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Inhibition of 5-HT$_3$ Receptors-activated Currents by Cannabinoids in Rat Trigeminal Ganglion Neurons*

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Summary: This study investigated the modulatory effect of synthetic cannabinoids WIN55,212-2 on 5-HT$_3$ receptor-activated currents ($I_{5\text{-}HT3}$) in cultured rat trigeminal ganglion (TG) neurons using whole-cell patch clamp technique. The results showed that: (1) The majority of examined neurons (78.70%) were sensitive to 5-HT (3–300 μmol/L). 5-HT induced inward currents in a concentration-dependent manner and the currents were blocked by ICS 205-930 (1 μmol/L), a selective antagonist of the 5-HT$_3$ receptor; (2) Pre-application of WIN55,212-2 (0.01–1 μmol/L) significantly inhibited $I_{5\text{-}HT3}$ reversibly in concentration-dependent and voltage-independent manners. The concentration-response curve of 5-HT$_3$ receptor was shifted downward by WIN55,212-2 without any change of the threshold value. The EC_{50} values of two curves were very close (17.5±4.5) μmol/L *vs.* (15.2±4.5) μmol/L and WIN55,212-2 decreased the maximal amplitude of $I_{5\text{-}HT3}$ by (48.65±4.15)%; (3) Neither AM281, a selective CB1 receptor antagonist, nor AM630, a selective CB2 receptor antagonist reversed the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2; (4) When WIN55,212-2 was given from 15 to 120 s before 5-HT application, inhibitory effect was gradually increased and the maximal inhibition took place at 90 s, and the inhibition remained at the same level after 90 s. We are led to concluded that-WIN55,212-2 inhibited $I_{5\text{-}HT3}$ significantly and neither CB1 receptor antagonist nor CB2 receptor antagonist could reverse the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2. Moreover, WIN55,212-2 is not an open channel blocker (OCB) of 5-HT$_3$ receptor. WIN55,212-2 significantly inhibited 5-HT-activated currents in a non-competitive manner. The inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 is probably new one of peripheral analgesic mechanisms of WIN55,212-2, but the mechanism by which WIN55,212-2 inhibits $I_{5\text{-}HT3}$ warrants further investigation.
Key words: WIN55,212-2; 5-HT3 receptor; CB1 receptor; CB2 receptor; trigeminal ganglion neuron; whole-cell patch clamp

Cannabis has been used in medical practice for thousands of years[1]. In the past 20 years, endocannabinoid neurotranmitter system has been identified with the discovery of endogenous cannabinoid anandamide (AEA), aminoguanidine (2-AG) and successful cloning of two types of endocannabinoids receptors (CB1 and CB2)[2, 3], both coupled to G-proteins[4, 5]. In peripheral system, CB1 receptors are located in primary sensory afferent neurons[6], whereas CB2 receptors are found in immune cells to reduce the inflammation of injured tissues[7]. Analgesic effect of systemic administration may be related to the corresponding cannabinoid receptor in central nervous system[8, 9]. However, it has also been observed that cannabinoid receptor agonist could produce a significant analgesic effect in the incomplete nerve ligation model[10], indicating that cannabinoid also, peripherally, plays a role in the modulation of pain. However, the peripheral analgesic effect by cannabinoids is yet to be elucidated. Understanding of its mechanism may help to develop cannabinoid analgesic agents and promote their clinical applications.

5-HT is one of the most important neurotransmitters and acts on different sub-types of 5-HT receptors. All sub-types of 5-HT receptors are metabotropic receptors with an exception that 5-HT$_3$ receptor is a Na^+/K^+ ligand-gated ion channel (LGIC)[11]. 5-HT is also a very important peripheral pain substance[12]. It works on 5-HT$_3$ receptors located in the primary sensory neurons (such as trigeminal ganglion), and activation of 5-HT$_3$ receptors then induces nociceptive signals that are transmitted from the periphery to the central nervous system and thus causes pain sensation.

Trigeminal ganglion is the location of primary sensory neurons, where 5-HT$_3$ receptors and cannabinoid CB1, CB2 receptors co-exist[13]. Reports demonstrated that CB1 receptor synthetic agonist, WIN55,212-2 blocked ATP-activated currents in trigeminal ganglion neurons[14] or inhibited nicotine-activated currents via the nicotinic acetylcholine receptor (nAChR) directly[15, 16]. However, little is known about whether cannabinoids

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modulate 5-HT_3 receptor-mediated nociceptive signals and thus modulate pain sensation in the trigeminal ganglia. This study explored whether cannabinoids influence 5-HT currents in TG neurons and the possible mechanisms by which cannbinoids modulate 5-HT-activated currents.

1 MATERIALS AND METHODS

1.1 Cell Culture

Adult Sprague-Dawley rats of both sexes, weighing 150–250 g were used in this study. The rats were anesthetized with 20% urethan (1.2 g/kg, i.p.) and then decapitated. Their trigeminal ganglia (TG) were dissected aseptically and washed twice in cold (4°C) Ca^{2+}-free Hank's balanced salt solution (HBSS). After TG were diced into small pieces, they were placed into a flask containing 3 mL HBSS together with papain 100 μL (Roche, 1090023, Swiss) and 5 mg L-cysteine and then incubated for 20–25 min at 37°C with 5% CO_2. Individual cells were dissociated by triturating the tissue through a fire-polished Pasteur pipette, followed by placing in a centrifuge tube containing 5 mL DMEM/F-12 solution supplemented with 10% fetal bovine serum. The cells were centrifuged 2 times for 5min at 1000 r/min and then were plated on the Poly-L-lysine (Sigma, p-2267, USA) cover-slips. The cover-slips were put into culture dishes (diameter: 35 mm) filled with 2 mL DMEM/F-12 solution containing 10% fetal bovine serum. The cells were cultured for 2–3 h at 37°C in 5% CO_2.

The HBSS contained (in mmol/L) 65 NaCl, 20 KCl, 26 $NaHCO_3$, 2.5 NaH_2PO_4, 20 D-glucose and EGTA1, adjusted to pH 7.4 with NaOH. All the animals were purchased from the Center of Experimental Animals of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

1.2 Electrophysiological Recordings

Whole-cell currents were recorded using patch clamp technique (amplifier, PCⅡC) (Yibo Co., HUST, China). According to the requirements, a whole-cell configuration was established and membrane currents were recorded and analyzed. Glass electrode was pulled with a tip diameter of 1–2 μm, and the resistance of the pitettes was 2–5 MΩ when filled with internal solution containing (in mmol/L): 140 KCl, 2 $MgCl_2$, 10 HEPES, 11 EGTA and 5 ATP, adjusted to pH 7.2 with KOH. Cells were bathed in an external solution containing (mmol/L) 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 D-glucose, adjusted to pH 7.4 with NaOH.

1.3 Drug Application

L-Cysteine, 5-HT, WIN55,212-2, HEPES, EGTA and Bicuculline were purchased from Sigma, USA. Papain was bought from Roche, USA. AM281 and AM630 were obtained from Tocris, USA. PMA and BIM came from Calbiochem, USA. DMEM/F-12 was purchased from Gibco, USA. The other chemicals, if not specifed, were procured from Chinese companies.

5-HT and Bicuculline were dissolved fresh in the external solution just before use. Stock solutions of WIN55,212-2, AM 281 and AM630 were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/L and diluted into concentrations required with the external solution before use. The final concentrations of DMSO were no more than 0.1% in external solution.

The drugs were held in a liner array of fused silica tubes (ID/OD: 200/500 μm) connected to a series of independent reservoirs. The drugs were delivered by gravity and rapid solution exchanges were achieved by shifting the tubes horizontally with a micromanipulator. The distance from the tube mouth to the cell examined was roughly 100 μm. Cells were constantly bathed in normal extra-cellular solution flowing from one tube connected to a larger reservoir between drug application intervals. The typical drug application paradigm for current recordings consisted of applying 5-HT in brief pulse 2–3 s with a 4 min intervals between drug applications.

1.4 Statistical Analysis

Statistical analysis of data was carried out by using Sigmaplot 2001 (SPSS Inc, USA). Data were presented as $\bar{x}\pm s$. Student's paired and unpaired *t*-test were used for difference evaluation and a P less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 The 5-HT-activated Currents in Rat TG Neurons

The majority of the neurons (78.70%, 85/108) examined responded to 5-HT (3–300 μmol/L) applied extra-cellularly with inward currents in a concentration-dependent manner (fig. 1B). The currents were characterized by fast activation and slow desensitization. The 5-HT-activated currents ($I_{5\text{-}HT3}$) could be blocked by ICS 205-930 (1 μmol/L), a specific antagonist of 5-HT_3 receptor (n=5) (fig. 1A). Only a minority of the cells (21.29%, 23/108) examined were not sensitive to 5-HT. To get another full 5-HT-activated current response recovery in the same neuron, a 4-min interval was needed.

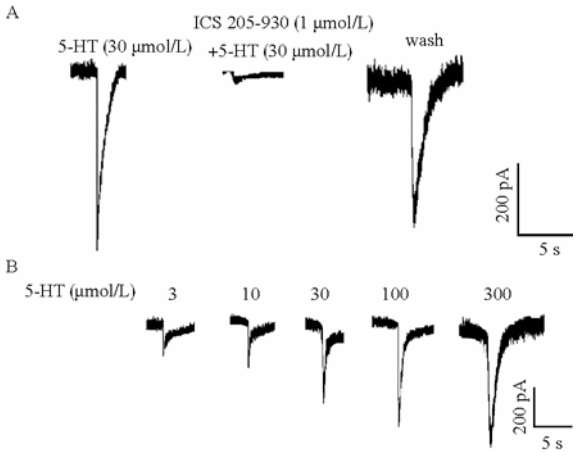

Fig. 1 $I_{5\text{-}HT3}$ recorded in rat trigeminal ganglion neurons
A: 5-HT-activated currents（30 μmol/L）were blocked by specific 5-HT_3 receptor antagonist ICS 205-930 (1 μmol/L); B: 5-HT at different concentrations (3–300 μmol/L) induced an inward current in a concentration-dependent manner. Membrane potential was held at –60 mV and the interval between drug application was 4 min. All recordings were from a single TG neuron.

2.2 Inhibition of 5-HT$_3$ Receptor-activated Currents by WIN55,212-2

No detectable membrane current was observed during application of WIN55,212-2 alone. Application of 5-HT (30 μmol/L) after pre-treatment of neurons with WIN55,212-2 for 90 s suppressed the peak value of $I_{5\text{-}HT3}$ significantly in most of the neurons examined (88.88%, 72/81). The inhibition was reversible after WIN55,212-2 washout. The inhibitory effect of WIN55,212-2 on $I_{5\text{-}HT3}$ was concentration-dependent. With the increment of WIN55,212-2 concentration from 0.01 μmol/L to 1 μ mol/L, the percentage inhibition on $I_{5\text{-}HT3}$ also increased and the maximum inhibition was (93.31±4.27)% (n=7) (fig. 2A and 2B). The IC_{50} value was 0.1 μmol/L (0.103 μmol/L).

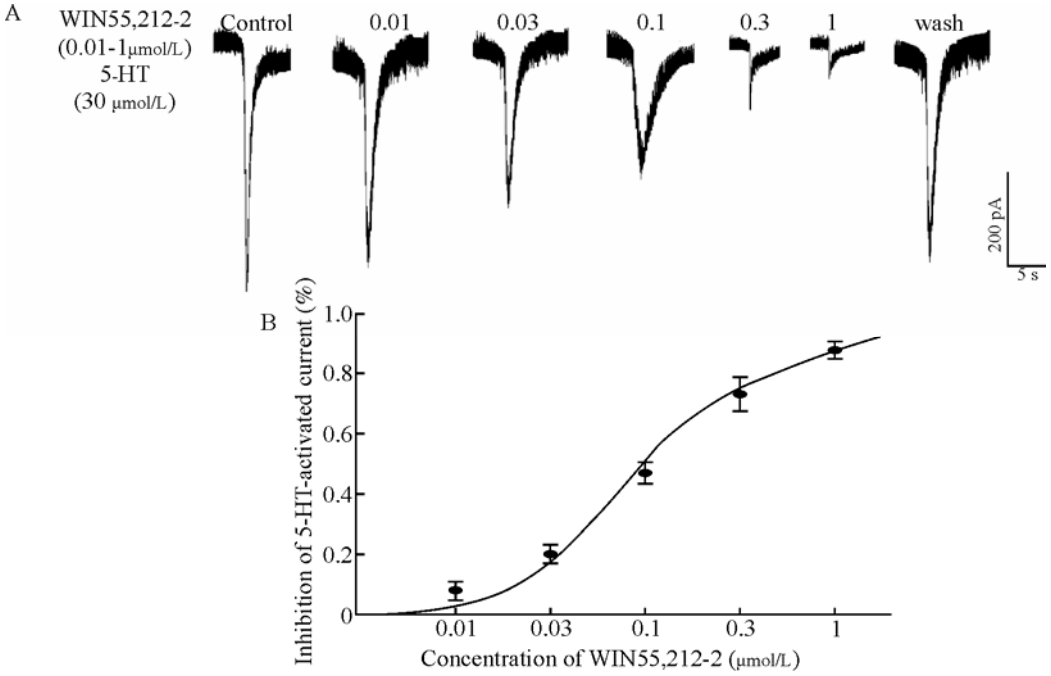

Fig. 2 Inhibition of 5-HT$_3$ receptor-activated currents by WIN55,212-2

A: The inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 at different concentrations (0.01–1 μmol/L); B: The inhibition percentage of 5-HT-activated currents by different concentrations of WIN55,212-2 (0.01–1 μmol/L) and the maximum inhibition rate was (93.31±4.27) % (n=7), IC50 was about 0.1 μmol/L (0.103 μmol/L)

2.3 The relationship between the Inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 and duration of WIN55,212-2 pre-application

To explore the relationship between the effect of WIN55,212-2 (0.1 μmol/L) on $I_{5\text{-}HT3}$ and the duration of WIN55,212-2 pre-application, different durations of WIN55,212-2 pre-application, ranging from 15 to 120 s were tested (fig. 3A and 3B). The result illustrated that the inhibition was increased gradually over the time of WIN55,212-2 pre-application and reached its maximum (75.76±5.27)% (n=6) at 90 s. When the pre-application time was longer than 90 s, the inhibition did not increase and stayed at a constant level.

2.4 Dose-response Relationship on the Inhibition of 5-HT$_3$ Receptor-activated Currents by WIN55,212-2

Fig. 4A shows the inhibitory effect of 0.1 μmol/L WIN55,212-2 on 5-HT-activated currents at different concentrations. Fig. 4B is the concentration-response curves of $I_{5\text{-}HT3}$ with and without pre-application of 0.1 μmol/L WIN55,212-2. Fig. 4B clearly indicates that WIN55,212-2 significantly shifted the concentration-response curve of 5-HT downwards without changing the threshold value and maximum response concentration. The EC_{50} value of two curves were very close [(17.5±4.5) μmol/L *vs.* (15.2±4.5) μmol/L] and WIN55,212-2 decreased the maximal amplitude of $I_{5\text{-}HT3}$ by (48.65±4.15)% (n=6).

2.5 Effect of Holding Potential on the Inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2

Fig. 5 shows the current-voltage (I-V) relationships for 5-HT (30 μmol/L)-activated currents with and without treatment with WIN55,212-2 (0.1 μmol/L). WIN55,212-2 suppressed $I_{5\text{-}HT3}$ at all holding potentials between –80 mV and +40 mV as shown by the I-V curve. The reversal potential of the currents activated by 5-HT alone was at –20 mV to 0 mV. After pre-treatment with WIN55,212-2 (0.1 μmol/L), the reversal potential of $I_{5\text{-}HT3}$ remained unchanged. The histogram in fig. 5 showed the percentage inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 at different voltages. There was no significant difference in the inhibition percentages among all holding potentials (P>0.05, n=5) (fig.5).

2.6 Effect of CB1 and CB2 Receptor Antagonists on the Inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2

To investigate whether cannabinoid receptors mediate the inhibitory effect of WIN55,212-2 on $I_{5\text{-}HT3}$, we tested the effects of AM281 (10 μmol/L), a selective antagonist of CB1 receptor and AM630, a selective antagonist of CB2 receptor on the maximal amplitudes of 5-HT (30 μmol/L)-activated currents. Application of 10 μmol/L AM281 alone had no significant effect on the amplitude of $I_{5\text{-}HT3}$ (P>0.05, n=4) (fig. 6A, 6C). When

AM281 was co-applied with WIN55,212-2 (0. 1 μmol/L), no significant change in percentage inhibition of WIN55,212-2 (P>0.05, n=4) (fig. 6B, 6C) was found. Application of AM630 (10 μmol/L) alone had no significant effect on the amplitudes of $I_{5\text{-}HT3}$ (P>0.05, n=4) (fig. 6A, 6C). When AM630 was co-applied with WIN55,212-2, no significant change in percentage inhibition of WIN55,212-2 (P>0.05, n=4) (fig. 6B, 6C) was noticed.

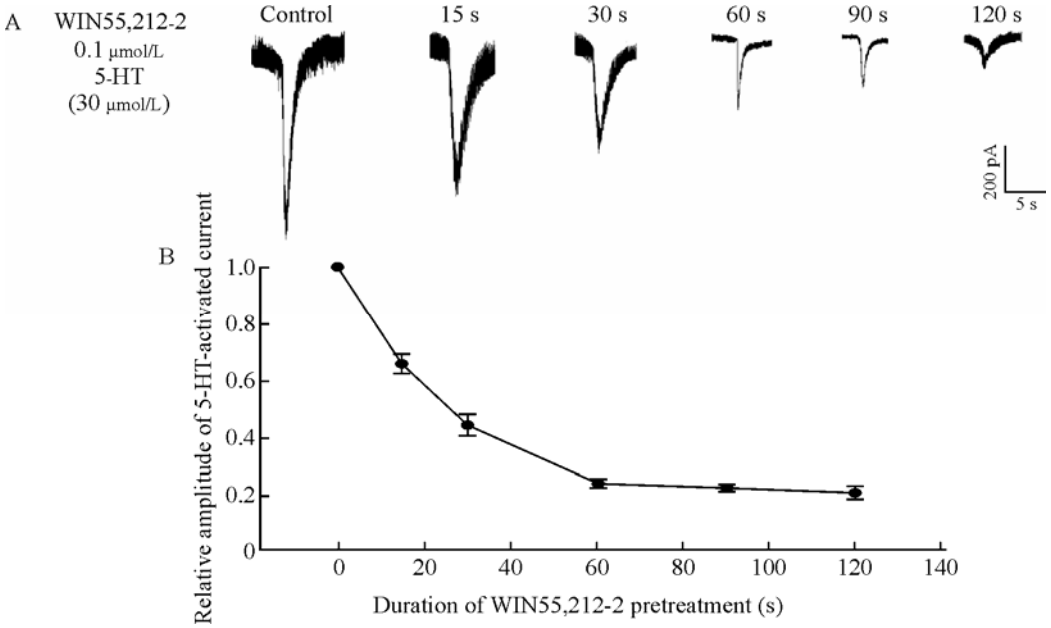

Fig. 3 The relationship between the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 and duration of WIN55,212-2 pre-application
A: Inhibition of 5-HT (30 μmol/L)-activated currents by WIN55,212-2 at different periods of pre-application time; B: The relative amplitude (n=6) of 5-HT (30 μmol/L)-activated currents at different time points (15 s to 120 s) of pre-application of WIN55,212-2 (0.1 μmol/L)

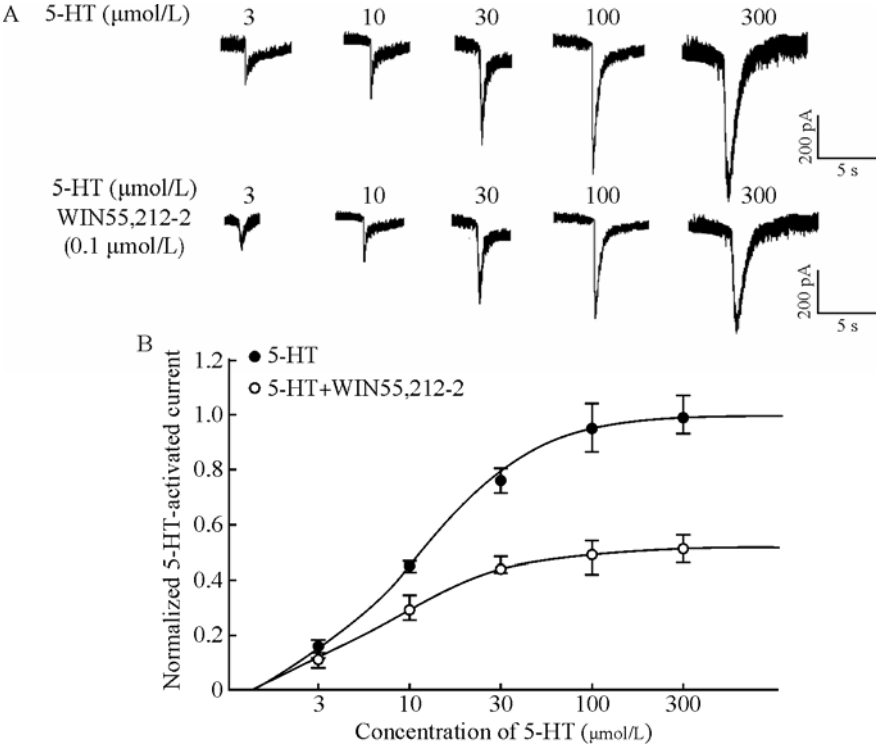

Fig. 4 Dose-response curve on the inhibition of 5-HT_3 receptor-activated currents with and without pre-application of WIN55,212-2
A: 5-HT-activated currents with and without pre-application of WIN55,212-2 (0.1 μmol/L); B: Pre-application of WIN55,212-2 (0.1 μmol/L) shifted dose-response curve of 5-HT-activated currents downward significantly without change in threshold and maximum response concentration, with EC_{50} being (15.2±4.5) μmol/L and (17.5±4.5) μmol/L respectively when WIN55,212-2 was pre-applied and 5-HT was given alone and maximum response being reduced to (48.65 ± 4.15)% (n=6).
In the graph, each point indicates the $\bar{x}\pm s$, curve meets Roger Frost equation $y=I_{max}/[I+(EC_{50}/C)n]$, where C represents the concentration of 5-HT, y is the amplitude of 5-HT-activated currents, EC_{50} is 5-HT concentration at which 5-HT-activated currents reached 50% of its maximum response, n is the Hill constant.

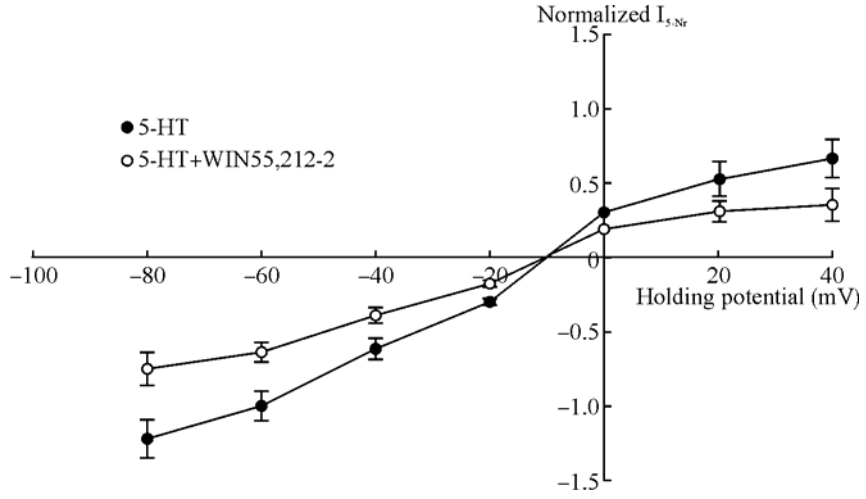

Fig. 5 Effect of holding potential on the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2

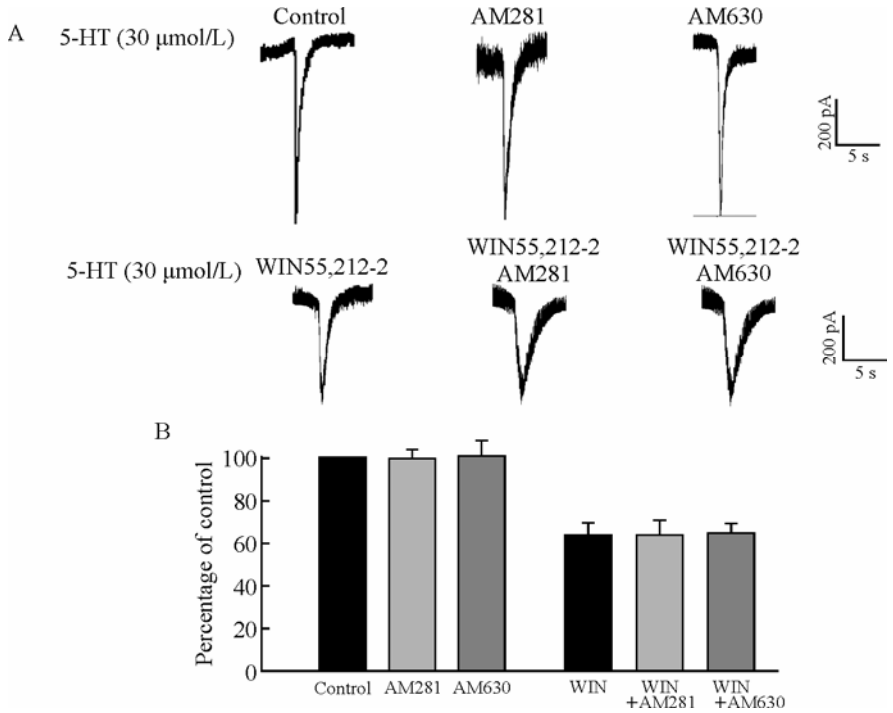

Fig. 6 Effect of CB1 and CB2 receptor antagonists on the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2

3 DISCUSSION

The recorded 5-HT-activated currents were mediated by 5-HT_3 receptors because the inward currents were blocked by 5-HT_3 receptor antagonist ICS 205-930 (1 μmol/L) in this experiment. The majority of examined TG neurons (78.70%, 85/108）were sensitive to 5-HT (30 μmol/L), which was well in accordance with result of Hu *et al*[11].

This experiment showed that synthetic cannabinoids WIN55,212-2 significantly inhibited 5-HT-activated currents in cultured TG neurons. With the increase of WIN55,212-2 concentration from 0.01 to 1 μmol/L, the inhibition of $I_{5\text{-}HT3}$ also increased, suggesting that the inhibitory effect of WIN55,212-2 was concentration-dependent. The concentration-response curve of 5-HT (30 μmol/L)-activated currents shifted downward significantly after pre-application of WIN55,212-2, without any change of the threshold value and the maximal response concentration. Moreover, EC_{50} of two curves was very close, and WIN55,212-2 significantly decreased current amplitude of the maximal response. The above results suggest that WIN55,212-2 inhibited 5-HT-activated currents in a non-competitive manner, that is, WIN55,212-2 and 5-HT did not act on the same site of 5-HT_3 receptor. The I-V curves for $I_{5\text{-}HT3}$ with and without pre-application of WIN55,212-2 indicates that the reversal potentials of two curves were virtually the same and changing the holding potential did not alter the inhibition by WIN55,212-2, suggesting that WIN55, 212-2 is not an open channel blocker (OCB) of 5-HT_3 receptor, that is, WIN55,212-2 does not work in the internal of the ion channel of 5-HT_3 receptors, because OCB usually enters the ion channel and blocks it when the channel opens. In another word, the inhibitory effect of OCB is volt-

age-dependent[17].

Peripheral CB1 receptors are mainly distributed in the peripheral nervous system, while CB2 receptors are found in immune cells, WIN55,212-2 is a CB1, CB2 receptor agonist[18]. To prove whether the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 was mediated via CB receptors, in this study, we used AM281, a selective CB1 receptor antagonist and AM630, a selective CB2 receptor antagonist. The results showed that neither AM281 nor AM630 reversed the inhibition of $I_{5\text{-}HT3}$ by WIN55212-2, suggesting that neither CB1 receptors nor CB2 receptors mediated the inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2 in the cultured TG neurons.

We then explored if WIN55,212-2 inhibited $I_{5\text{-}HT3}$ by directly acting on 5-HT_3 receptors. The present result demonstrated that the inhibition of $I_{5\text{-}HT3}$ by WIN55, 212-2 was non-competitive, ruling out the possibility that WIN55,212-2 binds competitively to the 5-HT binding sites of 5-HT_3 receptor. We could not, with present study, eliminate the possibility that WIN55,212-2 exerts its inhibitory effect by binding to special binding site on 5-HT_3 receptor rather than 5-HT binding site.

It is widely accepted that CB1 receptor modulates different chemically-gated ion channels and voltage-gated ion channels[19]. Therefore, there is possibility that CB receptor might participate in the inhibition of 5-HT_3 currents. Activation of CB1 receptor accounts for most of the endocanninoid actions in the brain. But here it is unlikely that WIN55,212-2 inhibited $I_{5\text{-}HT3}$ via CB1 receptors or via CB2 receptors although CB2 receptors are present in the peripheral nervous system. Our experimental results partially provided the answer. We demonstrated that the selective CB receptor antagonists did not reverse inhibition of $I_{5\text{-}HT3}$ by WIN55,212-2. Besides, the effect grade of the inhibition of 5-HT_3 receptors by cannabinoid and the effect grade of the inhibition of CB1 or CB2 are completely different, which negated impact of the interaction between the two types of receptors[19].

Several pathways have been proposed to underlie the endogenous AEA action on K^+ channels bypassing CB1 activation. Inside the cell, AEA can undergo metabolism via two possible pathways: hydrolysis and oxygenation. The products, arachidonic acid and ethanolamine or prostaglandins have been proved to mediate AEA inhibition on voltage-activated K^+ currents in dorsal root ganglion (DRG) neurons[20, 21]. Vignali *et al* suggested that lipid rafts and caveolae microdomains in endocannabinoid signaling in which AEA might interact with extracellular leaflet of the plasma membrane to exert its inhibitory action[22]. It was also reported that AEA might activate a non-CB1/CB2 cannabinoid receptor[23, 24]. In the present experiment, we do not know if synthetic WIN55,212-2 , like endogenous AEA can undergo these metabolic pathways, or interact with extra-cellular leaflet of the plasma membrane to exert its modulatory effect on $I_{5\text{-}HT3}$. We can not exclude the possibility that WIN55,212-2 acts through other receptors, such as orphan receptor GPR55 which can be activated by AEA, rather than CB1/CB2 receptor. Therefore, further investigation is warranted to explore the detailed mechanism by which synthetic WIN55,212-2 inhibits $I_{5\text{-}HT3}$.

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