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Blocking effect of tricyclopinate on nicotinic receptors in cultured sympathetic neurons[1]

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KEY WORDS ganglia; patch-clamp techniques; nicotinic receptors; nicotinic antagonists

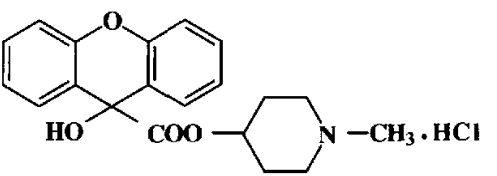
ABSTRACT

AIM: To investigate the mechanism of tricyclopinate, an antagonist of nicotinic receptor, on neuronal nicotinic acetylcholine receptors (nAChR). **METHODS**: A tight seal whole-cell recording patch-clamp technique was performed to record nicotine-evoked currents in the cultured sympathetic neurons from neonatal rat superior cervical ganglia (SCG). **RESULTS**: Tricyclopinate inhibited the nicotine-induced currents competitively and the inhibition was voltage-independent. The decay of the nicotine-induced current was accelerated significantly in the presence of tricyclopinate. **CONCLUSION**: Tricyclopinate inhibits neuronal nAChR by interacting with the allosteric sites rather than the open ionic channels or acetylcholine recognition site of the receptor.

INTRODUCTION

Tricyclopinate HCl (Fig 1), synthesized in our institute, is a xanthene-derived central anticholinergic agent. Previous work in our laboratory has shown that tricyclopinate could bind to muscarinic receptors from rat cerebral cortex with very high affinity[1]. Tricyclopinate could also displace the specific binding of [^{3}H]nicotine to nAChR in rat brain and antagonize the nicotine-induced convulsions in mice competitively[2]. The antimuscarinic effect of anticholinergic drugs is considered as crucial in the treatment of Parkinsonism, organophosphorus poisoning, and motion sickness. However, more and more evidences have confirmed that the antinicotinic activities of this kind of drugs also play an important role. For example, anticholinergic drugs could antagonize nicotine-induced convulsions in mice[3], and prevent soman-induced electro-encephalographic seizures in rats by blocking both the central muscarinic and nicotinic acetylcholine receptors[2,4]. However, the mechanism of anticholinergic drugs in blocking neuronal acetylcholine receptors (nAChR) is still not clear.

In the present experiment, the whole-cell patch-clamp technique was used to record the nicotine-induced currents in cultured neurons from rat superior cervical ganglia (SCG). The blocking mechanism of tricyclopinate on neuronal nAChR was investigated by observing its effects on the nicotine-induced currents. The action site of tricyclopinate on neuronal nAChR was analyzed.



Tricyclopinate HCl

MATERIALS AND METHODS

Cell culture Sympathetic neurons were isolated from SCG of neonatal (1 d) Wistar rat of either sex provided by medical experiment animal center of our institute. The dissociating and culture method was followed as described in our previous work[5]. Briefly, the SCG were cut into small pieces and digested with 0.25 % trypsin. The suspension was spun at 500 × *g* for 2 min. The pellet was resuspended in DMEM containing 10 % horse serum. The dissociated neurons were transferred to 35-mm tissue culture dishes and were cultured at 37 ℃ in 95 % O_2 + 5 % CO_2 environment. The experiments were done after the neurons were cultured for 7 to 10 d.

Current recording In the beginning of the ex-

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periment, the culture medium was replaced with the extracellular solution, which contained (mmol/L): NaCl 140, KCl 5, $CaCl_2$ 3, $MgCl_2$ 1, HEPES 10, glucose 10, and tetrodotoxin 0.001. The cultured SCG neurons were voltage-clamped with whole-cell patch-clamp technique[6]. The currents induced by nicotine were recorded with an amplifier (Axonpatch-1D, Axon instruments, USA). The patch-pipettes had a resistance of 1 – 5 MΩ and were filled with the solution containing (mmol/L): CsCl 140, HEPES 10, egtazic acid 10, and ATP 2. The junction potentials were cancelled using the Junction Null Control of the amplifier. Current signals were usually filtered at 2 kHz (– 3 dB) and sampled at a frequency of 2 kHz with a Labmaster TL-1 DMA interface (Axon instrument, USA). All experiments were carried out at room temperature (20 – 25 ℃).

Application of drugs Nicotine was purchased from Sigma Chemical Co. Tricyclopinate was synthesized in our institute.

Nicotine and the mixture of nicotine with tricyclopinate were dissolved in the extracellular solution and filled in a micro-manifold consisting of 3 microtubes, each of them had a diameter of 5 – 10 μm. The drugs were applied directly to the single neuron using a pressure injector (BH-2, Medical Systems Corp.). The microtube was placed approximately 20 – 30 μm from the cell and the puff pressure of N_2 (30 – 50 kPa) was adjusted to achieve rapid drug application while avoiding any mechanical disturbance in the recording of the electronic signal. One of the microtubes was filled with nicotine as a control and others with the mixture of nicotine and different concentrations of tricyclopinate as test groups. At the desensitization of neuronal nAChR, the amplitudes of nicotine-induced currents would decrease progressively when nicotine was applied repetitively at short intervals. It was found that over a 2.5-min interval was needed for the recovery of desensitized nAChR for a 1-s administration of nicotine, and 7 min for a 15-s administration. To ensure complete recovery of neuronal nAChR from desensitization, the interval was prolonged to 3 min for the 1-s administration of nicotine. When the desensitization of nAChR was observed, the time of administration would be prolonged to 15 s for adequate equilibrium with an interval of 8 min for full recovery of neuronal nAChR.

Calculation Data acquisition and analysis were controlled by pCLAMP 5.5.1 software (Axon instruments). All the data were expressed as $\bar{x} \pm s$. Our previous work demonstrated that the interaction of nAChR in rat SCG with its agonist fitted a single binding site model[5]. Therefore, the maximum effect (I_{max}) of nicotine on nAChR was determined by fitting the dose-response curve of nicotine with Clark's equation: $I = I_{max} \times D/(D + K)$. In the equation, I displayed the amplitude of currents induced by different concentrations of nicotine (D), and K the equilibrium dissociated constant. The decay rates of nicotine-induced currents were given by fitting the current signals with a double-exponential equation. The software of Origin (MicroCal Software) was used for curve fitting and graphic display. Prism2 (GraphPad software, Inc) was used to conduct two-way analysis of variance (ANOVA). $P < 0.05$ was considered as statistically significant.

RESULTS

Tricyclopinate depressed nicotine-induced currents When the gigaseal was formed between the tip of the microelectrode and the cell membrane, a swift pulse of suction and electronic zap were applied to rupture the path and establish a whole-cell recording configusation. The membrane potential was held at – 70 mV and the drugs were applied for 1-s. It was found that an inward current was evoked immediately while nicotine was ejected out into the cell. However, the current was depressed remarkably when the mixture of nicotine and tricyclopinate was ejected out to the same cell (Tab 1). The amplitudes of the currents increased from (0.93 ± 0.05) to (3.0 ± 0.8) nA at the concentration range of nicotine from 10 to 160 μmol/L. The inhibitory rates of tricyclopinate 10 μmol/L on nicotine-induced currents decreased from (70 ± 3) % to (34 ± 15) % with the increase of nicotinic concentrations.

Tab 1. Inhibition by tricyclopinate on nicotine-induced currents. $n = 5$ cells. $^bP < 0.01$ *vs* control group.

Nicotine /μmol·L^{-1}	Nicotine-induced currents/nA		Inhibition /%
	Control	Plus tricyclopinate /10 μmol·L^{-1}	
10	0.93 ± 0.05	0.28 ± 0.03^b	70 ± 3
20	1.4 ± 0.3	0.52 ± 0.25^b	64 ± 10
40	1.9 ± 0.8	0.89 ± 0.25^b	49 ± 13
80	2.4 ± 0.4	1.3 ± 0.3^b	46 ± 6
160	3.0 ± 0.8	2.0 ± 1.0^b	34 ± 15

The maximum amplitude (I_{max}) of nicotine-induced currents was determined by fitting the dose-response curve

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of nicotine with Clark's equation: $I = I_{max} \times D/(D + K)$. The obtained I_{max} was 3.359 nA and the equilibrium dissociated constant (K) was 28.52 μmol/L (Fig 1A). The kinetic property of tricyclopinate was analyzed by using the Lineweaver-Burk's double-inverse plot. According to the method, the two linear regression equations were obtained, which were $\hat{Y}_c = 1.07 + 25.88X$(control) and $\hat{Y}_t = 1.03 + 110.85X$ (plus tricyclopinate), respectively (Fig 2B). Both intercepts of the regressive lines on the ordinate were near 1, which suggested a competitive kinetic property illuminating the blocking effect of tricyclopinate on neuronal nAChR.

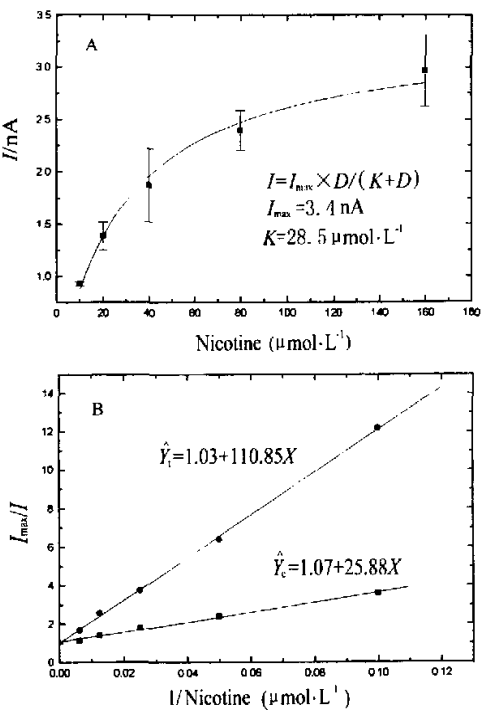

Fig 1. Kinetic property of tricyclopinate on neuronal nAChR. A: Fitting of the dose-response relationship of nicotine-induced currents with Clark's equation (n = 5 cells). B: Lineweaver-Burk's double-inverse plot. I_{max} was the largest value of nicotine-induced currents and I was the current induced by different concentrations of nicotine.

Voltage-independent blockage by tricyclopinate When the membrane potentials were held at −50, −70, and −90 mV, respectively, the currents evoked by 10 μmol/L nicotine were increased with hyperpolarization (Tab 2). When the mixture of 10 μmol/L nicotine and 10 μmol/L tricyclopinate was applied, the amplitudes of nicotine-induced currents were depressed at different membrane potentials. However, the inhibitory rates of tricyclopinate were not changed remarkably and they were (44 ± 3) %, (44 ± 5) %, and (46 ± 5) %, respectively. Two-way ANOVA revealed that the hyperpolarization of membrane potential increased the amplitudes of nicotine-induced currents (F = 15.64, P < 0.01), and tricyclopinate depressed the nicotine-induced current significantly (F = 49.63, P < 0.01). However, the change in holding membrane potentials had no significant interaction on the inhibition of tricyclopinate (F = 1.925, P = 0.1605).

Tab 2. Voltage-independent inhibition by tricyclopinate. n = 7 cells. ^bP < 0.01 *vs* control.

Holding Potential/mV	10 μmol/L Nicotine-induced currents/nA Control	10 μmol/L Nicotine-induced currents/nA plus tricyclopinate 10 μmol/L	Inhibition/%
−50	1.8 ± 0.4	0.98 ± 0.22^b	44 ± 3
−70	2.7 ± 0.6	1.5 ± 0.4^b	44 ± 5
−90	3.2 ± 0.6	1.8 ± 0.7^b	46 ± 5

Acceleration of the decay of nicotine-induced currents by tricyclopinate Due to the desensitization, nAChR lose sensitivity to its agonists when nAChR are exposed to agonists for a relatively longer time. In the present experiment, nicotine was applied for 15 s continuously for an adequate equilibrium. it was found that nicotine-induced currents rapidly reached the top and then decayed progressively (Fig 3). In the presence of 10 μmol/L tricyclopinate, the amplitudes of 100 μmol/L nicotine-induced currents decreased from (2.35 ± 0.3) nA to (1.5 ± 0.3) nA (n = 6 cells, P < 0.01) with an inhibition of (38.7 ± 0.8) %. The decay in nicotine-induced currents was fitted with double-exponential equation. The fast or short decay time constants of nicotine-induced currents did not show any significant deference between the control and test group (P > 0.05). However, the slow or long decay time constants were decreased remarkably from (10.6 ± 1.4) s to (3.68 ± 0.24) s (P < 0.01).

DISCUSSION

In the present experiment, it was found that an inward current was evoked at once in the cultured SCG neurons when nicotine was applied. It is suggested that the

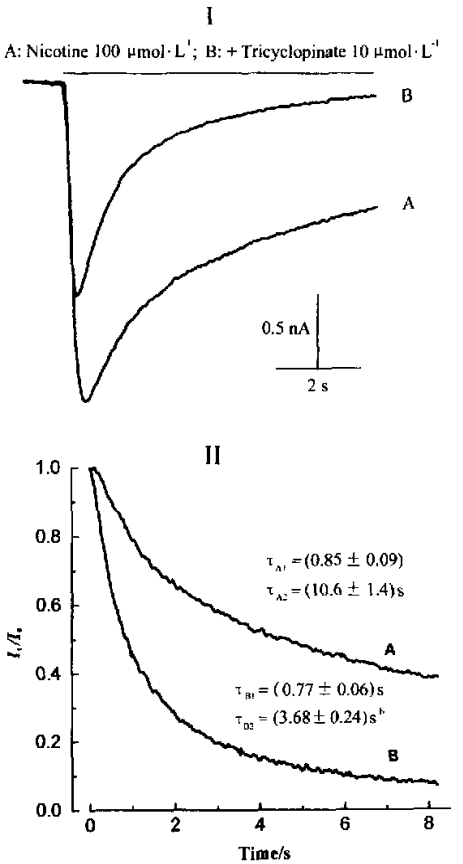

Fig 3. Effect of tricyclopinate (TCPN) on decay of nicotine-induced currents. The membrane potential was held at − 70 mV. I: both nicotine-induced currents were recorded from the same neuron in the presence (B) and absence of tricyclopinate (A). The horizontal bar displays the time of drug application. II: I_0 is the highest amplitude of nicotine-induced current; It is the currents recorded at different time. τ_1 and τ_2: the decay time constants of nicotine-induced current obtained by fitting the double-exponential curve. $^bP < 0.01$ *vs* τ_{A2}.

cultured sympathetic neurons express plenty of nAChR. Tricyclopinate could depress the nicotine-induced current and the inhibition decreased with increased concentration of nicotine. The Lineweaver-Burk's double-inverse plot showed that the kinetic property of tricyclopinate was competitive. In the presence of 10 μmol/L tricyclopinate, the slow decay time constants of nicotine-induced currents decreased remarkably ($P < 0.01$). It meant that tricyclopinate could speed up the desensitization of neuronal nAChR. The two-way ANOVA revealed that the amplitude of current induced by 10 μmol/L nicotine was significantly increased when the membrane potential was hyperpolarized from − 50 to − 90 mV, and the currents were inhibited remarkably by 10 μmol/L tricyclopinate at different potential levels ($P < 0.01$). However, the change of membrane potential did not play any significant interaction with the inhibitory effect of tricyclopinate ($P > 0.05$).

For neuronal nAChR, antagonists can block it by occupying the acetylcholine recognition sites[7] or interacting with the allosteric sites including the ionic channels[8,9]. Various pharmacological properties are displayed while antagonists interact with the different action sites on a receptor. In the past, the identification of different action sites of antagonists on receptors was primarily dependent on the drug kinetic properties. For example, an agonist recognition site would be considered if a receptor was inhibited in a competitive manner. However, evidences have showed that an antagonist could also block neuronal nAChR competitively by interacting with an allosteric[10]. Therefore, more data are needed to determine the action site of an antagonist besides its competitive kinetic property.

To identify the action site of tricyclopinate, we observed its effect on the receptor desensitization. The phenomenon of desensitization meant a reversible decline in the conductance response to agonists when the receptor exposed to its agonist for a longer time (Fig 3). In the presence of an antagonist interacting with the agonist recognition site, some receptors will be inhibited and stay in a refractory condition. Some others can still be competitively excited by agonists. For these activated receptors, their activities, such as the channel opening, closing, and desensitization will not be influenced by the antagonist any more. However, allosteric antagonists can influence the receptor desensitization as their binding sites on neuronal nAChR are different from agonist recognition sites[7,9,11]. As tricyclopinate could speed up the decay of nicotine-induced current, ie, accelerate the desensitization of neuronal nAChR, we deduced that tricyclopinate might depress nAChR by interacting with allosteric sites rather than acetylcholine recognition sites.

It is well known that the open-channel blocking mechanism underlies the voltage-dependent[9,11] and noncompetitive kinetic properties[10]. As the blockage by tricyclopinate is voltage-independent and competitive, it is reasonable to consider that tricyclopinate is not able to plug the open channels of nAChR in SCG neurons at the

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given range of the holding membrane potential.

According to the results and analysis above, we could speculate that tricyclopinate inhibits neuronal nAChR by interacting with the allosteric sites rather than the open ionic channels or acetylcholine recognition sites. Here, we provided a model to identify the different action sites for an antagonist of nAChR. Of course, the determination of action site of tricyclopinate was only based on the functional analysis, and final confirmation of the action site may need some other evidences.

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三环哌酯对培养交感神经元烟碱受体的阻断作用[1]

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关键词 神经节；膜片箝技术；烟碱受体；烟碱拮抗剂

目的：观察烟碱受体拮抗剂三环哌酯对烟碱诱发电流的作用. **方法**：用高阻抗封接膜片箝全细胞记录技术在培养的新生大鼠颈上神经节观察三环哌酯对烟碱诱发电流的作用. **结果**：三环哌酯竞争性地抑制烟碱诱发电流，其抑制作用没有电压依赖性，但能加速烟碱受体失敏. **结论**：三环哌酯抑制神经元烟碱受体的作用部位在变构位点而不是在离子通道或乙酰胆碱识别位点.

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