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Stimulating Effect of 6*R*-Tetrahydrobiopterin on CA^{2+} Channels in Neurons of Rat Dorsal Motor Nucleus of the Vagus

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We have recently found that 6R-tetrahydrobiopterin (6R-BH_4), a natural cofactor for aromatic L-amino acid hydroxylases and nitric oxide synthase, enhances dopamine release. Here, using a slice patch method, we examined the effect of 6R-BH_4 on Ca^{2+} channels in neurons of rat dorsal motor nucleus of the vagus, where dopaminergic neurons are densely located. 6R-BH_4 enhanced N-type Ca^{2+} channel currents, whereas 6S-BH_4, a diastereoisomer of 6R-BH_4, had little effect. Neither sodium nitroprusside, a nitric oxide generator, nor L-DOPA, a product of tyrosine hydroxylation, mimicked the effect of 6R-BH_4. These findings suggest that 6R-BH_4 enhances N-type Ca^{2+} channel currents in stereospecifically and independently of its cofactor activities as observed in its dopamine releasing action, and raise possibility that 6R-BH_4 enhances dopamine release by activating Ca^{2+} channels.

6R-Tetrahydrobiopterin (6R-BH_4) is a cofactor for hydroxylases of tyrosine [1], tryptophan [2] and phenylalanine [3], and also a cofactor for nitric oxide synthase (NOS) [4]. Recently, we have reported that 6R-BH_4 stimulates dopamine release *in vivo* from the striatum within the physiological concentrations [5]. The stimulating effect was independent of its cofactor activity [5, 6] and specific for the natural form (6R-form) of tetrahydrobiopterin, and its diastereoisomer, 6S-form (6S-BH_4), did not stimulate dopamine release or rather inhibited 6R-BH_4-induced dopamine release [7]. From these findings, we assume that 6R-BH_4 has the specific action site to stimulate dopamine release in the brain.

In the present study, to further investigate the mechanism of dopamine releasing action of 6R-BH_4, we studied the effect of 6R-BH_4 on Ca^{2+} channel currents in neurons of medial part of the dorsal motor nucleus of the vagus (DMNX), where dopaminergic neurons densely exist [8, 9, 10]. We report that 6R-BH_4 enhances voltage-gated N-type Ca^{2+} channel currents independently of its cofactor activity.

MATERIALS AND METHODS

A slice patch method used in this study was based on the method of Sekiyama et al. [11]. In brief, after brains were taken from 10–14 day old Wistar rats, brainstems were carefully isolated and placed in a chamber of a tissue slicer (DTK-1500, Dosaka Co., Kyoto, Japan) filled with oxygenated ice-cold Krebs solution (in mM); NaCl 113, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2, $MgCl_2$ 1. Cross-sectional slices of 120 μm thick were obtained, and the slices were incubated in oxygenated Krebs solution at 37°C for 1–2 h before recording.

After the incubation, a slice was placed in a chamber fixed to a microscope stage and continuously perfused with oxygenated Krebs solution at room temperature (23–25°C). Neurons were viewed using an upright microscope with a water-immersion objective lens' (× 40 Karl Zeiss, Germany, working distance of 1.5 mm) with Nomarski optics. According to the characteristics of dopaminergic neurons identified immunohistochemically [10], we investigated the effects of

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Abbreviations used: DMNX, dorsal motor nucleus of the vagus; NOS, nitric oxide synthase; 6R-BH_4, 6R-tetrahydrobiopterin; 6S-BH_4, 6S-tetrahydrobiopterin; SNP, sodium nitroprusside.

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6R-BH_4 on Ca^{2+} channels in ovoid-shape neurons (about 20 μm in long axis) with few processes located in the medial part of the caudal DMNX (caudal to the area postrema). After removing connective tissue overlying a neuron, tight-seal (>10 GW) whole-cell voltage-clamp recordings were performed with a EPC7 amplifier (List Electronics, Germany). Ca^{2+} currents were recorded in a solution (mM): $CaCl_2$ 2, $MgCl_2$ 1, tetraethylammonium (TEA) Cl 30, tetrodotoxin 0.0003, NaCl 86, $NaHCO_3$ 25, NaH_2PO_4 1, glucose 11, pH 7.4 adjusted by TEA-OH. DC resistance was 2–4 Mθ when a patch pipette was filled with an internal solution (in mM): CsCl 135, $MgCl_2$ 2, HEPES10, EGTA 10, ATPNa 3, 4-aminopyridine (4-AP) 0.3; pH 7.4 adjusted with CsOH. Fifteen minutes after disruptive of the patch membrane, we started recording of Ca^{2+} channel currents.

Pulse protocols and data-acquisition were controlled by a ADX-98H AD/DA interface (Canopus Electronics Co., Kobe, Japan) connected to an NEC 9801 based computer (Tokyo, Japan). Software was developed in our laboratory. The current recordings were filtered at 1 kHz and sampled at 2 KHz. The effects of drugs were assessed by peak current, which was expressed as % of mean value of three serial control peak current values prior to the drug application. Means ± SD % of three serial control peak current values in 10 experiments were 100.91 ± 3.31, 101.17 ± 1.53 and 97.71 ± 3.04, respectively. When changes in the peak current value after drug application were beyond 3SD of control values (9.93%), the effects of drugs were considered to be significant.

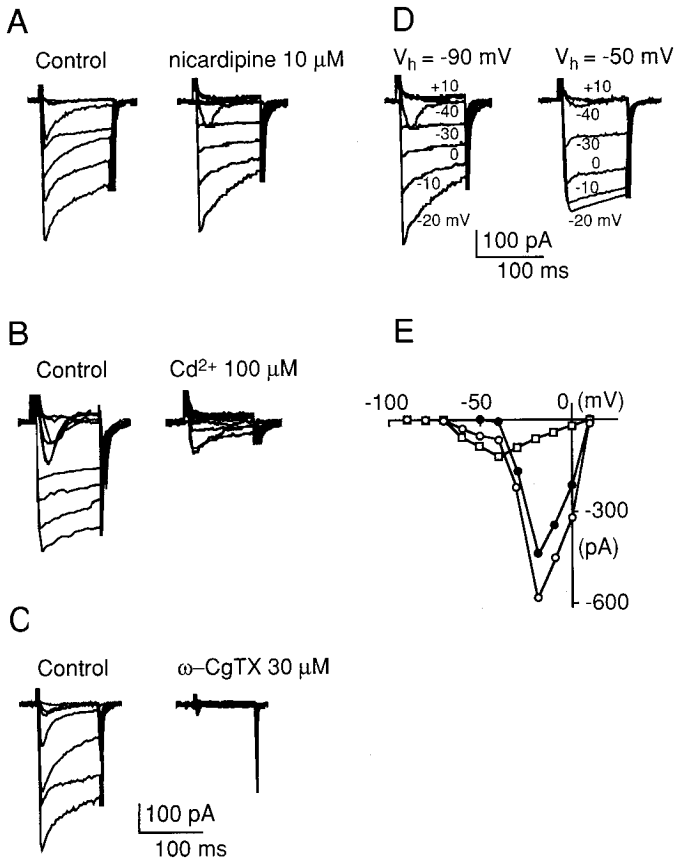

FIG. 1. Characterization of voltage-gated Ca^{2+} channels in DMNX neurons. Whole cell voltage clamp recording. The membrane potential was held at −90 mV unless otherwise indicated and depolarized to +10 mV by 10 mV-step pulses (duration, 100 ms). Drugs were bath-applied at indicated concentrations. Each experiment was performed in 4 cells and similar results were obtained in all cells. Representative recordings are shown. A: Effects of nicardipine on Ca^{2+} channel currents. Left panel, before administration of nicardipine. Right panel, after administration of 10 μM nicardipine. B: Effects of Cd^{2+} on Ca^{2+} channel currents. Left panel, before administration of Cd^{2+}. Right panel, after administration of 100 μM Cd^{2+}. C: Effects of ω-CgTX on Ca^{2+} channel currents. Left panel, before administration of ω-CgTX. Right panel, after administration of 30 μM ω-CgTX. D: Effects of holding potential (V_h) on Ca^{2+} channel currents. Left, $V_h = -90$ mV. Right panel, $V_h = -50$ mV. E: Current-voltage relationship of Ca^{2+} channel currents. Peak currents were plotted against voltages. ○, Ca^{2+} channel currents elicited by depolarization from −90 mV. □, Ca^{2+} channel currents elicited by depolarization from −90 mV in the presence of 100 μM Cd^{2+}. ●, Ca^{2+} channel currents elicited by depolarization from −50 mV.

Drugs were applied to the recording chamber by gravity. Perfusion rate was maintained at 2.2–2.5 ml/min and the bath volume was about 1.0 ml. 6R-$BH_4 \cdot 2HCl$ and 6S-$BH_4$2HCl were generous gifts from Suntory Institute for Biomedical Research, Osaka, Japan. All other chemicals are of the purest grade available from regular commercial sources.

RESULTS

We characterized the Ca^{2+} channel currents in neurons in DMNX by whole-cell voltage clamp mode (Fig. 1). When neurons were depolarized from a holding potential of −90 mV to +10 mV by 10 mV steps pulses (100 ms duration), inward voltage-gated Ca^{2+} currents were recorded (Fig. 1A, control). When nicardipine, a dihydropyridine type blocker of L-type Ca^{2+} channel [12], was administered to the bath solution at 10 μM, the Ca^{2+} currents were unchanged (Fig. 1A, n = 4), indicating that L-type Ca^{2+} channel is absent. Cd^{2+} is a blocker of L- and N-type Ca^{2+} channels but

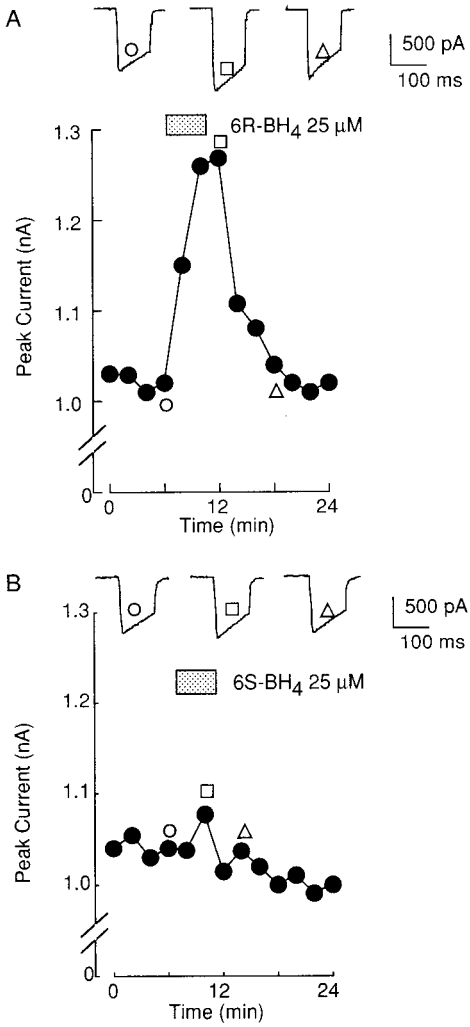

FIG. 2. Effects of 6R-BH_4 (A) and 6S-BH_4 (B) on Ca^{2+} channel currents in DMNX neurons. Whole-cell voltage-clamp recording. Ca^{2+} currents were evoked by depolarization to +10 mV (100 ms) from −50 mV every 2 min. Upper panel: Ca^{2+} channel currents recorded before (○), during (□) and after (△) application of 6R-BH_4 or 6S-BH_4. Lower panel: time course of peak currents. 6R-BH_4 or 6S-BH_4 was bath-applied at 25 μM for 4 min as indicated by bars. In case of 6R-BH_4, positive effects were observed in 5 cells out of 8 examined cells, and in 3 cells, no significant effect was observed. In case of 6S-BH_4, similar results were obtained in 6 examined cells. Representative recordings are shown.

not of T-type channels [12]. Bath application of 100 μM Cd^{2+} blocked high-voltage gated currents considerably but low-voltage gated currents persisted (Fig. 1B, n = 4). ω-Conotoxin (ω-CgTX) is a potent blocker of N-type Ca^{2+} channels and blocks moderately L- and T-type Ca^{2+} channels but does not block P-type Ca^{2+} channels [12]. ω-CgTX (30 μM) blocked all the Ca^{2+} channel (Fig. 1C, n = 4). These data show that T- and N-type voltage-gated Ca^{2+} channels are present in DMNX neurons. Because T-type Ca^{2+} channel is considered not to be involved in transmitter release [13, 14, 15, 16], we focused on N-type Ca^{2+} channels. When membrane potential was depolarized from a holding potential of −50 mV, T-type currents were selectively blocked (Fig. 1D, right) and N-type currents were evoked (Fig. 1D and E, n = 4). In the following experiments, holding potential was set at −50 mV and N-type Ca^{2+} current was evoked by depolarization to +10 mV every 2 min.

Bath application of 6R-BH_4 (25 μM) reversibly increased N-type Ca^{2+} channel currents in 5 neurons out of 8 examined neurons (Fig. 2A). Mean ± SD of the maximal peak currents after 6R-BH_4 application in five positive neurons was 124.34 ± 11.7% of control peak current value. In other three neurons, the maximal peak currents after 6R-BH_4 application did not significantly change (101.8, 104.3 and 103.4% of control peak current, respectively). Because excitatory effects on dopamine release is specific to the natural form (6R-form) of tetrahydrobiopterin [7], we examined whether the stimulating effect on Ca^{2+} channel currents is also specific for 6R-BH_4. When 25 μM 6S-BH_4, a diastereoisomer of 6R-BH_4, was bath-applied, Ca^{2+} currents hardly changed (Fig. 2B, n = 6).

Since 6R-BH_4 is a cofactor for NOS and tyrosine hydroxylase, we examined whether sodium

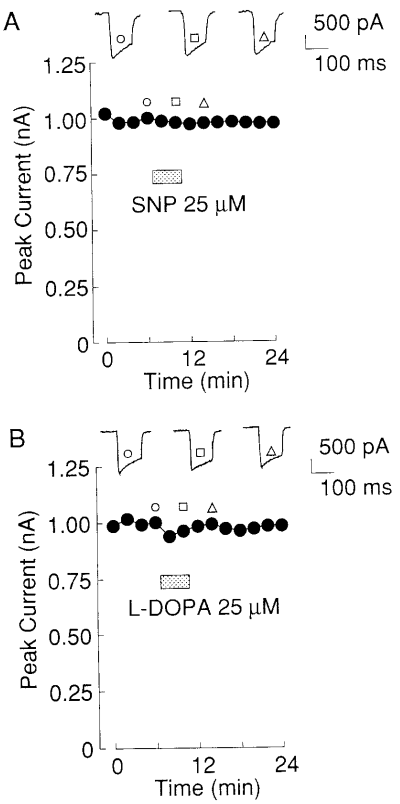

FIG. 3. Effects of SNP (A) and L-DOPA (B) on Ca^{2+} channel currents in DMNX neurons. Whole-cell voltage clamp recording. Upper panel: Ca^{2+} currents recorded before (○), during (□) and after (△) application of 25 μM SNP or L-DOPA. Lower panel; Time course of peak currents. SNP or L-DOPA was applied for 4 min as indicated by bars. Similar results were obtained in 6 cells for each drug. Representative recordings are shown.

nitroprusside (SNP), a nitric oxide generator, or L-DOPA, which is a product of tyrosine hydroxylation and can be taken up into the cells [17], mimics the effects of 6R-BH_4 on Ca^{2+} channel currents. When SNP or L-DOPA was added to the bath solution at 25 μM, Ca^{2+} channel currents were unchanged (Fig. 3, n = 6).

DISCUSSION

The present study shows that 6R-BH_4 increased N-type Ca^{2+} channel currents in DMNX neurons. The concentration of 6R-BH_4 used in the present study is comparable to the concentration in the extracellular fluid in our brain microdialysis study [5, 6, 7] and is considered to be within the physiological range [18]. In contrast to 6R-BH_4, 6S-BH_4, which is a diastereoisomer of 6R-BH_4 and has cofactor activities, had little effect on the Ca^{2+} channel currents, suggesting that the effect of 6R-BH_4 is specific for its stereostructure. These findings were consistent with our recent report that 6R-BH_4 stimulates dopamine release *in vivo* but that 6S-BH_4 has no effect [7]. Since neither SNP nor L-DOPA mimicked the effect of 6R-BH_4 on Ca^{2+} channel currents, it is likely that the stimulating effect of 6R-BH_4 on Ca^{2+} channels is not mediated by its cofactor activity for NOS and tyrosine hydroxylase. These results were again consistent with our previous reports that dopamine releasing action of 6R-BH_4 is independent of its cofactor activity [5, 6].

Taking account for the morphology (ovoid-shape with few processes) and localization (located in the medial part of caudal DMNX), the neurons examined in the present study are assumed to correspond to dopaminergic neurons as identified by immunohistochemical methods [8, 9, 10]. Thus the present study suggests that 6R-BH_4 activates N-type Ca^{2+} channels in dopaminergic neurons in DMNX in a same manner (specificity for the stereostructure and independency of the cofactor activities) as it stimulates dopamine release in the striatum [5, 6, 7], and raises possibility that 6R-BH_4 activates Ca^{2+} channels to induce dopamine release in the brain.

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