Urinary Bladder-Relaxant Effect of Kurarinone Depending on Potentiation of Large-Conductance Ca\(^{2+}\)-Activated K\(^+\) Channels

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Kurarinone Relaxes the Bladder by Potentiating BK Channels

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Abbreviations

ACh, acetylcholine; BK_{Ca} channel, large-conductance Ca^{2+}-activated K' channel; cRNA, complementary RNA; CTBIC, 4-chloro-7(trifluoromethyl)-10\(H\)-benzofuro[3,2-\(b\)]indole-1-carboxylic acid; DMSO, dimethyl sulfoxide; G-V, conductance-voltage; OAB, overactive bladder; OR, oocyte Ringer’s; \(P_{o}\), open probability; RFU, relative fluorescence unit; SHR, spontaneous hypertensive rat; UBSM, urinary bladder smooth muscle; \(V_{1/2}\), half-activation voltage; WKY, Wistar Kyoto
Abstract

The large-conductance calcium-activated potassium channel (BK<sub>Ca</sub> channel) plays critical roles in smooth muscle relaxation. In urinary bladder smooth muscle, BK<sub>Ca</sub> channel activity underlies the maintenance of the resting membrane potential and the repolarization of the spontaneous action potential triggering the phasic contraction. To identify novel BK<sub>Ca</sub> channel activators, we screened a library of natural compounds using a cell-based fluorescence assay and a hyperactive mutant BK<sub>Ca</sub> channel (Lee et al. 2013). From 794 natural compounds, kurarinone, a flavanone from <i>Sophora flavescens</i>, strongly potentiated BK<sub>Ca</sub> channels. When treated from the extracellular side, this compound progressively shifted the conductance-voltage relationship of BK<sub>Ca</sub> channels to more negative voltages and increased the maximum conductance in a dose-dependent manner. While kurarinone strongly potentiated the homomeric BK<sub>Ca</sub> channel composed of only the α subunit, its effects were much smaller on heteromeric channels co-assembled with auxiliary β subunits. While the activation kinetics were not altered significantly, the deactivation of BK<sub>Ca</sub> channels was dramatically slowed by kurarinone treatment. At the single-channel level, kurarinone increased the open probability of the BK<sub>Ca</sub> channel without affecting its single-channel conductance. Kurarinone potently relaxed acetylcholine-induced contraction of rat bladder smooth muscle, and thus decreased the micturition frequency of rats with overactive bladder symptoms. Thus, these results indicate that kurarinone can directly potentiate BK<sub>Ca</sub> channels and demonstrate the therapeutic potentials of kurarinone and its derivatives for developing anti-overactive bladder medications and/or supplements.
Introduction

The large-conductance Ca$^{2+}$-activated K$^{+}$ channel (BKCa channel) is activated by membrane depolarization and/or intracellular Ca$^{2+}$ and permeates K$^{+}$ ions across the cell membrane (reviewed in Cui et al. 2009; Yang et al. 2015). The BKCa channel is widely expressed in various types of excitable and non-excitatory cells, and is involved in the regulation of several important physiological processes including neurotransmitter release (Raffaelli et al. 2004), contraction of smooth muscle (Brenner et al. 2000; Herrera et al. 2000), and circadian behavioral rhythms (Meredith et al. 2006). Dysfunction of BKCa channels causes several diseases such as epilepsy (Lorenz et al. 2007; Du et al. 2005), erectile dysfunction (Werner et al. 2005), and overactive bladder (OAB) (Meredith et al. 2004).

OAB is a syndrome characterized by the presence of urinary urgency usually with increased day-time or night-time frequency (reviewed in Cerruto et al. 2012). OAB affects about 17% of the Western world population, both men and women, and its frequency increases with age (Coyne et al. 2013). There are several classes of medications to treat OAB that target different receptors in the bladder including antimuscarinics, mixed-action drugs, and β-adrenergic receptor agonists (Abraham et al. 2015). Although the mainstay of OAB treatment is antimuscarinic pharmacology, the decreasing efficacy and adverse reactions cause long-term compliance problems (Jayarajan et al. 2013). Thus, novel therapeutic methods for OAB that have direct effects on urinary bladder smooth muscle (UBSM) with fewer side effects need to be developed. Among emerging therapeutic targets for OAB, bladder K$^{+}$ channels have shown great potential in preclinical experiments. Thus far, however, activators or openers of the K$^{+}$ channels have yielded disappointing results in clinical research (Andersson et al. 2013).

The BKCa channel is one of the most physiologically important K$^{+}$ channels regulating UBSM function in health and disease (reviewed in Petkov et al. 2014). The BKCa channel is highly expressed in UBSM (Hristov et al. 2011). By being uniquely activated by both membrane depolarization and intracellular Ca$^{2+}$, BKCa channel activity underlies the maintenance of the resting membrane potential and the initial repolarization phase of the spontaneous action potential that triggers UBSM phasic contraction. Moreover, a series of studies reported that the BKCa channel significantly reduces cholinergic- and purinergic-induced contractility and suggested that expression of BKCa channels with altered function can contribute to OAB occurrence (Werner et al. 2007). Thus, it is possible to induce the relaxation of UBSM by chemically activating the endogenous BKCa channel. In fact, several activator compounds for BKCa channels have been reported for their relaxation effect on the urinary
bladder (dela Pena et al. 2009; Layne et al. 2010; Ahn et al. 2011; Park et al. 2014), further indicating the potential of this channel as a treatment target for OAB syndrome. However, the potency and specificity of BK_{Ca} channel activators remain a problem for their clinical usage (Nardi et al. 2006; Bentzen et al. 2014). Thus, there is a need to find novel BK_{Ca} channel activators of better efficacy with high specificity.

In the present study, we searched for novel natural activators of BK_{Ca} channels using a newly developed cell-based assay. Due to the greatly improved Ca^{2+} sensitivity of the mutant BK_{Ca} channel used in this assay, it was not necessary to increase the intracellular Ca^{2+} concentration for channel activation (Lee et al. 2013). Thus, we were able to utilize a thallium (Tl^{+})-based fluorescence assay, which is commercially available for voltage-gated K^{+} channels. By screening a library of natural compounds, we identified one natural compound, kurarinone (Fig. 2, inset) with a flavanone backbone, as a potent activator of BK_{Ca} channels. Isolated from Kushen, dried roots of *Sophora flavescens*, kurarinone activated BK_{Ca} channels in a dose-dependent manner from the extracellular side and strongly shifted the conductance-voltage (G-V) relationship of the channel to more negative voltages. At the single-channel level, kurarinone increased the open probability (P_{o}) of the channel without any effect on the single-channel conductance. Moreover, this compound significantly reduced acetylcholine (ACh)-induced contraction of rat urinary bladder strips and decreased the voiding frequency in a rat OAB model. Thus, we isolated a novel natural compound that activates BK_{Ca} channels and characterized its mode of activation and potential for anti-OAB activity.
Materials and Methods

Materials

A chemical library containing 794 natural compounds was obtained from the Korea Research Institute of Chemical Technology (KRICT, Daejeon, South Korea; www.chembank.org). Additional kurarinone and its derivatives were purified from Kushen, dried roots of *S. flavescens* (Jung et al. 2008). Kurarinone and other compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) as stock solutions. 4-Chloro-7-(trifluoromethyl)-10H-benzo[furo[3,2-b]indole-1-carboxylic acid (CTBIC) was also dissolved in DMSO.

Fluorescence Assay and Data Analysis

AD-293 cells, modified human embryonic kidney 293 cells stably expressing a mutant BKCa channel (G803D/N806K), were used for the cell-based assay (Lee et al. 2013). Cells were grown in Dulbecco’s Modified Eagle’s medium (Thermo, Waltham, MA) containing 10% fetal bovine serum (Thermo) and were selected with an antibiotic (1 mg/ml geneticin; Gibco/Life Technologies, Waltham, MA). Approximately 20,000 cells/well were seeded onto a 96-well clear-bottom, black-wall assay plate (Corning Incorporated, Corning, NY) coated with poly-D-lysine (Sigma-Aldrich). The FluxOR™ potassium channel assay (Invitrogen, Eugene, OR) was utilized for initial screening of the compound library and further analyses of candidate compounds. The experiments were performed following the manufacturer’s recommendations. Growth medium was replaced with 80 μl/well of loading buffer containing FluxOR™ fluorescent dye and incubated for 1 h in the dark. After incubation, loading buffer was replaced with 100 μl/well of assay buffer containing various concentrations of the compounds of interest and then incubated for 20–30 min. DMSO (1%) was used as a vehicle. CTBIC, a previously confirmed strong activator of BKCa channels (Gormemis et al. 2005; Lee et al. 2012), was used as a positive control. For fluorescence measurements, a Synergy H1 Hybrid multi-mode microplate reader (BioTek Instrument Inc., Winooski, VT) and Gen5 software were used for initial screening, and a FlexStation 3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA) and SoftMax® Pro software were used for additional assays. Fluorescence signals were acquired at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Membrane depolarization was induced by stimulus buffer containing Tl⁺ ions. Fluorescence signals were measured every 10 s for 2 min before treatment with stimulus buffer and then every
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10 s for 3 min after adding stimulus buffer for Synergy H1, and every 2 s for 20 s before treatment with stimulus buffer and then every 2 s for 160 s after adding stimulus buffer for FlexStation 3.

The change in fluorescence signals was analyzed by the relative fluorescence unit (RFU or F/F₀, where F₀ is the minimum fluorescence value of each fluorescence trace). To quantitatively compare the activation effects of kurarinone and its derivatives, the initial fluorescence increase was calculated using the first three points after treatment with stimulus buffer and the linear slope was estimated using OriginPro 9.1 (OriginLab Corp., Northampton, MA).

**Expression of a Functional BK_{Ca} Channel in Xenopus Oocytes**

*Xenopus laevis* oocytes were used for expression and electrophysiological recording of the rat BK_{Ca} channel α (Slo1), β1, and β4 subunits. Subcloning and functional expression of the rat BK_{Ca} channel α and β subunits using an oocyte expression vector (pNBC1.0 or pNBC2.0) were performed following previously described methods (Ha et al. 2006). The sequence information of these cDNAs is listed in GenBank under the accession numbers AF135265 (for the α subunit), FJ154955.1 (for the β1 subunit), and AY028605 (for the β4 subunit). Plasmid DNA were linearized using the NotI restriction enzyme, and then complementary RNA (cRNA) was synthesized from the linear forms of the plasmids using mMessage mMACHINE (Ambion, Austin, TX) supplemented with T7 RNA polymerase, nucleoside triphosphates, and the cap analog m7G(5')ppp(5')G.

Surgically obtained oocytes from ovarian lobes of *X. laevis* at stages V–VI (Xenopus One, Dexter, MI) were used for electrophysiological recording. After surgery, oocytes were transferred into Ca^{2+}-free oocyte Ringer’s (OR) culture medium (86 mM NaCl, 1.5 mM KCl, 2 mM MgCl₂, and 10 mM HEPES, pH 7.6) containing 3 mg/ml collagenases (Worthington Biochemicals, Freehold, NJ) and incubated for 1.5–2 h at room temperature to remove the follicular cell layer of oocytes. Thereafter, oocytes lacking the follicular cell layer were rinsed with Ca^{2+}-free OR medium and ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 50 g/ml gentamicin, pH 7.6). Washed oocytes were kept in ND-96 medium at 18°C. Oocytes were stabilized for at least 1 day before use. After stabilization, each oocyte was injected with approximately 50 ng of synthesized cRNA (prepared in 50 nl of nuclease-free water) for macroscopic current recordings and with 1 ng of synthesized cRNA (prepared in 50 nl of nuclease-free water) for single-channel recordings using a microdispenser (Drummond Scientific, Broomall, PA). In the coexpression experiments, β1
and β4 subunit RNAs were injected at a molar ratio to α subunit RNA of 12:1 to guarantee sufficient co-
assembly with the α subunit. After cRNA injection, oocytes were incubated for 1–3 days in ND-96 medium at
18°C. The vitelline membrane of oocytes was completely removed using fine forceps immediately before the
patch-clamp experiment.

**Electrophysiological Recordings and Data Analysis**

All macroscopic current recordings and single-channel recordings were performed using the gigaohm-seal
patch-clamp method in an outside-out configuration as described previously (Ha et al. 2006). In patch-clamp
experiments, glass pipettes fabricated from borosilicate glass (WPI, Sarasota, FL) and fire-polished to a
resistance of 3–5 MΩ were used. For single-channel recordings, patch pipettes were fire-polished to a resistance
of 4–8 MΩ. For electrical noise-reducing, the fore part of the patch pipettes was coated with beeswax. In the
recordings, the Axopatch 200B amplifier (Molecular Devices) was used to amplify the channel currents,
currents were low-pass filtered at 1 kHz using a four-pole Bessel filter, and currents were digitized at a rate of
10 or 20 points/ms using a Digidata 1200A (Molecular Devices).

In macroscopic current recordings, currents of BKCa channels were activated by voltage-clamp pulses ranging
from -80 to 200 mV in 10 mV increments when the resting potential was held at -100 mV. In single-channel
experiments, a single BKCa channel was readily activated by briefly delivering membrane potentials up to 150
mV before the recording. To analyze the single-channel recording, the threshold amplitude was set at half the
unitary current amplitude, and the open and closed states of the channel were determined. The mean amplitudes
of the unitary currents were estimated from histograms fitted with Gaussian distributions. Then, the mean
amplitudes were plotted against the transmembrane voltage, and the slope conductance of the linear regression
from each point was obtained. The distributions of the dwell-times of the open and closed states recorded for
single BKCa channels were obtained in a linear histogram using Clampfit (Molecular Devices) as described in a
previous study (Szoszkiewicz et al. 2008). The dwell-time distributions were fitted with a single-exponential
function using simplex-least-squares fitting methods (Clampfit), and the exponential time-constant (mean open
or closed time) was estimated.

The recording solutions contained 120 mM potassium gluconate, 10 mM HEPES, 4 mM KCl, and 5 mM
EGTA, pH 7.2. In this solution, gluconates can block the activation of endogenous calcium-activated chloride
channels. To calculate the amount of Ca\(^{2+}\) to add to the intracellular solution in order to achieve each free [Ca\(^{2+}\)], the MaxChelator program (Patton et al. 2004; http://maxchelator.stanford.edu/) was used. For data acquisition and analysis of both macroscopic and single-channel currents, commercial software packages such as Clampex 8.0 and Origin 9.1 (OriginLab Corp.) were used. Data were summarized as means ± S.E. (n = number of independent recordings) and compared using the paired Student’s t-test. A p-value of <0.05 was considered statistically significant.

**Isometric Tension Recording of UBSM**

Isometric tension recording of UBSM experiments was conducted as previously described (dela Peña et al. 2009; Kullmann et al. 2014). Briefly, male Sprague-Dawley rats (300–350 g) were euthanized by CO\(_2\) asphyxiation. Then, the urinary bladder was excised and divided longitudinally into four strips (approximately 2 × 8 mm). The isolated strips were clipped between a stationary mount and a force-displacement transducer and suspended in a temperature-controlled (37°C) organ bath containing 10 ml of Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 25.0 mM NaHCO\(_3\), 2.5 mM CaCl\(_2\), and 12.2 mM glucose; pH 7.35–7.40). The organ baths were continually bubbled with a mixture of 95% O\(_2\) and 5% CO\(_2\). Each UBSM strip was stretched to an optimal isometric tension of 1.0 g and equilibrated for 60 min. During the equilibration period, tissues were washed with fresh Krebs solution every 15 min and the basal tension was adjusted to 1.0 g. After equilibration, the strips were stabilized by repeated applications of ACh (1 μM) until constant responses were recorded. To investigate the relaxation effect of kurarinone, the tissues were preincubated for 30 min with kurarinone prior to the addition of ACh, and then ACh-induced contractile responses were repeated in the presence of kurarinone. The relaxation was expressed as a percentage of the decrease in the tension resulting from ACh-induced contractions. One strip in each series was allocated as the time control. Changes in isometric tension were recorded using a Power Lab Data Acquisition System (AD Instruments, Sydney, Australia) attached to a computer installed with Lab Chart Software (Version 7, AD Instruments). Data were summarized as means ± S.E. (n = number of detrusor smooth muscle strips) and compared using the paired Student’s t-test. A p-value of <0.05 was considered statistically significant.

**Voiding Behavior in Rats**
Spontaneously hypertensive rats (SHRs), as an OAB animal model, were used for the voiding behavior assay (Clemow et al., 1998; Persson et al., 1998; Patra et al., 2007; Jin et al., 2009). Wistar Kyoto (WKY) rats were used as control animals. WKY rats and SHRs weighing 300–350 g were used for behavior experiments. For experiments, animals were deprived of water for 15 h and fed 0.9% NaCl solution for 2 h. Afterwards, animals were placed in individual metabolic cages and had free access to saline solution and food. Animals were randomly separated into the control group and kurarinone group. Animals in the kurarinone group were orally administered 0.5 or 5 mg/kg kurarinone. The voiding behavior of the animals over 3 h was observed by an experimenter. After 3 h of observation, the total fluid intake and urine output were measured. Data are summarized as means ± S.E. (n = number of animals) and compared using the paired Student’s t-test. A p-value of <0.05 was considered statistically significant.
Results

Identification of BKCa Channel Activators Using a Tl⁺ Fluorescence Assay

To identify novel BKCa channel activators, 794 single compounds purified from natural sources were screened using a cell-based assay employing Tl⁺ fluorescence. At a final concentration of 5 μM, several compounds significantly increased Tl⁺ fluorescence compared with the vehicle (1% DMSO) (Fig. 1A). Among the compounds, the strongest fluorescence increase resulted from treatment with kurarinone (Fig. 2, inset), a flavanone compound from S. flavescens. Kurarinone increased Tl⁺ fluorescence in a dose-dependent manner (Fig. 2A), and the fluorescence increase was completely blocked by co-treatment with 1 μM paxilline, a selective BKCa channel inhibitor (Sanchez et al., 1996). Paxilline also completely blocked the fluorescence increase induced by higher concentrations of kurarinone (Supplemental Figure 1). The initial RFU increase induced by different concentrations of kurarinone is quantified in Figure 2B (n=4). The initial RFU increase induced by 5 μM kurarinone was approximately 70% of that induced by 5 μM CTBIC, the positive control (Supplemental Figure 2).

Kurarinone is a natural flavanone compound; therefore, we tested several related flavonoid compounds (Fig. 3A). Treatment with these compounds at a concentration of 10 μM (n=4) rendered differential effects on the Tl⁺ fluorescence increase. While kurarinone, leachianone G, and naringenin strongly increased fluorescence, other derivatives including kurarinol displayed much weaker effects. It is worth noting that kurarinol containing only an additional hydroxyl group in the aliphatic chain at the 8-position of the flavanone backbone showed a dramatic decrease in its efficacy. Because kurarinone showed the strongest increase in the initial Tl⁺-based fluorescence assay among the compounds tested, the subsequent functional studies were performed using this compound.

Effects of Kurarinone on Macroscopic Currents of the Cloned BKCa Channel

Although the cell-based Tl⁺ fluorescence assay was suited for high-throughput screening and provided initial candidates of BKCa channel activators, the activity of each compound needed to be validated and characterized using direct electrophysiology with the wild-type BKCa channel. Thus, we characterized the effects of kurarinone on the α subunit of the rat BKCa channel (rSlo1) heterologously expressed in Xenopus oocytes. The time-dependent effects on macroscopic channel currents were monitored using excised membrane patches.
in an outside-out configuration in the presence of 3 µM intracellular Ca\(^{2+}\) (Fig. 4). While small tail currents were evoked by each test pulse (Fig. 4, a), addition of 5 µM kurarinone to the extracellular side greatly potentiated the tail current (Fig. 4, b and c) in a time-dependent manner. Upon removal of kurarinone, the channel currents were gradually de-potentiated to the basal level (Fig. 4, d). It is worth noting that both the potentiation and de-potentiation of BK\(_{Ca}\) channels by kurarinone exhibited two phases, namely, the initial rapid increase within a few seconds, and the slower and steady increase that took minutes. When fitted with a double-exponential function, the association time-constant values were estimated as 1.47 ± 0.34 s for the fast phase (\(\tau_{fast}\)) and 64.9 ± 8.8 s for the slow phase (\(\tau_{slow}\)). Although two different phases were evident, the de-potentiation of BK\(_{Ca}\) channels took much longer than the potentiation, with the dissociation time-constants estimated as 2.78 ± 0.95 s and 90.06 ± 12.21 s, respectively. No significant change in the BK\(_{Ca}\) channel current was observed when kurarinone was added to the intracellular side using excised inside-out patch recording (data not shown). Thus, these results indicate that kurarinone can potentiate the activity of BK\(_{Ca}\) channels directly and reversibly from the extracellular side.

**Effects of Kurarinone on Macroscopic Currents of the BK\(_{Ca}\) Channel at Different Intracellular Ca\(^{2+}\) Concentrations**

We then studied the mechanism of kurarinone-induced potentiation of BK\(_{Ca}\) channels. BK\(_{Ca}\) channels were activated by a series of voltage pulses, and the macroscopic currents were recorded in the presence of increasing concentrations of extracellular kurarinone at different intracellular Ca\(^{2+}\) concentrations. The channel currents were activated at lower voltages and deactivated much more slowly as the concentration of kurarinone increased (Fig. 5A). In Figure 5B–F, the voltage-dependent activation of macroscopic BK\(_{Ca}\) channel currents is shown as the \(G-V\) relationship. Kurarinone progressively shifted the \(G-V\) curve to the left and increased the maximum conductance (\(G_{max}\)) in a dose-dependent manner at all intracellular Ca\(^{2+}\) concentrations. The kurarinone-dependent shift in the \(G-V\) relationship was further quantified and plotted in Figure 5F. In the presence of 20 µM kurarinone and 3 µM Ca\(^{2+}\), the half-activation voltage (\(V_{1/2}\)) shifted approximately 80 mV in the negative direction, from 107.4 ± 2.2 mV to 27.7 ± 3.0 mV, and a similar decline in \(V_{1/2}\) was observed at each intracellular Ca\(^{2+}\) concentration. Kurarinone also gradually increased the maximum conductance (\(G/G_{max}\)) of the channel in the presence of 20 µM kurarinone. The \(G_{max}\) was increased up to 16-fold by treatment with 20 µM kurarinone at [Ca\(^{2+}\)] of 1 µM under a membrane voltage of 150 mV. Thus, these results demonstrate that kurarinone
potentiates the BK$_{Ca}$ channel by activating it at more negative membrane voltages and increasing the maximum $P_o$ of the channel.

**Effects of β Subunits on Kurarinone-induced Potentiation of the BK$_{Ca}$ Channel**

The functional characteristics of BK$_{Ca}$ channels are altered by the auxiliary β subunits, and the efficacy of channel modulators can also be affected by the presence of β subunits. In Figure 6, the effect of kurarinone was compared between homomeric and heteromeric BK$_{Ca}$ channels. Oocytes were injected with cRNA for the rat α subunit (rSlo) alone or together with cRNA for either the β1 or β4 subunit. The co-assembly of α and β subunits was evident by the slower activation of channel currents. We then treated the extracellular side of membranes expressing homomeric (rSlo) or heteromeric (rSlo/rβ1 and rSlo/rβ4) BK$_{Ca}$ channels with kurarinone. While 20 µM kurarinone shifted the $G$-$V$ relationship of homomeric channels by approximately 80 mV in the negative direction, the effects were much smaller on heteromeric channels (-19.9 mV for rSlo/rβ1 and -22.8 mV for rSlo/rβ4; Fig. 6B; n=4). However, kurarinone still delayed heteromeric channel deactivation when the tail currents were compared between the vehicle-treated and 20 µM kurarinone-treated traces.

**Effects of Kurarinone on the Activation and Deactivation Kinetics of the BK$_{Ca}$ Channel**

As shown in the macroscopic current traces in Figure 5A, the deactivation of BK$_{Ca}$ channels appeared to be greatly affected by kurarinone. Thus, we investigated the effects of kurarinone on the gating kinetics of BK$_{Ca}$ channels in detail. In Figure 6, the activation (or opening) and deactivation (or closing) of BK$_{Ca}$ channels were analyzed. While the current level was increased in the presence of 20 µM kurarinone, the activation rate did not seem to change considerably (Fig. 7A). In fact, the activation time-constant ($\tau_{activation}$) was not increased significantly at three increasing concentrations of kurarinone (5, 10, and 20 µM) (Fig. 7C). On the other hand, the deactivation rate was dramatically decreased by kurarinone. Intriguingly, the slowing of deactivation by kurarinone was voltage-dependent in that kurarinone slowed the closure of the BK$_{Ca}$ channel more prominently when the channel had been activated by more positive voltages. Collectively, these results suggest that kurarinone stabilizes the open conformation of the BK$_{Ca}$ channel, and the binding affinity of kurarinone may be stronger with the channel conformation at a higher voltage.
Effects of Kurarinone on Single-Channel Currents of the BKCa Channel

To confirm and better understand the mechanism of kurarinone action, its effects were monitored at the single-channel level. Single BKCa channels were recorded in outside-out patches in the presence of 10 μM intracellular Ca2+. The membrane voltage was depolarized initially to higher than 80 mV in order to activate BKCa channels, and the number of channels in the patch membrane was counted. In this recording, patches containing only a single channel were used. Representative traces of single-channel recordings in the absence or presence of 5 μM kurarinone are shown in Figure 8A. As expected, the opening of single BKCa channels was increased by depolarizing membrane voltages. However, when 5 μM kurarinone was supplied to the extracellular side, the gating behavior was dramatically altered. While the BKCa channel rarely opened in the control solution at -25 mV, more frequent opening of the channel was evident upon application of kurarinone. At 50 mV, the channel stayed open for a prolonged period of time in the presence of kurarinone. The effects of kurarinone on single-channel conductance were also investigated by measuring the unitary current amplitudes of single BKCa channels at different membrane voltages in the absence and presence of the compound. Figure 8B shows the single-channel current-voltage relationships. The single-channel conductance was estimated as 221.1 ± 16.4 pS in the control and 238.3 ± 8.7 pS in the presence of kurarinone, indicating that the compound did not significantly alter the single-channel conductance of the channel. The effects of kurarinone on the Po of single channels were also analyzed. The Po was measured at several different voltages in the absence and presence of kurarinone and then fitted with a Boltzmann function (Fig. 8C). The voltage required for half-maximum opening, V1/2, was estimated as 68.7 ± 3.7 mV in the control and 43.7 ± 2.1 mV in the presence of 5 μM kurarinone (Fig. 8D). These results indicate that kurarinone can potentiate BKCa channels by increasing the Po of the channel without affecting the single-channel conductance, in good agreement with the aforementioned findings regarding macroscopic channel currents. We then analyzed the gating behavior of single BKCa channels in the presence of kurarinone. Because kurarinone increased the Po of single BKCa channels so dramatically, we restricted our analyses to single-channel recordings at 50 mV, at which open-close transitions could be compared in a reasonable time-scale (Fig. 8D). While the mean closed time was determined as 3.69 ± 0.80 ms and 3.60 ± 0.41 ms in the absence and presence of 5 μM kurarinone, respectively, the mean open times were estimated as 2.98 ± 0.34 ms and 5.77 ± 0.90 ms, respectively (Fig. 8E). These results suggest that the binding of kurarinone stabilizes the open conformation and thus decreases the closing rate of the channel without significantly affecting its opening transitions, further corroborating the results of the macroscopic current...
recordings.

Effect of Kurarinone on Rat Urinary Bladder Tissue

Kurarinone strongly potentiates a cloned BKCa channel expressed in a heterologous system; therefore, we wondered whether this natural compound can also relax bladder smooth muscle in vivo. To check the efficacy of kurarinone on ACh-induced contraction of UBSM, the isometric tension of rat detrusor muscle strips was recorded. While 1 μM ACh induced a peak tension followed by a decline to relatively stable plateau levels (Fig. 8A), pretreatment of tissues with kurarinone significantly inhibited ACh-induced contraction. The relaxant effect was 58.2% ± 6.2% with 100 μM kurarinone compared with vehicle treatment (p<0.05, n=6) (Fig. 8B). By contrast, there was no significant change in the contractile response in vehicle-treated time-matched control tissues.

Effect of Kurarinone on Micturition Behavior of WKY Rats and SHRs

To further validate the effects of kurarinone on bladder relaxation and voiding activity, the voiding behavior of WKY rats and SHRs, an animal model of OAB, was monitored. The cumulative voiding frequencies of WKY rats and SHRs orally administered kurarinone are shown in Figure 9A. A distinct difference in the voiding frequency was evident between WKY rats and SHRs upon kurarinone administration. While the voiding frequency of control WKY rats was not affected by kurarinone administration up to a dosage of 5 mg/kg, the compound reduced the voiding frequency of SHRs in a dose-dependent manner. The total voiding frequencies over 3 h are plotted in Figure 9B. Upon administration of 5 mg/kg kurarinone, a significant reduction in voiding frequency was observed in SHRs (10.9 ± 1.4 for vehicle and 6.9 ± 0.8 for kurarinone). No such reduction in voiding frequency was observed in WKY control rats. Together with the ex vivo isometric tension recording, these results further indicate that kurarinone has a bladder-relaxing activity by potentiating the detrusor muscle BKCa channel and is a valid candidate compound for improving OAB syndrome.
Discussion

In this study, we present the new $\text{BK}_{\text{Ca}}$ channel activators of plant flavonoids. In the screening of a library of natural compounds, we utilized a cell-based assay for the $\text{BK}_{\text{Ca}}$ channel that we developed recently (Lee et al. 2013). Using a Tl$^+$-based fluorescence assay that is commercially available for voltage-gated K$^+$ channels, we were able to screen a large number of compounds rapidly and robustly. Among the 794 single natural compounds tested, kurarinone, a plant flavanone, showed the strongest increase in Tl$^+$-induced fluorescence (Fig. 1).

Although kurarinone is the most abundant flavanone in the root of the medicinal herb $S. flavescens$, this plant also produces other flavonoids. Thus, the potentiation effects on the $\text{BK}_{\text{Ca}}$ channel were tested using natural compounds of flavanone and flavone backbones from $S. flavescens$ and other sources (Fig. 3). In a comparison of kurarinone, sophoraflavanone G, and lehmannin, the 5-methoxy group appears to be essential because changing it to a hydroxyl (sophoraflavanone G) or hydrogen (lehmannin) group significantly decreased the activity. In addition, the complete loss of activity in the case of hydroxylation of 8-lavandulyl, as in the structure of kurarinol, indicates that activity is dramatically perturbed by a hydrophilic substitution in this position. However, other derivatives with an 8-hydrogen or 8-prenyl group (such as naringenin and leachianone G, respectively) evoked considerable activation of $\text{BK}_{\text{Ca}}$ channels, even with a 5-hydroxyl group instead of the 5-methoxy group. One of the important modes of biological action of these flavanone derivatives seems to be related to the geometry of the flavonoid skeleton because the 3-hydroxyflavone derivatives kushenol C and morin showed a dramatic loss of activity. Thus, the difference in the bond angle between the dihydroxyphenyl groups versus flavanone and 3-hydroxyflavone rings (e.g., naringenin and morin) might distinguish the functional efficacy of channel activation. These results provide important information about the structure-activity relationship of various natural flavanones in potentiating $\text{BK}_{\text{Ca}}$ channels, and guide future studies for identifying and modifying flavanone compounds. As suggested by a previous study (Saponara et al. 2006), it is encouraging to find that naringenin, a predominant flavanone in grapefruit, shows an appreciable potentiation of $\text{BK}_{\text{Ca}}$ channel activity because the beneficial effects of this compound are well recognized as an antioxidant, a free radical scavenger, and an immune modulator (Cavia-Saiz et al. 2010; Du et al. 2009; Yilma et al. 2013).

Kurarinone potentiated the $\text{BK}_{\text{Ca}}$ channel currents in a dose-dependent manner from the extracellular side (Fig. 2). While kurarinone-induced channel activation was reversible, both the potentiation and de-potentiation showed two different components (Fig. 4). Because only the $\alpha$ subunit of the $\text{BK}_{\text{Ca}}$ channel (Slo1)
was expressed in Xenopus oocytes and the functional BK_{Ca} channel is assembled as a homo-tetramer, the two different time-components indicate either that there are two distinct binding sites, with a high and low affinity, of kurarinone in the functional channels or that the rapid initial binding of kurarinone to a single site can be stabilized by a subsequent conformational change over a slower time course. The binding site of kurarinone on BK_{Ca} channels and the exact mechanism of action remain to be further elucidated. Although numerous potent activators of BK_{Ca} channels have been reported, the binding site(s) has been localized for only a single compound with a benzofuroindole backbone (Lee et al. 2012). Kurarinone did not compete with a benzofuroindole, CTBIC, and a double-mutant BK_{Ca} channel (T273A/W275A) with a lower affinity for CTBIC did not significantly affect the potentiation (data not shown), strongly suggesting that kurarinone interacts at a binding site(s) distinct from that of benzofuroindoles. This suggestion is supported by the fact that, unlike benzofuroindoles that potentiate the homomeric and heteromeric channels equally (Ha et al. 2006), the effect of kurarinone was much weaker on heteromeric BK_{Ca} channels containing the β1 or β4 subunit.

At the macroscopic level, kurarinone potentiated the BK_{Ca} channel current in two different respects (Fig. 5). Kurarinone progressively shifted the G-V relationship of the BK_{Ca} channel, indicating that the binding of kurarinone stabilizes the open conformation of the channel. In the presence of 20 µM kurarinone at a [Ca^{2+}]_{i} of 3 µM, the G-V relationship shifted nearly 80 mV in the negative direction, corresponding to 3.39 kcal/mol stabilization of the open conformation by kurarinone binding. Because the V_{1/2} shift was nearly linear in the concentration range of kurarinone tested and did not show any sign of saturation even at 20 µM, the highest concentration feasible for electrophysiological recording (Fig. 5G), it was not possible to estimate the affinity of the compound in terms of the apparent dissociation constant (K_{d}^{app}) using V_{1/2} value shifts. The stabilizing effect of kurarinone on the open conformation of the channel was further validated at the single-channel level. In the presence of 5 µM kurarinone at 50 mV, the mean open time of single BK_{Ca} channels was increased 1.93-fold without any significant changes in their mean closed time. Because the reciprocal values of the mean open and closed times represent the closing and opening rates of the channel, respectively, these results indicate that single BK_{Ca} channels close 1.93-fold more slowly in the presence of kurarinone under the given conditions. In addition to the V_{1/2} shifts, kurarinone also increased the G_{max} of the channel, suggesting an increase in the maximum P_{o} of the channel at extreme positive voltages. The most dramatic effect of kurarinone on macroscopic BK_{Ca} channel currents was the deactivation kinetics of the channel. Kurarinone greatly decreased the deactivation rate (or closing rate) of macroscopic BK_{Ca} channels. It is intriguing that the slowing of
deactivation was also voltage-dependent (6.3-fold at 60 mV up to 19.3-fold at 200 mV). Given that kurarinone does not have any fixed charge, this result strongly suggests that kurarinone binds more tightly to the channel conformation induced at higher membrane voltages. Potentiation of BK$_{Ca}$ channels by kurarinone was also evident at the single-channel level. The $P_o$ of kurarinone significantly increased at all voltages tested without altering the single-channel conductance. At 25 mV, $P_o$ increased 17.5-fold (from 0.02 to 0.35) by treatment with 5 µM kurarinone.

In UBSM, BK$_{Ca}$ channel activity underlies the maintenance of the resting membrane potential and the initial repolarization phase of the spontaneous action potential triggering phasic contraction. Thus, we tested whether kurarinone, a potent activator of the BK$_{Ca}$ channel, can evoke relaxation of UBSM and thus affect micturition function. As expected, pretreatment with kurarinone resulted in relaxation of ACh-evoked contraction of rat UBSM strips. Upon pretreatment with 100 µM kurarinone, the muscle tension evoked by ACh was decreased to almost 60% compared with the control (Fig. 8). Moreover, when tested in a rat model of OAB, kurarinone also significantly improved micturition function. Oral administration of 5 mg/kg kurarinone significantly decreased the voiding frequency of SHRs nearly to the level of control WKY rats (Fig. 9). It is worth noting that the voiding frequency of WKY rats was not affected by kurarinone even at a dose of 5 mg/kg. The excitability and contractility of the normal bladder upon kurarinone treatment remain to be investigated in detail.

Isolated from the root of the medicinal herb *S. flavescens*, kurarinone has been studied for its anti-cancer and anti-inflammatory effects. Kurarinone has potent cytotoxic activity against human breast cancer cells (De Naeyer et al. 2004). This compound also inhibits the chemotactic migration of monocytes (Lee et al. 2005) and suppresses the differentiation of CD4$^+$ T cells (Kim et al., 2013). Kurarinone promotes apoptosis induced by tumor necrosis factor-inducing ligand (Seo et al. 2012). Thus, kurarinone and its derivatives have a high therapeutic potential and this study provides another target of these compounds.

In summary, by the successful application of a newly developed cell-based assay, we identified kurarinone and two other flavonoids as potent natural activators of the BK$_{Ca}$ channel. By characterizing the mechanism of action, we were able to show that kurarinone greatly potentiated the BK$_{Ca}$ channel by stabilizing its open conformation and thus slowing its closure. We also demonstrated the relaxation effect of kurarinone on UBSM and the beneficial effect of kurarinone on voiding behavior in rats with OAB symptoms. Thus, these results demonstrate the therapeutic potentials of kurarinone and its derivatives for developing anti-OAB
medications and/or supplements.
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Authorship Contributions

Participated in the conception and design of the study: S. Lee, B.C. Lee, and Park.

Interpretation of data and preparation of the manuscript: S. Lee, Kim, Cheong, and Park.

Acquisition and analysis of data: S. Lee, Chae, Cheong, S.W. Lee, and Park.

Preparation of kurarinone and other compounds isolated from Kushen: Choi.
References


succinyl macrolactin A against intestinal inflammation is mediated through PI3-kinase/Akt/mTOR and NF-kappaB signaling pathways. *Eur J Pharmacol* 735C: 184-192


Footnotes

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Figure Legends

Figure 1. Screening of BK_{Ca} channel activators using a cell-based fluorescence assay.

A library of natural compounds was screened for novel BK_{Ca} channel activators. AD-293 cells stably expressing a hyperactive mutant BK_{Ca} channel (G803D/N806K) were used for a Tl\(^+\)-based fluorescence (FluxOR\(^{TM}\)) assay. A. Representative traces of fluorescence change are shown for five different compounds as RFU. Each compound was added to each test well at a final concentration of 5 \(\mu\)M before the experiments, and stimulus buffer was added at 120 s. DMSO (■, 1%, vehicle) and kurarinone (●) are highlighted by filled symbols. The RFU value (B) and fold increase compared with DMSO (C) obtained at 240 s are shown for 14 different compounds. Kurarinone is highlighted in gray.

Figure 2. Concentration-dependent increase in fluorescence signals after kurarinone treatment.

AD-293 cells stably expressing the mutant BK_{Ca} channel were treated with different concentrations of kurarinone. A. Representative fluorescence traces. After obtaining the base line for 20 s, Tl\(^+\)-containing stimulus buffer was added. Cells were incubated in 1% DMSO (□) as a vehicle or in the presence of different concentrations of kurarinone (■, 3 \(\mu\)M; ●, 5 \(\mu\)M; ▲, 10 \(\mu\)M; and ▼, 30 \(\mu\)M). Cells were also incubated with 1 \(\mu\)M paxilline, a BK_{Ca} channel blocker, together with kurarinone (5 \(\mu\)M) (◆). B. Initial RFU increase at different concentrations of kurarinone. Error bars (S.E.M.) are indicated. Inset, chemical structure of kurarinone.

Figure 3. Structure-activity relationship study of flavonoid derivatives.

A. Structures of flavonoids used in the study. B. Representative traces of RFU upon treatment with each compound at a concentration of 10 \(\mu\)M. C. Initial RFU increase in the presence of each compound at a concentration of 10 \(\mu\)M. Error bars (S.E.M.) are indicated.

Figure 4. Reversible potentiation of macroscopic BK_{Ca} channel currents by kurarinone.

A representative diary plot of tail currents evoked by BK_{Ca} channels is shown as a continuous recording. The
channel currents were activated every second with 50 ms pre-pulses of 100 mV from the holding voltage of -100 mV, and the tail currents were evoked by step-pulses back to -100 mV. Tail current values were acquired at 0.8 ms after the initiation of -100 mV step-pulses. Each representative current trace (a–d) shows the current at the points indicated by arrows.

**Figure 5. Effects of kurarinone on current-voltage and G-V relationships of macroscopic BKCa channel currents at different intracellular Ca^{2+} concentrations.**

A. Representative traces of BKCa channel currents at a [Ca^{2+}]i of 0.3 μM are shown for different kurarinone concentrations. Ionic currents were elicited with 100 ms voltage step-pulses. Currents were recorded from -80 mV to 200 mV in 10 mV increments. The holding voltage was -100 mV. B. Effects of kurarinone on the G-V relationship at a [Ca^{2+}]i of 0.3 μM. Conductance was obtained from the peak tail currents. All currents were normalized by the maximum current of the 20 μM trace. Each symbol represents conductance at different kurarinone concentrations as follows: vehicle (■), 3 μM (●), 5 μM (▲), 10 μM (▼), and 20 μM (◆) (n=5). At each Ca^{2+} concentration, the same symbols were used to represent each kurarinone concentration. C. Effects of kurarinone on the G-V relationship at a [Ca^{2+}]i of 1 μM (n=6). D. Effects of kurarinone on the G-V relationship at a [Ca^{2+}]i of 3 μM. Vehicle (n=12), 3 μM (n=8), 5 μM (n=12), 10 μM (n=8), and 20 μM (n=4). E. Effects of kurarinone on the G-V relationship at a [Ca^{2+}]i of 10 μM, n=5. F. Effects of kurarinone on the G-V relationship at a [Ca^{2+}]i of 30 μM, n=5. G. Effects of kurarinone on V_{1/2}. Each symbol in the graph represents the mean and S.E.M. of V_{1/2} at different [Ca^{2+}], as follows: 0.3 μM (■), 1 μM (●), 3 μM (▲), 10 μM (▼), and 20 μM (◆). For statistical analyses, each experimental data set was independently fitted using the Boltzmann function, \( G/G_{\text{Max}} = \frac{(G_{\text{Max}} - G_{\text{Min}})(1 + \exp[(V_{1/2} - V)/k])}{1 + \exp[(V_{1/2} - V)/k]} + G_{\text{Min}} \), and the mean ± S.E.M. values of V_{1/2} and k were obtained. The k is the same as RT/zF, where R is the gas constant, T is temperature, F is the Faraday constant, and z is gating charge. RT/F was calculated as -26 in this experiment; therefore, k can be regarded as -26/z. In the graph, experimental data points were co-plotted with the Boltzmann function using V_{1/2} and k obtained from the statistical analyses (B–F).

**Figure 6. Effect of β subunits on kurarinone-induced potentiation of BKCa channel currents.**
Recordings were performed at a $[\text{Ca}^{2+}]_i$ of 3 μM with the rat BK$_{Ca}$ channel α subunit (rSlo) (A), rSlo with the rat β1 subunit (rβ1) (B), and rSlo with the rat β4 subunit (rβ4) (C). The traces on the first and second of each figure show representative traces at 200 mV in the absence and presence of kurarinone (20 μM), respectively. Ionic currents were elicited with 100 ms step-pulses when the resting potential was -100 mV. The graphs represent the G-V relationship in the absence (□) and presence (■, 20 μM) of kurarinone. Each data point represents mean ± S.E.M. (n=5).

Figure 7. Effect of kurarinone on activation and deactivation of macroscopic BK$_{Ca}$ currents.

**A**, **B**. Representative traces of activation (A) and deactivation (B) when vehicle (black) or 20 μM kurarinone (gray) was added. Current traces obtained at 100 mV were compared. **C**, **D**. Activation and deactivation time-constant values ($\tau$) at different concentrations of kurarinone. Symbols represent vehicle (□, n=12), 3 μM (■, n=8), 5 μM (●, n=12), 10 μM (▲, n=8), and 20 μM (◆, n=4). The time-constant values were obtained from fitting every independent data set using the exponential standard function ($y(t) = A_1 \exp(-t/\tau_1) + C$) with the Clampfit program.

Figure 8. Effect of kurarinone on single BK$_{Ca}$ channels.

**A**. Each graph shows typical single-channel current recordings of BK$_{Ca}$ channels at different membrane voltages. The concentration of intracellular Ca$^{2+}$ (pipette Ca$^{2+}$ concentration) was fixed at 10 μM. Currents were continuously recorded at different voltages initially in the absence of kurarinone and subsequently in the presence of kurarinone (5 μM). A solution containing kurarinone was perfused on the extracellular side. The solid lines represent the closed level and the dotted lines represent the open level of single BK$_{Ca}$ channels. **B**. Effects of kurarinone on single-channel conductance. The unitary current amplitude of the channel was determined using 10 μM intracellular Ca$^{2+}$ solution. The channel current was initially recorded in the absence of kurarinone (○), and then 5 μM kurarinone (●) was perfused. Membrane voltages were 75 mV (n=4), 50 mV (n=5), 25 mV (n=5), -25 mV (n=3), and -50 mV (n=2). Data presented in the graphs were obtained from all-points amplitude histograms fitted using the Gaussian function. The single-channel conductance was estimated using the slope fitted with the linear function. **C**. Effects of kurarinone on the voltage-dependent $P_o$ of single
BK<sub>Ca</sub> channels. \( P_o \) was measured using the same trace as in B in the absence of kurarinone (○) and in the presence of 5 µM kurarinone (●). Data points were fitted with the Boltzmann equation (\( P_o = \frac{[A_2+(A_1-A_2)]}{(1 + \exp\{(-V-V_{1/2})/k\})} \)). In the vehicle control, \( A_1=0.0002, A_2=0.64044, V_{1/2}=55.71185 \), and the slope factor \( k=9.03 \). Upon treatment with 5 µM kurarinone, \( A_1=-0.01278, A_2=0.95091, V_{1/2}=40.32 \), and \( k=21.98 \). D. Representative single-channel currents of BK<sub>Ca</sub> channels in an expanded time-scale in the absence (upper trace) and presence (lower trace) of 5 µM kurarinone at 50 mV. E. Effects of kurarinone on the mean open time and mean closed time of single BK<sub>Ca</sub> channels in the absence (empty bar) and presence (filled bar) of 5 µM kurarinone at 50 mV. Each bar graph represents mean ± S.E.M. (n=5).

Figure 9. Effect of kurarinone on the contractions induced by ACh in isolated rat urinary bladder strips.

A. Representative contraction trace induced by ACh without or with preincubation with kurarinone. B. Percentage relaxation of ACh-induced contraction induced by kurarinone based on the peak of ACh-induced contraction. Each bar graph represents mean ± S.E.M. of six experiments. Black denotes the control and white denotes the kurarinone treatment experiment.

Figure 10. Effect of kurarinone on the voiding behavior of rats.

A. Effect of kurarinone on the voiding frequency of WKY rats and SHRs after oral administration of kurarinone (0.5 and 5 mg/kg). Voiding frequency was observed over 3 h. B. Total numbers of voids. Each symbol or bar represents the mean ± S.E.M. of five (WKY rats) or seven (SHRs) animals (***, \( p<0.001 \)).
Figure 2

A

Fluorescence (RFU)

Time (s)

DMSO

3 μM

5 μM

10 μM

30 μM

Kurarinone 5 μM + Paxilline 1 μM

B

Initial RFU slope (RFU/s)

0.00

0.04

0.08

0.12

0.16

DMSO

3 μM

5 μM

10 μM

30 μM

Kurarinone
Figure 6

A  
Vehicle  
20 μM Kurarinone

B  
rslo

C  
rslo/rb1
Figure 9

A

![Graph showing relaxation response](image)

Control

Kurarinone 100 μM

B

![Bar graph showing relaxation](image)

Relaxation (%)

Control

Kurarinone

***
Figure 10

**A**

- **WKY: vehicle**
- **WKY: 0.5 mg/kg**
- **WKY: 5 mg/kg**
- **SHR: vehicle**
- **SHR: 0.5 mg/kg**
- **SHR: 5 mg/kg**

Voiding frequency vs. Time (h)

**B**

- **WKY**
- **SHR**

Voiding frequency

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*Indicates statistical significance:

* p < 0.05

** p < 0.01