superfusion with 20 μmol l^{-1} metoprolol followed by 100 μmol l^{-1} metoprolol, stronger hyperpolarization was observed during the 100 μmol l^{-1} metoprolol superfusion (-4.5 ± 2.2 mV *vs.* -3.5 ± 1.5 mV, not statistically significant; Figure 1b).

Of the 12 recorded bulbospinal RVLM neurons, 9 displayed hyperpolarization during superfusion with metoprolol dissolved in a low-Ca^{2+}, high-Mg^{2+} solution (Figure 1c, Table 1b). Furthermore, three of the four bulbospinal RVLM neurons also displayed hyperpolarization during superfusion with metoprolol dissolved in TTX solution (before -45.4 ± 2.2 mV, during -48.1 ± 2.2 mV, $P<0.01$; data not shown).

To examine the effects of β_1-AR agonists on the bulbospinal RVLM neurons, dobutamine solution was applied. Of the six recorded bulbospinal RVLM neurons, five displayed depolarization and increased FAPs in response to dobutamine superfusion (Figure 1d, Table 1a).

The effects of butoxamine and salbutamol on the bulbospinal RVLM neurons

Of the 16 recorded RVLM neurons, 11 showed depolarization and increased FAPs during butoxamine superfusion (Figure 2a, Table 1a). Of the eight bulbospinal RVLM neurons superfused with butoxamine dissolved in a low-Ca^{2+}, high-Mg^{2+} solution, seven showed

Table 1b Changes in the membrane potentials of bulbospinal RVLM neurons during superfusion with each of the drugs dissolved in a low-Ca^{2+}, high-Mg^{2+} solution

Drug			*MP (mV)*	*MR (MΩ)*
Metoprolol (20 μmol l^{-1})	Depolarization	Before	-43.7 ± 1.5	254 ± 21
	($n=2$)	During	-40.0 ± 0.1	266 ± 33
	No change	Before	-42.4	325
	($n=1$)	During	-43.0	333
	Hyperpolarization	Before	-43.5 ± 0.3	451 ± 45
	($n=9$)	During	-47.3 ± 1.0*	325 ± 50
Butoxamine (20 μmol l^{-1})	Depolarization		-43.0 ± 0.6	316 ± 44
	($n=7$)	During	-39.5 ± 0.7*	308 ± 58
	No change			
	($n=0$)			
	Hyperpolarization	Before	-42.5	357
	($n=1$)	During	-45.1	340

Abbreviations: FAP, frequency of action potential; MP, membrane potential; MR, membrane resistance; RVLM, rostral ventrolateral medulla. Values are mean ± s.e.m. *$P<0.01$ *vs.* before each drug superfusion.

Table 1a Changes in the membrane potentials of bulbospinal RVLM neurons during superfusion with each of the drugs

Drug			*MP (mV)*	*FAP (Hz)*	*MR (MΩ)*
Metoprolol (20 μmol l^{-1})	Depolarization	Before	-40	0.3	560
	($n=1$)	During	-37.2	0.8	440
	No change	Before	-46.9 ± 2.8	0.2 ± 0.1	426 ± 91
	($n=3$)	During	-47.0 ± 3.1	0.3 ± 0.1	374 ± 62
	Hyperpolarization	Before	-43.0 ± 0.9	1.9 ± 0.4	420 ± 43
	($n=16$)	During	-46.5 ± 1.0*	0.5 ± 0.2*	393 ± 43
Dobutamine (5 μmol l^{-1})	Depolarization	Before	-43.3 ± 2.2	0.8 ± 0.3	430 ± 11
	($n=5$)	During	-40.9 ± 2.8*	1.6 ± 0.3*	399 ± 12
	No change				
	($n=0$)				
	Hyperpolarization	Before	-41.6	0	490
	($n=1$)	During	-43.7	0	527
Butoxamine (20 μmol l^{-1})	Depolarization	Before	-46.2 ± 2.3	0.2 ± 0.1	418 ± 11
	($n=11$)	During	-44.0 ± 2.3*	0.9 ± 0.1*	389 ± 97
	No change	Before	-41.7 ± 1.7	0.2 ± 0.1	722 ± 11
	($n=2$)	During	-42.8 ± 1.0	0.3 ± 0.1	701 ± 16
	Hyperpolarization	Before	-44.0 ± 2.5	0.5 ± 0.2	336 ± 14
	($n=3$)	During	-47.0 ± 2.3[#]	0.1 ± 0.1	335 ± 14
Salbutamol (5 μmol l^{-1})	Depolarization				
	($n=0$)				
	No change				
	($n=0$)				
	Hyperpolarization	Before	-42.8 ± 1.7	1.1 ± 0.2	477 ± 32
	($n=8$)	During	-45.0 ± 1.8*	0.3 ± 0.1[#]	399 ± 32[#]

Abbreviations: FAP, frequency of action potential; MP, membrane potential; MR, membrane resistance; RVLM, rostral ventrolateral medulla. Values are mean ± s.e.m. *$P<0.01$, [#]$P<0.05$ *vs.* before each drug superfusion. The hyperpolarized neuron under butoxamine superfusion was a silent-type one.

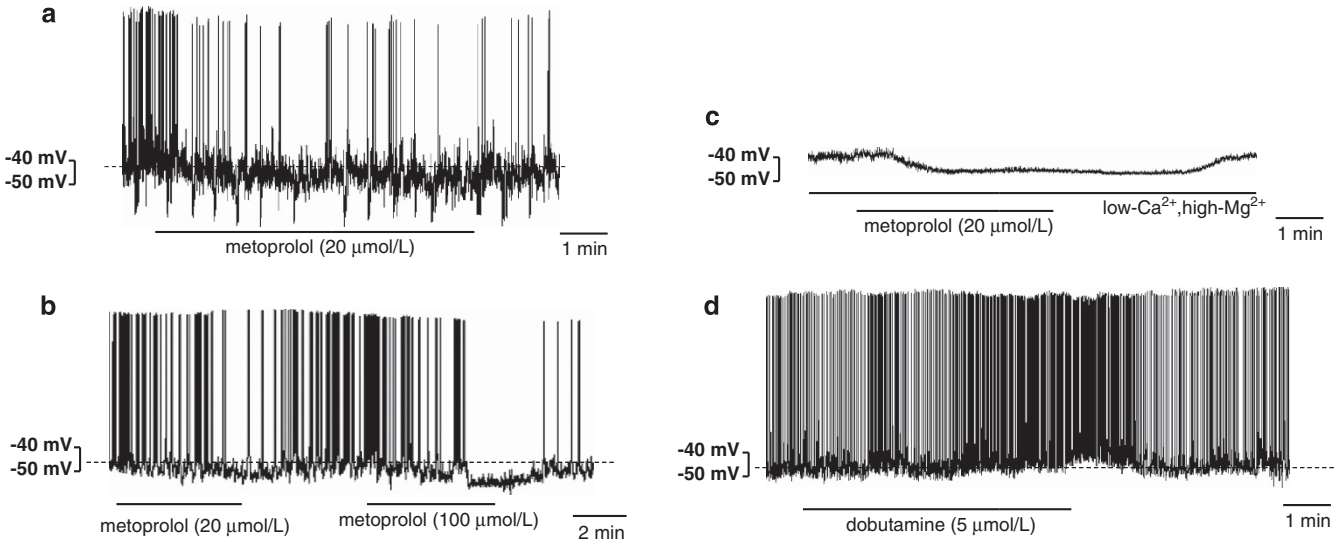

Figure 1 Changes in the membrane potentials (MPs) of the bulbospinal RVLM neurons during metoprolol (β_1-AR antagonist) and dobutamine (β_1-AR agonist) superfusion. (**a**) Bulbospinal RVLM neurons showed hyperpolarization during metoprolol superfusion. (**b**) While the MPs of the RVLM neurons were recorded, the cells were superfused with 20 μmol l^{-1} metoprolol followed by 100 μmol l^{-1} metoprolol; RVLM neuron hyperpolarization occurred in a dose-dependent manner. (**c**) Bulbospinal RVLM neurons showed hyperpolarization during superfusion with metoprolol dissolved in a low-Ca^{2+}, high-Mg^{2+} solution. This neuron showed an irregularly firing pattern but no spontaneous discharge given that it showed no APs during superfusion with a low-Ca^{2+}, high-Mg^{2+} solution. (**d**) Bulbospinal RVLM neurons showed depolarization during dobutamine superfusion.

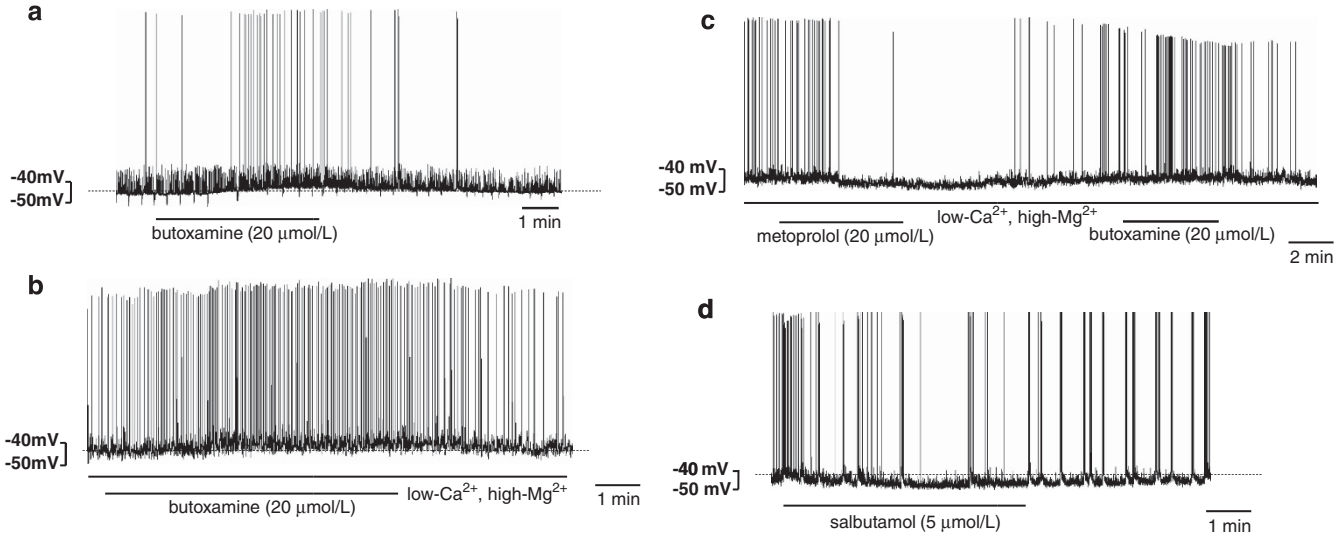

Figure 2 Changes in the MPs of the bulbospinal RVLM neurons during butoxamine (a β_2-AR antagonist) and salbutamol (a β_2-AR agonist) superfusion. (**a**) Bulbospinal RVLM neurons showed depolarization and an increase in the frequency of APs during butoxamine superfusion. (**b**) Bulbospinal RVLM neurons showed depolarization during superfusion with butoxamine dissolved in a low-Ca^{2+}, high-Mg^{2+} solution. This result suggests β_2-AR in these bulbospinal RVLM neurons. (**c**) Recording of the MPs of the bulbospinal RVLM neurons while the cells were superfused with metoprolol followed by butoxamine; both drugs were dissolved in a low-Ca^{2+}, high-Mg^{2+} solution. The neurons showed hyperpolarization during the metoprolol superfusion and depolarization during the butoxamine superfusion under the blockade of the synaptic transmissions. (**d**) Bulbospinal RVLM neurons showed hyperpolarization during salbutamol superfusion.

depolarization (Figure 2b, Table 1b). Additionally, all of the four bulbospinal RVLM neurons displayed depolarization during superfusion with butoxamine dissolved in TTX solution (before -45.3 ± 2.3 mV, during -41.2 ± 1.9 mV, $P<0.05$; data not shown).

As shown in Figure 3c, MPs of bulbospinal RVLM neurons ($n=5$) were recorded during superfusion with metoprolol dissolved in a low-Ca^{2+}, high-Mg^{2+} solution followed by superfusion with butoxamine dissolved in a low-Ca^{2+}, high-Mg^{2+} solution. In all the cases, the neurons displayed hyperpolarization during superfusion with metoprolol and depolarization during superfusion with butoxamine. To examine the effects of β_2-AR agonists on the bulbospinal RVLM neurons, salbutamol superfusion was performed. All of the eight recorded bulbospinal RVLM neurons displayed hyperpolarization and decreased FAPs during the salbutamol superfusion (Figure 2d,

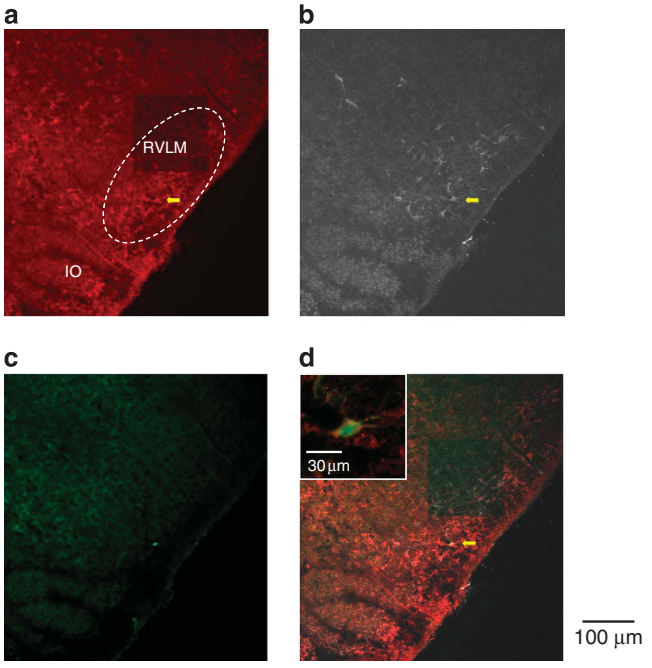

Figure 3 Histological examination of the bulbospinal RVLM neurons in transverse sections. (**a**) β_1-AR-immunoreactive neurons are indicated in red. Numerous β_1-AR immunoreactive neurons are visualized in the RVLM. The arrow indicates the recorded bulbospinal RVLM neuron. IO, inferior olivary nucleus. (**b**) The tyrosine hydroxylase (TH)-immunoreactive neurons in the RVLM are indicated in white. The arrow indicates the recorded bulbospinal RVLM neuron. (**c**) The Lucifer Yellow-stained bulbospinal RVLM neuron is presented in green. This neuron showed hyperpolarization during metoprolol superfusion. (**d**) Triple-merged image of panels (**a**–**c**). The arrow indicates the Lucifer Yellow-stained bulbospinal RVLM neuron exhibiting both β_1-AR and TH immunoreactivity. In the inset, the merged bulbospinal RVLM neuron is visualized under higher magnification.

Table 1a). The membrane resistance of the recorded RVLM neurons displayed significant changes during the salbutamol superfusion (Table 1a).

Immunoreactivity

Lucifer Yellow staining was performed after the whole-cell recordings of the bulbospinal RVLM neurons were completed (Figures 3–5). Five of the RVLM neurons displaying hyperpolarization during metoprolol superfusion were examined for β_1-AR immunoreactivity. All of these neurons were confirmed to be located in the RVLM and displayed β_1-AR immunoreactivity (Figures 3a, c, d and 4a). TH immunoreactivity was also observed in three of these neurons (Figures 3a–d). A triple-merged image revealed that approximately all of the TH-immunoreactive neurons in the RVLM area exhibited β_1-AR immunoreactivity (Figures 3a, b and d). Furthermore, two bulbospinal RVLM neurons that showed no change in MPs during metoprolol superfusion were examined for β_1-AR immunoreactivity and were negative for β_1-AR immunoreactivity (Figure 4b).

Three RVLM neurons that showed depolarization during butoxamine superfusion were examined for β_2-AR immunoreactivity; all of these neurons were confirmed to be located in the RVLM and display β_2-AR immunoreactivity (Figure 4c). Two of the neurons also displayed TH immunoreactivity. Approximately, all of the TH-immunoreactive neurons in the RVLM also exhibited β_2-AR immunoreactivity.

Five of the bulbospinal RVLM neurons that showed hyperpolarization during metoprolol superfusion and depolarization during the subsequent butoxamine superfusion (Figure 2c) were examined for β_1- and β_2-AR immunoreactivity, and all of these neurons exhibited both β_1- and β_2-AR immunoreactivity (Figures 5a–d). The number of β_1- and β_2-AR-immunoreactive RVLM neurons was ascertained in three 20-μm-thick transverse sections, including the Lucifer Yellow-stained neurons. The number of β_1- and β_2-AR-immunoreactive RVLM neurons (unilateral) was 199.0 ± 15.4 per section and 192.7 ± 20.8 per section, respectively. The number of RVLM neurons displaying both β_1- and β_2-AR immunoreactivity was 180.0 ± 21.6 per section. In the RVLM, most of the β_1-AR-immunoreactive neurons also exhibited β_2-AR immunoreactivity; however, complete overlap was not observed (Figures 5a, b and d).

DISCUSSION

β_1- and β_2-ARs on the bulbospinal RVLM neurons

RVLM neurons, including C1 catecholaminergic neurons, are considered to regulate peripheral sympathetic nervous activity and the BP.[28] However, few reports have examined β_1- or β_2-AR expression in the bulbospinal RVLM neurons. In this study, the clear effects of metoprolol, dobutamine, butoxamine and salbutamol on the activities of the bulbospinal RVLM neurons strongly suggested the existence and functional role of the β_1- and β_2-ARs in the bulbospinal RVLM neurons. We hypothesize that catecholamines in the RVLM may control the BP via the mediation of these receptors.

The role of catecholamines in the RVLM is not well understood. Abbott *et al.*[29] suggest that catecholaminergic neurons in the RVLM use glutamate as the primary neurotransmitter, and Morrison[30] also suggests that glutamate is the principal excitatory neurotransmitter from the RVLM to the sympathetic preganglionic neurons in the thoracic spinal cord. Moreover, Chen *et al.*[31] report that norepinephrine microinjection into the RVLM does not alter the resting systolic BP. However, in this study, bulbospinal RVLM neurons were hyperpolarized and depolarized by the β_1-AR antagonist and the β_2-AR antagonist, respectively. The results suggest that catecholamine may serve as a neurotransmitter in the RVLM. In addition to these receptors, the presence of α_2-ARs on RVLM neurons is known,[7–9] and its agonist hyperpolarizes RVLM neurons.[28] The effects of catecholamines depend on the type of ARs that catecholamines act on, and the catecholamines' effects on RVLM neurons may cancel each other through these types of ARs.

The effects of metoprolol and dobutamine on the bulbospinal RVLM neurons

Although Paschalis *et al.*[32] reported the existence of the β_1-AR in the neurons of the RVLM, the physiological roles of the β_1-AR expressed on the RVLM neurons remain unknown.

In this study, metoprolol induced hyperpolarization in 80% of the bulbospinal RVLM neurons (Figures 1a and b, Table 1a), and the percentage decreased to 75% under the blockade of synaptic transmissions (Figure 1c, Table 1b). These results suggest that metoprolol directly induces hyperpolarization of the bulbospinal RVLM neurons and that the β_1-AR is expressed on these neurons. Moreover, hyperpolarization of the RVLM neurons in response to metoprolol superfusion occurred in a dose-dependent manner (Figure 1b). Various previous studies suggest that orally administered metoprolol crosses the blood–brain barrier.[33,34] Given that metoprolol hyperpolarized RVLM neurons in this study, a portion of its BP-decreasing effect may be derived from reducing the activity of RVLM neurons.

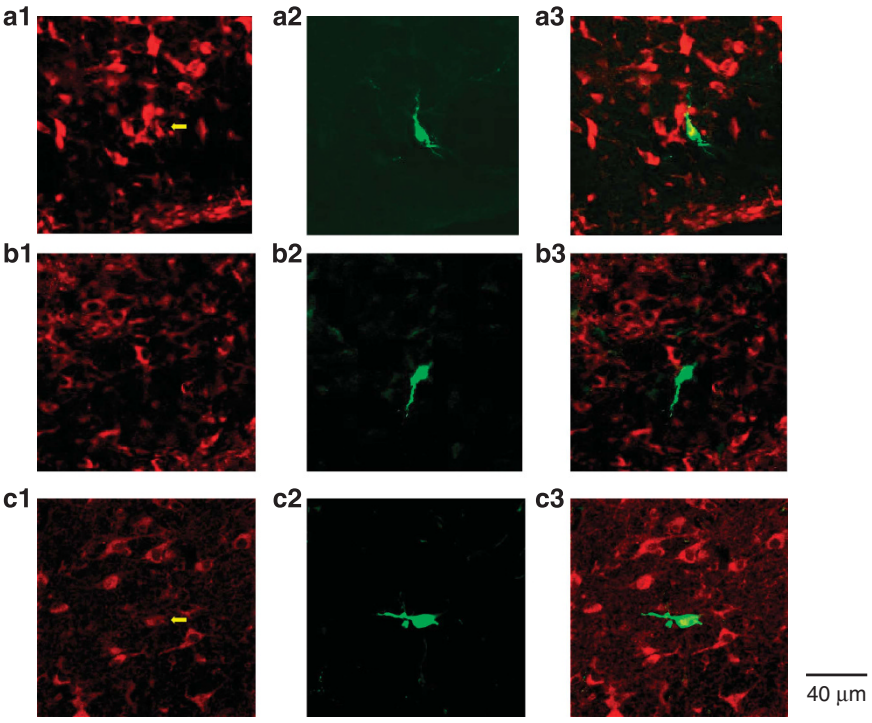

Figure 4 β_1-AR- and β_2-AR-immunoreactive neurons in the RVLM visualized under high magnification. (**a1**) β_1-AR-immunoreactive neurons in the RVLM. (**a2**) The Lucifer Yellow-stained bulbospinal RVLM neurons showed hyperpolarization during metoprolol superfusion. (**a3**) Merged image of panels **a1** and **a2**. The Lucifer Yellow-stained bulbospinal RVLM neurons showed β_1-AR immunoreactivity. (**b1**) β_1-AR-immunoreactive neurons in the RVLM. (**b2**) The Lucifer Yellow-stained bulbospinal RVLM neurons displayed no change in membrane potential during metoprolol superfusion. (**b3**) Merged image of panels **b1** and **b2**. The Lucifer Yellow-stained bulbospinal RVLM neurons did not show β_1-AR immunoreactivity. (**c1**) β_2-AR-immunoreactive neurons in the RVLM. (**c2**) The Lucifer Yellow-stained bulbospinal RVLM neurons showed hyperpolarization during butoxamine superfusion. (**c3**) Merged image of panels **c1** and **c2**. The Lucifer Yellow-stained bulbospinal RVLM neurons showed β_2-AR immunoreactivity.

To confirm the expression of the β_1-AR on these neurons, dobutamine superfusion, which induced depolarization in 83% of the RVLM neurons, was performed. These findings support our finding that blockade of the β_1-AR activity decreased the activity of the RVLM neurons.

In the brain, β_1-AR expression has been reported in neurons of the amygdala[35] as well as anterior hypothalamic[36] and paraventricular nuclei.[37] In these studies cited above, β_1-AR antagonists decreased the activity of these neurons. Other reports[38,39] have suggested that β_1-AR antagonists exert a neuroprotective effect. In many parts of the brain, β_1-AR antagonists may exert a neuroprotective effect by decreasing neuron activity. The decrease in bulbospinal RVLM neuron activity induced by metoprolol in this study suggests that β_1-AR antagonists may exert a protective effect on the RVLM neurons and reduce BP through decreasing RVLM neuron activity.

In two cases, the RVLM neurons exhibited depolarization during superfusion with metoprolol dissolved in a low-Ca^{2+}, high-Mg^{2+} solution (Table 1b). The mechanism underlying this unexpected finding remains unclear at present, and further study is needed.

The effects of butoxamine and salbutamol on the bulbospinal RVLM neurons

In the brain, β_2-AR expression has been reported in neurons of the cerebellum,[40] hippocampus[41] and amygdala.[42] Furthermore, various reports[43,44] also demonstrate that β_2-AR stimulation exerts neuroprotective effects by decreasing nuclear factor kappa B signaling, inflammation and apoptosis in the brain.[45] To the best of our knowledge, no reports on the existence or on the electrophysiological functions of the β_2-AR in RVLM neurons are available.

In this study, butoxamine induced depolarization of 69% of the bulbospinal RVLM neurons (Figure 2a, Table 1a), and the percentage increased to 88% under the blockade of synaptic transmissions (Figure 2b and Table 1b). These results suggest that the β_2-AR is expressed in the bulbospinal RVLM neurons and that blockade of the β_2-AR increases the activity of these neurons.

To confirm the opposing effects of butoxamine and metoprolol, metoprolol superfusion was followed by butoxamine superfusion, and both drugs were dissolved in a low-Ca^{2+}, high-Mg^{2+} solution (Figure 2c). Whereas the bulbospinal RVLM neurons showed hyperpolarization in response to superfusion with metoprolol, they exhibited depolarization in response to superfusion with butoxamine. These results suggest the presence of both β_1- and β_2-ARs in some bulbospinal RVLM neurons.

The β_2-AR agonist salbutamol produced hyperpolarization of the RVLM neurons in the majority of cases (Figure 2d, Table 1a). The hyperpolarizing effect of salbutamol lends support to the depolarizing effect of butoxamine.

These results suggest the existence and functional role of β_2-ARs in the bulbospinal RVLM neurons. β_2-AR agonists are generally known to dilate blood vessels and decrease BP.[46] The existence of β_2-ARs in the bulbospinal RVLM neurons was demonstrated in this study, and β_2-AR agonists are considered to reduce BP partially through decreasing bulbospinal RVLM neuron activity.

The membrane resistance of the RVLM neurons decreased only during salbutamol superfusion (Table 1a). β_2-AR agonists induce

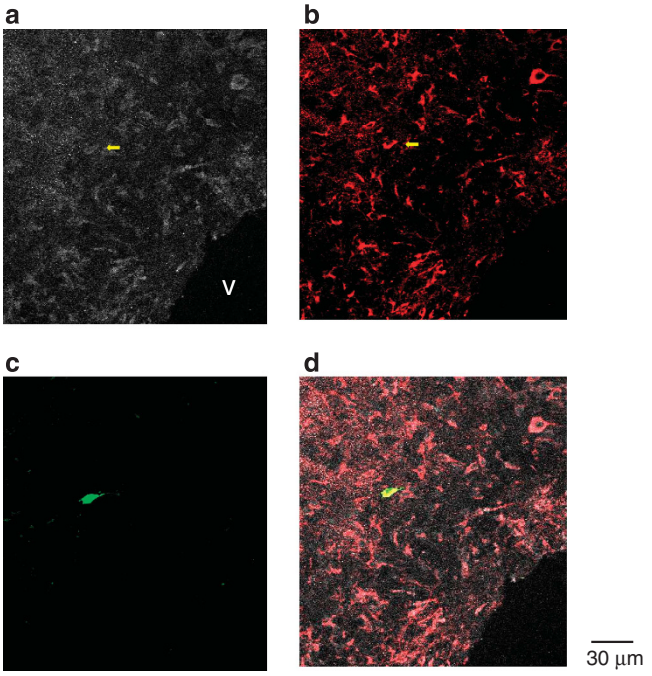

Figure 5 Triple-merged image of β_1-AR- and β_2-AR-immunoreactive neurons. (**a**) β_1-AR-immunoreactive neurons in the RVLM are shown in white. V, ventral side. (**b**) β_2-AR-immunoreactive neurons in the RVLM are shown in red. (**c**) The Lucifer Yellow-stained bulbospinal RVLM neurons exhibited hyperpolarization during metoprolol superfusion and depolarization during the subsequent butoxamine superfusion. (**d**) Triple-merged image of panels **a**–**c**. The Lucifer Yellow-stained bulbospinal RVLM neurons exhibited both β_1-AR and β_2-AR immunoreactivity. In the RVLM, approximately all of the β_1-AR-immunoreactive neurons also showed β_2-AR immunoreactivity.

Na^+–K^+ pump activation,[47] opening up of K^+(ATP) channels[48] and regulation of the L-type calcium channels Ca(v)1.2,[49] which may underlie the decrease of the membrane resistance of the RVLM neurons observed during salbutamol superfusion. The membrane resistances differ greatly among the neurons (from 100 MΩ to 1000 GΩ). Therefore, on the whole, no statistically significant changes of the membrane resistance were observed during superfusion with other drugs.

β_1- and β_2-ARs immunoreactivity in the bulbospinal RVLM neurons

In this study, all of the five neurons that displayed hyperpolarization in response to metoprolol superfusion exhibited β_1-AR immunoreactivity (Figures 3a, c, d and 4a). Given that RVLM neurons also include C1-catecholaminergic neurons,[3,4] we examined the TH immunoreactivity of the aforementioned five neurons, and three of these neurons exhibited TH immunoreactivity (Figures 3b, c and d). Approximately all of the TH-immunoreactive neurons in the RVLM also displayed β_1-AR immunoreactivity (Figures 3a, b and d), suggesting that catecholamines act on the RVLM neurons in an autocrine manner. Numerous β_1-AR-immunoreactive neurons were observed in the RVLM (Figure 3a). Paschilis *et al.*[32] reported the presence of β_1-AR-immunoreactive neurons in the RVLM, which supports our findings.

Bulbospinal RVLM neurons that displayed no response during metoprolol superfusion did not exhibit β_1-AR immunoreactivity (Figure 4b), indicating that not all bulbospinal RVLM neurons express β_1-ARs.

As shown in Figure 4c, all of the three neurons exhibiting depolarization in response to butoxamine superfusion displayed β_2-AR immunoreactivity. Two of the neurons also displayed TH immunoreactivity, and approximately all TH-immunoreactive neurons in the RVLM also exhibited β_2-AR immunoreactivity. Although there are few reports on the presence of β_2-AR-immunoreactive neurons in the RVLM, we demonstrated β_2-AR expression in the bulbospinal RVLM neurons both electrophysiologically and histologically in this study.

RVLM neurons that displayed depolarization during metoprolol superfusion and hyperpolarization during butoxamine superfusion (Figure 2c) were examined for β_1- and β_2-AR immunoreactivity. These neurons exhibited β_1- and β_2-AR immunoreactivity, and 90% of the neurons in the RVLM that exhibited β_1-AR immunoreactivity also displayed β_2-AR immunoreactivity (Figures 5a–d). β_1- and β_2-ARs mediate opposite effects on bulbospinal RVLM neuron activity. With regard to other parts of the brain, β_1- and β_2-ARs co-expression has been reported in the neurons of the cornu ammonis 1 (CA1) and CA3 regions of the rat hippocampus.[41]

In summary, the presence of β_1- and β_2-ARs in the bulbospinal RVLM neurons was demonstrated both electrophysiologically and histologically. Treatment with a β_1-AR antagonist decreased the activity of the bulbospinal RVLM neurons, whereas treatment with a β_2-AR antagonist increased their activity. A number of neurons in the RVLM displayed β_1- or β_2-AR's immunoreactivity, and the majority of TH-immunoreactive neurons exhibited both β_1- and β_2-AR immunoreactivity. Furthermore, β_1-ARs may co-exist with β_2-ARs on the bulbospinal neurons in the RVLM. Based on these results, β_2-AR antagonists or β_2-AR agonists may reduce the BP through decreasing the activity of bulbospinal RVLM neurons. However, the agonists or antagonists' effect on RVLM neurons *in vivo* is unknown, and the role of catecholamines in the RVLM is unclear. Further studies are expected.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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