

The inhibitory effect of α -methyl-5-HT on ATP-activated currents in rat dorsal root ganglion neurons

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Abstract

The purpose of this study was to explore the inhibitory effect of α -methyl-5-hydroxytryptamine (α -methyl-5-HT) on ATP-activated current (IATP) in rat dorsal root ganglion (DRG) neurons. Whole-cell patch clamp experiment was performed on cultured rat DRG neurons. One minute after treatment with α -methyl-5-HT, ATP (10^{-4} mol/L) activation current in rat DRG neurons was inhibited. However, this inhibitory effect was independent of the current caused by α -methyl-5-HT. The dose-response curve for IATP showed that α -methyl-5-HT significantly shifted it. The K_d values of ATP-activated currents before and after the pre-addition of α -methyl-5-HT were similar (4.23×10^{-5} mol/L vs. 6.81×10^{-5} mol/L). Furthermore, cyproheptadine (10^{-6} mol/L), an antagonist of 5-HT₂ receptor, can reverse the inhibition of α -methyl-5-HT. After intracellular dialysis of KN93 (CaMKII inhibitor) and H7 (PKC inhibitor), this inhibition was also completely eliminated. In conclusion, our results showed that α -methyl-5-HT inhibited ATP-activated current through activating the 5-HT₂ receptor and resulting in phosphorylation of the ATP receptor. It was caused by the activation of G protein coupled receptor and corresponding intracellular signaling transduction cascade.

Keywords: ATP-activated current; α -methyl-5-HT; DRG.

Practical Application: α -methyl-5-HT inhibited ATP-activated current through activating the 5-HT₂ receptor and resulting in phosphorylation of the ATP receptor. This study is of importance for understanding the interaction between ATP receptors and other receptors in DRG cells.

1 Introduction

The spinal dorsal horn is the primary center of sensory information integration. It is not only the primary gateway of peripheral nociceptive information transmission, but also the termination site of the descending system which originates from the brainstem and inhibits the noxious information transmission. It has a significant effect on transmission and regulation of nociceptive information at the spinal level. Spinal dorsal root ganglion (DRG) is the location of primary sensory neurons. It was reported that ATP transmitters released by DRG neurons and membrane ATP receptors mediated responses were related to the transmission and modulation of pain information (Hu et al., 2017; Lü et al., 2017).

ATP is widely present in and outside animal cells. In addition to its important function in cell metabolism, extracellular ATP and its metabolite adenosine can have a role in a series of biological processes, all of which are achieved through P1 and P2 receptors (Lü et al., 2017). ATP acts on primary sensory neurons as an excitatory neurotransmitter (Bele & Fabbretti, 2016). In 1983, Krishtal first confirmed the presence of ATP receptors in primary sensory neurons (Krishtal & Marchenko, 1986). Thereafter, the pharmacological properties of ATP receptors in DRG cell membrane (Bean, 1990), the kinetics of receptor activation (Krishtal et al., 1988), the ionic mechanism of ATP-activated current (Li et al., 1993) and its modulation (Hu & Li, 1996) were studied in depth. We have done a series of work on

the interaction between ATP receptors and other receptors in DRG cells (Wang et al., 2001; Skagerberg & Lindvall, 1985).

5-hydroxytryptamine (5-HT) and ATP play a role as neurotransmitters by directly activating cationic channels in the postsynaptic membrane, which are named 5-HT₃ and P2X receptors, respectively (Kamendi et al., 2008). Previous study suggested the inhibitory interactions between 5-HT₃ and P2X channels in submucosal neurons (Barajas-López et al., 2002). It also has been reported that 5-HT regulates the desensitization kinetics of P2X₁ responses by increasing their rate of recovery via the 5-HT_{2A} metabotropic receptor (Ase et al., 2005). Therefore, the purpose of this study was to explore the effects of α -methyl-5-HT on IATP in rat DRG neurons and its potential mechanism.

2 Materials and methods

2.1 Cell isolation and culture

All experimental procedures were carried out in accordance with Chinese legislation and National Institutes of Health (NIH) publications. The isolation method of rat DRG neuron specimens and whole-cell patch clamp experiment was performed as mentioned earlier with minor modifications (Wang et al., 2001). Sprague-Dawley (SD) rats, 4-5 week-old, were anesthetized with ether and put to death by dislocation. We dissected the thoracic

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and lumbar segments of vertebrate cylinder, and divided them into two parts longitudinally along the midline of dorsal and ventral sides. The DRG, dorsal root, ventral root and spinal nerve were removed from the inner side of each half of the dissected vertebrae and transferred to oxygen saturated Dulbecco's Modified Eagle's Medium (DMEM, Sigma) immediately under pH 7.4 and osmotic pressure 340 mOsm/kg. After removing the attached nerve and surrounding connective tissue, DRGs were cut up and put into the culture flask containing trypsin (type III, Sigma), collagenase (type I A, Sigma) and DNase (type III, Sigma). The culture flask was incubated in a constant temperature oscillating water bath (35 °C, 80 times/min) for 30-40 min. After adding soybean trypsin inhibitor (type II-s, Sigma) to stop trypsin digestion, the neurons were stored in a 35-mm culture dish for at least 30 min before the experiment. The neurons with diameter of 20-45 μM were selected in this study.

2.2 Whole-cell patch clamp

The experiment was performed at 22-25 °C. The patch/whole-cell clamp amplifier (CEZ-2400, Nihon Kohden) was used to perform whole-cell patch-clamp recording. The inner liquid component of the glass microelectrode included KCl 140, CaCl₂ 1, MgCl₂ 2, HEPES 10, EGTA 11 and ATP 4 (in mmol/L). The external solution contained NaCl 150, KCl 5, CaCl₂ 2.5, MgCl₂ 1, HEPES 10 and D-glucose 10 (in mmol/L). Sucrose was used to adjust osmotic osmolarity to 340 mosM/kg and KOH or NaOH was used to adjust pH to 7.4 in inner solution and external solution. The resistance of recording electrodes was between 2 and 4 MΩ. A small patch of membrane underneath the tip of the pipette was aspirated to form a gigaseal and a larger negative pressure was applied to rupture it to establish a whole-cell mode. Before recording the membrane current, the capacitance compensation and series resistance compensation were adjusted. The holding potential was set to -60 mV unless otherwise indicated. Membrane currents were filtered at 10 Hz (3dB). The pen recorder (Nihon Kohden) was used to record.

2.3 Reagent preparation

ATP (Sigma) and α-methyl-5-HT (an agonist of 5-HT2 receptor, RBI) were formulated with an external solution in which the pH was adjusted to 7.4 with 1 M NaOH. Cyproheptadine (an antagonist of 5-HT2 receptor, Sigma), KN93 (RBI) and H7 (RBI) were formulated with an internal solution in which the pH was adjusted to 7.2 with 1 M KOH.

2.4 Statistical analysis

Statistical analysis was performed by using SPSS 20.0 (SPSS Inc., USA). All data were expressed as means ± SEM. The differences between groups were analyzed by t test. P < 0.05 was considered to be statistically significant. The graph was drawn by using SigmaPlot software.

3 Results

3.1 ATP activated currents in DRG neurons

It is well known that the response of DRG neurons to externally applied ATP with inward currents is in a concentration dependent manner. In order to facilitate patch clamp recording, the experiment was carried out on cells with the diameter of 25-45 μm in this study. A total of 141 cells were detected in this study. 88.65% of cells (125/141) were sensitive to externally applied ATP (10^{-5} - 3×10^{-3} mol/L) with a response of inward current. In addition, 10.64% cells (15/141) were unresponsive, of which only one was an outward current (1/141, 0.71%).

The dual stimulation method was applied to detect the recovery time of ATP-activated current (IATP) desensitization, that was, the interval between the first and second ATP additions was 2 min, 3 min, 4 min, and 5 min, respectively. The results showed that the current caused by the application of ATP at 5 minutes was the same as that of the first time, indicating that the desensitization recovery time of ATP receptor was 5 minutes (Figure 1).

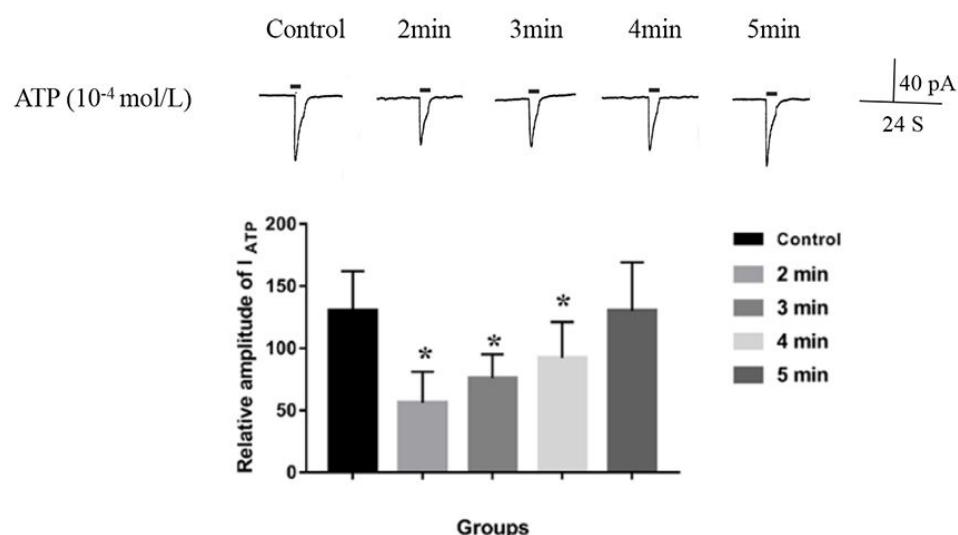


Figure 1. Time course of the recovery of ATP-activated currents. *P<0.05 vs. control group.

3.2 α -methyl-5-HT inhibited ATP activated current

There were at least 5 min interval between the first ATP test and the 2nd one. After pre-adding α -methyl-5-HT (10^{-10} - 10^{-5} mol/L) to ATP-sensitive cells, 72.4% of cells caused inhibition of ATP-activated current, a small part was increased (22.4%) and the rest did not respond (5.2%). This inhibitory effect was independent of whether α -methyl-5-HT itself caused membrane current. In other words, I_{ATP} can be inhibited in the case of α -methyl-5-HT itself whether it causes outward, inward or no response (Figure 2).

As shown in Figure 3, α -methyl-5-HT inhibited I_{ATP} with a concentration-dependent manner. From 10^{-10} mol/L,

the inhibition was gradually increased with the increase of concentration, reaching its peak at 10^{-7} mol/L. Interestingly, as the concentration continued to increase (10^{-6} and 10^{-5} mol/L), the inhibition was reduced, which may be a non-specific effect of high drug concentration (Figure 3).

In order to study the inhibitory effect of pre-addition time of α -methyl-5-HT (10^{-7} mol/L) on I_{ATP}, the inhibitory effects were recorded after 15 s, 30 s, 1 min and 2 min, respectively. The results suggested that the inhibition of α -methyl-5-HT was most pronounced at 1 min. (n=30, p<0.05) (Figure 4).

In addition, cyproheptadine, an antagonist of 5-HT2 receptor, can reverse the inhibition of α -methyl-5-HT on I_{ATP} (Figure 5).

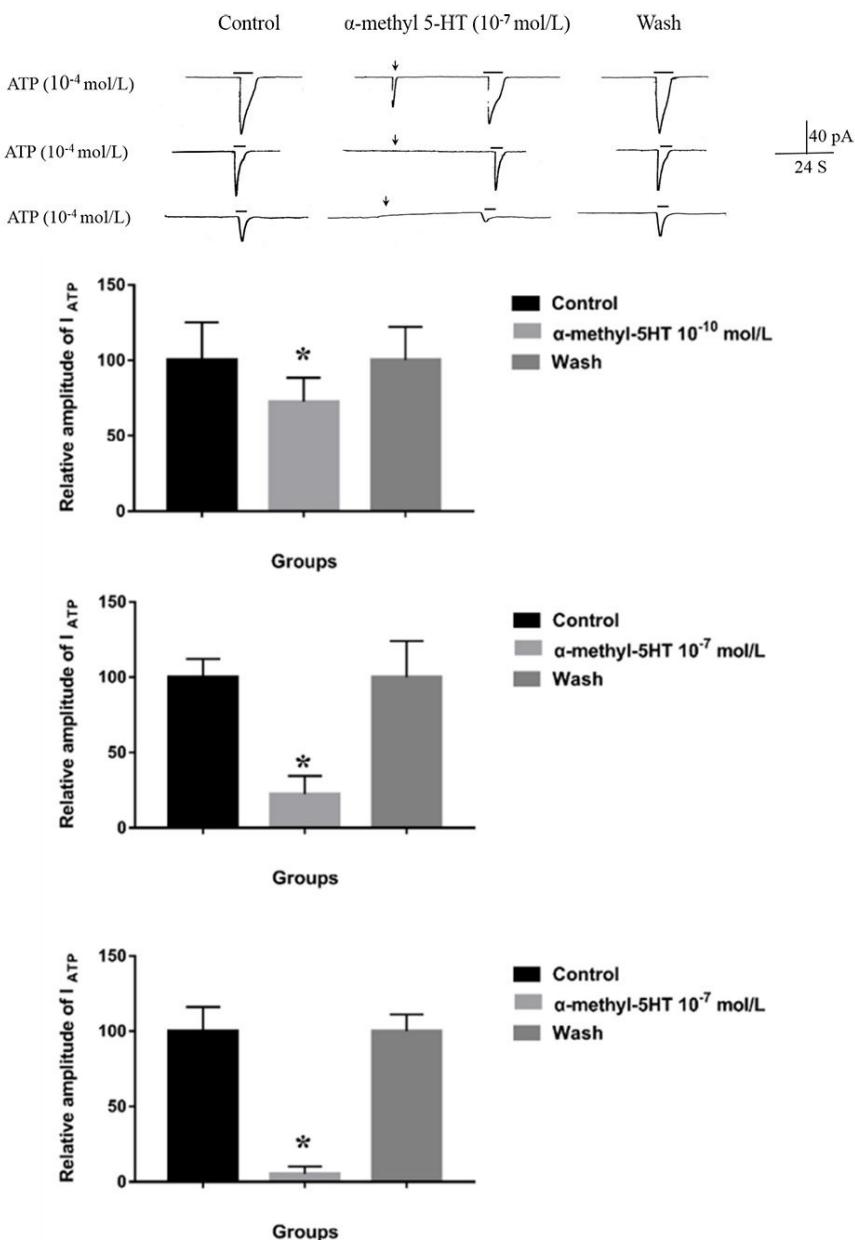


Figure 2. The relationship between α -methyl-5-HT induced response and α -methyl-5-HT inhibition on I_{ATP}. The three rows of current traces show that α -methyl-5-HT could inhibit I_{ATP} whether α -methyl-5-HT itself induced an inward current (middle row), outward current (low row), or no response (upper row). *P<0.05 vs. control group.

3.3 Concentration-response relationship of ATP-activated current with or without α -methyl-5-HT

Figure 6 showed the dose-effect curve of I_{ATP} (10^{-5} – 3×10^{-3} mol/L) after adding 10^{-7} mol/L α -methyl-5-HT for 1 min. The results suggested that: (1) The dose-effect curve of α -methyl-5-HT+ATP was significantly lower than that of control ATP; (2) After pre-adding α -methyl-5-HT, the maximum amplitude of ATP-activated current was inhibited up to 70%; (3) The Kd values of ATP-activated currents before and after pre-adding α -methyl-5-HT were similar, which were 4.23×10^{-5} mol/L and 6.81×10^{-5} mol/L, respectively; (4) The threshold concentration values of them are consistent (10^{-5} mol/L).

3.4 Effect of α -methyl-5-HT on the current-voltage relationship of ATP-activated current

Figure 7 was an I-V curve of pre-addition of 10^{-7} mol/L α -methyl-5-HT (1 min) to inhibit ATP-activated current. The results showed that: (1) The two curves of control ATP and α -methyl-5-HT+ATP were linear in the range of -100 to +40 mV; (2) In the negative voltage (-100 to 0 mV) or positive voltage (0 to +40 mV) interval, the voltage values of voltage points of α -methyl-5-HT+ATP curve were smaller than those of control ATP curve; (3) Compared with control ATP, the reversal potential value of α -methyl-5-HT+ATP curve was basically inconvenient, both of which were about 0 mV.

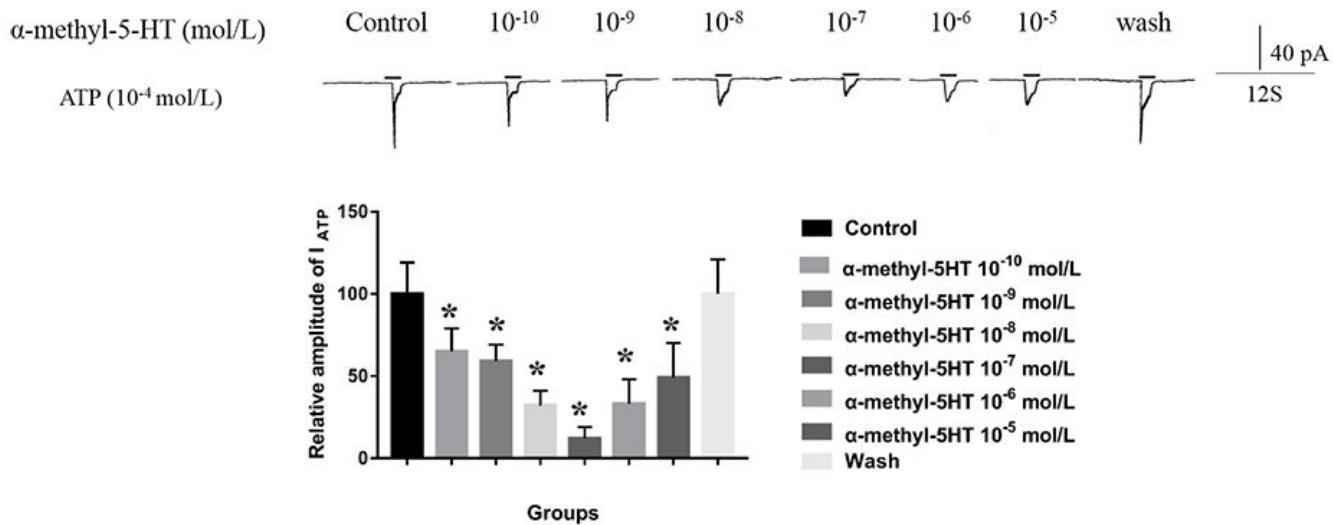


Figure 3. Dose-response curve for the inhibition of I_{ATP} by α -methyl-5-HT. An example of single neuronal recording of I_{ATP} (10^{-4} mol/L, HP = -60 mV) pretreated with six different concentrations of α -methyl-5-HT was illustrated both in the top. The bottom graph dose-response curve for the inhibition by α -methyl-5-HT of ATP-activated currents. Data points are the mean \pm SEM of 5-HT. *P<0.05 vs. control group.

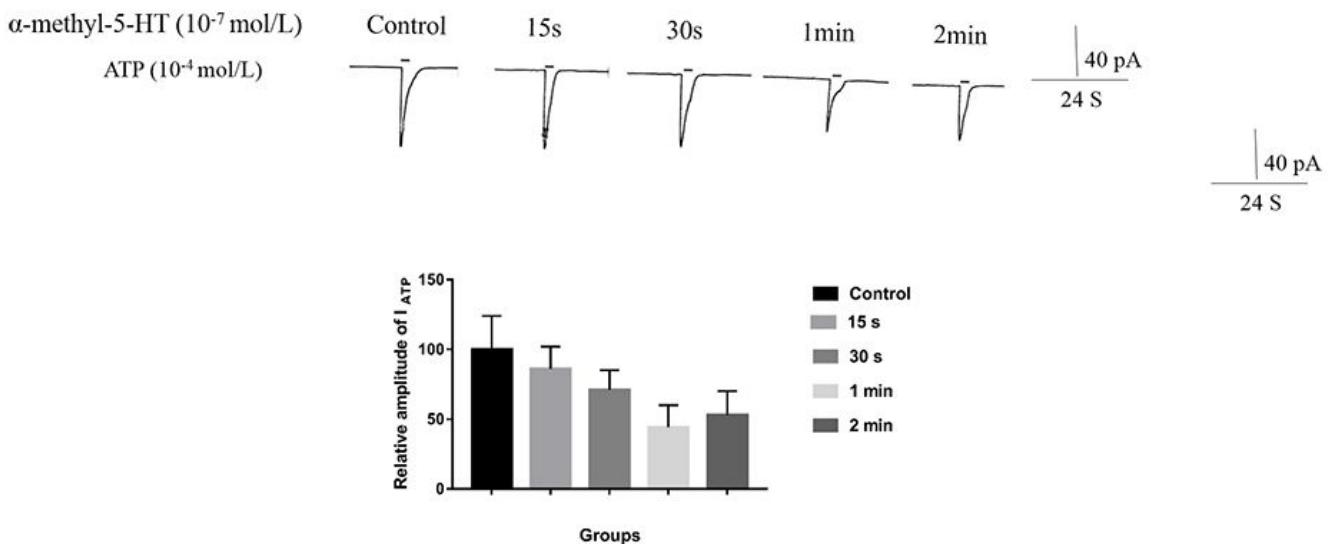


Figure 4. Time course of α -methyl-5-HT inhibition on I_{ATP}.

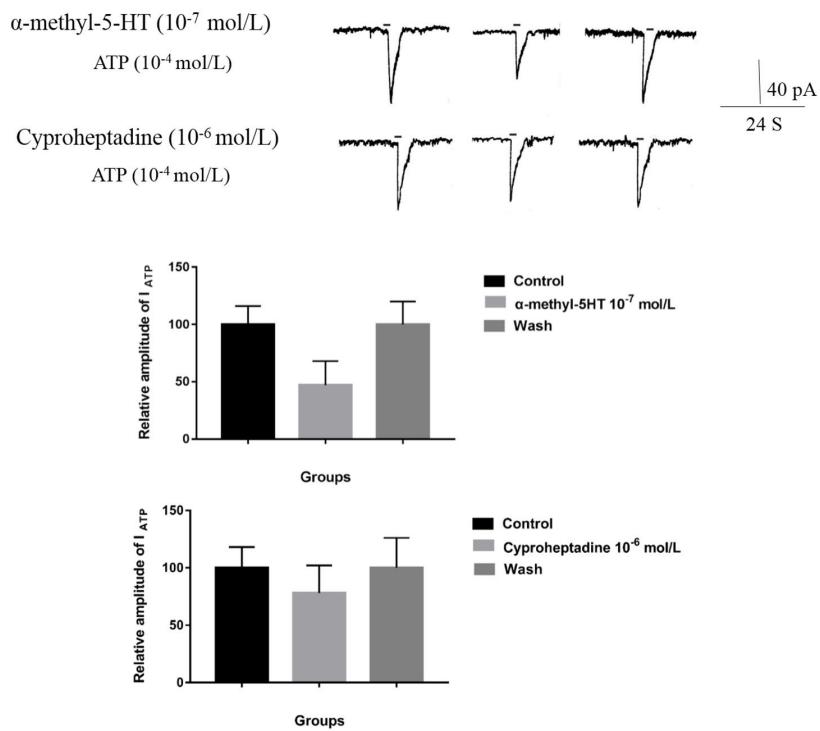


Figure 5. Reversal by cyproheptadine of α-methyl-5-HT inhibition on I_{ATP}

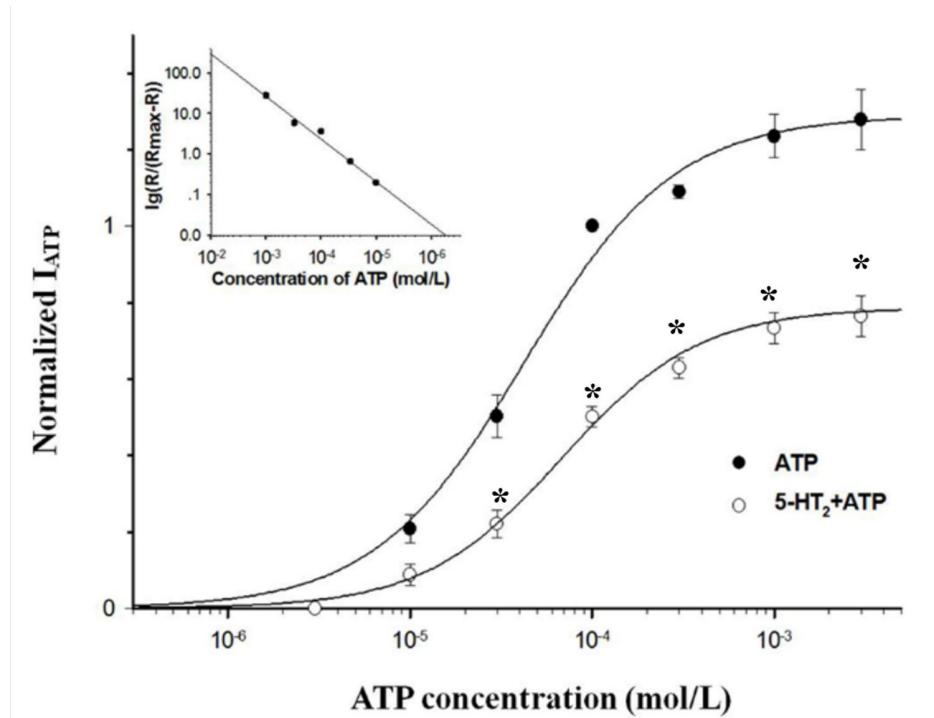


Figure 6. Concentration-response relationship for ATP currents with (○) and without (●) preapplication of α-methyl-5-HT. The graph shows the concentration-response curves for ATP currents with and without reapplication of α-methyl-5-HT (10⁻⁷ mol/L). Each point represents the mean ± SEM. of I_{ATP} of 4-7 neurons. All ATP-activated currents were normalized to the peak current induced by 10⁻⁴ mol/L ATP (marked with asterisk). Holding potential was set at -60 mV. The curve for ATP alone is a good fit of data to the logistic equation $Y = E_{max} / [1 + (Kd/C)^n]$, where C is the concentration of ATP; Y, the normalized currents expressed as fraction of the maximum current response value. Kd, the dissociation constant of ATP receptor. The curve was drawn according to the equation described above assuming Hill coefficient is 0.67 (which was determined by Hill plotting). The curve for I_{ATP} with α-methyl-5-HT pretreatment was drawn by eye. *P<0.05 vs. control group.

3.5 Intracellular transduction mechanism of α -methyl-5-HT inhibiting ATP- activated current

As shown in Figure 8, the inhibition of ATP-activated current by α -methyl-5-HT could be completely eliminated by KN93 (CaMK II inhibitor, 10 μ mol/L) in comparison with the control record ($n=6$, $p < 0.05$). In addition, when combined with 200 μ mol/L H7 (inhibitor of PKC) and 10⁻²

mmol/L BAPTA, the inhibition of α -methyl-5-HT on ATP can also be blocked.

4 Discussion

In our study, 88.65% freshly isolated neurons (125/141) were sensitive to externally applied ATP. After pre-adding α -methyl-5-HT to ATP-sensitive cells, 72.4% of cells caused

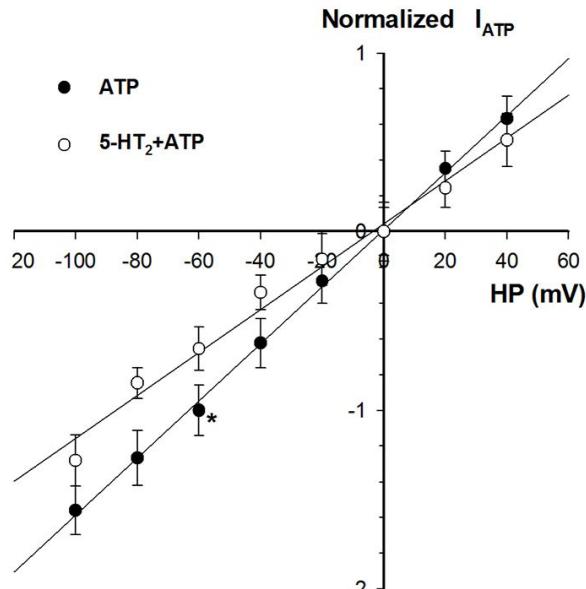


Figure 7. Current-voltage (I-V) relationship for I_{ATP} without pretreatment of α -methyl-5-HT. Graph shows the I-V relationship for I_{ATP} (10^{-4} mol/L) with and without preapplication of α -methyl-5-HT (10^{-7} mol/L), in which the values of reversal potentials in both cases are basically the same (around 0 mV). All currents recorded were normalized to the peak value of I_{ATP} without pre-treatment of α -methyl-5-HT voltage clamped at -60 mV (mark with asterisk). With pre-application of α -methyl-5-HT the inhibition of I_{ATP} is linear. HP: H Potential.

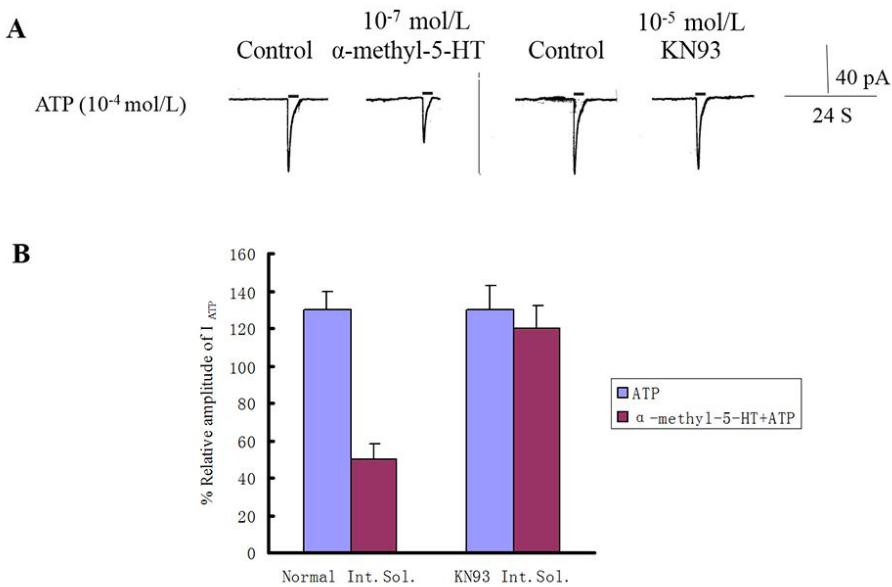


Figure 8. Evidence for the abolishment of inhibition of α -methyl-5-HT on I_{ATP} by intracellular dialysis of KN93. (A) The left pair of current traces show the inhibition by α -methyl-5-HT (10^{-7} mol/L) of ATP (10^{-4} mol/L) activated current with the micropipette filled with normal internal solution. Whereas, as can be seen from the right pair of current traces there is no significant difference between the amplitudes of ATP (10^{-4} mol/L) activated currents with and without pre-application of α -methyl-5-HT (10^{-7} mol/L) when the micropipette was filled with KN93 internal solution; (B) Histogram demonstrates the removal of inhibition by α -methyl-5-HT of I_{ATP} by intracellular dialysis of KN93. * $P < 0.05$ vs. control group.

inhibition of ATP-activated current. The inhibitory effect of α -methyl-5-HT on IATP was concentration-dependent, and was most pronounced at 1 min.

As one of the main neurotransmitters of primary sensory neurons, ATP has a significant effect on the generation, regulation and transmission of nociceptive information (Nishida et al., 2014). ATP receptors belong to purine receptors, and there are two categories: one is adenosine receptor (P1) and the other is ATP receptor (P2) (Nishida et al., 2014). There are a variety of receptors that bind to ATP, and there are two known: P2Y receptor, which activates phospholipase C through G-protein coupling, P2X receptor, which is ligand-gated ion channels (Ralevic & Burnstock, 1998). Seven subunits of P2X have been cloned (North & Surprenant, 2000; Ying et al., 2017), which contain two transmembrane domains, M1 and M2, and the extracellular loop is connected between M1 and M2, and the N and C ends are located intracellularly. The intracellular phosphorylation sites are located at the C-terminal (Van Eck et al., 2005).

The most noteworthy function of the P2X receptor in primary sensory neurons is related to pain perception. P2X3 receptors only exist in sensory neurons and are only expressed in small diameter DRG cells related to nociception. Therefore, ATP receptors, especially the P2X3 receptor, have an effect on the production and transmission of pain sensation (Van Eck et al., 2005). In our experiment, the reversal potential for activation of P2X receptors was close to 0 mV. Our results suggested that the reversal potential of ATP-activated current was close to 0 mV with or without 10^{-7} mol/L α -methyl-5-HT (1 min) (Liang et al., 2005), which indicated that ATP-activated current in our study might mainly activated P2X receptors.

The 5-HT receptor can be classified into 5-HT1-5-HT7 and other types. Except that 5-HT3 receptor is a Ligand-gated ion channel receptor superfamily, the rest are G-protein coupled receptor (Kluess et al., 2005). In this experiment, it was observed that α -methyl-5-HT, a 5-HT2 receptor agonist, sometimes caused an obvious inward current (21.5%, 3/14), but no matter what reaction α -methyl-5-HT caused, it had nothing to do with its inhibition of ATP.

From the I-V curve of Figure 8, it can be seen that compared with the control ATP curve, the current value of α -methyl-5-HT+ATP curve was linearly decreased, that was to say, the suppression of ATP-activated current by α -methyl-5-HT had nothing to do with the change of voltage. It indicated that this inhibition was not due to channel blockage. Figure 7 showed that the dose-effect curve and Kd value of ATP-activated current after pre-addition of α -methyl-5-HT was similar to that of the control ATP curve, but the current amplitude was significantly decreased at the maximum concentration and the α -methyl-5-HT curve moved down. It suggested that the inhibitory effect of α -methyl-5-HT was non-competitive, that was, α -methyl-5-HT was not the result of competitive action on ATP receptor agonist binding sites. At the same time, the inhibitory effect was shifted by cyproheptadine, a 5-HT2 receptor antagonist, which proved once again that α -methyl-5-HT acted through 5-HT2 receptor.

As mentioned above, α -methyl-5-HT receptor is a G-protein coupled receptor. Therefore, we can imagine that phospholipase C (PLC) can be activated by G-protein after 5-HT2 receptor

activation. PLC can decompose PIP2 (phosphatidylinositol 4, 5-diphosphate) to produce two important second messengers, IP3 (inositol triphosphate) and DC (glycerol diester). DC may phosphorylate ATP receptors by activating PKC (protein kinase C). In future research, we can further explore this mechanism.

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