Activation of Large-Conductance Calcium-Activated Potassium Channels by Puerarin

-The Underlying Mechanism of Puerarin-Mediated Vasodilation

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d) **ABBREVIATIONS:** BK<sub>Ca</sub> channel, the large-conductance calcium-activated potassium channel; BK-α, the α subunit of BK<sub>Ca</sub> channels; BK-α+β1, the α and β1 subunit of BK<sub>Ca</sub> channels; slo, the α subunit of BK<sub>Ca</sub> channels; mslo, mouse slo; dslo, drosophila slo; hβ1, human β1; NA, noradrenaline bitartrate; eNOS, endothelial nitric oxide synthase; cAMP, 3',5'-cyclic adenosine monophosphate; TEA, tetraethylammonium; IbTX, iberiotoxin; HEK cells, human embryonic kidney cells; NP<sub>O</sub>, the open probability for a multi-channel patch; EC<sub>50</sub>, the half-maximal concentration; MeSO<sub>3</sub>H, methanesulfonic acid; HEDTA, hydroxyethylenediaminetriacetic acid; RCK, regulator of conductance for K<sup>+</sup>.

e) This work belongs to the “Cardiovascular” section.
Abstract

Puerarin is the main isoflavone found in *Pueraria lobata* (Willd) Ohwi, which has been used in therapy for various cardiovascular diseases. The present study examined the effects of puerarin on the large-conductance voltage- and Ca\(^{2+}\)-activated potassium (BK\(_{Ca}\)) channel and on rat thoracic aortas. BK\(_{Ca}\) channels encoded with either α (BK-α) or α/β subunits (BK-α+β1) were heterologously expressed in *Xenopus* oocytes or HEK293 cells. The activities of BK\(_{Ca}\) channels were measured using excised patch-clamp recordings. Puerarin activated BK-α+β1 currents with a half-maximal concentration (EC\(_{50}\)) of 0.8 nM and a Hill coefficient of 1.11 at 10 µM Ca\(^{2+}\), and with EC\(_{50}\) of 12.6 nM and a Hill coefficient of 1.08 at 0 µM Ca\(^{2+}\). Puerarin (1 nM) induced a 16 mV leftward shift in the conductance-voltage curve for BK-α+β1 currents at 10 µM Ca\(^{2+}\), and at 100 nM induced a 26 mV leftward shift at 0 µM Ca\(^{2+}\). Puerarin mainly increased the BK-α+β1 channel open probability without changing the unitary conductance. Activation was also detected in the absence of the β1 subunit. A deglycosylated analog of puerarin, daidzein, also activated BK\(_{Ca}\) channels with weaker potency. In addition, puerarin (0.1 to 1000 µM) caused concentration-dependent relaxations of rat thoracic aortic rings contracted with 1 µM noradrenaline bitartrate (NA) (EC\(_{50}\) = 1.1 µM). These were significantly inhibited by 50 nM iberiotoxin, a specific blocker of BK\(_{Ca}\) channels. This is the first study demonstrating that puerarin activates BK\(_{Ca}\) channels, especially BK-α+β1 channels. The activation of the BK\(_{Ca}\) channel probably contributes to the puerarin-mediated vasodilation action.
Introduction

Puerarin (7-Hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β-D-glucopyranoside), Fig. 1) is the main isoflavone isolated from the Chinese medicinal herb Ge-gen (also known as “Kudzu” in the West), the root of the wild leguminous creeper, Pueraria lobata (Willd) Ohwi. Puerarin has multiple pharmacological activities (Gao, 2003) and has been used to treat many cardiovascular diseases such as hypertension (Song et al., 1988; Wu et al., 2006), angina (Chen, 2004; Wang et al., 2006) and myocardial infarction (Xiao et al., 2005; Zhang et al., 2006). The vascular relaxing action of puerarin is one of the focuses of research on its pharmacology. It involves a variety of molecular mechanisms such as activation of endothelial nitric oxide synthase (eNOS) (Ma et al., 2003) and the cAMP pathway (Yeung et al., 2006). Some researches (Sun and Li, 2002; Dong et al., 2004) have shown that the potassium channel blocker tetraethylammonium (TEA) can significantly block puerarin-induced vasodilation. Other studies have shown that puerarin blocks the L-type Ca^{2+} channel and the K^{+} channel in isolated guinea pig ventricular myocytes (Miao et al., 1998; Qian et al., 1999; Zhang et al., 2001), and inhibits the Na^{+} current in rat ventricular myocytes (Zhang et al., 2003). However, there are no reports of effects of puerarin on K^{+} channels in smooth muscle cells.

The BK_{Ca} channel, the large-conductance voltage- and Ca^{2+}-activated potassium channel, abundantly expressed in vascular smooth muscle cells, plays a critical role in controlling vascular tone. Activation of BK_{Ca} channels leads to hyperpolarization of the cell membrane, which causes deactivation of voltage-dependent calcium channels and vasodilation (Brenner et al., 2000). Deletion of the BK_{Ca} channel leads to high blood pressure in small arteries of mice (Sausbier et al., 2005). The smooth muscle BK_{Ca} channels are typically composed of four pore-forming α subunits.
and an unknown number of regulatory β1 subunits. The accessory β1 subunits of BKCa channels play a vital role in coupling Ca^{2+} sparks to BKCa channel activation in vascular smooth muscles (Chang et al., 2006). Its down-regulation has been shown to play an important role in hypertension (Amberg et al., 2003; Pluger et al., 2000).

In this study, we investigated the effects of puerarin on cloned BKCa channels and examined the vasodilation effects of puerarin in the presence or absence of the specific BKCa channel blocker iberiotoxin (IbTX). These results may help to understand the underlying mechanisms of puerarin-mediated vasodilation.
Materials and Methods

Materials. Puerarin and daidzein (7,4’-dihydroxyisoflavone) were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (purity ≥ 99%). Daidzein was dissolved with dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%, which did not alter the kinetic properties of BKCa channels (data not shown). All of the other chemicals were obtained from Sigma-Aldrich except indicated.

Expression of mslo and hβ1 Channels in Xenopus Oocytes. Methods of expression of mslo and hβ1 channels in stage V–VI Xenopus oocytes were as described previously (Xia et al., 1999; Yao et al., 2005). In brief, oocytes were digested by treatment with 2 mg/ml collagenase I in zero calcium ND-96 solution. Between 2 and 24 h after digestion, 1–2 ng (mslo and hβ1) cRNA (a gift of Dr. Christopher Lingle, Washington University, St. Louis, MO) was injected into Xenopus oocytes using a Drummond Nanoject II (Drummond Scientific Co.). After injection, oocytes were then incubated in ND-96 solution at 18°C. Currents were recorded 2–7 days after RNA injection. ND-96 solution (pH 7.5) contained the following concentrations (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, 2.5 sodium pyruvate, and 10 H+-HEPES. It was supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin for incubation.

Expression of dslo Channels in HEK293 Cells. Human embryonic kidney (HEK293) cells were cultured in DMEM (GIBCO) with 10% FBS (McCartney et al., 2005). HEK293 cells grew on 24 well plates at a density of ~1×10⁴/well, and were transfected with 0.6 µg of GFP/pcDNA3.1 and 0.6 µg of dslo/pcDNA3.1 (a gift of Dr. Christopher Lingle, Washington University, St. Louis, MO) using Lipofectamine 2000 (Invitrogen). Cells were used for electrophysiological recordings in 1–2 days after transfection.
**Electrophysiology.** Patch pipettes were pulled from borosilicate glass capillaries with a resistance between 2 and 5 megaohms. All experiments in excised patch configurations were performed and recorded using a PC2C patch clamp amplifier (Inbio Life Science Instrument Co., Ltd., China) and PClamp software (Axon Instruments, Inc.). Currents were typically digitized at 20 kHz. Macroscopic records were filtered at 10 kHz during digitization. Single-channel records were filtered at 5 kHz and digitized at 10 kHz. For an inside-out patch experiment, the intracellular solution contained (in mM): 160 MeSO₃K, 10 H⁺-HEPES, and 2 MgCl₂, adjusted to pH 7.0 with methanesulfonic acid (MeSO₃H). The bath solution contained (in mM): 160 MeSO₃K, 10 H⁺-HEPES, and 5 N-hydroxyethylenediaminetriacetic acid (HEDTA) with added Ca²⁺ to make 10 µM free Ca²⁺, as defined by the EGTAETC program (E. McCleskey, Vollum Institute, Portland, OR), with the pH adjusted to 7.0.

During recording, drugs and control/wash solutions were puffed locally onto the cell via a puffer pipette containing seven solution channels. The tip (~300 µm diameter) of the puffer pipette was located about 120 µm from the cell. As determined by conductance tests, the solution around a cell under study was fully controlled by the application solution with a flow rate of 100 µl/min or greater. All experiments were done at room temperature, 22–25 °C.

**Vasoreactivity Measurements.** Male Wistar rats (weighing 200~250 g, obtained from the Animal Center, Institute of Health and Epidemic Prevention, Hubei, China) were sacrificed by decapitation under ether anesthesia. The thoracic aortas were excised and cleaned of adherent connective tissue, and were cut into 3 mm ring segments. The endothelial layer was removed in some experiments by gently rubbing the intimal surface of the vessels with a hypodermic needle. Conversely, in other cases, the endothelium was maintained. Arterial rings were mounted on two
stainless steel hooks in 5 ml organ baths filled with Krebs-Ringer buffer (pH 7.4) of the following composition (mM): 119 NaCl, 25 NaHCO₃, 11.1 Glucose, 1.6 CaCl₂, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, and gassed with a mixture of 95% O₂: 5% CO₂. The rings were equilibrated for 45 min at +37 °C with a resting tension of 1.0 g. During this period, the bathing solution was replaced every 15 min and, if necessary, the basal tone was readjusted to 1.0 g. After the equilibration, the endothelial removal/integrity was confirmed by the administration of acetylcholine (ACh) (10 µM) to noradrenaline bitartrate (NA, 1 µM)-precontracted vascular rings. A relaxation <10% of the NA-induced contraction was considered the representative of an acceptable lack of the endothelial layer, whilst a relaxation ≥70% of the NA-induced contraction was considered the representative of an acceptable integrity of the endothelium. Rings were then washed in pre-warmed Krebs-Ringer solution until the baseline tone was regained. The rings were then contracted with 1 µM NA to the maximal contraction. Following washout of NA, cumulative concentration-response curves to stepwise cumulative addition of puerarin (0.1 ~ 1000 µM) with or without 50 nM IbTX were established. Each new addition of puerarin was made after the response to the previous addition had attained a steady state. The force of contraction was measured with an isometric force-displacement transducer and registered with a polygraph (RM6240 transducer, RM6240B/C Polygraph; Chengdu Instrument Co., China). Relaxation responses were expressed as a percentage of NA-induced contraction. All animal experiments were approved by the Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996).

**Data Analysis.** Data were analyzed with Clampfit (Axon Instruments, Inc.), SigmaPlot
(SPSS Inc.), and QUB (State University of New York, Buffalo) software. Unless indicated, the data are presented as means ± STD; statistical significance between two groups and among multiple groups was tested using Student’s t test and one-way analysis of variance, respectively. Differences in the mean values were considered significant at a probability of < 0.05. Dose-response relationships for puerarin activating BK\text{Ca} channels and relaxing rat thoracic aortic rings were fitted to a Hill equation of the following form: \( f = f_{\text{max}}/(1 + (\text{EC}_{50}/[\text{puerarin}])^n) \), where \( f \) is activation percentage of BK\text{Ca} currents or relaxation percentage of rat thoracic aortic rings, \( f_{\text{max}} \) is maximum value of \( f \), and [puerarin] is the concentration of puerarin. \( \text{EC}_{50} \) and \( n \) denote the puerarin concentration of half-maximal effect and the Hill coefficient, respectively. G-V curves were fitted to a Boltzmann equation of following form: \( G/G_{\text{max}} = 1/(1 + \exp ((V-V_{50})/k)) \), where \( G \) is the conductance of the channel, \( G_{\text{max}} \) is the maximal \( G \), \( V \) is the holding potential, \( V_{50} \) is the voltage for half-maximal activation, and \( k \) represents the slope factor. G-V curves were generated from steady-state currents. Single channel analysis was performed utilizing QUB. Single channel amplitudes were measured by using an all-points histogram of current records. \( \text{NPo} \) (the open probability for a multi-channel patch) was determined over at least 10 s of recording. \( \text{NPo} \) values were calculated from the area under the curve (AUC) of the Gaussian fit of all-points amplitude histograms. Assuming a Poisson distribution, \( \text{NPo} = \sum \text{Xi}/\text{n} \) with \( i = 1 \ldots n \), where \( n \) is the maximum number of simultaneous conducting channels during the observation period, and \( \text{Xi} \) is the relative AUC corresponding to each opening.
Results

**Puerarin Activating Macroscopic BK-α+β1 Channel Currents.** Macroscopic BK$_{Ca}$ currents in *Xenopus* oocytes coexpressing mslo and hβ1 (mslo+hβ1) were obtained at different potentials in excised patches. Puerarin does not activate BK$_{Ca}$ channel currents when applied to the outside of excised cell membrane patches even at 100 µM concentration (data not shown). However, puerarin potently activated channels when applied to the cytoplasmic side of excised cell membrane in the presence of 0 and 10 µM intracellular Ca$^{2+}$ (Fig.1). Figure 1A shows the voltage dependence of puerarin-induced enhancement of BK$_{Ca}$ currents at 10 µM Ca$^{2+}$. Application of 1 nM puerarin significantly increased the currents at negative potentials, and the activation was partially reversible. The *middle panel* summarizes the relationships between conductance and voltage. 1 nM puerarin resulted in a 16 mV leftward shift of the V$_{50}$ of G-V curves. The *bottom panel* shows the enhancement percentage of BK$_{Ca}$ currents by puerarin at different potentials. The activation of BK$_{Ca}$ channel currents by puerarin was markedly inverse voltage dependent, i.e., there was a larger increase at lower potentials (76.6 ± 13.5% and 27.5 ± 6.1% at -40 and -20 mV, respectively, p < 0.05, versus control, n =6). Figure 1B summarizes the activation by 100 nM puerarin at 0 µM Ca$^{2+}$. 100 nM puerarin also stimulated BK$_{Ca}$ currents and induced a 26 mV leftward shift in the V$_{50}$ of G-V curves.

Currents of mslo+hβ1 in *Xenopus* oocytes shown in Fig.2 were recorded in inside-out patches at -20 and +100 mV with 10 and 0 µM Ca$^{2+}$, respectively. In Fig. 2A, 1 nM puerarin increased BK$_{Ca}$ currents, and the time courses of the currents, which were activated at various concentrations of puerarin, indicate that the whole process goes in a rapid and partially reversible manner. The dose-response curve is fitted to a Hill equation with an EC$_{50}$ of 0.8 nM and a Hill
coefficient of 1.11. Similarly, puerarin increased the BK$_{Ca}$ currents at +100 mV and zero Ca$^{2+}$ concentration (Fig. 2B). However, the EC$_{50}$ and the Hill coefficient derived from the dose-response curve in this case are 12.6 nM and 1.08, respectively, indicating that Ca$^{2+}$ facilitates activation by puerarin.

**The Effect of Puerarin on Single BK-α+β1 Channel Currents.** Single-channel currents of mslo+hβ1 were recorded in inside-out patches at 10 µM free Ca$^{2+}$ concentration. Fig. 3 and supplementary Figure 1 show the effect of puerarin on the single BK-α+β1 channel currents at –20 mV and +50 mV, respectively. In Fig. 3A, the initial NP$_{O}$ was 0.51, and after the application of 1 nM puerarin the NP$_{O}$ was increased to 0.70. The single channel amplitude was almost unchanged after the application of 1 nM puerarin. A summary of the effects of 1nM puerarin on NP$_{O}$ and the unitary amplitude of single BK$_{Ca}$ channels at –20 mV is shown in Fig. 3B. Puerarin induced a 37 ± 7% enhancement in NP$_{O}$ and slight changes in unitary amplitude at –20 mV ($n = 6$). To further confirm any possible change in single BK$_{Ca}$ channel conductance caused by puerarin, a series of experiments was performed at several holding potentials ranging from –60 to +60 mV with 10 µM Ca$^{2+}$. In Fig. 3C, the current-voltage plots show that there is no significant change in conductance before and after the application of 1 nM puerarin (242.8± 12.4 and 248.5 ± 16.0 picosiemens, control and plus puerarin, respectively, $n = 6$).

**Puerarin Activating Currents of BK-α (mslo) Channel.** One often asks whether the auxiliary β1 subunit of BK$_{Ca}$ channels plays a critical role in the above stimulating effect (Giangiacomo et al., 1998; Perez, 2005; Zakharov et al., 2005). Consequently we have further explored the effect of puerarin in the absence of the β1 subunit. A series of experiments shown in Fig. 4 was conducted at zero Ca$^{2+}$ concentration. Under those conditions, puerarin also shows an
activation effect on mslo currents, but at higher concentrations. Figure 4A shows that 1 µM puerarin significantly activates mslo currents with an 18 mV leftward shift in the V_{50}. The dose-response curve was fitted to a Hill equation with an EC_{50} of 166.6 nM and a Hill coefficient of 0.72 (Fig. 4B).

**Puerarin Has No Effect on dslo Currents.** To estimate the domain of BK_{Ca} channels that puerarin may bind to, the BK_{Ca} encoded with dslo subunit expressed in HEK293 cells was used. Puerarin has no effect on the dslo currents at 0 µM Ca^{2+} (data not shown) and 10 µM Ca^{2+} (Fig. 5). Therefore, we infer that the possible interaction domain may locate at the S0-S1 and S8-S9 linkers, because the BK_{Ca} dslo channel contains the above regions significantly different from that of the BK_{Ca} mslo channel.

**Daidzein, an Analog to Puerarin, Activating mslo Currents.** Daidzein is an aglycone of puerarin, in other words, is a hydrolysate of puerarin. To investigate the role of β-D-glycosyl residue at 8-position of the puerarin isoflavone core in the activation process of BK_{Ca} channels, the effect of daidzein on BK_{Ca} mslo channels was examined at zero Ca^{2+} concentration. In Fig. 6, 1 µM daidzein also increases BK_{Ca} currents in inside-out patches with an 11 mV leftward shift in the V_{50} which is smaller than that of puerarin of 18 mV shift as shown in Fig. 4A. That means that the β-D-glycosyl residue also plays a role in enhancing channels.

**Puerarin Relaxing Rat Thoracic Aortic Rings.** Puerarin (0.1 to 1000 µM) caused concentration-dependent relaxations in endothelium-intact and endothelium-denuded rat thoracic aortic rings contracted with NA (1 µM) (Fig. 7). The EC_{50} was 1.1 µM and 2.2 µM, and the maximal relaxation was 45.9 ± 5.1% (n = 5) and 33.5 ± 4.6% (n = 6) to endothelium-intact and endothelium-denuded aortic rings, respectively, indicating that endothelium was involved in
puerarin-induced vasodilation. The specific BKCa channel blocker, IbTX (50 nM), significantly inhibited puerarin-induced relaxations on both endothelium-intact and endothelium-denuded aortic rings, the fractional inhibition at puerarin concentration of 1 µM on endothelium-intact and endothelium-denuded aortic rings was 34.0% and 48.0%, respectively, suggesting that the activation of BKCa channels also contributes to the puerarin-mediated vasodilation action.
Discussion

The major findings of this study are as follows. Puerarin potently activated BK-α+β1 currents in the nanomolar concentration range. It increased the activity of BK-α+β1 channels with no change in single-channel conductance. Puerarin also stimulated BK-α currents with weaker potency. A puerarin analogue, daidzein, activated BK-α currents with weaker potency than puerarin. Puerarin dilated rat thoracic aortic rings in a concentration-dependent manner, and this relaxation response could be inhibited by IbTX.

Mechanisms of the Effect of Puerarin on BKCa Channels. Puerarin potently activated cloned BK-α+β1 channels, shifting the G-V relationship to the left in cell-free patches. Compared with many other natural BKCa channel openers including BMS-204352 (EC50 ≥ 300 nM) (Gribkoff et al., 2001), dehydrosoyasaponin-I (EC50 ≥ 100 nM) (Giangiacomo et al., 1998) and mallotoxin (effective concentration ≥ 500 nM) (Zakharov et al., 2005), the effective concentration range for puerarin is considerably lower (EC50 values: 0.8 nM at 10 µM Ca2+ and 12.6 nM at 0 µM Ca2+). The effect of puerarin on BKCa channels is in a voltage and Ca2+-dependent manner. It is very notable that the effect of puerarin is inversed-voltage dependent, i.e. the activation of BKCa channels by puerarin is significantly larger at negative potentials, which is similar to another BKCa channel opener-12, 14-Dichlorodehydroabietic Acid (Sakamoto et al., 2006). The EC50 of puerarin on BKCa channels is a 16-fold augmentation at zero Ca2+ compared with at 10 µM Ca2+, which may imply that Ca2+ facilitates the activation of puerarin. On the other side, Ca2+ produced more leftward shift in V50 of BKCa channels in the presence of puerarin than that in the absence of puerarin, e.g. the V50 of BK-α+β1 was -27 ± 3 mV in the absence of 1 nM puerarin but -43 ± 4 mV in the presence of 1 nM puerarin at 10 µM Ca2+, indicating that puerarin
was able to increase the Ca\textsuperscript{2+}-sensitivity of BK\textsubscript{Ca} channel gating. Single channel recordings showed that puerarin mainly increased the BK\textsubscript{Ca} channel NP\textsubscript{0} without changing the unitary conductance. In addition, the Hill coefficients of dose-response curves were approximately unity both at 10 \( \mu \text{M} \) Ca\textsuperscript{2+} and 0 \( \mu \text{M} \) Ca\textsuperscript{2+}, suggesting only one site for interaction between puerarin and the BK\textsubscript{Ca} channel protein.

The Role of the \( \beta \)1 Subunit. Puerarin activating BK\textsubscript{Ca} channels was also observed in the absence of the \( \beta \)1 subunit, which indicates that \( \beta \)1 subunit is not required for the activation. However, puerarin has a stronger effect on the BK-\( \alpha \)+\( \beta \)1 channels than BK-\( \alpha \) channel alone. In the absence of the \( \beta \)1 subunit, the EC\textsubscript{50} of puerarin is greater by 13 times, suggesting that the role of the \( \beta \)1 subunit is probably to facilitate puerarin binding. The \( \beta \)1 subunit is composed of two transmembrane domains, a long extracellular loop, and two short intracellular segments (Knaus et al., 1994), leaving little to construct an intracellular binding site for puerarin. Since puerarin can activate the BK\textsubscript{Ca} channels only when applied to the cytoplasmic side of cell membrane, we infer that puerarin binding sites are located on the intracellular side of the \( \alpha \) subunits. The N terminus and S0 of the BK-\( \alpha \) subunit have been supposed to be the possible regions to regulate the gating of BK\textsubscript{Ca} channels via interacting with \( \beta \)1-subunits (Wallner et al., 1996). We examined the effect of puerarin on dslo, interestingly, no action was observed. Aligning the sequences of mslo and dslo, we find that there is main difference in two cytoplasmic motifs, i.e. the S0-S1 linker and S8-S9 linker. The sites for puerarin binding to BK\textsubscript{Ca} channels may be located in one of these domains. More experiments are needed to determine the precise locations. As we know, both dslo and mslo channels contain the same calcium binding sites in RCK1 (regulator of conductance for K\textsuperscript{+}) domain and calcium bowl regions, but have very different Ca\textsuperscript{2+}-sensitivity (Xia et al., 2002).
Furthermore, considering that the S0-S1 and S8-S9 linkers are the major differences in the sequences between dslo and mslo channels, we speculate that they could be the candidates of locations affecting Ca$^{2+}$-sensitivity of BK$_{Ca}$ channels.

**The Function of the Glycosyl Residue of Puerarin.** Puerarin is an isoflavone glycoside with a β-D-glucopyranoside at 8-position and two hydroxyl groups at 4’, 7-positions. The isoflavone nucleus is a rigid and hydrophobic structure, while the glycosyl residue and hydroxy group are hydrophilic. Daidzein, an analog of puerarin, has no glycosyl residue and activates BK$_{Ca}$ channels with weaker potency than puerarin. These results indicate that the β-D-glucopyranoside at 8-position plays an important role in puerarin activating BK$_{Ca}$ channels. Thus, engineering in new BK$_{Ca}$ channel openers should focus on the modification of the number or location of glycosyl group in the isoflavone nucleus.

**Vasodilation and the Activation of BK$_{Ca}$ Channels Induced by Puerarin.** Puerarin caused concentration-dependent relaxations in isolated rat thoracic aortic rings contracted with NA. IbTX, a specific BK$_{Ca}$ channel blocker, markedly inhibited puerarin-induced relaxations. It is likely, therefore, that the mechanism of this response to puerarin involves opening of BK$_{Ca}$ channels. In the absence of endothelium, puerarin produced less relaxation than that in the presence of endothelium, indicating that endothelium was also involved in puerarin-mediated vasodilation. However, the inhibition of relaxation by IbTX was not decreased in the absence of endothelium, implying that the contribution of BK$_{Ca}$ channels to puerarin-mediated vasodilation is independent to the endothelium. It is worth noting that the EC$_{50}$ of puerarin required to relax aortic rings (1.1 µM) was ~1000-fold higher than that required to directly activate BK$_{Ca}$ channels. This difference is attributed to the fact that puerarin hardly penetrates the cell membrane as mentioned above.
However, puerarin could be taken into the cytoplasm through endocytosis. The average diameter of a vesicle is about 140 nm (Zhang et al., 1995), so each vesicle could carry drugs in $3 \times 10^{-6}$ of the extracellular concentration into a 10 µm cell in diameter. There are about 200 vesicles taking part in one secretion event (Gillis et al., 1996), and from this we can calculate that the concentration of puerarin in the cytoplasm of vascular smooth muscle cells would be in $6 \times 10^{-4}$ of the extracellular concentration, which would be the concentration activating the $\text{BK}_{\text{Ca}}$ channels.

The plasma concentration of puerarin is in the range 1-1000 µM in humans and animals (Deng et al., 2004; Jin et al., 1991; Wu et al., 2004), so the concentration used in this study is of therapeutic relevance.

In summary, we have provided direct evidences for the activation of cloned $\text{BK}_{\text{Ca}}$ channels by puerarin, and shown that $\text{BK}_{\text{Ca}}$ channel activation contributes to puerarin-induced vasodilation, which is likely to be a mechanism by which puerarin exerts its action on rat thoracic aortas, in addition to its activation of eNOS and cAMP pathway, as demonstrated by other investigators (Ma et al., 2003; Yeung et al., 2006). The present findings are of interest for understanding the contribution of $\text{BK}_{\text{Ca}}$ openers to lowering blood pressure and improving other cardiovascular symptoms.
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Footnotes

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Legends for Figures

**Fig. 1.** Activation of mslo+hβ1 channels coexpressed in *Xenopus* oocytes by puerarin. A, all traces were recorded in inside-out patches at 10 µM intracellular Ca²⁺. *Top panel*, representative traces under conditions of control, 1 nM puerarin and washout as indicated. Currents were evoked by voltage steps in the range from -180 to +100 mV for 30 ms in 20 mV steps, after a prepulse to -180 mV as shown at bottom right. *Middle panel*, G-V curves from individual patches were normalized and then averaged. The fitted values for the V₅₀ of control and in the presence of 1 nM puerarin are -27 ± 3 mV and -43 ± 4 mV (p < 0.01, n = 6), respectively. *Bottom panel*, the voltage dependence of the activation of mslo+hβ1 currents by 1 nM puerarin at 10 µM Ca²⁺ calculated from the data in the *middle panel*. *, p < 0.05 versus control, n = 6. B, all traces were recorded in inside-out patches at 0 µM intracellular Ca²⁺. *Top panel*, representative traces are shown under conditions of control, 100 nM puerarin and washout as indicated. *Middle panel*, the fitted values for the V₅₀ of control and 100 nM puerarin are 164 ± 6 mV and 138 ± 11 mV (p < 0.01, n = 5), respectively. The inset shows the chemical structure of puerarin. *Bottom panel*, the voltage dependence of the activation of mslo+hβ1 currents by 100 nM puerarin at 0 µM Ca²⁺ was reevaluated from the data shown in *middle panel*. *, p < 0.05 versus control, n = 5.

**Fig. 2.** Concentration dependence of puerarin activation of mslo+hβ1 currents. A, traces show the activation of BKCa currents coexpressed with mslo and hβ1 subunits in *Xenopus* oocytes by 1 nM puerarin, in presence of 10 µM internal Ca²⁺. *Top panel*, example traces show the BKCa currents from an inside-out patch before, during and after application of 1 nM puerarin at ~20 mV. The dashed line represents zero current. *Middle panel*, plots show the time course of activation of
BK_Ca currents, which were normalized to control, from four different inside-out patches in the presence of 0.1, 0.5, 1 and 5 nM puerarin at -20 mV. Currents were recorded every 5 seconds.

Bottom panel, the solid line is a fit to the Hill equation. The EC50 of the dose-response curve of puerarin activation of BK_Ca currents was 0.8 nM with a Hill coefficient 1.11 (*, p < 0.05 versus control, n = 4). B, the time course and the dose-response curves of puerarin activation of BK_Ca currents are shown in the middle and bottom panels, at +100 mV and zero intracellular Ca^{2+}. The time course was obtained from an inside-out patch during application of 5, 10, 20 and 100 nM puerarin. Currents were recorded every 15 seconds. The EC50 value was 12.6 nM with a Hill coefficient 1.08 (*, p < 0.05 versus control, n = 4).

Fig. 3. Effects of puerarin on single-channel characteristics of BK_Ca channels coexpressed with mslo and h\(\beta_1\) subunits in Xenopus oocytes at 10 \(\mu\)M Ca^{2+}. A, single-channel traces were obtained from an inside-out patch before (Control) and after application of 1 nM puerarin. Channels were held at -20 mV in symmetrical 160 KCl solutions. The letters c, o1 and o2 indicate the close, open 1 and open 2 levels, respectively. Records a and b are insets of square section. Each histogram (bottom panel) illustrates all possible change in open probabilities and unitary conductance after applying puerarin. B, summary of the effect of 1 nM puerarin on NP_O (left panel) and unitary amplitude (right panel) of single BK_Ca channels at -20 mV (**, p < 0.01 versus control, n = 6). C, single channel current-voltage (I-V) curves are plotted in the absence and presence of 1 nM puerarin (n = 6).

Fig. 4. Activation by puerarin of BK_Ca channels in the absence of the \(\beta_1\) subunit at zero Ca^{2+}
concentration. A, upper panel, representative traces of BK<sub>Ca</sub> currents expressed with mslo subunits in <i>Xenopus</i> oocytes were obtained from an inside-out patch before (left) and after (right) application of 1 µM puerarin. The voltage protocol is shown in the inset. Lower panel, the V<sub>50</sub> of G-V curves for control and 1 µM puerarin are 169 ± 3 mV and 151 ± 1 mV (p < 0.01, n = 5), respectively. B, the concentration-dependent activation curve of mslo obtained from five different patches at +100 mV. The experimental points were fitted to a Hill function (solid line) with a Hill coefficient of 0.72. The EC<sub>50</sub> obtained from the fit was 166.6 nM.

**Fig. 5.** Lack of effect of puerarin on BK<sub>Ca</sub> currents expressed with dslo subunits in HEK293 cells. A, example current traces of dslo are shown before (left) and after (right) application of 1 µM puerarin at 10 µM internal Ca<sup>2+</sup>. Currents were activated at potentials from -180 through +200 mV for 150 ms in 20 mV increment, after a prepulse to -180 mV as shown in inset. B, the V<sub>50</sub> of G-V curves are as following: for control (open symbols), V<sub>50</sub> control = 95 ± 6 mV; and for 100 nM puerarin (filled symbols), V<sub>50</sub> puerarin = 100 ± 7 mV (n = 5). Error bars represent STD.

**Fig. 6.** Activation of mslo currents by daidzein, an analog to puerarin. A, example traces of mslo channels expressed in <i>Xenopus</i> oocytes were obtained from an inside-out patch before (left) and after (right) application of 1 µM daidzein at zero Ca<sup>2+</sup>. The voltage protocol is shown at the right bottom. B, the V<sub>50</sub> of G-V curves are V<sub>50</sub> control = 159 ± 9 mV (Control, open symbols) and V<sub>50</sub> daidzein = 148 ± 5 mV (1 µM daidzein, filled symbols) (p < 0.01, n = 5). Error bars represent STD. The chemical structure of daidzein is shown in inset.
**Fig. 7.** Puerarin-induced relaxation of isolated rat thoracic aortas. Rings were contracted with NA (1 µM) before puerarin (0.1 to 1000 µM) was added cumulatively. Concentration-response curves were obtained on endothelium-intact (*circles*) or endothelium-denuded (*diamonds*) aortic rings, in the absence (*filled symbols*) or in the presence (*open symbols*) of 50 nM IbTX. *, versus endothelium-intact group, $p < 0.05$; #, versus endothelium-denuded group, $p < 0.05$. The *inset* shows the fractional inhibition of relaxation by 50 nM IbTX at puerarin concentration of 1, 10 and 100 µM on endothelium-intact (*filled bars*) or endothelium-denuded (*open bars*) vessels.
Figure 1

A mslo+hb1
(10 μM Ca²⁺)

Control
Puerarin
Wash

10 ms 2 nA

100 mV
-180 mV
-120 mV

B mslo+hb1
(0 μM Ca²⁺)

Control
Puerarin
Wash

10 ms 4 nA

100 mV
-180 mV
-120 mV

G/Gₘₐₓ(Con)
Voltage (mV)

G/Gₘₐₓ(Con)
Voltage (mV)

○ Control
● 100 nM Puerarin
○ 1 nM Puerarin

(\frac{I_{Pue}}{I_{Con}})⁻¹*100
Voltage (mV)

(\frac{I_{Pue}}{I_{Con}})⁻¹*100
Voltage (mV)

*
Figure 3

**mslo+hβ1 10 μM Ca^{2+}**

A

Control (-20 mV)

1 nM Puerarin

**B**

NPO/NPO_{Con} **C**

Amplitude (pA)

pA vs. mV

- Control
- 1 nM Puerarin
Figure 4

A  mslo  0 Ca\(^{2+}\)

10 ms  2 nA

+200 mV

+120 mV

+180 mV

B

\[ \frac{G}{G_{max}} (\text{Con}) \]

1.0

0.5

0.0

-200 -100 0 100 200

Voltage (mV)

-200 -100 0 100 200

1 µM Puerarin

Control

\[ \left( \frac{I_{\text{Puer}}}{I_{\text{Con}}} \right) \]

100

80

60

40

20

0

0 0.01 0.1 1 10

Puerarin (µM)
Figure 5

A  dslo 10 μM Ca^{2+}

Control  1 μM Puerarin

50 ms 0.5 nA

+200 mV  -120 mV

-180 mV

B

G/G_{max} (Con)

0.0  0.5  1.0

-200 -100  0  100  200

Voltage (mV)

○ Control

● 1 μM Puerarin
Figure 6

A  mslo 0 µM Ca^{2+}

Control  1 µM Daidzein

10 ms  2 nA

+200 mV

-180 mV  -120 mV

B

1.5

HO

1.0


G/G_{max} (Con)

0.5

0.0

-200  -100  0  100  200

Voltage (mV)

○ Control

● 1 µM Daidzein
Figure 7

The graph shows the effect of puerarin concentration on fractional inhibition and relaxation percentage in the presence or absence of endothelium. The data points are labeled with symbols indicating different treatments:

- **+Endothelium**: Solid black circles, n=5
- **-Endothelium**: Diamond, n=6
- **IbTX 50nM +Endothelium**: Open circles, n=6
- **IbTX 50nM -Endothelium**: Open diamonds, n=5

The x-axis represents puerarin concentration (µM) ranging from 0.1 to 1000, while the y-axis represents the fractional inhibition percentage and relaxation percentage.