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Cholinergic Activity Related to Cardiovascular Regulation in Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats

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The hyperactivity of cholinergic system in the RVLM of spontaneously hypertensive rats (SHR) may contribute to the sustained elevation of blood pressure. However, the hyperactivity mechanisms of cholinergic system are controversial. Thus, to clarify the mechanisms of cholinergic hyperactivity in RVLM of the SHR, we studied the activities of enzymes that participate in the biosynthesis and degradation of acetylcholine (ACh) and the density of muscarinic receptors in RVLM of the 14- to 18-week-old SHR and age-marched Wistar Kyoto rats (WKY). Choline acetyltransferase activity was far greater in RVLM of SHR than that of WKY. [^{3}H]ACh release from RVLM was also greater in SHR than in WKY. Acetylcholinesterase activity and [^{3}H]NMS binding of RVLM slice of SHR were not significantly different from that of WKY. These results suggest that the enhanced cholinergic mechanisms in the RVLM of SHR is due to the enhanced presynaptic cholinergic tone rather than the altered postsynaptic mechanisms.

Key Words: SHR, Rostral ventrolateral medulla (RVLM), Cholinergic activity

INTRODUCTION

A group of neurons in the surface of the rostral ventrolateral medulla (RVLM) is important in cardiovascular control (Reis et al, 1987). The RVLM is essentially a subdivision of the nucleus paragigantocellularis lateralis, which includes in its rostral boundaries cells containing phenylethanolamine-N-methyltransferase (PNMT) activity, the so-called C-1 region (Reis et al, 1987). The RVLM provides direct tonic vasoconstrictor activity to spinal preganglionic neurons and plays a key role in mediating the reflex hypotensive response to baroreceptor activation (Granata et al, 1985). The RVLM has been also demonstrated to receive innervation from cholinergic fibers, and stimulation of muscarinic receptors in this region evokes a hypertensive response; alternatively, muscarinic blockade results in a fall in blood pressure, even in normotensive animals (Morrison et al, 1988; Giuliano et al, 1989; Arneric et al, 1990; Lee et al, 1991; Kubo et al, 1995).

The imbalance in central cholinergic systems has been suggested to contribute to the development and maintenance of hypertension in spontaneously hypertensive rats (SHR) (Lee et al, 1991; Kubo et al, 1995). The intravenous injection of the centrally acting cholinesterase inhibitor physostigmine evoked an enhanced hypertensive response in SHR as compared with normotensive Wistar Kyoto rats (WKY) (Makari et al, 1989). Because an important action site of intravenously administered physostigmine is the RVLM (Punnen et al, 1986; Arneric et al, 1990), it was proposed that there is the hyperactivity of cholinergic system in the RVLM of SHR and that it may contribute to the sustained elevation of blood pressure in the SHR (Arneric et al, 1990; Lee et al, 1991; Kubo et al, 1995). However, the hyperactivity mechanisms of cholinergic system are yet to be established. Lee et al (1991) proposed that hyperactivity of cholinergic system in RVLM is due to the enhanced postsynaptic mechanisms, but Kubo et al (1995) suggested that the enhanced presynaptic mechanisms as a reason of hyperactivity. However, all of them failed to provide a supportive evidence for their argument.

Therefore, the present study was performed to clarify the mechanisms of cholinergic hyperactivity in RVLM of the SHR.

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METHODS

Animals

Experiments were performed on 14- to 18-week-old male SHR and age-matched male Wister-Kyoto rats (WKY). They were kept under alternative 12-h periods of dark and light and given standard rat chow and tap water *ad libitum*.

Preparation of RVLM micropunches

Rats were decapitated by the scaffold. The entire brain, from the first cervical segment of the spinal cord to the forebrain, was rapidly removed and placed in ice-cold modified Kreb's bicarbonate buffer (NaCl 118 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, D-glucose 11 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.2 mM, choline chloride 1 μM). All subsequent procedures were performed at 4°C. The pia-arachinoid membranes were carefully removed from the brain. The cerebellum was separated from the hindbrain, and the forebrain was dissected at the level of the inferior colliculus. The remaining hindbrain was placed on a McIlwain tissue chopper, and, beginning at the calamus scriptorius, slices (0.5 mm thick) were made as the tissue was moved in a rostral direction. The slices rostral 1.0~2.0 mm to calamus scriptorius were used for experiments. With a stereomicroscope, bilateral micropunches (1.0 mm o.d.) were made of the regions corresponding to and surrounding the C1 area of the RVLM (Fig. 1) using the technique of Palkovits (1985).

Release of [^{3}H]acetylcholine ([^{3}H]ACh) from RVLM punches

Release of [^{3}H]ACh was analyzed using the radiochemical method by Hadhazy and Szerb (1977) with some modifications. Four punches from each rat were incubated for 40 min at 37°C in Krebs bicarbonate buffer containing 10 μCi/ml [^{3}H]choline chloride gased with 95% O_2/5% CO_2. Tissue punches were washed twice with gassed Krebs bicarbonate buffer to remove any excess [^{3}H]choline and transferred to perfusion chamber of Brandel superfusion system. Superfusion was done with gassed (95% O_2/5% CO_2) Krebs bicarbonate buffer containing hemicholinium-3 (10 μM) and atropine (100 nM) at the rate of 0.5 ml/min. Superfusate was collected into scintillation mini-vial at 5 min intervals. After 80 min of superfusion, [^{3}H]ACh release was evoked by exposing the punches to K^+ (30 mM) for 5 min. Increased K^+ concentrations were compensated by equimolar reductions in Na^+ concentration. At the end of superfusion, the punches were solubilized in 0.5 ml of tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene). The radioactivities in the superfusates and punches were determined by liquid scintillation counter.

Measurement of choline acetyltransferase (ChAT) activity

The activity of ChAT was measured according to the method of Fonnum (1975). The RVLM punches were sonicated for 5 s in 75 μl of 5 mM KH_2PO_4 (pH 7.0) containing 0.2% triton X-100 and 5 mM EDTA. The supernatant after centrifugation at 10,000 *g* for 20 min was used.

The incubation mixture contained [^{3}H]acetyl CoA (20 mCi/mmol) 0.2 mM, NaCl 300 mM, EDTA 20 mM, choline bromide 8 mM, physostigmine 0.1 mM and sodium phosphate buffer 50 mM (pH 7.4). The supernatant (8 μl) was placed in a scintillation mini-vial and the incubation mixture (20 μl) was added. The solution was mixed and incubated for 15 min at 37°C. The incubation was terminated by addition of 1 ml ice-cold sodium phosphate buffer (10 mM, pH 7.4). 0.4 ml of acetonitrile containing 2 mg Kalignost and 2 ml of toluene scintillation mixture (0.5% PPO, 0.01% POPOP) was added. and the vial was shaken lightly for a minute. The ACh was extracted into the toluene phase whereas the acetyl-CoA was left in the aqueous phase. The scintillation vial was placed in the scintillation counter and two layers were allowed to separate for 10 min. The radioactivities of [^{3}H]ACh in toluene phase were counted.

Measurement of acetylcholinesterase (AChE) activity

AChE activity was measured by the method of

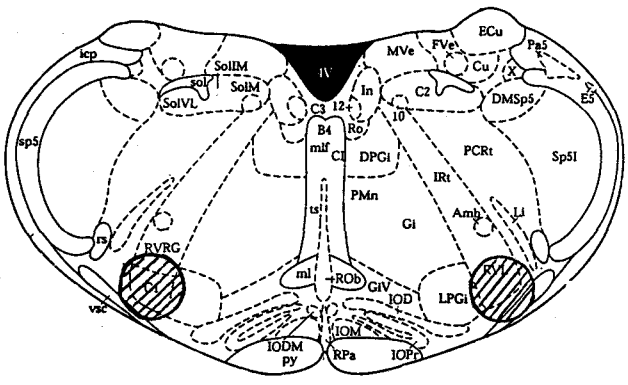

Fig. 1. Region selected for punches of the rostral ventrolateral medulla.

Ellman et al (1961). Micropunches of RVLM were homogenized in 0.08 M Tris-HCl buffer (pH 8.0). Aliquotes of homogenates were added to an incubation mixture, consisting of 3.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 3 mM acetylcholine iodide. The reaction was followed spectrophotometrically at 412 nM.

Receptor binding assay

Micropunches of RVLM were homogenized in Na/K-phosphate buffer (pH 7.4) and incubated with 2 nM [^{3}H]NMS for 1 hr at 37°C. Homogenate was filtered through G/F filter by using cell harvester and washed twice with ice-cold buffer. Radioactivities of filter disc were counted. Nonspecific binding was determined in the presence of 5 μM atropine.

Protein concentration was determined by the method of Lowry et al (1951).

Chemicals

[^{3}H]acetyl CoA, [^{3}H]choline and [^{3}H]NMS were purchased from Amersham (UK), and other chemicals from Sigma (U.S.A.).

Statistics

Data were expressed as mean±SEM. Differences between groups were determined by Student's *t*-test.

RESULTS

Arterial pressure of rats

The mean systolic blood pressure of SHR in the resting state was 201.6±6.2 mmHg, and that of WKY was 148.3±6.5 mmHg ($P<0.001$).

Cardiovascular responses to cholinergic agents microinjected into the RVLM

Unilateral microinjection of physostigmine, an acetylcholinesterase inhibitor, into the RVLM caused a pressor response in WKY and SHR. The pressor response to physostigmine in SHR was significantly greater than that in WKY (Table 1, $P<0.001$). Bilateral microinjection of atropine, a muscarinic acetylcholine receptor antagonist, into the RVLM produced a depressor response in WKY and SHR. The depressor response was significantly greater in SHR than in WKY (Table 1, $P<0.01$)

Table 1. Effects of cholinergic agents microinjected into RVLM on blood pressure of rats

	⊿B.P. (mmHg)	
	WKY	SHR
Physostigmine (300 pmol)	9.3±1.5	19.4±1.2***
Atropine (1 nmol)	17.5±2.0	27.0±1.6**

Each value represents the mean±S.E.
RVLM: rostral ventrolateral medulla
WKY: Wistar-Kyoto rats
SHR: spontaneously hypertensive rats
**: $p<0.01$, ***: $p<0.001$

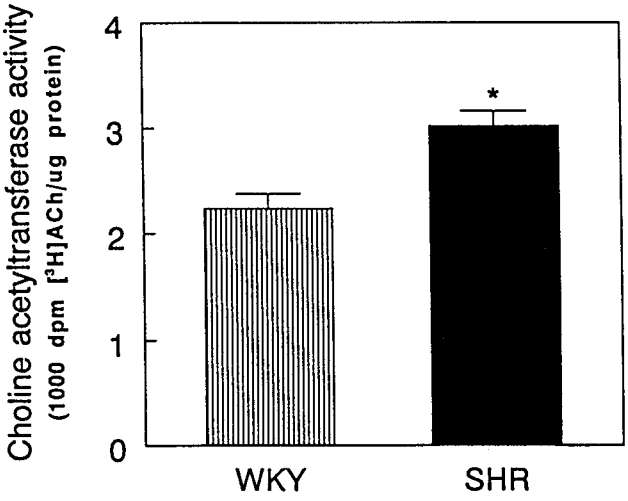

Fig. 2. Choline acetyltransferase activity of rostral ventrolateral medulla of rats. Each value represents the mean ±S.E.. WKY: Wistar-Kyoto rat, SHR: Spontaneously hypertensive rat, *$P<0.05$

Release of [^{3}H]ACh

The basal release rates were 0.98±0.13% of total dpm/min in SHR and 0.82±0.11% of total dpm/min in WKY. Depolarization of the tissue with 35 mM K^+ resulted in a stimulus-evoked release of [^{3}H]ACh. The increase of [^{3}H]ACh release by high K^+ was greater in SHR than in WKY (Fig. 2, $P<0.05$).

Choline acetyltransferase (ChAT) activity

The activity of ChAT in the RVLM of SHR was 3020±141 dpm [^{3}H]ACh/μg protein, and that of WKY was 2236±141 dpm [^{3}H]ACh/μg protein. The significant activation of ChAT was shown in SHR

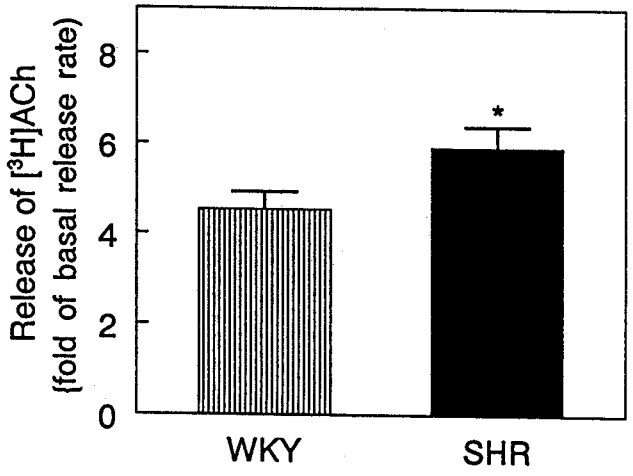

Fig. 3. Release of [^{3}H]acetylcholine from rostral ventrolateral medulla of rats. Each value represents the mean ± S.E.. WKY: Wistar-Kyoto rat, SHR: Spontaneously hypertensive rat, *P < 0.05

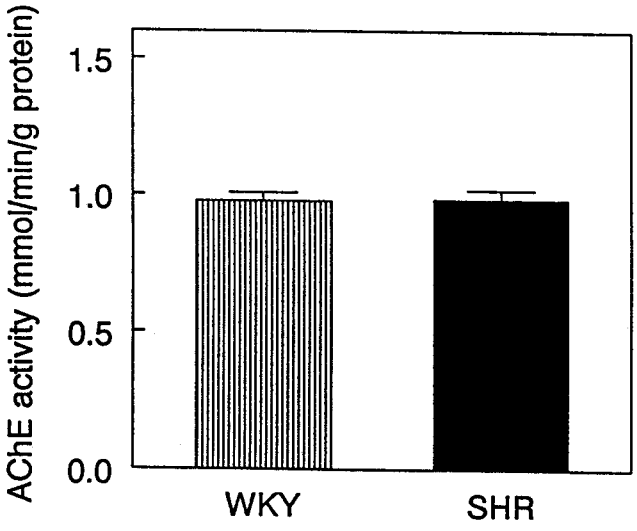

Fig. 4. Acetylcholinesterase activity (AChE) of rostral ventrolateral medulla of rats. Each value represents the mean ± S.E.. WKY: Wistar-Kyoto rat, SHR: Spontaneously hypertensive rat

(Fig. 3, $P < 0.05$).

Acetylcholinesterase (AChE) activity

The activity of AChE in the RVLM was 0.982 ± 0.034 μmol/min/mg protein in SHR and 0.976 ± 0.031 μmol/min/mg protein in WKY, respectively. There was no difference in AChE activity between the two groups (Fig. 4).

Muscarinic receptor density

The densities of [^{3}H]NMS binding were 0.50 ± 0.03 pmol/mg protein in SHR and 0.41 ± 0.03 pmol/mg protein in WKY. The muscarinic receptor density was not significantly different between the two groups (Fig. 5).

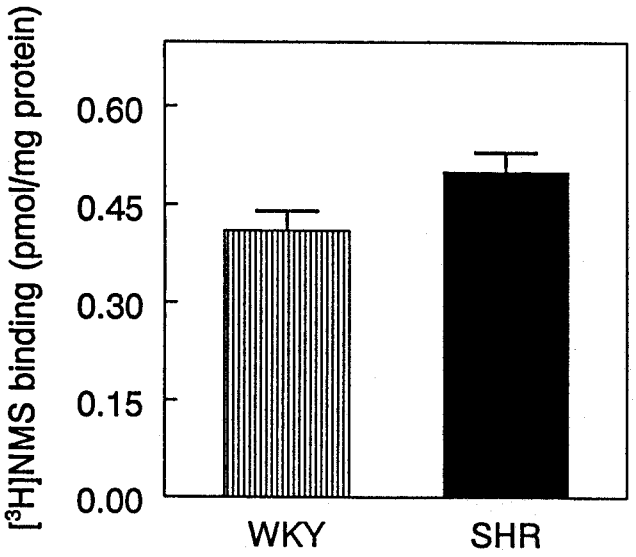

Fig. 5. [^{3}H]NMS binding in rostral ventrolateral medulla slice of rats. Each value represents the mean ± S.E.. WKY: Wistar-Kyoto rat, SHR: Spontaneously hypertensive rat

DISCUSSION

Several reports showed that the microinjection of cholinergic agents into the RVLM of the rats produced increases in blood pressure and confirmed that the cholinergic mechanisms in the RVLM are involved in central blood pressure regulation (Sundaram & Sapru, 1988; Lee et al, 1991; Kubo et al, 1995). The pressor response to the acetylcholinesterase inhibitor physostigmine injected into the RVLM was greater in SHR than in WKY, and the depressor response to the muscarinic receptor antagonist atropine injected into the same site was greater in SHR than in WKY (Lee et al, 1991; Kubo et al, 1995). These results suggest that cholinergic mechanisms in the RVLM are enhanced in SHR. However, there is no consistence in the pressor response to ACh and carbachol, cholinergic agonists directing ACh receptors. In the report of Lee et al (1991), the pressor response to carbachol was significantly greater in SHR than in WKY. However, there was no difference in the pressor responses to ACh and carbachol between the two strains in the report of Kubo et al (1995). The enhanced cholinergic mechanisms can be elicited by the enhanced release of ACh, the lower activity of AChE, and/or the higher density of muscarinic receptors, but

the reason(s) of the enhanced cholinergic mechanisms is not clear.

In the present study, the activity of ChAT, an enzyme responsible for the synthesis of ACh, in the RVLM was higher in SHR than in WKY. Although these data suggest that cholinergic neuronal activity in the RVLM is enhanced in SHR, they do not necessarily reflect enhanced ACh release in this region in SHR since ChAT is present not only in nerve terminals but also in cell bodies of cholinergic neurons. Thus, we determined the rate of ACh release from RVLM slices. The rate of ACh release was higher in SHR than in WKY. These results suggest that the enhanced ACh release in SHR is at least partly responsible for the enhanced cholinergic mechanisms.

Because the enhanced AChE activity affects the ACh contents in synaptic cleft, we measured the AChE activity in the RVLM. The AChE activities in the RVLM of SHR and of WKY were not significantly different from each other. It means the enhanced cholinergic activity in SHR is not due to the difference in the degradation rate of ACh.

In addition, the density of muscarinic receptors was measured to determine the another possibility for the enhanced cholinergic mechanisms. The density of muscarinic receptors of RVLM of SHR was somewhat greater than of WKY, but it was not significant. It means that there is no significant difference in postsynaptic mechanisms of cholinergic neuronal pathway of RVLM between SHR and WKY.

Alterations in cholinergic mechanisms responsible for blood pressure regulation in SHR may occur also in brain regions other than the RVLM, since the pressor response to intravenous injection of physostigmine still partly remains after bilateral microinjections of the muscarinic receptor antagonist scopolamine into the RVLM of rats (Giuliano et al, 1989; Arneric et al, 1990). Possible regions may include the hypothalamus (Brezenoff & Xiao, 1989; Brezenoff et al, 1990), the lateral septal area (Scheucher et al, 1991), and the spinal cord (Buccafusco & Magri, 1990)

The results of the present study suggest that the enhanced cholinergic mechanisms in the RVLM of SHR is due to the enhanced presynaptic cholinergic tone rather than the altered postsynaptic mechanisms.

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