Inhibition of Ion Channels by Hirsutine in Rat Pheochromocytoma Cells

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Received July 18, 1991 Accepted September 4, 1991

ABSTRACT — Effects of hirsutine, an alkaloid that produces a potent ganglion blocking effect, were investigated using rat pheochromocytoma PC12 cells. Hirsutine (1 to 10 μM) suppressed dopamine-release evoked by 100 μM nicotine. In voltage-clamped cells, hirsutine (1 to 10 μM) inhibited the inward current activated by 100 μM nicotine. Hirsutine was equipotent to hexamethonium in blocking the nicotine-activated current. The voltage-dependency of the nicotine activated current was not modified by hirsutine. Effects of hirsutine on other ion channels were tested to determine its selectivity. Inward currents mediated through ATP-activated channels were scarcely affected by hirsutine (up to 100 μM). However, hirsutine (10 μM) inhibited Ba currents passing through Ca channels and K currents activated by depolarizing voltage steps. The results suggest that hirsutine potently blocks nicotinic receptor-channels, but hirsutine also inhibits voltage-gated Ca and K channels. Roles of the inhibition of these channels in the pharmacological effects of hirsutine were discussed.

Hirsutine (Fig. 1) is one of the indole alkaloids isolated from Uncaria genus (1), and its ganglion blocker-like effects have been reported. Hirsutine inhibited the preganglionic nerve-mediated contractions of guinea pig (2) and dog urinary bladder (3) without affecting the contractions induced by exogenous acetylcholine in situ. Action potentials of postganglionic nerves in response to preganglionic nerve-stimulation were suppressed by hirsutine in rat superior cervical ganglion. As hirsutine was less potent in blocking neuromuscular transmission in rat limbs (4), hirsutine may affect responses mediated through ganglion-type nicotinic receptors preferentially over those mediated through muscle-type nicotinic receptors as is the case for hexamethonium (C₆).

PC12 cells, a cell line derived from a rat pheochromocytoma (5), has retained the ability to release catecholamines in response to

Fig. 1. Chemical structure of hirsutine.

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stimulations including exogenous nicotinic stimulation (e.g., 6, 7), so they are widely used in studies on neurosecretion. PC12 cells are known to possess ganglion-type nicotinic receptors (8) and are suitable for studying mechanisms underlying ganglion-blocking effects.

In the present study, we used PC12 cells to determine whether hirsutine inhibits nicotinic acetylcholine receptor-channels. The nicotinic receptor-channels are primarily responsible for neurotransmission in ganglions (9) and targets of C₆ (10, 11). We also tested the effects of hirsutine on other ion channels in these cells.

MATERIALS AND METHODS

Cell culture

PC12 cells were prepared as previously described by Inoue and Kenimer (7). In brief, PC12 cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 7% fetal bovine serum, 7% heat-inactivated horse serum, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate at 37°C in an atmosphere of 10% CO₂, and then plated onto collagen-coated polystyrene dishes at 1 X 10⁶ cells per dish. After an additional 2 to 3 day-culture, the cells were used for the experiments.

Dopamine-release

Release of dopamine from PC12 cells was measured by a modification of a previously reported procedure (7). 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 Na₂HPO₄, 10 mM glucose, 0.1 mM EDTA, and 2.5 mM HEPES (pH adjusted to 7.4 with NaOH) for 1 hr at room temperature. After washing once with BS (1 ml), the cells were treated with the desired concentration of hirsutine or C₆ for 30 sec. The cells were then stimulated by ATP in the absence or presence of hirsutine or C₆. The incubation solution was removed 1 min after the ATP-application and mixed with 0.25 ml of 1 N perchloric acid for measurement of the dopamine released into the solution. 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 for measurements of the dopamine content.

Dopamine content was determined with a high-pressure liquid chromatography-electrochemical detector (HPLC-ECD) system (Bioanalytical Systems). Ten microliters 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 (Shimadzu C-R4A).

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 (12). The cells were continuously superfused with an extracellular solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 11.1 mM glucose and 10.0 mM N,N,N',N'-tetraacetic acid (EGTA) (pH was adjusted to 7.4). 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 glycolediamine-N,N,N',N'-tetraacetic acid (EGTA) (pH was adjusted to 7.3 with CsOH). In the measurements of K currents, CsCl in the in-
tracellular solution was replaced with KCl, and the pH was adjusted with KOH. For the measurements of Ba currents permeating through voltage-gated Ca channels, extracellular CaCl₂ was replaced with BaCl₂ (10.8 mM). Cd (300 μM) was applied at the end of each experiment to block the Ca channels completely (13). The amplitude of the Ba current was determined by subtracting the current traces obtained with 300 μM Cd. Membrane currents were monitored on a storage oscilloscope (Nihon Kohden VC-11) via a patch clamp amplifier (Nihon Kohden CEZ-2200). 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).

Hirsutine was a gift from Prof. Shinichiro Sakai of Chiba University. (−)-Nicotine hydrogen tartrate salt and adenosine 5'-triphosphate disodium salt were purchased from Sigma (St. Louis, MO, U.S.A.). Hexamethonium bromide was obtained from Wako Pure Chemicals (Osaka, Japan). All drugs were applied to cells by superfusion. In estimating the effects of hirsutine, the cells were only exposed to nicotine or ATP for a brief period (about 7 sec) to avoid desensitization of nicotinic-receptor channels (14, 15) or ATP-activated channels (16, 17).

RESULTS

Effects of hirsutine on nicotine-evoked dopamine-release

We first tested whether hirsutine blocks nicotine-evoked dopamine-release from PC12 cells. Both hirsutine (1 to 10 μM) and C₆ (10 μM) inhibited dopamine-release evoked by 100 μM nicotine (Fig. 2). The inhibition induced by 10 μM hirsutine was larger than that induced by 10 μM C₆. We regarded these cells as a model for postganglionic cells in nicotinic neurotransmission and used them to clarify the mechanisms underlying the ganglionic blocking actions of hirsutine described in the following sections.

![Fig. 2. Effects of hirsutine and hexamethonium (C₆) on nicotine-evoked dopamine release from PC12 cells. The cells were stimulated with 100 μM nicotine for 1 min. The cells were treated with hirsutine or hexamethonium 30 sec before the nicotine-stimulation. Each column represents the mean from 3 dishes tested. Bars show the S.E.M. Asterisks indicate a statistically significant difference from the control (100 μM nicotine, alone) determined by analysis of variance and Duncan's test for multiple comparisons (*P < 0.05).](image-url)

Effects of hirsutine on nicotine-activated channels

Figure 3A shows the inhibitory action of hirsutine (1 μM) on the inward current activated by 100 μM nicotine. Hirsutine 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 hirsutine (not shown). A full recovery was observed about 2 min after the rinse with a drug-free solution (Fig. 3A). Hirsutine inhibited the nicotine-activated current in a concentration-dependent manner (Fig. 3B). The inhibition induced by 1 μM hirsutine was comparable to that induced by 1 μM C₆ (Fig. 3B). The inhibition induced by hirsutine was slightly overcome by an increase of nicotine; the inward current remaining after inhibition by 1 μM hirsutine was 57.3 ± 2.7% of the control (mean ± S.E.M., n = 7) for a higher concentration (1 mM) of nicotine, which was larger than the value (40.2 ± 2.9% of control, n =
We tested whether hirsutine affects these channels in a voltage-dependent manner. First, we applied to the cells hyperpolarizing test pulses from -30 mV to -100 mV periodically (not shown). The rate of reduction by 1 μM hirsutine of the nicotine (100 μM)-activated inward current was not different between -30 and -100 mV: the current was reduced to 43.6 ± 7.8% of the control at -30 mV and 38.5 ± 8.2% of the control at -100 mV (mean ± S.E.M., n = 4). Next we applied a ramp pulse to the cells to determine the reversal potential of the nicotine-activated current (Fig. 4). The nicotine-activated current under the control condition was reversed around 0 mV, which might reflect a poor ion selectivity of nicotinic receptor channels (9). The reversal potential was not affected by hirsutine (1 μM). These results suggest that hirsutine does not affect the nicotine-activated current in a voltage-dependent manner.

Effects of hirsutine on other channels

We tested the selectivity of the effects of hirsutine among various types of ion channels. First, we examined its effect on ATP-activated channels which we previously found and characterized in PC12 cells (16–18). Figure 5 shows the inward current activated by 100 μM ATP. The ATP-activated current in the presence of 10 μM hirsutine was 103.8 ± 5.0% of the control (mean ± S.E.M., n = 7) and that in the presence of 100 μM hirsutine was 84.3 ± 7.1% of the control (n = 3). This result suggests that hirsutine can discriminate between these ligand-gated channels and nicotinic receptor-channels.

We then examined the effects of hirsutine on voltage-gated channels. Figure 6 shows the effects of hirsutine on Ca channels. Ba (10.8 mM) was used as a charge-carrier to obtain a large inward current (13). Hirsutine (1 to 100 μM) inhibited the Ba current. The inhibition was rapid in onset (Fig. 6B). The concentration-dependency of the inhibition of the Ba current (Fig. 6C) was not much different from that of the inhibition of the nicotinic receptor-
Hirsutine, for example, reduced the Ba current to 68.3% of the control (Fig. 6C), whereas the drug reduced the nicotine-activated current to 40.2% of the control (Fig. 3C). Hirsutine did not significantly affect the voltage-dependency of the Ba current (Fig. 6D).

Figure 7A shows an outward current activated by a depolarizing pulse to +10 mV obtained from a K-loaded cell. The outward current is mediated through K channels (14, 19). Ca channels were not blocked because the amplitude of the Ca current with 1.8 mM Ca in these cells (ca. 20 pA, ref. 14) was negligible as compared with the apparent outward current (more than 500 pA, Fig. 7). Hirsutine also inhibited the K current (Fig. 7A). Hirsutine (10 μM) inhibited the K current to the same extent as the nicotine activated current (Fig. 3C) or the Ba current (Fig. 6C) (52.1 ± 2.9% of the control, mean ± S.E.M., n = 6). The inhibition of the K current was rapid in onset (Fig. 7B) as was the case for that of the Ba current (Fig. 6).

Fig. 4. Voltage-dependency of the nicotine (100 μM)-activated current in the absence (A) or the presence (B) of hirsutine (1 μM) obtained from a PC12 cell. The cell was held at -70 mV, and a ramp pulse to +30 mV (+100 mV/sec) was applied every 2 sec. The current trace during the ramp pulse near the peak nicotine-activated current was superimposed on the trace just before the nicotine-application in each panel. Each application of nicotine was separated by 1 min. Hirsutine was present 10 sec before and throughout the nicotine-application (B).

Fig. 5. Lack of effects of hirsutine on ATP-activated current. ATP (100 μM) was applied every 30 sec. Each panel shows successive recordings of current responses to the ATP-application obtained from a cell. The holding potential was -60 mV. Hirsutine (100 μM) was present 10 sec before and throughout the ATP-application (middle panel).
DISCUSSION

We have characterized the effects of hirsutine, an alkaloid which exerts ganglion blocking-like action (2, 3, 20), on ion channels using pheochromocytoma PC12 cells. Hirsutine inhibited nicotinic receptor channels at concentrations of μM order, which is comparable to the inhibition induced by C₆. The results suggest that components of herbal drugs like hirsutine can also block neuronal nicotinic receptor-channels as well as synthetic positively charged molecules such as C₆ or toxins such as d-tubocurarine (21). Hirsutine also blocked voltage-gated Ca and K channels, suggesting that hirsutine inhibits non-selectively a variety of ion channels.

Hirsutine inhibited nicotine-evoked dopamine-release from PC12 cells more potently than C₆ (Fig. 2) in spite of the fact that these antagonists were equipotent in blocking the nicotine-activated current (Fig. 3). A consider-
able part of nicotine-evoked catecholamine-release depends on Ca-influx mediated through Ca channels secondly activated by depolarization in PC12 cells (22, 23). Thus, the potent inhibition by hirsutine of the dopamine-release may be due to blockade by hirsutine of Ca channels (Fig. 6) in addition to nicotinic receptor-channels (Fig. 3). In smooth muscle tissues, nerve-mediated contraction of guinea pig (2) and dog urinary bladder (3) was inhibited equally by hirsutine and C₆. The blockade of Ca channels by hirsutine may not play a role in the ganglionic blocking actions in these smooth muscle tissues.

Ascher et al. (10) compared blocking properties of various nicotinic antagonists in rat parasympathetic ganglion cells. They showed that some nicotinic antagonists including C₆ preferentially inhibit the inward current mediated through nicotinic receptor-channels at negative potentials, and they concluded that this phenomenon is due to blockade of ion permeation through the open-state of the channels. In the present study, we did not find such voltage-dependent inhibition by hirsutine of the nicotine-activated current (Fig. 4). Our results may indicate that hirsutine is not such an “open-channel blocker”. The attenuation of hirsutine-induced inhibition by an increment of nicotine concentration suggests that hirsutine affects agonist-binding to nicotinic receptors.

We have shown that hirsutine, an alkaloid isolated from Uncaria genus, affects nicotinic acetylcholine channels as well as voltage-gated Ca and K channels. Hirsutine may not be usable as a pharmacological tool because of its non-selective nature of ion channel inhibition. However, combined inhibition of nicotinic receptor-channels and Ca channels may work cooperatively in some cases, which may be relevant to the pharmacological effects of hirsutine. For example, hirsutine exerts antihypertensive effects, which is one of the major purposes of clinical application of Uncaria genus (24). These antihypertensive effects may be due to such a cooperative inhibition by hirsutine of nicotinic receptor-channels and Ca channels: the nicotinic receptor-channel inhibition may result in a decrease in catecholamine-release from the adrenal medulla, and the Ca channel inhibition may result in attenuation of vascular smooth muscle tone. Contribution of Ca channel inhibition by other indole alkaloids extracted from Uncaria genus, which have similar structures to hirsutine, to the antihypertensive effects was also suggested from measurements of mechanical responses of isolated blood vessels (25). Channel inhibition may also be related to other pharmacological actions of hirsutine or Uncaria genus including those on central nervous systems (26).

Fig. 7. Effects of hirsutine on K current. A. K current recorded from a PC12 cell. The K current was activated by a test pulse from −60 mV to +10 mV every 5 sec. The K currents just before (control) and 1 min after application of 10 μM hirsutine (+ hirsutine) were superimposed. B. Time course of inhibition by hirsutine of the K current obtained from another cell. Hirsutine (10 μM) was applied at time indicated with an arrow and was continuously present afterwards.
Acknowledgments

We are grateful to Prof. S. Sakai of Chiba University for supplying hirsutine. We also thank Mrs. T. Obama for cell culture with her skilled techniques. This work was partly supported by the Japan Health Sciences Foundation.

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