# ORIGINAL PAPER

# Inhibitory effects of honokiol on the voltage-gated potassium channels in freshly isolated mouse dorsal root ganglion neurons

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**Abstract**Voltage-gated potassium (KV) currents, subdivided into rapidly inactivating A-type currents (*I*A) and slowly inactivating delayed rectifier currents (*I*K), play a fundamental role in modulating pain by controlling neuronal excitability. The effects of Honokiol (Hon), a natural biphenolic compound derived from *Magnolia officinalis*, on KV currents were investigated in freshly isolated mouse dorsal root ganglion (DRG) neurons using the whole-cell patch clamp technique. Results showed that Hon inhibited *I*A and *I*K in concentration-dependent manner. The IC50 values for block of *I*A and *I*K were 30.5 μM and 25.7 μM, respectively. Hon (30 μM) shifted the steady-state activation curves of *I*A and *I*Kto positive potentials by 17.6 mV and 16.7 mV, whereas inactivation and recovery from the inactivated state of *I*A were unaffected. These results suggest that Hon preferentially interacts with the active states of the *I*A and *I*K channels, and has no effect on the resting state and inactivated state of the *I*A channel. Blockade on K+ channels by Hon may contribute to its antinociceptive effect, especially anti-inflammatory pain.

**Keywords** Honokiol· Dorsal root ganglion neurons· Patch clamp· Voltage-gated potassium channels

**Introduction**

Honokiol (Hon - see Fig. 1), a biphenolic compound extracted from the Chinese medicinal herb *Magnolia officinalis*, has been used as an anti-bacterial, anti-neoplastic, anti-allergic, anti-spasmodic and anti-asthmatic compound [[1-5](#_ENREF_1)]. Hon has been shown to be able to inhibit platelet-activating factor production in human neutrophils [[6](#_ENREF_6)], scavenge hydroxyl radicals *in vitro* [[7](#_ENREF_7)], decrease  ventricular arrhythmia induced by coronary ligation [[8](#_ENREF_8)], and suppress the aggregation and release reaction of rabbit platelets induced by collagen and arachidonic acid through the inhibition of thromboxane formation [[9](#_ENREF_9)]. Recently, Hon has displayed a neuroprotective effect. It has therapeutic potential in anxiety, pain, epilepsy, cerebrovascular injury , and cognitive disorders including Alzheimer’s disease [[10-15](#_ENREF_10)]. The neuroprotective effects of Hon may be attributed toa wide range of mechanisms, but the effects of Hon on ion channels in neuroprotection, especially in pain, are unclear.

Peripheral nerve damage elicits hyperactivity of dorsal root ganglion (DRG) neurons to contribute toward peripheral neuropathic pain. Increased excitability of DRG neurons is attributable to alterations in the expression and biophysical properties of voltage-gated Na+, K+ and Ca2+ channels [[16](#_ENREF_16), [17](#_ENREF_17)]. Voltage-gated potassium (KV) channel currents are crucial determinants of neuronal excitability  for spike threshold and firing frequency control and are subdivided into rapidly inactivating A-type K+ current (*I*A) and slowly inactivating delayed-rectifier K+ current (*I*K) [[18-21](#_ENREF_18)]. Recent reports have demonstrated that both *I*A and *I*K were significantly reduced in the chronic constriction injury (CCI) rat [[22](#_ENREF_22)]. The changes of *I*K currents in the chronic compression (CCD) model obtained from different studies are inconsistent [[23](#_ENREF_23), [24](#_ENREF_24)]. These discrepancies may be because of differences in cell-size. But a reduction in *I*K and/or *I*A currents is observed in DRG neurons under various pathological conditions, including chronic bladder inflammation, spinal cord injury and chronic pancreatitis [[25-27](#_ENREF_25)]. These findings indicate that the hyperexcitability is associated with a decreased density of KV current.

In the present study, we investigated the effects of Hon on KV current in DRG neurons using the whole-cell patch clamp technique and determined whether Hon affected the activity and kinetic properties of KV channels. We first found that Hon inhibited *I*A and *I*K in a concentration- dependent manner in DRG neurons. In addition, Hon shifted the steady-state activation curves of *I*A and *I*K to more positive potentials, without altering inactivation and recovery from the inactivated state of *I*A.

**Materials and Methods**

**Animals**

Kunming mice (25-30g), obtained from the Qinglongshan Animal Center of Nanjing, were caged with a 12 h natural light-dark cycle with proper food and water. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee ofChina Pharmaceutical University and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the suffering and number of animals used during the experiments.

**Cell Preparation**

Dorsal root ganglion cells were freshly isolated on the basis of the method described previously [[28](#_ENREF_28)]. Briefly, mice were killed by decapitation and ganglia were dissected from the vertebral column. Ganglia were submerged in oxygenated Tyrode's solution (containing in mM: NaCl 137, MgCl2 1.2, KCl 5.4, NaH2PO4 1.2, CaCl2 1, HEPES 10, glucose 10, pH 7.4) and minced with iridectomy scissors. Minced ganglia were incubated at 37℃ for 60 min in Tyrode's solution containing collagenase (1 mg/ml, type Ⅱ; Worthinton, USA). Then the DRGs were dissociated by triturating through a fire-polished Pasteur pipette. The isolated DRGs were washed for 3 times to suspend digestion using [extracellular](javascript:void(0);) solutions and then plated on Poly-D-lysine coated 35 mm culture dishes containing standard external solution (containing in mM: NaCl 137, MgCl2 1.2, KCl 5.4, NaH2PO4 0.33, HEPES 10, glucose 10, CaCl2 1, Nifedipine 0.005, TTX 0.001, pH 7.4).

**Electrophysiological recordings**

Whole-cell K+ currents were recorded from DRG neurons with a patch-clamp amplifier (HEKA EPC 10, HEKA Instruments, Germany). Stimulation protocols and data acquisition were controlled with Pulse software (HEKA Pulse, HEKA Instruments). Pipettes were fabricated from 1.5 mm out diameter borosilicate glass capillaries (Institute of Biophysics, Chinese Academy of Sciences, China) using a Sutter P-97 puller (Sutter Instrument, USA). Fire-polished pipette resistance was 3~5 MΩ when filled with the intracellular solution (containing in mM: KCl 140, CaCl2 1, MgCl2 1, Na2ATP 2, EGTA 10, HEPES 5, adjusted to pH 7.2 with KOH). Current signals were filtered at 3 kHz, digitized at 10 kHz. Series resistance was compensated (>70%). The capacitances (c-fast and c-slow) were compensated automatically by the software online during recording. When needed, linear leak current artifacts were removed by online leak subtraction. To block Ca2+ and TTX-sensitive Na+ currents, nifedipine and TTX were added to the extracellular solution, respectively. Recordings were obtained from small neurons with diameters less than 30 μm. All experiments were performed at room temperature (22~25℃).

**Chemicals**

TTX, Tetraethylammonium Chloride (TEA-Cl), 4-Aminopyridine (4-AP), EGTA and HEPES were purchased from Sigma (St Louis, MO, USA), and others were analytical reagents made in China. Stock solution of TTX was prepared in distilled water. Hon (purity 98%) was purchased from the National Institute for Food and Drug Control (Beijing, China). A stock solution of Hon was prepared in DMSO (Sigma, USA) and then diluted in extracellular solution to attain the desired working solutions. Drug was applied to the bath through the perfusion system.

**Data Analyses**

All data were analyzed by pulse (HEKA electronic, Germany), Igor pro 6.3 (WaveMetrics, USA) and Sigmaplot (Jandel Scientific, USA) softwares. Data were expressed as mean ± S.E.M. Statistical analysis was performed using paired Student’s t-test to evaluate the statistical differences between two groups, and one-way ANOVA was used for multiple groups. A *P* value < 0.05 was considered to be statistically significant.

**Results**

**Effects of Hon on KV currents**

Cells were held at ‒100 mV, voltage-gated potassium (KV) channel currents were elicited by a series of 400 ms test pulses ranging from‒60 mV to +80 mV in 10 mV increments at 0.5 Hz. Activated KV currents include transient current (*I*A), sensitive to 4-AP (Fig. 2a, b), and sustained outward current (*I*K), sensitive to TEA (Fig. 2c, d)*.* In this experiment, 5 mM 4-AP and 50 mM TEA suppressed KV currents by 57.0 ± 3.3% and 64.7 ± 2.2% (*n* = 5), respectively.

Upon the administration of Hon , blocking action of Hon on KV current occurred in 1~2 min, and reached a maximum and steady value in about 7 min. In order to ensure the maximum effect of Hon, KV current was recorded after application of Hon for 10 min. *I*A was estimated as the peak of the transient component, and *I*K was measured at the ending point of 400 ms step depolarizations (Fig. 2e, f). As shown in Fig. 2g, h, during exposure to 30 μM Hon, the maximal peak *I*A and *I*K were reduced by 46.8 ± 2.8% and 48.4 ± 1.7% (*n* = 14), respectively. Hon inhibited the peak amplitudes of *I*A and *I*K in a concentration-dependent manner. The data were fitted well with the following Hill equation, *y* =1/[1+(IC50/*C*)*n*], where IC50 is the drug concentration causing the half-maximum response, *C* is the concentration of drug, *y* and *n* are the fraction of the maximum inhibition percentage and the Hill coefficient. The IC50 values for block of *I*A and *I*Kwere 30.5 μM and 25.7 μM (*n* = 6), respectively. The maximal suppressions of Hon on *I*A and *I*K were 90.0 ± 2.1% and 91.5 ± 2.7% at 300 μM, respectively. The Hill coefficients were 1.04 and 1.15, respectively (Fig. 3a, b). These results suggest that the inhibition of Hon on KV current is preferential for *I*K.

**Effects of Hon on the current–voltage curves** **of** ***I*A and *I*K**

Total K+ currents (KV currents) were elicited by a series of 400 ms test pulses ranging from ‒60 mV to +80 mV in 10 mV increments, preceded by a holding potential of ‒100 mV (Fig. 4a, b). When the voltage was held at ‒40 mV, the depolarization pulses activated *I*K and inactivated *I*A (Fig. 4c, d). Subtraction of the *I*K from the total KV current attained *I*A (Fig. 4e, f). The peak current-voltage (*I*-*V*) curves for *I*A and *I*K in control and after the exposure to 30 µM Hon are shown in Fig. 4g, h. Hon markedly reduced *I*A and *I*K by 43 ± 5% and 62 ± 3% at +80 mV, respectively(*n* = 10, *P* < 0.05).

**Effects of Hon on the activation characteristics of *I*A and *I*K**

Activation of K+ channel was evaluated by a conductance transform of the peak current–voltage relationship. The conductance was calculated from the peak current value at each test pulse potential using the equation: *G* = *I*/(*V*-*V*rev), where V is the membrane potential at which *I* was recorded, *V*rev is reversal potential (calculated as ‒86 mV for the potassium concentrations used). The normalized conductance was fitted well with a Boltzmann equation: *G*/*G*max = 1/{1+exp[(*V*1/2-*V*)/*k*]}, where *G*max is the maximal conductance, *V*1/2 is the half activation voltage and *k* is a slope factor. The steady-state activation curves for *I*A and *I*K are shown in Fig. 5a, b. The values of *V*1/2for activation of *I*Ain control and in the presence of 30 μM Hon were ‒25.9 ± 3.8 mV and ‒8.3 ± 4.8 mV (*n* = 10, *P* < 0.05), with *k* values of 19.7 ± 2.4 mV and 26.0 ± 0.9 mV (*n* = 10, *P* < 0.05), respectively. Thus Hon produced a 17.6 mV positive shift in the *V*1/2 of the steady-state activation curve. For *I*K, Hon markedly shifted *V*1/2 from 6.9 ± 1.7 mV for control neurons to 23.6 ± 2.5 mV for 30 μM Hon treated neurons (*n* = 10, *P* < 0.05). In contrast, no significant difference in the slope factor kwas observed between control (20.5 ± 1.9 mV) and 30 μM Hon treated neurons (22.9 ± 0.7 mV, *n* = 10, *P* > 0.05).

**Effects of Hon on the inactivation of *I*A**

To determine the voltage dependence of the steady-state inactivation of *I*A, two-pulse voltage protocols were employed in this experiment. The holding potential was at ‒100 mV, a 1 s conditional prepulse of various voltages from ‒120 to ‒30 mV in 10 mV increment was followed by a 400 ms testing pulse to +60 mV. The inactivation curve was fitted by the Boltzmann equation : *I*/*I*max = 1/{1+exp[(*V*-*V*1/2)/*k*]}, where *I* is the peak current evoked from the conditional potential *V*, *I*max is the maximal peak current, *V*1/2 is the half inactivation voltage, and *k* is a slope factor. The *V*1/2 and k of *I*A were ‒78.0 ± 2.3 mV and 13.7 ± 1.6 mV in control , and were ‒81.8 ± 3.2 mV and 15.4 ± 1.9 mV in the presence of 30 μM Hon (*n* = 7, *P* > 0.05), respectively. Hon slightly shifted the steady-state inactivation curve of *I*A towards negative potential, but not statistically significant (Fig. 6). These results suggested that Hon did not obviously affect the steady-state inactivation of *I*A.

**Effects of Hon on the recovery of *I*A from inactivation**

The time course of *I*A recovery from inactivation was assessed using a standard two-pulse protocol. A 400 ms prepulse depolarized to +60 mV from a holding potential of ‒100 mV was followed by recovery gap potential at ‒100 mV for variable length and then by a 400 ms test pulse to +60 mV. The ratio of test pulse currents to prepulse currents plotted againstrecovery time. The recovery process of *I*A could be well fitted by a single exponential function, with recovery time constant of 79.9 ± 5.2 ms and 89.7 ± 7.5 ms before and after application of 30 µM Hon (*n* = 7, *P* > 0.05) , respectively. The results showed that Hon had no significant effect on the time course of recovery of *I*A from inactivation (Fig. 7).

**Effects of Hon on the frequency-dependence of *I*A**

To study the effect of Hon on frequency-dependence, KV currents were elicited by 20 consecutive 400 ms depolarizing pulses to +80 mV from a holding potential of ‒100 mV at different frequencies (1, 5 and 10 Hz). *I*A was measured as the peak of the transient component, and *I*K was measured at the ending point of 400 ms voltage steps. Data were normalized with respect to the first step. As shown in Fig. 8, the responses of *I*A and *I*K to depolarizing pulses remained unchanged at different frequencies before and after application of 30 µM Hon (*n* = 6). Thus, Hon did not show a frequency-dependent effect on KV currents (*I*A and *I*K).

**Discussion**

In the present study, inhibitory effects of Hon on voltage-dependent K+ channels were investigated in mouse dorsal root ganglion neurons. The results indicated that Hon significantly inhibited two important voltage-gated K+ (KV) channel currents, the transient A-type K+ current (*I*A) and the sustained delayed rectifier K+ current (*I*K) , in a concentration-dependent manner. IC50 were 30.5 µM and 25.7 for *I*A and *I*K, respectively. In addition, Hon shifted the activation curves of *I*K and *I*A to more positive potentials. However, Hon had no effect on inactivation, recovery from the inactivated state and frequency-dependence of *I*A.

Voltage-gated K+ channels play a  critical role in the control of electrophysiological properties and excitability of neurons [[29](#_ENREF_29)]. A-type K+ channels have been implicated in the delay of the spike onset and the decrease in the action potential (AP) firing frequency, while the sustained delayed rectifier K+ channels have been involved in action potential threshold and the number of APs of neurons, implying that the two types of K+ channels in DRG may play different roles in neuronal excitability [[22](#_ENREF_22), [30](#_ENREF_30)]. In this study, we confirmed that Hon significantly inhibited *I*K and *I*A in a concentration-dependent manner. It is thus likely that Hon regulates the excitability of DRG neurons by suppressing two types of K+ channels. However, It is noteworthy that Hon produces varying effects on both types of K+ currents, the blocking potency of Hon on *I*K (IC50: 25.7 μM)is stronger than on *I*A (IC50: 30.5 μM). This difference may be due to the possibility that there is the different sensitivity for Hon to *I*K and *I*A. Functionally, *I*A was relatively small and inactivated fast , repolarizing phases of action potentials were usually slow and mainly formed by *I*K in DRG neurons [[31](#_ENREF_31)]. Consequently, the modification of *I*K by Hon may affect membrane potential and thereby cause changes in the neuronal activity.

The state-dependent K+ channel blockade of Hon may help to understand its kinetic mechanism. Voltage-gated K+ channels have three distinct states: closed or resting, open, and inactivated. In present study, Hon shifted the activation curves of *I*A to more positive potentials, without altering inactivation and recovery from the inactivated state and frequency-dependence of *I*A. The positive shift of the activation curve of *I*A indicates that Hon preferentially binds to the activated state of the A-type K+ channel. According to Modulated receptor hypothesis for the state-dependent interaction[[32](#_ENREF_32)], Hon displayed higher binding affinity to the open state than to the resting state or inactivated state of the A-type K+ channel. Additionally, *I*K, a slowly inactivating delayed rectifier current, was also suppressed. The activation curve of *I*K was shifted to more positive potential by Hon, but frequency-dependent inhibition of *I*K was not found in the presence of Hon, suggesting Hon had a higher affinity for the open state of the sustained delayed rectifier K+ channel.

Pain arising from intense or damaging stimuli has an important physiological role, protecting the body and promoting healing from injury. It is divided into physiological pain, inflammatory pain and neuropathic pain [[33](#_ENREF_33)]. Previous studies have shown that Hon produces the antinociceptive action in inflammatory pain models induced by formalin, substance P and prostaglandin, and do not display analgesia in physiological pain determined by the tail-flick, hot-plate paw-shaking tests [[11](#_ENREF_11), [33](#_ENREF_33)]. After peripheral inflammation or nerve injury, voltage-gated K+ currents are changed in DRG cells. In many neuropathic pain models, the two types of voltage-gated K+ currents were downregulated or unaltered in DRG neurons, respectively [[22](#_ENREF_22), [23](#_ENREF_23), [25-27](#_ENREF_25)]. On the contrary, the increases in *I*K and *I*A were observed in the model of peripheral inflammatory pain induced by an inflammatory agent (zymosan) [[34](#_ENREF_34)], this is consistent with the antinociceptive effects of Hon in inflammatory pain models. However, the reasons for these differences between these various models are not yet clear. The relationships between voltage-gated K+ currents and different types of pains are required to be further studied. Still, we speculate that the anti-inflammatory pain of Hon may be mainly attributed to its blocking effect on KV channels. The latest study reported that diclofenac, a nonsteroidal anti-inflammatory drug with anti-nociceptive action, inhibited *I*K and increasedM-type K+ current (*I*K(M)) in DRG neurons, and decreased rhythmic firing of APs in hippocampal CA1 pyramidal neurons [[35](#_ENREF_35)]. These results powerfully support our viewpoint that anti-inflammatory pain is associated with blocking potassium currents.

Hon, including most flavonoid compounds, has strong antioxidant activity and other bioactivity because of containing phenolic hydroxyl groups in its chemical structure. In an earlier report,piceatannol, a derivative of resveratrol , possessed an additional hydroxyl group in the 3′ position of resveratrol, which made it exerting more potent than resveratrol in cardiac ion channel inhibition, including *I*Na, *I*to and *I*Kss, in parallel with the antiarrhythmic activity [[36](#_ENREF_36)]. This result suggested that increasing hydroxyl groups were beneficial to enhance inhibitory effect of drug on ion channels. Therefore, we think that the strong potencies of Hon in anti-oxidative, anti-inflammatory, and neuroprotective activities mainly depend on phenolic hydroxyl groups in its chemical structure.

In conclusion, our data demonstrate that Hon exhibits concentration- dependent blocking effects of *I*A and *I*K. It preferentially interacts with the active states of the *I*A and *I*K channels, without apparent effect on inactivation and recovery from the inactivated state of *I*A. Currently, based on the knowledge what we know about analgesic effects of Hon, we propose that the antinociceptive action of Hon may be related to its blocking effect on K+ channels, Hon may possess the therapeutic potential in the treatment of inflammatory pain. But, the regulations of K+ channels in different pain models still remain to be investigated in future experiments.

**Compliance with Ethical Standards**

**Conflict of interest**  The authors declare that they have no conflict of interest.

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**Legends for Figures**

**Fig. 1** Structure of Hon

**Fig. 2** Hon inhibited voltage-gated K+ (KV) currents in DRG neurons. The total KV currents were elicited by 400 ms depolarization from holding potential ‒100 mV, ranging from ‒60 mV to +80 mV in 10 mV increments. Representative traces of KV currents were shown. **a** and **b** The total KV currents and 4-aminopyridine (4-AP)-sensitive A-type K+ currents (*I*A) (*n* = 5). **c** and **d** The total KV currents and tetraethylammonium chloride (TEA)-sensitive delayed rectifier K+ currents (*I*K) (*n* = 5). **e** and **f** The total KV currents before and after application of 30 μM Hon. **g** and **h** Current-voltage relationships for *I*A and *I*K in the absence and presence of 30 μM Hon. *I*A was measured as the peak of the transient component, and *I*K was measured at the ending point of 400 ms voltage steps. (*n* = 14, \**P* < 0.05 *vs* control).

**Fig. 3** Concentration-response curves for inhibition of *I*A (**a**) and *I*K (**b**) by Hon. Data were fitted with the Hill equation, *y* =1/[1+(IC50/*C*)*n*], where IC50 is the drug concentration causing the half-maximum response, *C* is the concentration of drug, *y* and *n* are the fraction of the maximum inhibition percentage and the Hill coefficient (*n* = 6).

**Fig. 4** Effects of Hon on current–voltage (*I*-*V*) relationships of *I*A and *I*K. The current was evoked by a series of 400 ms stimulating pulses from ‒60 mV to +80 mV in 10 mV steps. **a** and **b** Representative current traces for total KV currents, cell was held at ‒100 mV before and after the exposure to 30 µM Hon. **c** and **d** Representative current traces for *I*K, cell was held at ‒40 mV before and after the exposure to 30 µM Hon. **e** and **f** Representative current traces for *I*A were obtained by subtracting *I*K in **c**and **d**from total KV currents in **a**and **b**, respectively. **g** and **h** Current-voltage relationships for *I*A and *I*K (*n* = 10, \**P* < 0.05 *vs* control).

**Fig. 5** Effects of Hon on steady state activation curves of *I*A (**a**) and *I*K (**b**). Activation curves of K+ currents were obtained from the data of *I-V* curves and were fitted by the Boltzmann equation.

**Fig. 6** Effects of Hon on steady state inactivation of *I*A. Current curves were obtained by a 1 s conditional prepulse of various voltages from ‒120 to ‒30 mV in 10 mV increment was followed by a 400 ms testing pulse to +60 mV, cell was held at ‒100 mV. Steady state inactivation curves were fitted by the Boltzmann equation (*n* = 7).

**Fig. 7** Effects of Hon on recovery of *I*A from inactivation. Current traces were recorded using a standard two-pulse protocol. A 400 ms prepulse depolarized to +60 mV from a holding potential of ‒100 mV was followed by recovery gap potential at ‒100 mV for variable length and then by a 400 ms test pulse to +60 mV. Data were fitted with a single exponential equation (*n* = 7).

**Fig. 8** Frequency-dependent inhibition of Hon (30 µM) on KV currents. KV currents were elicited by 20 consecutive 400 ms pulses depolarized from a holding potential of -100 to +80 mV at 1, 5 and 10 Hz. *I*A was measured as the peak of the transient component, and *I*K was measured at the ending point of 400 ms voltage steps. *I*A and *I*K were normalized with respect to the value obtained for the first step (*n* = 6).