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Torilin from *Torilis japonica* (Houtt.) DC. Blocks hKv1.5 Channel Current

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Torilin was purified from *Torilis japonica* (Houtt.) DC., and its effects on a rapidly activating delayed rectifier K^+ channel (hKv1.5), cloned from human heart and stably expressed in Ltk$^-$ cells, as well as the corresponding K^+ current (the ultrarapid delayed rectifier, I_{KUR}) were assessed in human atrial myocytes. Using the whole cell configuration of the patch-clamp technique, torilin was found to inhibit the hKv1.5 current in time and voltage-dependent manners, with an IC_{50} value of 2.51±0.34 μM at +60 mV. Torilin accelerated the inactivation kinetics of the hKv1.5 channel, and slowed the deactivation kinetics of the hKv1.5 current, resulting in a tail crossover phenomenon. Additionally, torilin inhibited the hKv1.5 current in a use-dependent manner. These results strongly suggest that torilin is a type of open-channel blocker of the hKv1.5 channel.

Key words: hKv1.5 channel blocker, Torilin, *Torilis japonica* (Houtt.) DC

INTRODUCTION

Torilis japonica (Houtt.) DC. (Umbelliferae) is widely distributed in Korea, Japan and China, and has been used as a folk medicine for the treatment of impotence, infertility, women's diseases, chronic diarrhea and carbuncle (Lee, 1996; Sung *et al.*, 1998). The isolation of several types of sesquiterpenoids, guaiane, humulene, germacrane and eudesmane have been reported from the methanolic extract of the fruits (Itokawa *et al.*, 1983a, 1983b, 1986; Kitajima *et al.*, 1998, 2002; Ryu and Jeong, 2001). Torilin has been reported to have anti-inflammatory (Lee *et al.*, 1999), testosterone 5α-reductase inhibitory (Park *et al.*, 2003), hepatoprotective (Oh *et al.*, 2002) and anti-cancer activities (Kim *et al.*, 2000, 2001; Park *et al.*, 2006).

Kv channels represent a structurally and functionally diverse group of membrane proteins, which play an important role in determining the length of the cardiac action potential, and are the targets for antiarrhythmic drugs (Colatsky *et al.*, 1990). Multiple *Shaker*-like K^+ channel α and β subunit genes have been cloned from human myocardium, which contribute to its electrical activity (Deal *et al.*, 1996). One of these, Kv1.5, has been identified as one of the more cardiovascular-specific Kv channel isoforms, although it has also been found in other tissues (Tamkun *et al.*, 1991; Overturf *et al.*, 1994). Cloned from human heart tissue, it forms the molecular basis for an ultrarapid delayed rectifier K^+ current (I_{KUR}). hKv1.5 currents expressed in heterologous expression systems are similar in their biophysical and pharmacological properties to those of I_{KUR} recorded in human atrial myocytes (Wang *et al.*, 1993; Feng *et al.*, 1997). Accordingly, Kv channels have become major targets for the treatment of arrhythmia.

Our studies have focused on the development of anti-arrhythmic drug from natural plants, and we previously reported that papaverine (Choe *et al.*, 2003), oxypeucedanin (Eun *et al.*, 2005a) and psoralen (Eun *et al.*, 2005b) inhibited the hKv1.5 current.

In the present study, torilin, one of the extracts from *T. japonica*, was found to block the hKv1.5 channel current expressed in Ltk$^-$ cells in a concentration-dependent manner.

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MATERIALS AND METHODS

General procedure

All the ^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. The EI/MS (70 eV) spectrum was determined on a VG-VSEQ mass spectrometer (VG Analytical, UK). The TLC was carried out on pre-coated silica gel F_{254} plates (Merck, Darmstadt, Germany), and Kiesel gel 60 (230-400 mesh, Merck) was used as the silica gel for the column chromatography. The column used for LPLC was a Lobar A (Merck Lichroprep Si 60, 240-10 mm). All the other chemicals and solvents were of analytical grade, and used without further purification.

Plant materials

The fruits of *T. japonica* were collected and air-dried at Wanju, Chonbuk, Korea in June 2003. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-03-045).

Extraction and isolation

The shade dried plant material (500 g) was extracted three times with MeOH at room temperature and then filtered. The extracts were combined and evaporated *in vacuo* at 40°C. The resultant methanolic extract (55 g) was partitioned three times with methylene chloride to afford a methylene chloride-soluble syrup on drying (20 g). The methylene chloride fraction showed the most significant inhibitory activity on the hKv1.5 current, and this fraction (7 g) was subjected to chromatography on a silica gel column, and eluted with $CHCl_3$-MeOH, 30:1, to give three fractions (MC1-MC3). Fraction MC1 (3 g) was subjected to chromatography on a silica gel column chromatography, and eluted with $CHCl_3$-MeOH, 35:1, to afford five sub-fractions (MC11-MC15). Sub-fraction MC12 (600 mg) was further subjected to chromatography on silica gel, and eluted with *n*-hexane-EtOAc, 3:1, and purified on a Lobar-A column eluted with *n*-hexane-EtOAc, 3:1, to give compound 1 (Fig. 1, 170 mg).

Compound 1 (Torilin, Fig. 1)

Colorless needles (MeOH); 78-79°C, EI/MS *m/z*: 376[M]$^{+}$, 316, 234, 216, 188, 83, ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.60 (dd, *J* = 18.4, 6.0 Hz, H-2a), 2.07 (dd, *J* = 18.4, 3.0, H-2b), 2.88 (d, *J* = 13.6, H-6a), 2.53 (m, H-6b), 2.42 (m, H-1), 2.41 (dd, *J* = 10.2, 3.5, H-7), 5.46 (m, H-8), 2.25 (dd, *J* = 14.5, 7.7, H-9a), 1.62 (m, H-9b), 1.50 (m, H-10), 1.52 (s, H-12), 1.54 (s, H-13), 1.03 (d, *J* = 6.5, H-14), 1.73 (s, H-15), 6.09 (m, H-3'), 2.01 (d, *J* = 7.2, H-4'), 1.91 (s, H-5'), 1.99 (s, H-2"), ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 51.6 (C-1), 41.6 (C-2), 208.9 (C-3), 135.4 (C-4), 174.7 (C-5), 26.2 (C-6), 46.7 (C-7), 71.1 (C-8), 40.9 (C-9), 33.8 (C-10), 84.8 (C-11), 25.0 (C-12), 24.4 (C-13), 23.2 (C-14), 8.5 (C-15), 167.1 (C-1'), 128.1 (C-2'), 138.7 (C-3'), 16.2 (C-4'), 21.1 (C-5'), 170.7 (C-1"), 23.1 (C-2").

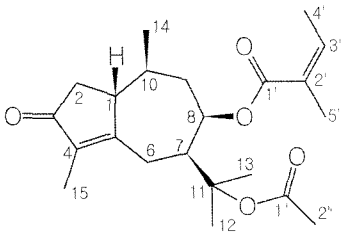

Fig. 1. Chemical structure of Torilin

Cell culture and transfection

The method used to establish the expression of hKv1.5 in a clonal mouse Ltk^{-} cell line was the same as described previously (Snyders *et al.*, 1993). The transfected cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 0.25 mg/mL of G418, under a 5% CO_2 atmosphere. Before the experiment, the sub-confluent cultures were incubated for 12 h with 2 μM dexamethasone to induce the expression of hKv1.5 channels. The cells were removed from the dish with a rubber policeman, a procedure that left the majority of the cells intact.

Electrical recording

The currents of the hKv1.5 channels were recorded using the whole cell configuration of the gigaohm-seal patch clamp techniques (Kwak *et al.*, 1999), with the electrical signals amplified with a patch clamp amplifier (Axon Instruments, Axopatch-1D, Foster, U.S.A.). The currents were digitized using a signal converter (Digidata 1200, Axon Instruments) and stored on the hard disk of a computer. A micropipette, with a resistance of 1~2MΩ (Kimax-51, 1.5-1.8 × 10 mm), used for current recording, was pulled out by a 2-stage pipette puller (Narishige, PP-83). The intracellular pipette-filling solution for use in the whole cell mode contained 100 mM KCl, 10 mM HEPES, 5 mM K_4BAPTA, 5 mM K_2ATP and 1 mM $MgCl_2$ (pH 7.2). The extracellular solution contained 130 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.35). The current traces were recorded in the Ltk^{-} cells with a depolarizing pulse of +50 mV, from a holding potential of -80 mV, followed by a repolarizing pulse of -50 mV. To observe the concentration-dependent block of the hKv1.5 channel currents due to torilin, steady-state currents, taken at the end of the depolarizing pulse of +50 mV, were normalized to the control obtained in the absence of torilin.

Statistical analysis

The results are expressed as the mean ± S.E.M. The Student's *t*-test and analysis of variance (ANOVA) were used to calculate statistical significant, with a value of $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Mammalian Kv channels are divided into nine subfamilies, Kv1~Kv9. Of these, the Kv1 subfamily is the most diverse, including at least eight subclasses, Kv1.1~Kv1.8 (Grissner, 1997). Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv2.1, Kv4.2 and Kv4.3 of the Kv channel genes have been cloned from cardiac tissue (Snyders *et al.*, 1993). The main Kv channel genes expressed in human heart are the hKv1.4, hKv1.5 and hKv4.3, as well as HERG genes. All these genes are highly expressed in both atrium and ventricle tissues and; in particular, the hKv1.5 gene is preferentially expressed in human atrium tissue. The current generated by hKv1.5 channels is similar in physiological and pharmacological sensitivity to the very rapidly activating rectifier K^+ current recorded in human atrial myocytes (I_{KUR}) (Wang *et al.*, 1993) and dog ventricle tissue (Jeck and Boyden, 1992). In particular, selective block of the hKv1.5-like current in human atrial myocytes results in a significant prolongation of the action potential (Wang *et al.*, 1994). Blocking of the cardiac K^+ channels increases the duration of the action potential (Deal *et al.*, 1996).

The selective block of the hKv1.5-like current in human atrial myocytes results in a significant prolongation of the action potential (Wang *et al.*, 1993, 1994). Blocking of the cardiac K^+ channels increases the duration of the action potential (Roden, 1993). The ^1H- and ^{13}C-NMR data of compound **1** obtained in this study were in good agreement with the literature values (Kang *et al.*, 1994). The inhibitory component of the hKv1.5 current in *T. Japonica* was torilin. Fig. 2 shows the concentration-dependent inhibitory effects of torilin on the hKv1.5 channel currents expressed in the Ltk^- cells. The membrane potential was held at -80 mV and 250-ms, with depolarizing pulses from -80 to +60 mV, in 10 mV steps, were applied every 20 s. The outward currents were followed by decaying outward tail currents on repolarization to -50 mV. Under control conditions, a depolarization positive to -40 mV elicited outward currents, which progressively increased with further depolarizations. At +60 mV, after the current reached a maximum, it declined slowly during maintained depolarization (Fig. 2Aa). In the presence of torilin (10 μM), the inhibition of the hKv1.5 currents was characterized by acceleration in the current decay, with relatively fewer effects on the peak amplitude (Fig. 2Ab). Fig. 2B shows the effect of torilin (10 μM) on the steady-state current-voltage (I-V) relationship for the hKv1.5 channel, as constructed by plotting the current amplitudes at the end of 250 ms depolarizations as a function of the test pulse voltage. Torilin reduced the peak and steady-state currents

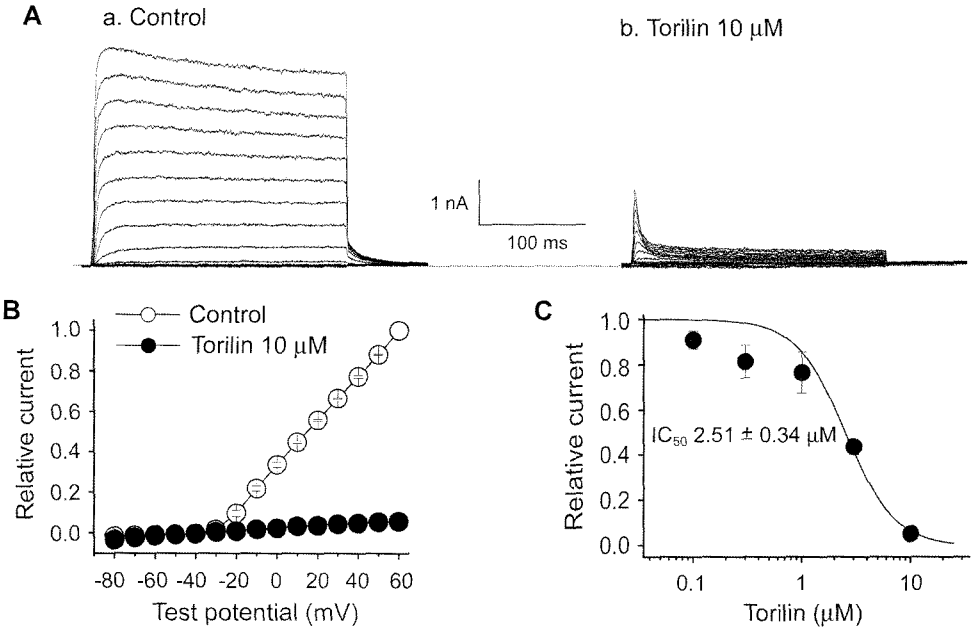

Fig. 2. Effects of torilin on the hKv1.5 current expressed in a Ltk^- cell line. hKv1.5 current traces were recorded before (Aa) and 20 min after exposure to 10 μM torilin (Ab). The voltage protocol consisted of 250-ms depolarizing pulses, ranging from -80 to +60 mV, at 10-mV increments, from a holding potential of -80 mV, with repolarizing to -50 mV for 400 ms. (B) The resultant I-V relationship of the steady-state current taken at the end of the depolarizing pulses. (C) Concentration-response relationships of hKv1.5 block by torilin. Each point with a vertical bar denotes the mean ± S.E.M.

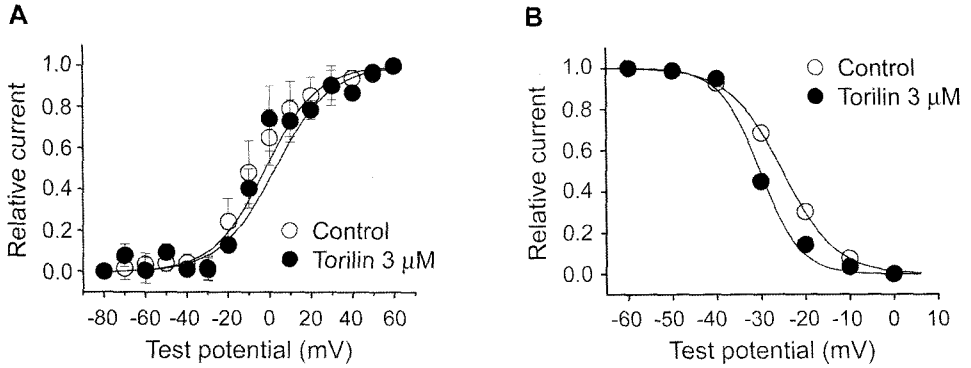

Fig. 3. Effects of torilin on the activation and inactivation of the hKv1.5 current. The activation curve was obtained from the deactivating tail current amplitudes at -50 mV, following 250 ms depolarizing steps to potentials between -80 to +60 mV, in steps of 10 mV, from a holding potential from -80 mV. The inactivation curve was obtained from the depolarizing steps to potentials between -60 mV to 0 mV, in steps of 10 mV. Each point with a vertical bar denotes the mean ± S.E.M.

elicited by pulses to +60 mV (n = 7). Block of the hKv1.5 channel by torilin was in a concentration-dependent manner, as shown in Fig. 2C. The half-maximal inhibitory concentration (IC_{50}) and Hill coefficient were 2.51 ± 0.34 μM and 1.29, respectively (n=7). Drugs that block ion channels often alter the voltage dependence. Therefore, the voltage dependence of activation (Fig. 3A) and inactivation (Fig. 3B), from the peak amplitude of the decaying tail currents, were analyzed in the absence or presence of torilin (3 μM). The half-activation voltages were -12.7 ± 3.2 and -13.4 ± 3.1 mV (n=6) without and with torilin, respectively. The half-inactivation voltages were -25.1 ± 3.0 and -30.2 ± 3.3 mV (n=6) without and with torilin, respectively. To quantify the voltage dependence of torilin block, the relative current $I_{torilin}/I_{control}$ was plotted as a function of the membrane potential (Fig. 4, n=5). In the presence of torilin (3 μM), the blockade increased steeply between -30 mV and 0 mV, which corresponds to the voltage range of channel opening (Snyders *et al.*, 1993). These data suggest that torilin primarily binds to the open state of the hKv1.5 channel. As shown in Fig. 5, the effects of torilin on the deactivation kinetics of hKv1.5 were determined during a repolarizing step of -50 mV, following a depolarizing step to +60 mV. In the presence of torilin (1 μM), the initial tail current was reduced, with the subsequent slower decline resulting in a "crossover" phenomenon with the control tracing. The use-dependence of the torilin-induced inhibition of the hKv1.5 channel was also investigated. Original current traces, under control conditions and in the presence of 3 μM torilin, were produced by 20 repetitive applications of depolarizing pulses at three different frequencies, 1, 2 and 3 Hz (Fig. 6). As shown in Fig. 6, the peak amplitude of the hKv1.5 current decreased slightly at a frequency of 1-3 Hz under control conditions. In the

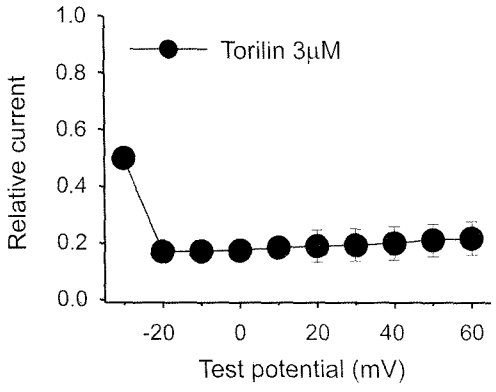

Fig. 4. Voltage-dependent block of hKv1.5, due to torilin, expressed in Ltk⁻ cells. The voltage protocol consisted of 250-ms depolarizing pulses from -30 to +60 mV, in 10-mV increments, from a holding potential of -80 mV, with repolarization to -50 mV for 400 ms. Relative currents were obtained from the $I_{torilin}/I_{control}$ ratio at each depolarizing potential in the absence and presence of torilin. Each point with a vertical bar denotes the mean ± S.E.M.

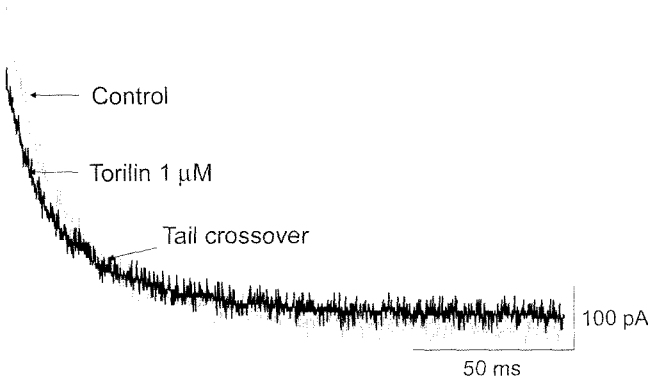

Fig. 5. The effect of torilin on the deactivation kinetics of hKv1.5 current expressed in Ltk⁻ cells. Deactivation kinetics were investigated during a repolarizing step of -50 mV for 400 ms, after a 250 ms depolarizing step to +60 mV, from a holding potential of -80 mV. By superimposing the tail currents in the absence and presence of torilin, a tail crossover phenomenon (indicated by the arrow) was observed.

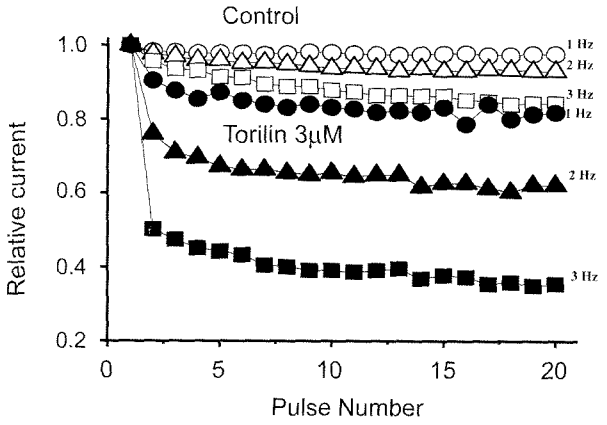

Fig. 6. Use-dependent inhibition of hKv1.5, due to torilin, expressed in Ltk⁻ cells. The plot of the normalized peak amplitudes of the currents, under control conditions and in the presence of torilin, at every pulse versus the pulse numbers in the pulse train.

presence of 3 μM torilin, the peak amplitude of the hKv1.5 current was not significantly reduced after the first pulse. The subsequent trace showed a progressive decrease in the peak amplitude of the hKv1.5 current to a steady level. The extent of the steady-state block was increased at 1, 2 and 3 Hz (n=4); thus, torilin exhibited a use-dependent inhibition of the hKv1.5 current, with little tonic block.

Torilin initially induced a fast decline of the hKv1.5 current during depolarization, suggesting that torilin binds to the open state of the hKv1.5 channels. Also, the interaction of torilin with the hKv1.5 channels was highly voltage-dependent within the voltage range of the channel's opening. Furthermore, torilin slowed the time course of deactivating tail currents; thus, inducing the tail crossover phenomenon, which is typically detected with open channel blocks. Taken together, these findings indicate that torilin may be an ideal antiarrhythmic drug for atrial fibrillation.

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