Non-Competitive Antagonism by Hirsuteine of Nicotinic Receptor-Mediated Dopamine Release from Rat Pheochromocytoma Cells

Tomokazu Watano, Ken Nakazawa, Tomoko Obama, Mayumi Mori, Kazuhide Inoue, Kannosuke Fujimori and Akira Takanaka

1 Division of Pharmacology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158, Japan
2 Kampo Research Laboratories, Kanebo Co., Ltd., 1-5-90 Tomobuchi-cho, Osaka 534, Japan

Received November 25, 1992 Accepted January 14, 1993

ABSTRACT—Effects of hirsuteine, an indole alkaloid extracted from Uncaria genus, on nicotine- and high K+-induced responses were investigated in rat pheochromocytoma PC12 cells. Hirsuteine (300 nM–10 μM) inhibited dopamine release evoked by 100 μM nicotine in a concentration-dependent manner. Hirsuteine did not produce a parallel shift of the concentration-response relationship curve for nicotine, but reduced maximal dopamine release. Dopamine release evoked by 60 and 155 mM KCl was also inhibited by hirsuteine, but the concentration necessary for significant inhibition was higher (more than 10 μM). Under whole cell voltage-clamp, hirsuteine reversibly inhibited inward currents activated by 100 μM nicotine. The current inhibition was slightly accelerated by hyperpolarization. The results suggest that hirsuteine non-competitively antagonizes nicotine-evoked dopamine release from PC12 cells, and that the blockade of nicotinic receptor channel complexes, but not that of voltage-gated Ca channels, plays an obligatory role in the antagonism.

Keywords: Hirsuteine, PC12 cells, Nicotinic receptor, Dopamine release

Hirsuteine as well as hirsutine (Fig. 1) is an indole alkaloid isolated from the Uncaria genus (1). The pharmacological effects of hirsutine such as ganglion-blocker-like effects (2–5) and a hypotensive effect (2) have been reported. As for hirsuteine, Ozaki (6, 7) showed that the pharmacological profile of hirsuteine is similar to that of hirsutine. We previously investigated the effects of hirsutine in PC12 cells, a cell line derived from a rat pheochromocytoma (8), to clarify the mechanisms underlying its ganglion-blocking action, and found that hirsutine blocks several types of ion channels in these cells. In the present study, we used a similar approach to determine the effects of hirsuteine on neurosecretion. Our results suggest that hirsuteine non-competitively antagonizes nicotine-evoked dopamine release from PC12 cells, and that the blockade of nicotinic receptor channels, but not that of voltage-gated Ca channels, plays an obligatory role in the antagonism.

Fig. 1. Chemical structures of hirsutine and hirsuteine.

MATERIALS AND METHODS

Cell culture

PC12 cells were prepared as previously described by Inoue and Kenimer (9). In brief, PC12 cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 7%
Dopamine release

Release of dopamine from PC12 cells was measured according to the previous reports (9, 10). The dishes containing cultured PC12 cells were incubated with 1 ml of balanced salt solution (BS) containing: 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 10 mM glucose, 0.1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and 2.5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (pH adjusted to 7.4 with NaOH) for 1 hr at room temperature. After washing once with BS (1 ml), the cells were stimulated by nicotine in the absence or presence of hirsuteine. The incubation solution was removed from the dishes 1 min after the nicotine-application and mixed with 0.25 ml of 1 N perchloric acid. The cells remaining in the dishes were disrupted by sonication after adding 1 ml of 0.2 N perchloric acid. Both the incubation solution and the sonicated cellular solutions were centrifuged (at 5°C for 2 min, 1000 x g), and the supernatants were collected.

Dopamine content was determined with a high-performance liquid chromatography-electrochemical detector (HPLC-ECD) system (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Ten-microliter aliquots of the supernatants were applied to the HPLC system, which consisted of a reverse phase column (4.6 mm x 150 mm, ODS 3 μm, Bioanalytical Systems) and an ECD with the electrode potential set at +0.7 V versus the Ag/AgCl reference electrode. The mobile phase consisted of a monochloroacetate buffer (140 mM, pH 3.05) containing 10% methanol, 30 mg/l sodium 1-octanesulfonate and 1.8 mM EDTA (flow rate, 0.9 ml/min). The data was analyzed with a chromatographic data processor (C-R4A, Shimadzu, Kyoto).

Percent secretion was calculated using the values obtained for dopamine content in the incubation solution (A) and dopamine content remaining in the cells (B) by the following equation: % of total dopamine/min = 100 x A/(A+B).

Electrophysiological study

Membrane potentials and currents were measured using whole-cell recordings of improved patch-clamp techniques (11). The cells were continuously superfused with an extracellular solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 10.0 mM HEPES (pH adjusted to 7.4 with NaOH). The heat-polished patch pipettes had tip resistances of about 5 MΩ when filled with an intracellular solution containing 150 mM CsCl, 10.0 mM HEPES and 5.0 mM glycochelidiamine-N,N',N''-tetraacetic acid (EGTA) (pH adjusted to 7.3 with CsOH). For the measurements of Ba currents permeating through voltage-gated Ca channels, extracellular CaCl2 was replaced with BaCl2 (10.8 mM) to obtain a large inward current. Cd (300 μM) was applied at the end of each experiment. The amplitude of the Ba current was determined as the current component blocked by 300 μM Cd (12). Membrane currents were monitored on a storage oscilloscope (VC-11, Nihon Kohden, Tokyo) via a patch clamp amplifier (CEZ-2200, Nihon Kohden). Electrical signals were filtered at 1 kHz and stored on magnetic tape for later analysis. The experiments were performed at room temperature (about 25°C).

Hirsuteine was prepared at the Kampo Research Laboratories of Kanebo Co., Ltd. (-)-Nicotine hydrochloride tartrate salt and adenosine 5’-triphosphate disodium salt were purchased from Sigma (St. Louis, MO, U.S.A.). All drugs were applied to cells by superfusion. In estimating the effects of hirsuteine, the cells were only exposed to nicotine or ATP for a brief period (about 5 sec) to avoid desensitization of nicotinic-receptor channels (13, 14) or ATP-receptor channels (15, 16)

RESULTS

Effects of hirsuteine on nicotine-evoked dopamine release

We first tested whether hirsuteine blocks nicotine-evoked dopamine release from PC12 cells. Hirsuteine (300 nM – 10 μM) inhibited the dopamine release evoked by 100 μM nicotine in a concentration-dependent manner (Fig. 2A). Figure 2B shows changes in the concentration-response relationship for the nicotine-evoked dopamine release in the presence of hirsuteine. Hirsuteine (1 – 100 μM) did not shift the relationship toward a higher concentration range, but reduced the maximal dopamine release.

Effects of hirsuteine on nicotine-activated channels

Figure 3A shows the inhibitory action of hirsuteine (1 μM) on the inward current activated by 100 μM nicotine. Hirsuteine was present 10 sec before and during the application of nicotine in this case. The inhibition was not augmented with a longer (1 min) pretreatment with hirsuteine (not shown). The current inhibited by hirsuteine was almost fully recovered 1 min after the rinse with a drug-free solution (Fig. 3A). Figure 3B shows the concentration-dependence of the current inhibition. The current was more sensitive to hirsuteine than the dopamine release (Fig. 2A): for example, 1 μM hirsuteine reduced the dopamine release only to about 80% of the control,
whereas it suppressed the current to as low as 30% of the control. The extent of inhibition by hirsuteine (100 nM) on the current activated by 30 pM nicotine (32.6±4.2% of control, n=9) was not different from that on the current activated by 100 pM nicotine (30.9±2.9% of control, n=6).

We tested whether hirsuteine affects nicotine-activated currents in a voltage-dependent manner. We held the cells at −30 mV and applied hyperpolarizing test pulses to −100 mV periodically (Fig. 4A). As shown in Fig. 4B, the inhibition by 10 nM hirsuteine of the nicotine (10 nM−10 μM) on the nicotine-activated current. The nicotine-activated currents were measured as in A, and the amplitude of the nicotine activated current in the presence of hirsuteine was normalized to the control. Each column represents the mean from 3 to 6 tested cells. Bars show the S.E.M.

Effects of hirsuteine on dopamine release by other stimulation and other channels

Figure 5 shows the effects of hirsuteine on dopamine release evoked by 60 and 155 mM KCl and voltage-gated Ca channels in a voltage-dependent manner. We held the cells at −30 mV and applied hyperpolarizing test pulses to −100 mV periodically (Fig. 4A). As shown in Fig. 4B, the inhibition by 10 nM hirsuteine of the nicotine (100 μM)-activated inward current was accelerated by hyperpolarization, although the difference was not statistically significant (Student’s t-test, P > 0.05).
Fig. 4. Voltage-dependence of the blockade by hirsuteine (10 nM) of nicotine (100 μM)-activated current. A. Current response. Nicotine (100 μM) was applied to the cell every 1 min (solid bars). The cells were held at −30 mV, and a 200 msec test pulse to −100 mV was applied every 1 sec. Hirsuteine was present 10 sec before and throughout the nicotine-application (right panel). B. Summarized data. The currents at −30 and −100 mV were measured as in A. The amplitude of the nicotine-activated current in the presence of hirsuteine was normalized to the current before the application of hirsuteine. Each column represents the mean from 6 tested cells. Bars show the S.E.M.

Fig. 5. Effects of hirsuteine on dopamine release evoked by high concentrations of KCl (60, 155 mM: A) and Ba current permeating through voltage-gated Ca channels (B, C). A. Dopamine release. The cells were stimulated with high concentrations of KCl (60, 155 mM) for 1 min in the absence or the presence of hirsuteine. Each column represents the mean from 3 tested dishes. Bars show the S.E.M. B. Ba current recorded from a PC12 cell. The current was activated by a 400-msec test pulse to +10 mV every 5 sec from a holding potential of −60 mV. The current traces just before (control) and 1 min after application of 10 μM hirsuteine (+hirsuteine) were superimposed. C. Concentration-dependency. Ba currents were obtained as in B. Peak Ba current was normalized to that just before the hirsuteine application. Each column represents the mean from 4 tested cells. Bars show the S.E.M.
channels in PC12 cells. Hirsuteine (10–100 μM) partially inhibited dopamine release evoked by 60 and 155 mM KCl (Fig. 5A). The extent of the inhibition by hirsuteine was larger for the release evoked by 60 mM KCl than for the release evoked by 155 mM KCl. Ba currents flowing through the voltage-gated Ca channels were suppressed by lower concentrations of hirsuteine (Fig. 5B, C) as compared with the dopamine release, but the currents were still less sensitive than nicotine-activated currents (Fig. 3B).

We previously characterized extracellular ATP-evoked dopamine release and ATP-activated channels in PC12 cells (15–17). The dopamine release (19.96 ± 1.60% of total, n = 3) evoked by 100 μM ATP was about 3-fold larger than that evoked by 100 μM nicotine (5.43 ± 0.30% of total, n = 3). Recent investigations suggested that the large catecholamine release from PC12 cells by ATP is not related to voltage-gated Ca channels secondarily activated by ATP-evoked depolarization, but due to a large Ca-influx through ATP-activated channels themselves (15, 18). Hirsuteine (100 μM) did not affect the dopamine release evoked by 100 μM ATP (102.8 ± 7.4% of control, n = 3). The ATP-activated current was also not affected by 100 μM hirsuteine (102.0 ± 7.5% of total, n = 4).

**DISCUSSION**

We have characterized the inhibition by hirsuteine of nicotine-activated dopamine release from PC12 cells. The reduction by hirsuteine of the maximal dopamine release (Fig. 2B) suggests that the inhibition is non-competitive. The inhibition by hirsuteine of the nicotine-activated current was not augmented with decreased nicotine concentration, suggesting that the current inhibition is also non-competitive. The non-competitive antagonism is not due to an irreversible blockade of receptors because the nicotine-evoked current inhibited by hirsuteine was readily reversed by a rinse with drug-free solution (Fig. 3A). Perhaps, hirsuteine blocks ion permeation through nicotinic receptor channels and thereby reduces depolarization. The voltage-dependence of a channel block varies according to the locations of the binding sites for the blocking particles (19). The binding site of hirsuteine may be close to the outer surface of the cell membrane, judging from the weak voltage-dependence of the current inhibition (Fig. 4). We speculate that the binding site is in the channel pore, although a possibility of allosteric sites cannot be excluded at present.

Hirsuteine inhibited nicotine-evoked dopamine release at micromolar concentrations (Fig. 2A), whereas it inhibited the nicotine-activated current at a concentration as low as 10 nM (Fig. 3B). One possibility is that the decrease in current amplitude does not directly reflect the reduction in Ca-influx necessary for dopamine release. The current remaining after blockade by lower concentrations (less than 1 μM) of hirsuteine may be sufficient to produce a large Ca-influx during exposure to nicotine for as long as 1 min (see "Materials and Methods"). A similar discrepancy in concentration-dependence was previously reported for hirsutine and hexamethonium (10). It may also be argued that the nicotine-activated current remaining after the blockade by hirsuteine can supply Ca because neuronal nicotinic receptor channels are Ca-permeable (20, 21). However, this component may not contribute to the dopamine release reported here because nicotine-evoked catecholamine release is abolished by Ca-channel antagonists (9).

Dopamine release evoked by 60 and 155 mM KCl (Fig. 5A) was not largely affected by 10 μM hirsuteine. A considerable part of the nicotine-evoked catecholamine release depends on Ca-influx mediated through Ca channels secondarily activated by depolarization in PC12 cells (22, 23). Although hirsuteine (1–10 μM) inhibited Ba currents (Fig. 5B), the lack of significant effects of 10 μM hirsuteine on the dopamine release may exclude any major contribution of the blockade of Ca channels to the antagonism to nicotine-evoked dopamine release. The antagonism may be essentially attributable to the blockade of nicotinic receptor-channels.

The effects of hirsuteine reported here were similar to those of hirsutine (10). Both alkaloids inhibited nicotine-evoked dopamine release and inward currents, and Ba currents flowing through Ca channels. These results may be related to antihypertensive effects which are one of the major purposes of clinical application of Uncaria genus. The alkaloids may decrease blood pressure by inhibiting the nicotinic receptor-channels in ganglions, which results in reduction of stimulatory regulation by sympathetic neurons of vascular tones and/or a decrease in catecholamine release from the adrenal medulla. Ozaki (7) proposed that vasodilation induced by these alkaloids accounts for their antihypertensive effects. The Ca channel inhibition by these alkaloids may be responsible for the vasodilation. However, in the antihypertensive effects, the Ca channel inhibition may be secondary to the ganglion-blocking action, judging from the concentration-dependence in the present study.

**Acknowledgments**

The authors thank Dr. A. Suzuki, Director of the Kampo Research Laboratories of Kanebo Co., Ltd. and Prof. I. Kitagawa of Osaka University for providing T. Watano with the opportunity to study at the National Institute of Health Sciences; and we also thank Prof. K. Momose of Showa University for providing M. Mori with the opportunity to study at the National Institute of Health Sciences. This work was partly supported by the Japan Health Sciences Foundation.
REFERENCES

1. Haginiwa, J., Sakai, S., Aimi, N., Yamanaka, E. and Shinma, N.: Studies of plants containing indole alkaloids: II. On the alkaloids of Uncaria rhynchophylla Miq. Yakugaku Zasshi 93, 448–452 (1973) (Abs. in English)

2. Ozaki, Y., Harada, M. and Sakai, S.: Pharmacological studies on Uncaria and Amsonia alkaloids. J. Pharmocol. 30, Supp. 137P (1980)

3. Harada, M., Ozaki, Y. and Ohno, H.: Effects of indole alkaloids from Gardneria nuts Seib. et Zucc. and Uncaria rhynchophylla Miq. on a guinea pig urinary bladder preparation in situ. Chem. Pharm. Bull. (Tokyo) 27, 1069–1074 (1979)

4. Ozaki, Y. and Harada, M.: Site of ganglion blocking action of garderamine and hirsutine in the dog urinary bladder in situ preparation. Japan. J. Pharmacol. 33, 463–471 (1983)

5. Harada, M. and Ozaki, Y.: Effects of indole alkaloids from Gardneria genus and Uncaria genus on neuromuscular transmission in the rat limb in situ. Chem. Pharm. Bull. (Tokyo) 24, 211–214 (1976)

6. Ozaki, Y.: Pharmacological studies of indole alkaloids obtained from domestic plants, Uncaria rhynchophylla Miq. and Amsonia elliptica Roem. et Schult. Folia Pharmacol. Japon. 94, 17–26 (1989) (Abs. in English)

7. Ozaki, Y.: Vasodilatative effects of indole alkaloids obtained from domestic plants, Uncaria rhynchophylla Miq. and Amsonia elliptica Roem. et Schult. Folia Pharmacol. Japon. 95, 47–54 (1990) (Abs. in English)

8. Greene, L.A. and Tischler, A.S.: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U.S.A. 78, 3245–3249 (1976)

9. Inoue, K. and Kenimer, J.G.: Muscarinic stimulation of calcium influx and norepinephrine release in PC12 cells. J. Biol. Chem. 263, 8157–8161 (1988)

10. Nakazawa, K., Watano, T., Ohara-Imaizumi, M., Inoue, K., Fujimori, K., Ozaki, Y., Harada, M. and Takanaka, A.: Inhibition of ion channels by hirsutine in rat pheochromocytoma cells. J. Pharmocol. 57, 507–515 (1991)

11. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J.: Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. Pflugers Arch. 391, 85–100 (1981)

12. Nakazawa, K., Inoue, K., Ohara-Imaizumi, M., Fujimori, K. and Takanaka, A.: Inhibition of Ca-channels by diazepam compared with that by nicardipine in pheochromocytoma PC12 cells. Brain Res. 553, 44–50 (1991)

13. Nakazawa, K., Fujimori, K., Takanaka, A. and Inoue, K.: Existence of muscarinic suppression of a K current in PC-12 pheochromocytoma cells. Am. J. Physiol. 257, C1030–C1033 (1989)

14. Ifune, C.K. and Steinbach, J.H.: Regulation of sodium currents and acetylcholine responses in PC12 cells. Brain Res. 506, 243–248 (1990)

15. Inoue, K., Nakazawa, K., Fujimori, K. and Takanaka, A.: Extracellular adenosine 5'-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. Neurosci. Lett. 106, 294–299 (1989)

16. Nakazawa, K., Fujimori, K., Takanaka, A. and Inoue, K.: An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. J. Physiol. (Lond.) 428, 257–272 (1990)

17. Nakazawa, K., Inoue, K., Fujimori, K. and Takanaka, A.: ATP-activated single-channel currents recorded from cell-free patches of pheochromocytoma PC12 cells. Neurosci. Lett. 119, 5–8 (1990)

18. Nakazawa, K. and Inoue, K.: Roles of Ca influx through ATP-activated channels in catecholamine release from pheochromocytoma PC12 cells. J. Neurophysiol. 68, 2026–2032 (1992)

19. Hille, B.: Mechanisms of block. In Ionic Channels of Excitable Membrane 2nd ed., pp. 390–423, Sinaure Associates, Inc., Sunderland (1992)

20. Vernino, S., Amador, M., Luetje, C.W., Patrick, J. and Dani, J.A.: Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron 8, 127–134 (1992)

21. Muller, C., Choquet, D., Korn, H. and Changeux, J.P.: Calcium influx through nicotinic receptor in rat central neurons: its relevance to cellular regulation. Neuron 8, 135–143 (1992)

22. Ritchie, A.K.: Catecholamine secretion in a rat pheochromocytoma cell line: two pathways for calcium entry. J. Physiol. (Lond.) 286, 541–561 (1979)

23. Stallcup, W.: Sodium and calcium fluxes in a clonal nerve cell line. J. Physiol. (Lond.) 286, 525–540 (1979)