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RESEARCH ARTICLE

Relaxant effect induced by wogonin from *Scutellaria baicalensis* on rat isolated uterine smooth muscle

Huey-Chuan Shih[1] and Ling-Ling Yang[1,2,3]

[1]*School of Pharmacy, College of Pharmacy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan,*
[2]*Center of Translational Research on Traditional Medicine, China Medical University Hospital, Taichung, Taiwan, and*
[3]*Graduate Institute of Clinical Medical Science, College of Medicine, China Medical University, Taichung, Taiwan*

Abstract

Context: Wogonin is a flavone derivative isolated from *Scutellaria baicalensis* Georgi (Labiatae) root, which is a traditional Chinese drug used as an anti-inflammatory and for management of dysmenorrhea.

Objective: The effect of wogonin on the uterus has not yet been examined. We investigated the relaxant effects of wogonin on contractile activity of isolated uterine strips of rats.

Materials and methods: The effect of wogonin on spontaneous uterine contraction, and uterine contraction induced by agonists, K^+-depolarization and oxytocin in Ca^{2+}-free solution was observed. To clarify the type of potassium channel, we tested the effects of 4-aminopyridine, tetraethylammonium and glibenclamide.

Results: Wogonin reduced the contractile amplitude of uterine strip smooth muscle of rats in a dose-dependent manner. The concentration of wogonin for reducing the contraction amplitude by 50% (IC_{50}) on spontaneous contractions was 60.5 μM. Wogonin also inhibited the contraction induced by three agonists (oxytocin, prostaglandin $F_{2\alpha}$ and acetylcholine). For the uterine strips pretreated with oxytocin in Ca^{2+}-free solution or K^+-depolarization, wogonin showed relaxant effect on the induced uterine contractions. In addition, whereas the inhibitive effect of wogonin on the contraction of uterine smooth muscle in rats could be partly blocked by 4-aminopyridine and tetraethylammonium, it was not influenced by glibenclamide.

Discussion and conclusion: Wogonin significantly inhibited the contraction of rat uterine smooth muscle probably through the inhibition of the inflow of extracellular calcium into cells via cell membrane, and intracellular release of calcium ions. In addition, the relaxant effect induced by wogonin might be due in part to the opening of voltage-dependent and large conductance Ca^{2+}-activated K^+ channels.

Keywords: Relaxant effect, extracellular calcium, intracellular calcium, Ca^{2+}-activated K^+ channel

Introduction

Dysmenorrhea, classified in primary and secondary forms, is a common problem for women of child-bearing age, and may occur in up to 50% of menstruating women. Primary dysmenorrhea refers to menstrual cramping pain in the lower abdomen caused by enhanced uterine contractions, in the absence of underlying pelvic pathology (Tonini, 2002). Nonsteroidal anti-inflammatory drugs (NSAIDs) are the main treatment in clinical medicine for dysmenorrhea, but they have many side effects (Lefebvre et al., 2005).

Scutellaria baicalensis Georgi (Labiatae) is a medicinal herb widely used for the treatment of various inflammatory diseases, hepatitis, tumors, allergic reactions, diarrhea, and dysmenorrhea in East Asian countries including China, Korea, Taiwan, and Japan (Kubo et al., 1994; Lin et al., 2005). One of the active components in the plant is 5, 7-dihydroxy-8-methoxyflavone, also known as wogonin (Figure 1). Wogonin is a flavonoid and has been shown to exert antioxidant (Gao et al., 1999), antiviral (Ma et al., 2002; Guo et al., 2007), antithrombotic (Kimura et al., 1997) and anti-inflammatory (Chen et al., 2001; Guo et al., 2007) activities. However, the relaxant effect of wogonin on the uterus contractions has not yet been examined. In this paper, we analyzed the relaxant

Address for Correspondence: Prof. Ling-Ling Yang, School of Pharmacy, College of Pharmacy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. Tel: +886 2 2736 1661. Fax: 886-2-27375440. E-mail: llyang@tmu.edu.tw

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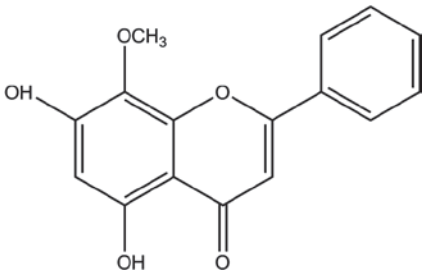

Figure 1. Chemical structure of wogonin. Molecular formula: $C_{16}H_{12}O_5$; molecular weight: 284.26.

effect and physiological mechanisms of wogonin on the rat uterine contractions.

Materials and methods

Plant material and extraction

Wogonin was extracted from dried *S. baicalensis*. The dried roots of *S. baicalensis* were purchased from SanYan Medicine Corporation (Taipei, Taiwan). Authenticity of the plant species was validated by the specific morphological and anatomical features and thin-layer chromatography (Lin et al., 1990). The voucher specimen was deposited at the laboratory in the Graduate Institute of Pharmacognosy, Taipei Medical University.

In brief, dried *S. baicalensis* roots were cut into small pieces, immersed, and extracted with 10-fold v/w 50% aqueous ethanol twice at room temperature for 2 weeks. After filtration, the residues were reflux-extracted with 4-fold v/w of 50% aqueous ethanol twice for 6 h. The 50% aqueous ethanol extracts were subjected to column chromatography on silica gel eluted with $CHCl_3$ and $CHCl_3$-MeOH, and chromatographed on silica gel eluted with hexane-acetone to yield wogonin. The compound was identified by direct comparison of its electrospray ionization (ESI) mass, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopic data with authentic samples. Purity tests of wogonin were performed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu model LC-10AT system (Kyoto, Japan) equipped with a Shimadzu model SIL-9A autoinjector and a Shimadzu model SPD-10 a detector (Shimadzu, Kyoto, Japan). The samples were separated on a Supelocosil TMLC18 (4.6 mm × 250 mm, 5 μm) with CH_3OH-CH_3COOH-H_2O (5:5:90, A) and CH_3OH-CH_3COOH-H_2O (90:5:5, B) as mobile phase at flow rate of 1.0 ml/min gradient elution, with UV detection at 275 nm, 26°C in 0–40 min (A:B = 6:4–2:8). The results showed the three kinds of active components could be separated completely in 30 min. The purity of all compounds exceeded 99.0% (Li et al., 2004).

Reagents

Oxytocin (OXT), acetylcholine HCl (ACh), dimethyl sulfoxide (DMSO), 4-aminopyridine (4-AP), tetraethylammonium (TEA) and glibenclamide (Glib) were purchased from Sigma, St. Louis, MO, USA. Diethylstilbestrol was purchased from China Chemical & Pharmaceutical, (Taiwan), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from Ono Pharmaceutical (Japan). The stock solutions of all drugs were diluted to the desired concentrations with a physiological salt solution.

Animals

We used non-pregnant female Wistar rats weighing 250–350 g purchased from the Center of Experimental Animals, National Taiwan University, Taipei, Taiwan. All experiments were performed in accordance with guidelines for animal experiments of Taipei Medical University and the guiding principles for the care and use of laboratory animals approved by the Chinese Society of Laboratory Animal Sciences, Taiwan. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Isolation of rat uterus

Diethylstilbestrol (0.5 mg/kg) was subcutaneously injected to female Wistar rats 24 h before operation to induce estrus and to increase drug sensitivity of uterus. Thereafter, animals were sacrificed by decapitation, and the uterus was excised and cut into two muscle strips (each 1 cm). The uterine strips were immediately removed and placed in a flask containing Locke's solution of the following composition (in mM): NaCl 154, KCl 5.63, $NaHCO_3$ 1.79, $CaCl_2{\cdot}2H_2O$ 2.55, and glucose 5.55. Experiments commenced within 5 min of removal of tissue samples. Preparations were placed in isolated organ baths, incubated in Locke's solution at 37 ± 1°C and bubbled with gas (95% O_2, 5% CO_2). The preload was 1 g, and the equilibration period was no less than 45 min (Zafra-Polo et al., 1993). Contractions were recorded by force displacement transducers (Kent Scientific Corporation, USA) using MP100 Biological System workstation software (Biopac Systems, Inc, USA) on a PC.

Experimental procedure

Experimental observation of wogonin effect on spontaneous contractions

Rat uterine strips were equilibrated in Locke's solution for 50 min. Cumulative amounts of wogonin (1–100 μM) were added every 15 min. Control experiments were performed with the vehicle (0.1% DMSO). The amplitude of spontaneous contractions on the uterine smooth muscle was recorded.

Experimental observation of wogonin effect on agonist-induced contractions

Uterine strips were incubated in the Locke's solution and equilibrated for 50 min, then treated with OXT (0.01 U/ml bath concentration), $PGF_{2\alpha}$ (0.1 μM bath concentration) and ACh (1 μM bath concentration), respectively, for 15 min, and further treated with wogonin (1–100 μM), respectively, every 15 min in the presence of OXT, $PGF_{2\alpha}$

and ACh. Control experiments were performed with the vehicle (0.1% DMSO).

Experimental observation of wogonin effect on OXT-induced Ca^{2+}-free contractions

Uterine smooth muscle strips were equilibrated for 1 h in Locke's solution. The solution was then replaced with Ca^{2+}-free solution containing 3 mM EDTA, and incubation continued for 50 min. Subsequently, the solution was replaced by a Ca^{2+}-free solution containing 1 mM EDTA, and the uterus was incubated for an additional 20–30 min. After a sustained contractile response to OXT (0.01 U/ml) was obtained, cumulative concentrations of wogonin (1–100 μM) was added every 15 min, respectively, in the presence of OXT. Tensions of uterine smooth muscle strips were recorded over a 10 min period after administration.

Experimental observation of wogonin effect on contractions induced by K^+-depolarization

The organ was immersed in Jalon–Ringer solution and equilibrated for 50 min. This solution was replaced by a high K^+ condition (KCl 56.3 mM) that caused a rapid contraction, followed by slight relaxation and a prolonged contraction plateau. When the plateau was reached, cumulative concentrations of wogonin (1–100 μM) were administered, and concentration-related relaxations were observed.

Experimental observation of wogonin effect involved in the potassium channels

In order to test the involvement of potassium channels in the mechanism of action of wogonin, we used three kinds of potassium channel blockers including 4-AP [a voltage-dependent potassium channel (K_V) blocker; 5 mM], TEA [a large conductance Ca^{2+}-activated potassium channel (BK_{Ca}) blocker; 1 mM] and Glib [an adenosine triphosphate (ATP)-dependent potassium channel (K_{ATP}) blocker, 30 μM]. These blockers were respectively applied to the uterine strips once at the beginning of experiment. After 15 min, wogonin was added every 15 min in a cumulative way. The cumulative concentration-response curves of wogonin for each case were then constructed and compared with those obtained with uterine smooth muscle strips that had not been treated with these inhibitors.

Statistical analysis

The results are expressed as the mean ± SE of several preparations (n) from different animals. The amplitude of contractions occurring over a 10 min period before adding wogonin was taken as the maximum contraction amplitude. Relaxation was expressed as a percentage of reduction in the contraction amplitude from the maximum to the value after wogonin treatment. According to the dose–response curves, the concentration of wogonin for producing 50% of relaxation (IC_{50}) was estimated. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA) and Student's t-test. Significant differences with controls are shown as $^*p < 0.05$ and $^{**}p < 0.01$.

Results

Effects of wogonin on spontaneous contractions of the rat uterus

Application of wogonin, in a cumulative fashion, inhibited spontaneous contractions of the rat uterus in a dose-dependent manner, whereas the addition of vehicle alone (0.1% DMSO) had no effect. Different concentrations of wogonin could attenuate contractile activity of uterine strips and degrade the amplitude of contraction wave. The IC_{50} value of wogonin on the amplitude of spontaneous uterine contractions was 62.03 μM (Figure 2).

Effects of wogonin on uterine contractions induced by agonists

Wogonin exhibited a dose-dependent relaxant effect on the rat isolated uterus precontracted with $PGF_{2\alpha}$ (0.1 μM), ACh (1 μM) and OXT (0.01 U/ml). The IC_{50} values of wogonin on $PGF_{2\alpha}$-, ACh-, and OXT-induced contractions were 17.97, 22.80 and 35.65 μM, respectively. This indicates that wogonin had stronger effect in inhibiting $PGF_{2\alpha}$-induced uterine contractions (Figure 3).

Effect of wogonin on OXT-induced uterine contractions in the Ca^{2+}-free solution

A uterine smooth muscle strip was incubated in a Ca^{2+}-free solution containing 3 mM EDTA for 50 min, and then in a Ca^{2+}-free solution containing 1 mM EDTA for an additional 20–30 min. A sustained contractile response to OXT (0.01 U/ml) was obtained, and cumulative amounts of wogonin were added. It was found that with the increase in the concentration of wogonin, uterine contractions were gradually inhibited (Figure 4). The inhibitory effect

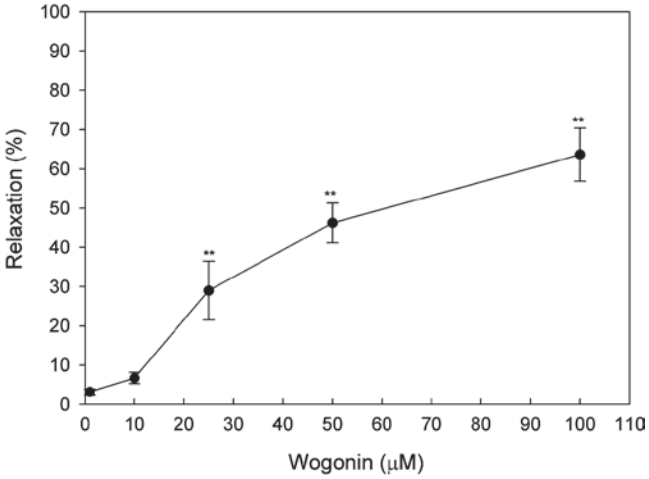

Figure 2. Effect of wogonin on the spontaneous contractions in uterine smooth muscle strips isolated from non-pregnant rats. Data are expressed as the mean ± SE ($n = 4$). *Significantly different from the control ($^{**}p < 0.01$).

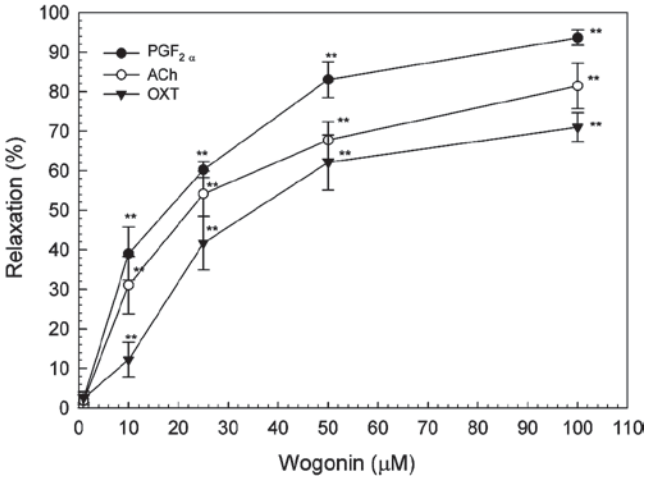

Figure 3. Effects of wogonin on contractions of rat uterus induced by OXT (0.01 U/ml), $PGF_{2\alpha}$ (0.1 μM) or ACh (1 μM). Data are expressed as the mean ± SE ($n=4$).*Significantly different from the control (**$p<0.01$).

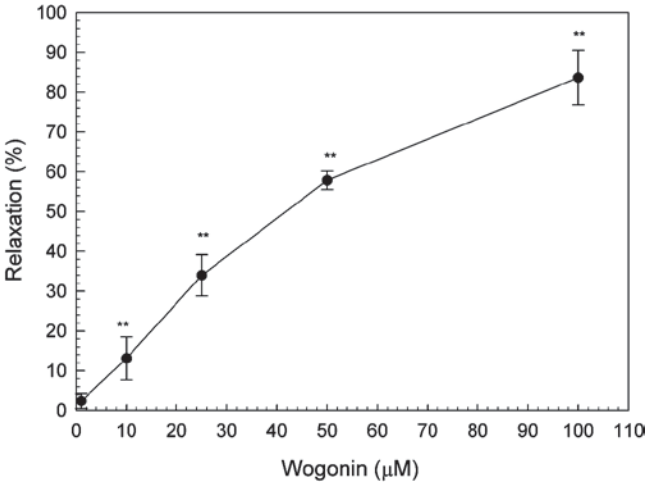

Figure 4. Effects of wogonin on contractions of rat uterus induced by OXT (0.01 U/ml) on Ca^{2+}-free solution. Data are expressed as the mean ± SE ($n=4$). *Significantly different from the control (**$p<0.01$).

of wogonin was significant at the doses of 10, 25, 50 and 100 μM ($p<0.01$).

Effect of wogonin on uterinei contractions induced by K^+-depolarization (high K^+ condition)

The effect of wogonin on a high concentration of potassium (KCl 56.3 mM)-induced uterine contractions was examined. As shown in (Figure 5) the uterine contractions induced by K^+ depolarization were gradually inhibited with increasing concentrations of wogonin reaching the significant level at 10, 25, 50 and 100 μM ($p<0.01$). The IC_{50} of the wogonin was 52.37 μM.

Effects of potassium channel blockers on wogonin-induced relaxation in uterine smooth muscle

To clarify the type of potassium channel, we tested the effect of 4-AP (a K_V blocker; 5 mM), TEA (a BK_{Ca} blocker; 1 mM) and Glib (a K_{ATP} blocker; 30 μM). TEA and 4-AP

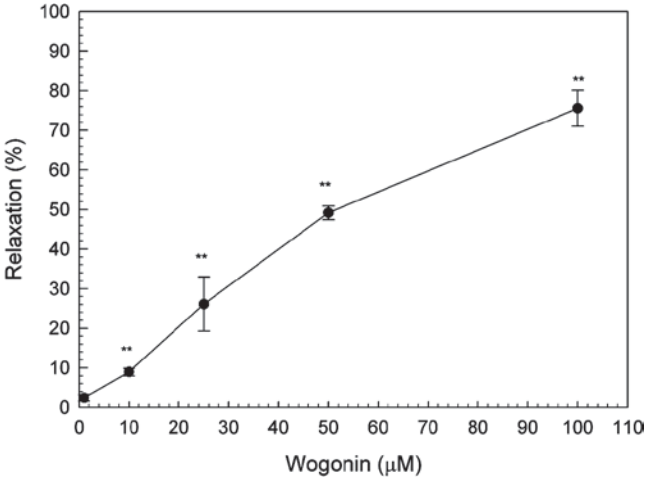

Figure 5. Effects of wogonin on tonic contractions of rat uterus induced by KCl (56.3 mM). Data are expressed as the mean ± SE ($n=4$). *Significantly different from the control (**$p<0.01$).

could partially block the inhibitive effect of wogonin on the contraction of uterine smooth muscle in rats. Comparing with the control group the influences of TEA and 4-AP had significant difference in the contractile amplitude after variance analysis ($p<0.01$), while Glib had no obvious blocking effect on the inhibition of contractile activity of uterine smooth muscle in rats (Figure 6).

Discussion

There are several studies which evaluated the effect of Chinese medicinal prescriptions for the dysmenorrhea. Calixto et al. (1991) investigated the effect of *Leonotis nepetaefolia* R. Br. (Labiatae) on rat uterus contractions *in vitro*; Perez-Vallina et al. (1995) investigated the effect of nonsteroid anti-inflammatory drugs on rat uterus contractions induced by $PGF_{2\alpha}$ or KCl *in vitro*. Our group has investigated the effect of Chinese medicinal prescriptions for dysmenorrhea (Hsu et al., 2003, 2006). Our results in this study (Figures 2 and 3) demonstrate that wogonin, one of the major components of the herb *S. baicalensis*, exerted significant relaxant effects on spontaneous and agonist (OXT, $PGF_{2\alpha}$ and ACh)-induced uterine contractions. It may contribute to its important role in treating dysmenorrhea.

When the intracellular Ca^{2+} ion concentration in uterus smooth muscle exceeds 10^{-6} M, uterine contractions will be induced (Fritsch & Murdoch, 1997). There are four different Ca^{2+} influx channels which regulate the intracellular Ca^{2+} ion concentration: receptor-operated channels (ROCs), voltage-operated channels (VOCs), second messenger-operated channels and store-operated channels (SOCs) (Bolton, 1979; D'Ocon et al., 1991; Hurwitz, 1986; Triggle et al., 1989).

Recently, some other factors have been described as contributing to this contraction such as increase of intracellular concentration of inositol triphosphate

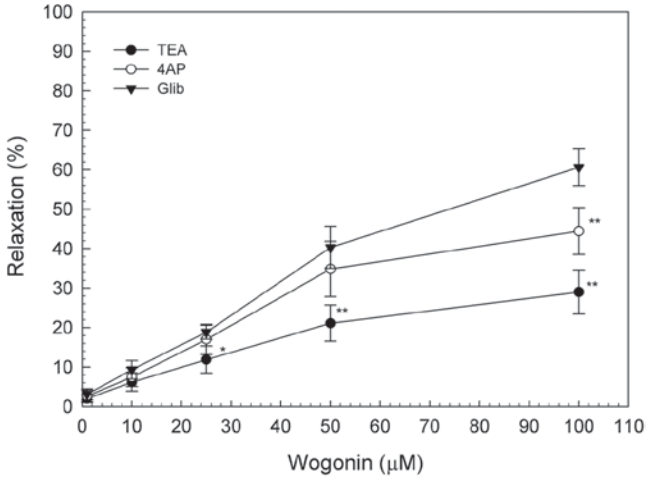

Figure 6. Effect of the potassium channel inhibitors, TEA (1 mM), 4-AP (5 mM), and Glib (30 μM) on wogonin-induced uterine relaxation. Data are expressed as the mean ± SE ($n = 4$). *Significantly different from the control ($^{*}p < 0.05$, $^{**}p < 0.01$).

(IP3) or arachidonic acid production, which then increases the intracellular Ca^{2+} concentration (Phillippe & Chien, 1995). On the other hand, rat uterus OXT-induced contraction results from increases of cytosolic Ca^{2+} originating from IP3-sensitive intracellular stores and of calcium influx via ROCs (Trujillo et al., 2000). In our experiment using a Ca^{2+}-free solution, the Ca^{2+} influx channels will not be the causes for the increase of intracellular Ca^{2+} concentration. Therefore, our experimental results of wogonin effect on OXT-induced uterine contractions in the Ca^{2+}-free solution (Figure 4) show that wogonin is able to alleviates uterine contractions through inhibiting the release of Ca^{2+} from intracellular stores.

The intracellular Ca^{2+} ion concentration is also affected by membrane potential because depolarization of membrane potential opens the voltage-gated Ca^{2+} channels and then the extracellular Ca^{2+} ions migrate into the cell, resulting in the induction of uterine contractions (D'Ocon et al., 1991). High K^{+} condition changes membrane potential, and therefore we also investigated the effects of wogonin on K^{+} depolarization-induced uterine contractions in this study. The findings shown in Figure 5 reveal that wogonin at 10, 25, 50 and 100 μM could significantly inhibit the uterine contraction induced by K^{+} (56.3 mM KCl) depolarization. This suggests that wogonin might have a role to stabilize the membrane potential of the uterine smooth muscle cells, and subsequently decrease uterine contractions by decreasing the membrane action potential.

At lease three types of potassium ion currents have been described in rat myometrium: a fast transient current and two calcium-dependent non-inactivating currents. A single-channel recording experiment revealed that large-conductance calcium-dependent potassium channels appear in the myometrium of both pregnant and non-pregnant (Triggle, 1996) and their activation results in cell hyperpolarization and suppression of the concentration of intracellular Ca^{2+}. Therefore, a potassium channel opener is a strong uterine relaxant.

Potassium channels are composed of diverse groups of membrane channels including a voltage-dependent potassium channel (K_V), a large conductance Ca^{2+}-activated potassium channel (BK_{Ca}) and an ATP-dependent potassium channel (K_{ATP}). Opening of potassium channels causes hyperpolarization of the plasma membrane by increasing potassium conductance and reduces cell excitability by shifting the membrane potential away from the threshold for excitation (Koji et al., 1999). In the present study, we used potassium channel blockers, including 4-AP, TEA and Glib. These results (Figure 6) showed that wogonin-induced relaxation of the uterus was blocked by TEA and 4-AP but not by Glib. The present findings suggested that the relaxation effect induced by wogonin may be partially due to BK_{Ca} and K_V in isolated uterine smooth muscle strips.

Conclusion

The present findings clearly show that wogonin has multiple effects on the uterine smooth muscles. The mechanism may be related to that it can inhibit extracellular calcium inflowing into cells via cell membrane and intracellular release of calcium ions. In addition, wogonin induced relaxation responses in uterus smooth muscle may be dependent on the activation of Kv and BK_{Ca} potassium channels. These results indicate that wogonin is one of the active ingredients of *S. baicalensis* and has the potential to be developed into an effective drug for the treatment of primary dysmenorrhea.

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Declaration of interest

The authors report no conflicts of interest.

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