Puerarin facilitates Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release triggered by KCl-depolarization in primary cultured rat hippocampal neurons

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Abstract

The effects of puerarin on behaviour and brain neuronal activity in animal studies have been described previously. However, molecule mechanisms underlying these effects were poorly understood. Here, we examined the regulation of puerarin on the Ca\textsuperscript{2+} signals in primary rat hippocampal neurons using Fura-2 based calcium imaging techniques. Application of puerarin had no effect on the basal intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}), but potentiated the KCl-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transient in 87% of recorded neurons. Dantrolene or ruthenium red, the inhibitors of ryanodine receptors, completely blocked this potentiation induced by puerarin. Moreover, in Ca\textsuperscript{2+}-free solution, pre-application of puerarin significantly augmented the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} evoked by caffeine (3 mM), which is a specific agent to activate the ryanodine receptors. In contrast, nifedipine failed to prevent the potentiation induced by puerarin. Similarly, in the experiments of whole-cell patch-clamp recording, puerarin did not show any effect on calcium currents generated by depolarization pulses. These data demonstrated that the potentiation induced by puerarin was attributed to the facilitation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) via ryanodine receptors, rather than extracellular Ca\textsuperscript{2+} influx. Using estrogen receptor antagonist ICI 182780 and tamoxifen, we further demonstrated that the potentiation induced by puerarin was mediated by the estrogen receptor. Furthermore, the membrane-permeant inhibitor of protein kinase A (PKA) H89 completely inhibited this potentiation. However, U-73122, the inhibitor of phospholipase C (PLC) had no effect, indicating that the cyclic AMP/PKA signaling pathway was involved in the activation of CICR by puerarin.

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Keywords: Puerarin; Calcium; Hippocampal neuron; Estrogen receptor

1. Introduction

Puerarin (7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β-D-glucopyranoside)), the main isoflavone glycoside extracted from the root of Pueraria lobata (Jiang et al., 2005), has been used for various medicinal purposes in traditional Chinese medicine for thousands of years (Qicheng, 1980). Recently, it has been reported that puerarin improved the learning and memory behavior in ovariectomized mice and D-galactose induced aging mice (Xu and Zhang, 2002; Xu et al., 2004). Puerarin also attenuated impairment of inhibitory avoidance performance induced by mecamylamine, p-chloroamphetamine, and dizocilpine in the rats (Hsieh et al., 2002). However, the intracellular signaling mechanisms underlying these therapeutic actions remain unclear.

Previous studies show that puerarin is a kind of phytoestrogen based on its isoflavone structure. For instance, Boue et al. (2003) observed that puerarin induced MCF-7 cells line proliferation although its potency was weaker than that of β-estradiol, which is an important sex hormone and exerts their biological action through either genomic or non-genomic pathways. Non-genomic effects of estrogen are involved in the plasma membrane-
associated estrogen receptors that initiate intracellular signaling cascades (Kelly and Levin, 2001; Moss et al., 1997). An important non-genomic effect of estradiol is to alter the intracellular calcium concentration ([Ca$^{2+}$]). For example, it can induce a fast rise in the intracellular free Ca$^{2+}$ concentration via influx of external Ca$^{2+}$ and/or release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores (Audy et al., 1996; Morley et al., 1992). Due to the estrogen-like activity of puerarin, it is possible that puerarin may influence cellular [Ca$^{2+}$], via similar mechanisms to estrogen. Therefore, the present study was designed to investigate whether puerarin influenced the basal [Ca$^{2+}$], and the KCl-evoked [Ca$^{2+}$], transient by a non-genomic effect in primary cultured rat hippocampal neurons, and, if so, to determine whether the cyclic AMP/protein kinase A (PKA) signaling pathway was involved in this sensitizing effect of puerarin.

2. Materials and methods

2.1. Materials

7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-([β-D-glucopyranoside]) (puerarin, molecular weight: 416.37, purity above 98%) was obtained from National Institute for the Control of Pharmaceutical and Biological Products, China. Dantrolene, ruthenium red, Fulvestrant (ICI 182780), tamoxifen, N-[2-[p-bromocinnamylamino]ethyl]-5-isouquinolesulfonamide dihydrochloride (H89), 1-[6-[[17]-3-methoxyestr-1,3,5(10)trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), caffeine, nifedipine, 4-aminopyridine (4-AP) and tetraethylammonium chloride (TEA-Cl) were purchased from Sigma (St. Louis, MO, USA). Fura-2 AM was obtained from Biotium (Hayward, CA, USA). Modified Dulbecco’s Eagle’s medium (DMEM)/F12 and B27 supplement were obtained from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Other general agents were available commercially.

All the drugs were prepared as stock solutions. Puerarin, H89, U-73122 and Fura-2/AM were dissolved in dimethylsulfoxide (DMSO). ICI 182780 and tamoxifen were made in ethanol. These stock solutions were diluted to the final concentrations with the extracellular solution before application. The final concentration of DMSO or ethanol did not exceed 0.1%. No detectable effect of the vehicles was found in our experiments.

2.2. Cell culture

The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. Primary cultures of hippocampal neurons were prepared as described in our previous studies (Ming et al., 2006; Yermolaieva et al., 2001). Briefly, hippocampi were dissected from the brains of neonatal Sprague–Dawley rats (day 0–3). Brain tissues were treated with 0.125% trypsin in Hank’s balanced salt solution for 25 min at 37 °C and mechanically dissociated using a fire-polished pasteur pipettes. Cell suspension was centrifuged at 1000 × g for 8 min and the cell pellets were resuspended in the DMEM and F-12 supplement (1:1) with 10% fetal bovine serum, 5 U/ml penicillin, 5 μg/ml streptomycin, 0.5 mM glutamine. For [Ca$^{2+}$], imaging analyses, cells (20,000–40,000) were seeded on poly-d-lysine coated coverslips and kept at 37 °C in 5% CO$_2$ (SHELLAB, Oregon, USA). After 24 h, the culture medium was changed to DMEM medium supplemented with 2% B27 and the hippocampal neurons were fed with fresh medium twice weekly. Microscopically, glial cells were not apparent in hippocampal cultures employing this protocol. The neurons were maintained for 7–10 days in primary culture until used for [Ca$^{2+}$], measurements or whole-cell patch-clamp recording.

2.3. Measurement of intracellular free calcium

For [Ca$^{2+}$], measurements, cultured hippocampal neurons were rinsed three times with HEPES-buffered solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 glucose and 10 HEPES (pH 7.3). Then, cells were incubated with 2 μM fura-2 AM for 30 min at 37 °C and subsequent washed three times with HEPES to remove the excess extracellular fura-2 AM. Coverslips were mounted on a recording chamber and perfused with HEPES-buffered solution using a peristaltic pump at a rate of 1.5–4 ml/min. Experiments were performed at room temperature. [Ca$^{2+}$] changes were measured by a Ratio Vision digital fluorescence microscopy system (TILL Photonics GmbH, Germany). Fura-2/AM loaded cells were illuminated at 340 nm for 150 ms and 380 nm for 50 ms at 1 s intervals using a TILL Polychrome monochromator. Fura-2 fluorescence emission was imaged at 510 nm by an intensified cooled charge coupled device (TILL Photonics GmbH). F340/F380 fluorescence ratios were generated by TILLVISION 4.0 software. The contours of a single neuron were used to define a region of interest (ROI) from which the mean fluorescence was measured. Paired F340/F380 fluorescence ratio images of ROI were acquired every second. The intracellular free calcium concentration was presented as the ratio of the fluorescence signals obtained (340/380 nm). All experiments were repeated three times using different batches of cells and at least the 3–4 dishes with cells were used for recording in different batches of cells.

2.4. Whole-cell patch-clamp recording

The procedure for whole-cell patch-clamp recording was described in our previous study (Chen et al., 2002). The bath solution contained (in mM): 110 NaCl, 5 KCl, 5 CaCl$_2$, 1 MgCl$_2$, 11 glucose, 10 HEPES, 5 4-AP, 25 TEA-Cl and 1 μM tetrodotoxin. Recording pipettes were filled with the following solution (in mM): 64 CsF, 0.1 CaCl$_2$, 2 MgCl$_2$, 10 EGTA, 10 HEPES, and 5 Tris–ATP. To isolate the voltage-dependent calcium currents, tetrodotoxin (1 μM), 4-AP (5 mM) and TEA-Cl (25 mM) were added in the bath solution. The resistance of the recording pipette was in the range of 2–5 MΩ. After establishing a whole-cell configuration, the adjustment of capacitance compensation and series resistance compensation was done.
before recording. The current signals were sampled by 10 kHz and filtered at 3 kHz thereafter. Whole-cell patch-clamp recordings were recorded with EPC-10 amplifier (HEKA, Lambrecht, Germany). Data acquisition was performed with Pulse/PulseFit software (HEKA, Southboro, Germany). Drug actions were measured only after steady-state conditions reached, which were judged by the amplitudes and time courses of currents remaining constant. All the recordings were made at 20–22 °C under conditions optimized so as to ensure the isolation of $I_{\text{Ca}}$ from other voltage-activated currents. All experiments were repeated three times using different batches of cells and at least the 3–4 dishes with cells were used for recording in different batches of cells.

2.5. Statistical analysis

The amplitude of $[\text{Ca}^{2+}]_i$ transient represents the difference between baseline concentration and the transient peak response to the stimulation. Quantification of mean data was achieved by expressing each KCl-evoked $[\text{Ca}^{2+}]_i$ transient as a percentage (%) of the first KCl-induced response in the same cell. Statistical significance between the multiple groups was determined using paired t test or one-way ANOVA when appropriate followed by post hoc comparisons (SPSS 10.0 software). Data are presented as means±S.E.M. Differences at the $P<0.05$ level were considered statistically significant.

3. Results

3.1. Puerarin potentiates KCl-evoked $[\text{Ca}^{2+}]_i$ rise in cultured rat hippocampal neurons

To investigate the effects of puerarin on $[\text{Ca}^{2+}]_i$ in hippocampal neurons, we first characterized the $[\text{Ca}^{2+}]_i$ responses evoked by the KCl-depolarization as control. Fast superfusion (approximately 40 s) with 25 mM KCl caused transient $[\text{Ca}^{2+}]_i$ increases in the majority of tested neurons ($n=47$). Repeated KCl stimulation produced equal $[\text{Ca}^{2+}]_i$ responses and these responses were fully reversed after a 10 min washout with normal extracellular solution (Fig. 1A). Cells were perfused with puerarin for 5 min prior to measuring the $[\text{Ca}^{2+}]_i$ response. Puerarin by itself had no effect on basal $[\text{Ca}^{2+}]_i$, but potentiated the KCl-induced $[\text{Ca}^{2+}]_i$ transients in 87% of recorded neurons. On average, pretreatment with 30 μM puerarin potentiated the KCl-evoked $[\text{Ca}^{2+}]_i$ response to 163.7±8.5% of control. This potentiation effect of puerarin was reversible. After a 10 min washout period, the third KCl-induced $[\text{Ca}^{2+}]_i$ transient was equal to the first (Fig. 1B, $n=31$). The potentiation effect of puerarin (1–100 μM) was in a dose-dependent manner (Fig. 1C). The EC$_{50}$ for potentiation effect was 19.1±2.5 μM (fitted with Sigma Plot version 3.0 to the Hill equation). Each experiment is the mean of at least 9 neurons.

3.2. The puerarin-induced potentiation of KCl-evoked $[\text{Ca}^{2+}]_i$, rise is mediated by CICR via ryanodine receptors

It is generally accepted that $[\text{Ca}^{2+}]_i$, transient triggered by KCl has two components. The first is attributed to the opening of voltage-gated calcium channel (VGCC) in response to membrane depolarization and the second is attributed to the $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (CICR) via ryanodine receptors.

To determine whether intracellular calcium release was involved in the potentiation effect of puerarin, we employed the dantrolene and ruthenium red, which are used as ryanodine receptor antagonists. Consistent with previous reports, dantrolene (10 μM, 8 min) partially decreased the KCl-evoked $[\text{Ca}^{2+}]_i$ transients to the 61.6±7.3% of control ($P<0.05, n=14$). Furthermore, pretreatment with dantrolene almost completely blocked the potentiation of KCl-induced $[\text{Ca}^{2+}]_i$, rise induced by puerarin (30 μM) in hippocampal neurons (Fig. 2A and B, $n=11$). Similarly, ruthenium red (10 μM, 8 min) also significant suppressed the effect of puerarin (Fig. 2B, $n=9$). These results thus demonstrated that the puerarin-induced potentiation of the KCl-evoked $[\text{Ca}^{2+}]_i$, rise is due to the facilitation of CICR via ryanodine receptors.
To evaluate the role of extracellular Ca\(^{2+}\) influx via VGCC in the potentiation effect of puerarin, nifedipine was used to block L-type VGCC. As shown in Fig. 2B, application of nifedipine (10 μM, 8 min) partially inhibited KCl-induced [Ca\(^{2+}\)]\(_i\) transients to the 56.8±3.5% of control (\(P<0.05\), \(n=16\)). However, in the presence of nifedipine, puerarin (30 μM, 5 min) did not inhibit the potentiation of puerarin (Puer), whereas this potentiation was completely blocked by dantrolene or ruthenium red. The number of cells recorded in the experiments is indicated in each bar. All experiments were repeated three times using different batches of cells. * \(P<0.05\) compared to control (Cont). # \(P<0.05\) nifedipine vs. nifedipine + puerarin (one-way ANOVA followed by post hoc analysis).

Fig. 2. The potentiation of puerarin is attributed to the facilitation of CICR via ryanodine receptors. (A) Representative trace showing pretreatment with dantrolene (10 μM, 8 min) completely abolished the potentiation of puerarin (30 μM, 5 min) on KCl-evoked [Ca\(^{2+}\)]\(_i\) transients in hippocampal neurons (\(n=11\)). (B) Summary data revealed that respectively application of nifedipine (Nif, 10 μM), dantrolene (Dant, 10 μM) or ruthenium red (Ruth red, 10 μM) partly decreased the KCl-evoked [Ca\(^{2+}\)]\(_i\) increase by themselves. Pretreatment with nifedipine (10 μM, 5 min) did not inhibit the potentiation of puerarin (Puer), whereas this potentiation was completely blocked by dantrolene or ruthenium red. The number of cells recorded in the experiments is indicated in each bar. All experiments were repeated three times using different batches of cells. * \(P<0.05\) compared to control (Cont). # \(P<0.05\) nifedipine vs. nifedipine + puerarin (one-way ANOVA followed by post hoc analysis).

To evaluate the role of extracellular Ca\(^{2+}\) influx via VGCC in the potentiation effect of puerarin, nifedipine was used to block L-type VGCC. As shown in Fig. 2B, application of nifedipine (10 μM, 8 min) partially inhibited KCl-induced [Ca\(^{2+}\)]\(_i\) transients to the 56.8±3.5% of control (\(P<0.05\), \(n=16\)). However, in the presence of nifedipine, puerarin (30 μM, 5 min) still augmented KCl-induced [Ca\(^{2+}\)]\(_i\) transients to the 137.9±11.4% of control (Fig. 2B, \(n=12\)), suggesting that the potentiation effect of puerarin may not be resulted from the enhanced Ca\(^{2+}\) entry via VGCC. To confirm this, we employed the whole-cell recording of patch-clamp technology to examine the effect of puerarin on calcium current (\(I_{Ca}\)) elicited by a depolarizing pulse in the hippocampal neurons. The inward Ca\(^{2+}\) currents, which achieved by stepping from −50 mV to 50 mV for 300 ms every 10 s from a holding potential of −80 mV, consist of three components: L-, P/Q-, and R-type channels. As illustrated in Fig. 3A and B, pretreatment the neuron with puerarin (30 μM) did not show any effect on the amplitude of \(I_{Ca}\) (\(P>0.05\), \(n=7\)), which further indicated that the VGCC was not essential for the puerarin-induced potentiation of KCl-induced [Ca\(^{2+}\)]\(_i\) transients.

3.3. Puerarin potentiates caffeine-evoked [Ca\(^{2+}\)]\(_i\) increase

To further explore the effect of puerarin on CICR in hippocampal neurons, caffeine, a specific agent to activate CICR, was used. As shown in Fig. 4A, caffeine (3 mM, 40 s) produced equal [Ca\(^{2+}\)]\(_i\) responses with a 10 min interval between stimuli in hippocampal neuron (\(n=13\)) in the absence of extracellular Ca\(^{2+}\). Caffeine-evoked [Ca\(^{2+}\)]\(_i\) transients were significantly enhanced by pretreatment with puerarin (30 μM, 5 min) (\(P<0.05\), \(n=9\)). The effect was reversible, as illustrated by a third caffeine stimulation following 10 min washout period. The experiments were repeated three times using different batches of cells.

Fig. 3. Effects of puerarin on VGCC calcium currents in rat hippocampal neurons. Cells were held at a potential of −80 mV, and currents were elicited by a depolarizing test step from −50 mV to 50 mV. The voltage steps were applied for 300 ms at intervals of 10 s. (A) Represent recording showing that application of puerarin (30 μM) failed to affect amplitude of \(I_{Ca}\) (closed circles, \(n=11\)) and \(I_{Ca}\) in the presence of puerarin 30 μM (open circles, \(n=7\)). * \(P<0.05\) compared to control. The experiments were repeated three times using different batches of cells.

Fig. 4. Puerarin potentiates caffeine-evoked [Ca\(^{2+}\)]\(_i\) transients. (A) Addition of caffeine (3 mM, 40 s) produced equal [Ca\(^{2+}\)]\(_i\) responses with a 10 min interval between stimuli in hippocampal neuron (\(n=13\)) in the absence of extracellular Ca\(^{2+}\). (B) Representative trace showing pretreatment with puerarin (30 μM, 5 min) significantly enhanced the caffeine (3 mM)-induced [Ca\(^{2+}\)]\(_i\) transient in the absence of extracellular Ca\(^{2+}\) (92.4±21.8%, \(n=9\)). The effect was reversible, as illustrated by a third caffeine stimulation following 10 min washout period. The experiments were repeated three times using different batches of cells.
Ryanodine receptors, was used. In the Ca^{2+} free medium, repeated stimulations with caffeine (3 mM, 40 s) evoked equal \([\text{Ca}^{2+}]_i\) changes when the interval between applications was more than 10 min (Fig. 4A, \(n=13\)). As shown in Fig. 4B, in the absence of extracellular Ca^{2+}, pretreatment with puerarin (30 \(\mu\)M) greatly enhanced the caffeine-induced \([\text{Ca}^{2+}]_i\), transient to the 192.4 \(\pm 21.8\)% of control (\(P<0.01, n=9\)) in 91% of recorded neurons.

The potentiation effect of puerarin was reversible. After a 10 min washout period, the third caffeine-induced \([\text{Ca}^{2+}]_i\) transient was equal to the first. These results thus further support the deduction that the sensitization of CICR contributed to the potentiation effect of puerarin.

### 3.4. Puerarin-induced potentiation of KCl-evoked \([\text{Ca}^{2+}]_i\) transients is mediated by estrogen receptors

Using estrogen receptor-specific antagonist ICI 182780, we therefore investigated the role of estrogen receptor on the potentiation effect of puerarin. As shown in Fig. 5A and D, preincubation with ICI 182780 (1 \(\mu\)M, 8 min) abolished the potentiation effect of puerarin (30 \(\mu\)M) on KCl-evoked \([\text{Ca}^{2+}]_i\) transients (from 163.7 \(\pm 8.5\)% to 103.1 \(\pm 7.9\)% (\(n=21\))). Moreover, the mixed estrogen receptor agonist/antagonist tamoxifen (1 \(\mu\)M, 8 min) also significantly attenuated this potentiation effect (Fig. 5D, \(n=14\)), indicating that the activation of estrogen receptors is involved in the potentiation induced by puerarin.

### 3.5. PKA pathway contributes to potentiation induced by puerarin

Membrane-associated estrogen receptor is thought to be linked to activation of cyclic AMP/PKA and phospholipase C (PLC)/IP3 signaling cascades (Gu and Moss, 1996; Shingo and Kito, 2002, 2005; Le Mellay et al., 1999). To identify the signaling pathways through which puerarin potentiated the KCl-evoked \([\text{Ca}^{2+}]_i\) transients, a membrane-permeant PKA inhibitor H89 and a PLC inhibitor U-73122 were used. As shown in Fig. 5B and D, pretreatment with H89 (10 \(\mu\)M, 8 min) prevented the potentiation effect of puerarin (30 \(\mu\)M) on KCl-evoked Ca^{2+} transients in hippocampal neurons (from 163.7 \(\pm 8.5\)% to 95.6 \(\pm 10.4\)% (\(n=18\))). In contrast, U-73122 (10 \(\mu\)M, 8 min) failed to inhibit this potentiation effect of puerarin (Fig. 5C, D, 157.5 \(\pm 12.5\)%). These results suggest that the potentiation effect of puerarin is dependent on the activation of PKA.

### 4. Discussion

In the present study, we found that puerarin did not influence the basal \([\text{Ca}^{2+}]_i\), but substantially increased the peak of KCl-evoked Ca^{2+} transient in cultured rat hippocampal neurons. It is well documented that the stimulating effect of KCl presumably results from membrane depolarization with subsequent activation of VGCC and further Ca^{2+}-induced Ca^{2+} release from the ryanodine-sensitizing Ca^{2+} store (Friel and Tsien, 1992; Shmigol et al., 1994). Indeed, our results showed that application of KCl elevated \([\text{Ca}^{2+}]_i\) in hippocampal neurons. This response was partially attenuated by L-type VGCC antagonist nifedipine. Dantrolene or ruthenium red, the CICR inhibitors against ryanodine receptors, also decreased the Ca^{2+} transients evoked by KCl. Furthermore, dantrolene or ruthenium red completely blocked the potentiation effect of puerarin on the KCl-evoked \([\text{Ca}^{2+}]_i\) transients, whereas nifedipine failed to prevent this \([\text{Ca}^{2+}]_i\) response, suggesting that the potentiation was attributed to the facilitation of CICR via ryanodine receptors, rather than the
enhancement of Ca\(^{2+}\) entry via VGCC. Moreover, using whole-cell patch-clamp recording in cultured rat hippocampal neurons, puerarin failed to affect calcium currents generated by a depolarization pulse, which is similar with KCl-induced depolarization. This result further confirmed that VGCC was not involved in the potentiation effect of puerarin. In line with these findings, puerarin also markedly potentiated the neuronal [Ca\(^{2+}\)], response induced by lower concentration of caffeine in the absence of extracellular Ca\(^{2+}\). Caffeine is known as a specific agent that acts on the ryanodine receptor and could directly evoke intracellular Ca\(^{2+}\) release from the ryanodine-sensitizing Ca\(^{2+}\) store in Ca\(^{2+}\)-free solution (Zacchetti et al., 1991; Weber et al., 2002). Therefore, this provides further conceivable evidence for the CICR facilitating action of puerarin.

It is widely accepted that CICR plays a critical role in regulation of various neuronal functions. Numerous evidences confirmed that CICR from ER was involved in the process of synaptic plasticity, morphological plasticity and neuronal growth (Verkhratsky, 2005). As demonstrated by Lauri et al. (2003), long-term potentiation at mossy fiber-CA3 synapses recorded in hippocampal slices were critically dependent on CICR, which was prevented by ryanodine. Moreover, caffeine-activated release of Ca\(^{2+}\) from the ER triggers rapid elongation of spines in cultured embryonic hippocampal neurons (Korkotian and Segal, 1999). On the other hand, CICR was shown to regulate the neuronal excitability by influencing the activity of K (Ca) channels and Ca\(^{2+}\)-dependent Cl\(^{-}\) channels, which control postsynaptic hyperpolarization (AHP) and depolarization afterpotentials (DAPs) respectively in the different neurons (Li and Hatton, 1997; Martinez-Pinna et al., 2000; Sah and McLachlan, 1991). Previously, Xu et al. (2004) observed that, in ovariectomized mice, puerarin ameliorated learning and memory deficits through affecting the activity of the glutamergic/GABAergic system in the hippocampus. Moreover, in the aging mice induced by p-galactose, puerarin showed an improvement effect against the memory impairment and altered synaptic structure of hippocampus (Xu and Zhang, 2002). It has also been shown that puerarin attenuated the deficits of inhibitory avoidance performance induced by mecamylamine, p-chloroamphetamine, and dizocilpine, the effects were related to modulating cholinergic activity and serotonergic neuronal activity (Hsieh et al., 2002). Since facilitation of CICR by puerarin in the neurons may modulate the synaptic plasticity, morphological plasticity and regulate neuronal excitability in the neuron circuit, we postulate that this might be the underlying molecule mechanism for the therapeutic effects of puerarin in these animal studies (Xu and Zhang, 2002; Xu et al., 2004; Hsieh et al., 2002). However, further studies are required to detail this mechanism.

As the natural isoflavone isolated from P. lobata, puerarin was shown to possess the phytoestrogen activity in recent reports (Benihabib et al., 2002; Zhang et al., 2005). In the present study, the facilitation of CICR by puerarin appears to be a rapid non-genomic effect mediated by membrane-associative estrogen receptors, because preincubation with the estrogen antagonist IC1182780 or tamoxifen completely blocked potentiation of puerarin on KCl-evoked [Ca\(^{2+}\)], transient. Further-
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