

Full Paper

Electrophysiological Characterization of Nicotine-Induced Excitation of Dopaminergic Neurons in the Rat Substantia Nigra

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Abstract. The electrophysiological characteristics of nicotine-induced excitation of dopaminergic neurons in the rat substantia nigra was investigated using the whole-cell patch clamp technique under the voltage-clamp mode. Nicotine (0.01–100 μ M) induced inward currents corresponding to nicotine-induced depolarization with an increase in firing in a dose-dependent manner. This current was inhibited by dihydro- β -erythroidine, a selective antagonist for the $\alpha 4\beta 2$ type neuronal nicotinic receptor. Nicotine directly acts on postsynaptic $\alpha 4\beta 2$ type nicotinic receptors and induces inward currents, resulting in excitation of dopaminergic neurons in the substantia nigra and subsequent enhancement of dopamine release in the corpus striatum.

Keywords: nicotine, patch clamp, dopamine, substantia nigra, Parkinson's disease

Introduction

Clinical investigations have revealed that smoking improves the symptoms of Parkinson's disease at the early stage of its progression (1, 2). In order to clarify the mechanism underlying this beneficial property of smoking, we have been trying to investigate the physiological functions and significance of neuronal nicotinic acetylcholine receptors (nAChRs) in the extrapyramidal tract, especially the nigrostriatal dopaminergic nervous system.

Nicotinic receptors in the central nervous system are located on not only post-synaptic cell bodies and/or their dendrites, but also on pre-synaptic axon terminals (3–5). Our previous *in vivo* and *in vitro* experiments demonstrated that nicotine induced excitation of striatal neurons by facilitating the release of excitatory neurotransmitters from the nerve terminals in the corpus striatum (6, 7), suggesting that nicotine pre-synaptically modulates the functions of striatum neurons.

In contrast, in the substantia nigra, previous studies using conventional extracellular and/or intracellular recordings have shown that either systemic or iontophoretic application of nicotine increased the firing rate

of substantia nigral neurons, and this increase was blocked by mecamylamine or dihydro- β -erythroidine (DH β E), antagonists for nAChRs (8–12). Additionally, immunohistochemical studies indicate that dopaminergic neurons in the substantia nigra compacta (SNC) receive the cholinergic input from the pedunculopontine nucleus (13, 14). Although it is important to clarify how nicotine acts on dopaminergic neurons in the substantia nigra that project to the striatum, it still remains unclear whether nicotine exerts its effects post- or pre-synaptically in this area. Furthermore, the neuronal nicotinic receptor subtype that contributes to the cholinergic transmission in this area is still controversial. In this study, we performed a more precise examination of the properties of nicotinic receptors on nigral neurons using the slice patch clamp method.

Materials and Methods

Slice patch clamp study

After decapitation of young rats (10–20-day-old), a block of tissue containing the substantia nigra was trimmed and kept in ice-cold artificial cerebrospinal fluid bubbled with O₂-CO₂ (95%-5%). The slices (150- μ m-thick) including the substantia nigra were cut horizontally from the block using a microslicer (DTK-

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1000; Dosaka, Kyoto) and incubated at 34°C for 1 h before the experiments. Then, the nigral slices were placed in a recording chamber perfused continuously with artificial cerebrospinal fluid at 2 ml/min. The ionic composition of the artificial cerebrospinal fluid for dissection and perfusion was as follows: 113 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4 , 25 mM NaHCO_3 , 11 mM glucose, 2 mM CaCl_2 , and 1 mM MgCl_2 . The pH of the solution was 7.4 when bubbled with $\text{O}_2\text{-CO}_2$ (95%-5%). Perfusion solution with 0.3 μM tetrodotoxin was used for the voltage clamp experiments. The drugs were applied into the bath by switching the perfusion line manually. The experiments were carried out at room temperature (21–25°C). The nigral neurons were observed under Nomarski optics with a water-immersion lens ($\times 40$, BX50WI; Olympus, Tokyo).

Under the current-clamp and voltage-clamp modes, membrane potentials and currents were recorded from these nigral neurons according to the standard whole cell patch clamp technique using an Axopatch 200B patch clamp system (Axon Instruments, Foster City, CA, USA) (15). Patch pipettes were pulled from borosilicate capillary glass (o.d. 1.5 mm; World Precision Instrument-Japan, Tokyo) and had resistances between 3 and 7 M Ω when filled with an internal solution of the following ionic composition: 130 mM K-gluconate, 10 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 10 mM HEPES, and 10 mM EGTA (pH adjusted to 7.4 with KOH). The signals of membrane potentials and currents were monitored on an oscilloscope (VC-11; Nihon Kohden, Tokyo), recorded directly on a Thermal Array Recorder (RTA-1100, Nihon Kohden) and sampled at 1 kHz by a microcomputer using the program pCLAMP (Axon Instruments) or stored for off-line analysis on video cassettes after passage through a pulse-code modulation device (VR-10B; Instrutech Corporation, Great Neck, NY, USA). The membrane potentials were corrected for the liquid-junction potential between the pipette and artificial cerebrospinal fluid by adjusting the pipette offset of the Axopatch 200B at the beginning of the experiments.

Data analysis

Statistical analysis was performed by Student's *t*-test or ANOVA method.

Drugs used

Nicotine tartrate dihydrate (Nacalai Tesque, Kyoto), dihydro- β -erythroidine HBr (DH β E) (RBI, Natick, MA, USA), and tetrodotoxin (TTX) (Wako Chemical, Osaka) were purchased from the sources shown. All other chemicals were of analytical grade.

Results

Electrophysiological identification of dopaminergic neurons

Conventional whole-cell recording was made from visually identified substantia nigral neurons in the prepared mid-brain slices. Dopaminergic neurons were characterized by their electrophysiological properties as reported previously (12, 16). In brief, dopaminergic neurons were characterized by the appearance of action potentials with long-duration (>2 ms), spontaneous firing with low frequency ($<2-3$ Hz) and prominent after-hyperpolarization. One of the most defined characteristics of dopaminergic neurons is a voltage- and time-dependent sag of the membrane potential in response to step pulses of hyperpolarizing current, resulting from the activation of a current through I_h (Fig. 1). Using these electrophysiological criteria, 38 dopaminergic neurons (size: 34.6 ± 0.6 μm , resting membrane potential: -56.5 ± 0.7 mV) were identified and used for the following experiments.

Excitation of dopaminergic neurons by nicotine in whole-cell current-clamp mode

After identification of dopaminergic neurons, bath application of nicotine induced depolarization of dopaminergic neurons with an increase in firings in whole-cell current-clamp recording (Fig. 2). Nicotine (0.01, 0.1, 1, and 10 μM) depolarized membrane potentials by 0.2 ± 0.4 ($n = 4$), 3.9 ± 0.3 ($n = 4$), 6.8 ± 0.7 ($n = 6$), and 9.5 ± 0.9 ($n = 7$) mV from the resting membrane potentials, respectively. This nicotine-induced excitation of dopaminergic neurons was still observed when the Ca^{2+} in the perfusion medium was substituted with Mg^{2+} (5 mM) (Fig. 3). Nicotine (10 μM) induced depolarization of 7.7 ± 0.8 ($n = 4$) mV in normal perfusion solution, while it depolarized the neurons by 6.6 ± 0.8 ($n = 4$) mV even in Ca^{2+} free solution. There was no significant difference in the depolarizing effects of nicotine on dopaminergic neurons between normal and Ca^{2+} free conditions.

Inward currents induced by nicotine in whole-cell voltage-clamp mode

To investigate the mechanism underlying nicotine-induced excitation, the effects of nicotine on dopaminergic neurons in the substantia nigra were examined in the whole-cell voltage-clamp mode at a holding potential of -60 mV. Bath application of nicotine induced the inward currents in a dose-dependent manner (Fig. 4A and Table 1). To characterize the relationship between nicotine-induced current and membrane potential, 6-s voltage ramps ranging from -120 to 0 mV were applied to a whole-cell patched dopaminergic neuron in

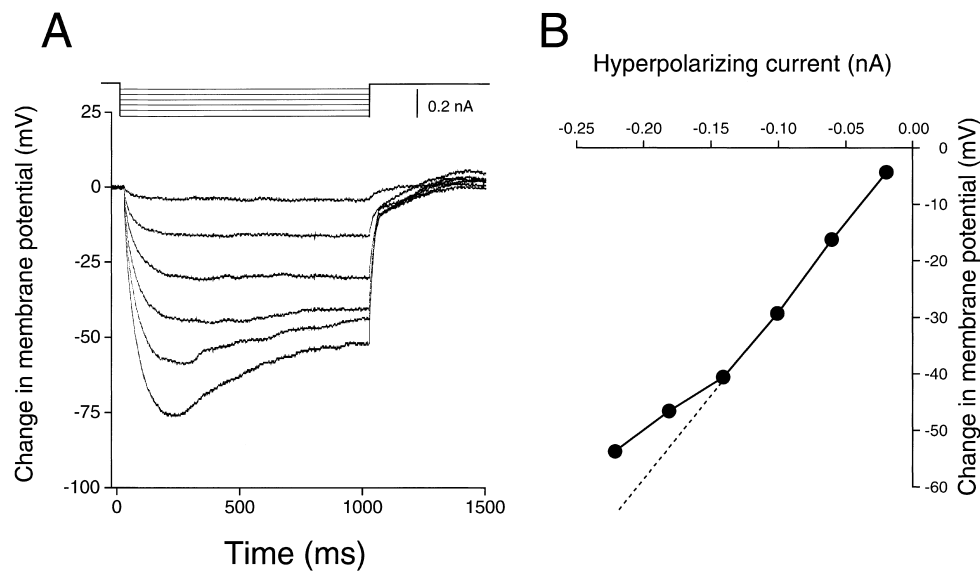


Fig. 1. Voltage- and time-dependent sag of a dopaminergic neuron in the substantia nigra. **A:** Hyperpolarizing step currents were applied to a dopaminergic neuron from the patch pipette in the whole-cell current-clamp mode. The voltage- and time-dependent sag was observed in the voltage traces when 1-s step pulses hyperpolarized the cell membrane by more than 50 mV from the resting membrane potential. **B:** The relationship between hyperpolarizing currents and change in membrane potential 900 ms after each application of a step pulse current is shown. Linearity of the relationship deviated when the extent of hyperpolarization was more than 50 mV.

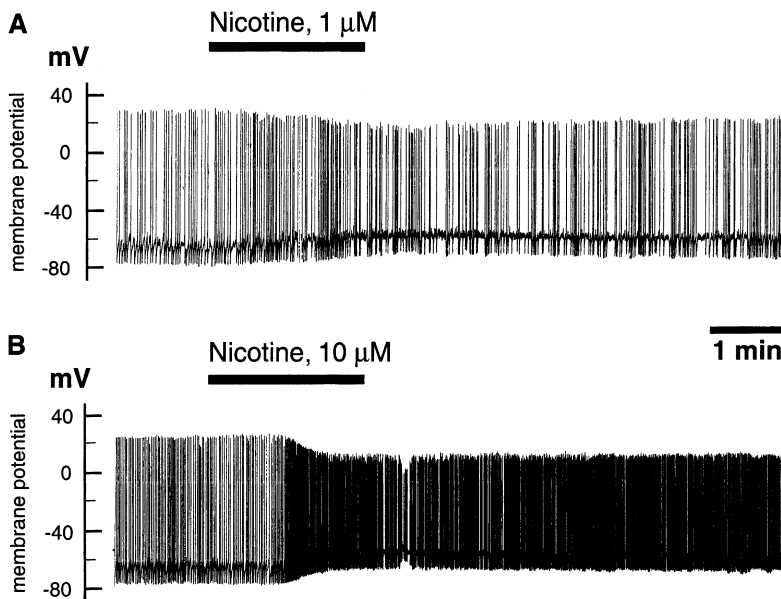


Fig. 2. Depolarization of dopaminergic neurons with an increase in firings induced by nicotine. **A:** The resting membrane potential was depolarized and firings were increased by 2-min bath application of nicotine (1 μ M) to a dopaminergic neuron in the whole-cell current-clamp recording. **B:** Nicotine (10 μ M) more clearly depolarized the membrane potential and increased firings when it was applied to the same neuron observed in (A) 15 min after the preceding application.

the absence and presence of nicotine (10 μ M) (Fig. 4B). The nicotine-induced net current was observed by subtracting currents in the presence of nicotine from those in the absence of nicotine (Fig. 4C). The reversal potential of this net current was -24.2 ± 3.9 ($n = 4$) mV. The relationship between the net current and voltage was linear at the membrane potential of less than -20 mV; however, it was rectified at the membrane potential of

more than -20 mV.

Selective antagonist for $\alpha 4\beta 2$ nicotinic receptor blocked nicotine-induced inward current

To clarify the nicotinic receptor subtype that mediated this inward current, we examined the effects of DH β E, a specific antagonist for $\alpha 4\beta 2$ type nAChR. After confirming that nicotine (10 μ M) elicited an inward current

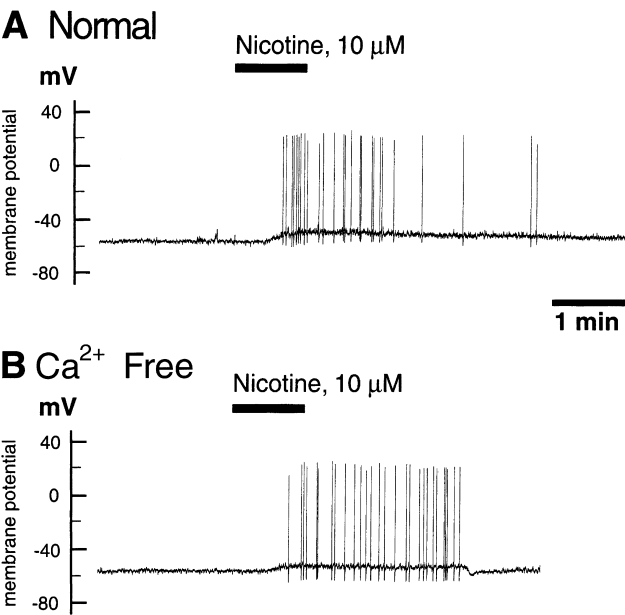


Fig. 3. Nicotine-induced excitation was independent of extracellular calcium ions. **A:** Nicotine (10 μ M) depolarized the membrane potential and increased firings of a dopaminergic neuron in the normal perfusion solution with calcium (2 mM) and magnesium (1 mM). **B:** After the first application of nicotine in normal conditions, the second application of nicotine (10 μ M) also depolarized and induced excitation of the same neuron observed in (A), even in the perfusion solution containing no calcium (0 mM) and a high level of magnesium (5 mM).

Table 1. Dose-dependent nicotine-induced inward currents of dopaminergic neurons in the substantia nigra and inhibition of nicotine-induced currents by DH β E

	Concentration (μ M)	Peak current (pA)	N
Nicotine alone	0.01	0.5 ± 1.5	5
	0.1	7.9 ± 1.4	6
	1	18.5 ± 1.7	5
	10	28.3 ± 2.6	7
	100	28.5 ± 2.8	4
Nicotine (10 μ M) + DH β E (1 μ M)		$4.0 \pm 1.2^{**}$	4

Peak amplitude of nicotine-induced inward currents at each dose is shown as the mean \pm S.E.M. N represents number of neurons tested at each dose. ANOVA analysis indicates a dose-dependent increase in nicotine-induced current ($P < 0.01$). Nicotine (10 μ M)-induced currents were significantly inhibited by DH β E (1 μ M), a selective antagonist for $\alpha 4 \beta 2$ type neuronal nicotinic receptors. ($**P < 0.01$, Student's *t*-test).

in the examined dopaminergic neuron, DH β E (1 μ M) was applied. DH β E alone had no effect on the dopaminergic neuron; however, simultaneous application of DH β E with nicotine significantly inhibited nicotine-induced currents (Fig. 5) (Table 1).

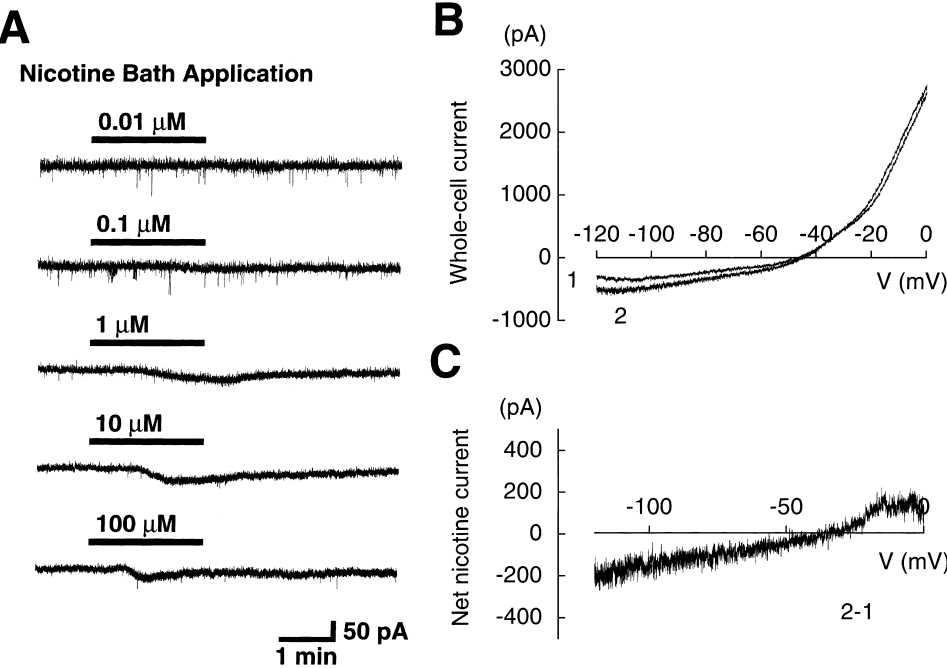


Fig. 4. Dose-dependent nicotine-induced inward currents of dopaminergic neurons in the substantia nigra. **A:** Nicotine was applied to a dopaminergic neuron in the slice preparation for 2 min at each dose every 10 min. Nicotine (0.01–100 μ M) induced inward currents in a dose-dependent manner at the holding potential of -60 mV. **B:** Current-voltage relationship in the absence and presence of nicotine when a voltage ramp was applied. In the voltage-clamp mode, 6-s voltage ramp waves ranging from -120 to 0 mV were applied to a whole-cell patched dopaminergic neuron. Traces 1 and 2 indicate the current-voltage relationships in the absence and presence of nicotine (10 μ M), respectively. **C:** Net nicotine-induced current-voltage relationship is shown by subtracting trace 1 from trace 2 current. Nicotine-induced current was reversed around -25 mV and rectified at the membrane potential more than -20 mV.

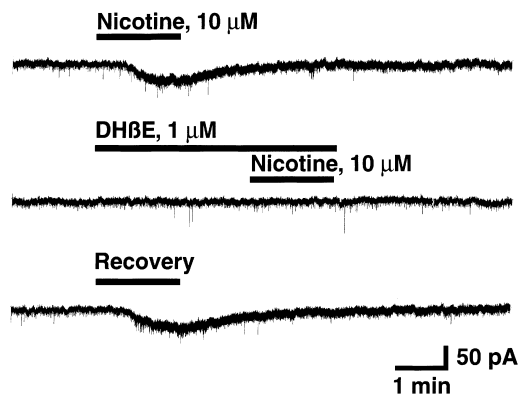


Fig. 5. Inhibition of nicotine-induced current of a dopaminergic neuron by DH β E. Bath application of nicotine (10 μ M) for 1.5 min induced inward current at the holding potential of -60 mV. This current was completely abolished in the presence of DH β E (1 μ M), a selective antagonist for $\alpha 4\beta 2$ type neuronal nicotinic receptors. The inhibitory effect of DH β E on nicotine-induced current was reversible since the current was recovered after the washout of DH β E.

Discussion

Previous reports have demonstrated that nicotine or acetylcholine exerts excitation on dopaminergic neurons in the substantia nigra (5, 8, 9), which receives cholinergic inputs from the pedunculopontine nucleus (17). Since both nicotinic and muscarinic inputs could induce excitation (14), the acetylcholine receptor subtype that mainly mediates excitation in this area has been unclear. A recent study using conventional intracellular recording revealed that nicotine induced the excitation of dopaminergic neurons in the substantia nigra through the activation of postsynaptic $\alpha 4\beta 2$ type nicotinic acetylcholine receptors; however, the ionic mechanism involved in this excitation has remained unclear (11).

In our whole-cell slice-patch experiments, bath application of nicotine depolarized dopaminergic neurons with an increase in firing in a dose-dependent manner under the current-clamp configuration. In addition, our voltage-clamp experiments showed that application of nicotine induced inward currents that were characterized by inward rectification and blockade by DH β E, a selective antagonist for the $\alpha 4\beta 2$ type nAChR. The current-voltage relationship observed in the present study was very similar to that of $\alpha 4\beta 2$ type nAChR as shown previously (18). These results suggest that the nicotine-induced excitation observed in the substantia nigra is mainly mediated by postsynaptic $\alpha 4\beta 2$ nAChRs. Furthermore, as the nicotine-induced depolarization was not affected by the removal of Ca^{2+} in perfusion solution, Ca^{2+} may be dispensable as an ionic component through this channel.

In addition to the present electrophysiological evidence that directly showed the existence of $\alpha 4\beta 2$ receptors in dopaminergic neurons of the substantia nigra, a recent study suggested the existence of $\alpha 7$ type nAChR as well as $\alpha 4\beta 2$ nAChR in this area (19). This discrepancy may be due to the technical limitations of drug application used in this study. It is known that bath application of nicotine cannot induce the current mediated by $\alpha 7$ type nAChRs since the deactivation phase of this current is too fast. An examination using a fast drug application system is necessary to elucidate the contribution of $\alpha 7$ receptors to the nicotine-induced excitation in this area.

It is concluded that nicotine can increase the release of dopamine in the striatum by activation of nicotinic receptors on the soma and dendrites of dopaminergic neurons in the substantia nigra in addition to activation of nicotinic receptors on the nerve terminals of dopaminergic neurons in the striatum.

Acknowledgments

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