INTRODUCTION

A large percentage of women (>50%) suffer from dysmenorrhea (1). It is generally accepted that primary dysmenorrhea is a repercussion of decreased uterine blood flow caused by the hypercontractility of the uterus smooth muscle and local contractions of the uterine vessels (2). Up to date, the cause of dysmenorrhea remains unclear, but the prostaglandins includes prostaglandin E2 and F2α are important factors of the regulation of uterine contractility. Up to date, the cause of dysmenorrhea remains unclear, but the prostaglandins includes prostaglandin E2 and F2α are important factors of the regulation of uterine contractility. Also, it has been recently emphasized that peroxisome proliferator activated receptors are mediators of prostaglandin E2 synthesis/accumulation in porcine endometrium (3). Dysmenorrhea is mainly treated with nonsteroidal anti-inflammatory drugs (NSAIDs) which suppress prostaglandins production by inhibition of cyclooxygenase. However, NSAIDs are not successful in all patients and they have remarkable adverse effects involving cardiovascular undesirable events, disorders of kidney and digestive system (1, 2). Accordingly, the ideal agent for the prevention and treatment of uterine abnormal contractility has not yet been found. Therefore, development of effective and safe agents is an important research topic.

The effect of resveratrol (1–100 µM) on contractility of non-pregnant rat uterus: the contribution of K+ channels

This study was aimed to evaluate resveratrol (1–100 µM) effect on the spontaneous rhythmic contractions (SRC), oxytocin-induced (0.2 nM, POxC) phasic and tonic (20 nM, TOxC) contractions of isolated rat uterus. The SRC and POxC were more sensitive to resveratrol than TOxC (pD₂ values: 4.53 and 4.66 versus 4.06). Different blockers of K⁺ channels (glibenclamide, tetraethylammonium, iberiotoxin, 4-aminopyridine) antagonized the response to resveratrol on the SRC and phasic contractions, but did not antagonize the effect of resveratrol on the TOxC. In order to compare the relaxant activities of resveratrol on the TOxC with that of potassium channel openers, a separate experiments with NS 1619, a highly specific big Ca²⁺-sensitive K⁺ (BKCa) channels opener and pinacidil, a predominant opener of ATP-sensitive K⁺ (KATP) channels were done. NS 1619 (10–100 µM) and pinacidil (10–100 µM) produced more potent inhibition of TOxC than resveratrol (pD₂ values were 6.00 and 5.29). Iberiotoxin, a highly selective BKCa channels blocker, antagonized the response to NS 1619 and glibenclamide, a highly selective KATP channels blocker, antagonized the response to pinacidil on the TOxC. To test K⁺ and extracellular Ca²⁺- independent mechanism(s) of resveratrol on TOxC, a K⁺-rich, Ca²⁺-free solution was used. Under this condition, only high concentrations (≥30 µM) of resveratrol inhibited TOxC. Western blots analysis confirmed expression of Kir6.1, Kir6.2, KCa1.1, Kv2.1 and Kv4.2. channel proteins in myometrium. Thus, the effect of resveratrol is dependent on the types of contractions. The inhibitory response of resveratrol on the SRC and phasic contractions involves different myometrial K⁺ channels. When applied in high concentrations, resveratrol has an additional K⁺ channels independent mechanism(s) of action. As the effects of NS 1619, pinacidil and resveratrol on the TOxC are different, we can conclude that resveratrol does not behave as a classical potassium channel opener.

Key words: non-pregnant rat uterus, contractility, potassium channels, resveratrol, calcium, oxytocin, calcium channel activator, potassium channel blocker
blocked K_{Ca} and Kv, but activated BKCa channels (12). This discrepancy in the results obtained on the various experimental models strongly suggests tissue and species selectivity of resveratrol. However, despite the increasing interest for the effects of resveratrol on various smooth muscles, the influence of resveratrol on the contractility of uterus is not entirely defined. According to our knowledge, there is only one study dealing with resveratrol effect on the non-pregnant uterus. Hsia et al. demonstrated that resveratrol inhibited prostaglandin F_{2alpha}-oxytocin-, acetylcholine-, and carbachol-induced uterine contractions in rats. Also, it inhibited contractions induced by high K^+ concentration and by Ca^{2+} channel activator (Bay K 8644). They concluded that resveratrol suppress the increases in intracellular Ca^{2+} concentration ([Ca^{2+}]_i) induced by different receptor agonists and blocks influx of external Ca^{2+} (13).

It is obvious that uterine contractility is a complex and dynamic physiological process which consists of phasic and prolonged tonic contractions. In order to determine whether resveratrol influences myometrium contractility, we investigated three types of uterine contractions: spontaneous rhythmic contractions (SRC), oxytocin-induced phasic contractions and oxytocin-induced tonic contractions. It is most commonly considered that SRC are the result of depolarization of membrane and consequent opening L-type Ca^{2+} channels. Intracellular Ca^{2+} transients produced by Ca^{2+} entry generate phasic spontaneous contractions; however the true nature of these formations is not fully understood (14). Previously it has been confirmed that low nanomolar concentration of oxytocin (≤1 nM) induces phasic contractions of rat uterus in oestrus, but higher concentrations of oxytocin (≥20 nM) induce tonic contractions (15). Therefore, here we used 0.2 nM and 20 nM of oxytocin for generation of phasic and tonic contractions, respectively. Phasic contractions are essential for the successful progression of labor, but they are undesirable for dysmenorrhea. The prolonged tonic contractions of the uterus may reduce blood flow in the myometrium and cause hypoxia.

Opening of K^+ channel is crucial for achieving the hyperpolarisation and lowering of the [Ca^{2+}]_o, resulting in relaxation of myometrium. It has been shown that potassium channels openers inhibited uterus smooth muscle contractility by activation of different K^+ channels (16-18). To date, several types of K^+ channels have been identified by means of pharmacological and electrophysiological methods in the uterus smooth muscle. The most studied include the K_{Q}, Kv, and BKCa channels (19-22). However, K^+ channel family includes more than 100 various protein subunits (22). The understanding of the most subtypes of K^+ channel and detection of their subunits/proteins in the smooth protein subunits (22). The understanding of the most subtypes of K^+ channels openers (16-18). To date, several types of K^+ channels have been identified by means of pharmacological and electrophysiological methods in the uterus smooth muscle. The most studied include the K_{Q}, Kv, and BKCa channels (19-22). However, K^+ channel family includes more than 100 various protein subunits (22). The understanding of the most subtypes of K^+ channel and detection of their subunits/proteins in the smooth muscle of uterus is important. Changes in the expression or activity of K^+ channels can translate into changes in the excitability, contractility and relaxation of smooth muscles of the uterus.

Therefore, this study was aimed to investigate: 1) the influence of resveratrol on contractility of rat non-pregnant uterus and 2) the contribution of different K^+ channel subtypes in the resveratrol action on myometrial contractility.

Western blot study with specific antibodies for Kir6.1 and Kir6.2 subunit of K_{Q} channels, α- subunits Kv2.1 and Kv4.2 subtype of Kv channels and KCa1.1 subunit of BKCa channels was performed in order to confirm their presence in the smooth muscle of the non-pregnant rat uterus in oestrus.

**MATERIALS AND METHODS**

**Animals**

Experiments were carried out on the virgin female Wistar rats (200–250 g) obtained from the animal facilities of the Faculty of Medicine, University of Belgrade. This study has been care out in accordance with the Directive 2010/63/EU and the Guide for the Care and Use of Laboratory Animals (8th ed), as adopted and promulgated by the United States National Institute of Health. It has been approved by Ethics Committee of Faculty of Medicine, University Belgrade (No 4211/2).

The oestrus phase of the oestrous cycle was determined by microscopic examination of a daily vaginal smear (23). All rats were killed by cervical dislocation. Uterine horns were cut into longitudinal segments of approximately 5 mm length and mounted in 10 ml volume organ bath containing Krebs-Ringer solution aerated with 95% O_{2} and 5% CO_{2} at 37°C. Each strip was attached with one end via surgical thread to a force transducer and the other one was held fixed. The preparations were mounted for isometric recording under 1 g tension and equilibrated for 45 min. Isometric tension was measured with TSZ-04/1.2 isolated tissue bath system and force transducer (Experimetria, Budapest, Hungary). Data were recorded via computer using IsolAB software (Elunit, Belgrade, Serbia).

**Experimental procedure**

After equilibration, some preparations (37%) had spontaneous rhythmic contractions. The preparations were allowed to stabilize for at least 30 min before adding drugs. In a separate series of experiments, uterus strips were stimulated with oxytocin 0.2 nM or 20 nM to induce contractions and allowed for 30 min period to assess control contractile performance. In both types of experiments, concentration-response curves were constructed by adding resveratrol (1–100 µM) directly to the bathing solution in a cumulative way, taking the amplitude of the response immediately before addition of a drug as the control contraction. The results are expressed as the percentage inhibition of the control contraction. Increasing concentrations of resveratrol were added only after the previous concentration had produced an equilibrium response or after 20 min if no response was obtained.

In order to compare the effect of resveratrol with the effects of K^+ channels openers, NS 1619 and pinacidil were used. The uterus strips were stimulated with oxytocin (20 nM) to induce tonic contractions and allowed for 30 min period to assess control contractile performance. In both types of experiments, concentration-response curves were constructed by adding NS 1619 (10–100 µM) or pinacidil (10–100 µM) directly to the bathing solution in a cumulative way. Increasing concentrations of K^+ channels openers were added only after the previous concentration had produced an equilibrium response or after 20 min if no response was obtained.

To test the involvement of K^+ channels in a mechanism of action of resveratrol, NS 1619 and pinacidil, the different K^+ channel blockers were examined. In separate experiments, glibenclamide, iberiotoxin, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP) were added into the bathing solution 20 min before exposure to resveratrol, NS 1619 and pinacidil. The concentration- response curves to resveratrol and potassium channel openers were obtained in the presence of K^+ channel blockers.

To investigate the effect of resveratrol on contractions elicited only with intracellular source of Ca^{2+} the protocol was used:

1. Uterus strips were bathed in Krebs solution.
2. The tissue samples were bathed in K^-rich, Ca^{2+}-free solution in which the concentration of KCl was raised to 60 mM by isosmotic replacement of NaCl.
3. Strips were stimulated with oxytocin (20 nM) to produce control contraction.
4. The tissue was returned to Krebs solution for a 30 min.
5. The tissue samples were bathed in K^-rich, Ca^{2+}-free solution, again.
6) A single concentration of resveratrol (1 µM, 3 µM, 10 µM, 30 µM and 100 µM) was added separately to the medium for 10 minutes and the preparation was stimulated with oxytocin. The control contraction to oxytocin was taken as 100%. Vehicle matched control experiments were conducted.

**Western blot analysis**

Rat uterine tissue samples were homogenized in RIPA buffer with protease inhibitors (Roche Applied Science, Mannheim, Germany). Cell lysate was centrifuged for 20 minutes, 11,000 rpm at 4°C and the supernatant was transferred to a new tube. Protein concentration was measured at 595 nm with spectrophotometer using the Bio-Rad Protein Assay, based on the method of Bradford. Prior to SDS PAGE, LDS Sample Buffer and Reducing Agent were added to the sample, denaturated for 10 minutes at 70°C and loaded into precast 4–12% Bis-Tris gel (Life Technologies, Carlsbad, CA, USA). After electrophoresis, separated proteins were transferred to a nitrocellulose membrane. A membrane was blocked with 5% nonfat dry milk and 0.1% Tween 20 for one hour at room temperature and probed with primary antibody for Kir6.1 and Kir6.2 subunit of KATP channels, α-subunits Kv2.1 and Kv4.2 subtype of Kv channels and KCa1.1 subunit of BKCa channels (Anti-Kir6.1; Anti-Kir6.2; Anti-Kv2.1; Anti-Kv4.2; Anti-KCa1.1; Alomone Labs, Jerusalem, Israel) overnight at 4°C. Membranes were probed with HRP-conjugated antirabbit secondary antibody for 1.5 h at room temperature. Western blotting kit (Roche, Applied Science, Mannheim, Germany) was used for the chemiluminescent detections of proteins. After visualization, the membranes were stripped with 0.2 M NaOH, blocked and reprobed with anti-β-actin antibody (Abcam, Cambridge, England) and visualized. Protein loading was normalized to β-actin. ImageQuant software was used for quantitative analysis (24).

**Drugs and solutions**

All chemicals were obtained from the Sigma-Aldrich Inc. St Louis, MO, USA. Resveratrol and NS 1619 were dissolved in 70% v/v ethanol with further dilution in distilled water before use. Working concentrations of ethanol in the bath were <0.01% (v/v). Glibenclamide was dissolved in polyethylene glycol. Stock solution of pinacidil was dissolved in dilute acetic acid solution (0.1 N HCl) with further in distilled water before use. Iberitoxin, TEA, 4-AP, and oxytocin were dissolved in distilled water. The Krebs-Ringer solution had the following composition (mmol/l: NaCl 120, KCl 5, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, glucose 11). Krebs-Ringer K+-rich, Ca2+-free solution was prepared by omitting CaCl2 and adding EGTA (1 mM). All drugs were added directly to the bath in a volume of 50 µL and the concentrations given are the calculated final concentrations in the bath solution.

**Treatment of data and statistics**

The amplitude of the tonic contractions was measured from the baseline to plateau, and the amplitude of the phasic contractions were measured from the baseline to the end of the spike. The frequency of SRC and oxytocin-induced contractions was calculated as a number of cycles in a 10 minutes period of time. The mean amplitude and frequency during the control period was taken as 100%.

EC50 value is defined as the concentration of resveratrol required to produce 50% of the maximum response of elicited and spontaneous contractions, and it was determined for each curve by using a non-linear least square fitting procedure of the individual experimental data, and presented as pD2 (pD2 = –log EC50). The results are expressed as the means ± standard error of the mean (S.E.M.); n refers to the number of experiments. Statistical difference between means was determined by

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**Fig. 1.** Original recordings show the effects of resveratrol (1–100 µM) on contractile activity of the non-pregnant rat myometrium: SRC (A), phasic contractions provoked by low concentration of oxytocin (0.2 nM, square) (B), tonic contractions provoked by high concentration of oxytocin (20 nM, triangle) (C). SRC, spontaneous rhythmic contractions; Oxy, oxytocin.
Student’s t-test; a value of P<0.05 was considered statistically significant. Data analysis was performed in Graph Pad Prism (Graph Pad Software Inc., San Diego, USA).

RESULTS

Effects of resveratrol on spontaneous rhythmic contractions

Longitudinal muscle strips of the rat uterus exhibited the SRC of constant amplitude 2.29±0.9 g and frequency 10.07±1.20 contractions per 10 minutes, as shown in Fig. 1A (n=12). Resveratrol (1–100 M) inhibited amplitude of SRCs in a concentration-dependent manner with a pD2 value of 4.53±0.20, maximal response 93.64±2.53 %, n=12 (Fig. 1A). Used in the same concentrations, resveratrol significantly inhibited the frequency of SRC (pD2 value was 4.34±0.66, n=12, Fig. 1A).

Glibenclamide (10 µM), a selective KATP channels blocker produced a significant rightward shift of the concentration-response curve of resveratrol (pD2 values: 4.53±0.20 in the absence versus 4.33±0.40 in the presence of glibenclamide, P<0.05, n=7). Glibenclamide failed to produce a suppression of the maximal response of resveratrol on SRC (93.64±2.53% in the absence versus 90.50±5.21% in the presence of glibenclamide, P<0.05, n=7, Fig. 2A).

TEA, a selective inhibitor of calcium-sensitive K+ channels used in concentration of 1 mM significantly affected resveratrol-induced inhibition (pD2 values: 4.53±0.20 in the absence versus 4.40±0.30 in the presence of TEA, P<0.05, n=7). TEA produced a significant suppression of the maximal response of resveratrol on SRC (93.64±2.53% in the absence versus 77.50±3.08% in the presence of TEA, P<0.05, n=7, Fig. 2A).

Iberiotoxin (100 nM), a highly selective blocker of BKCa channels, significantly affected resveratrol-induced inhibition (pD2 values: 4.53±0.20 in the absence versus 4.20±0.51 in the presence of iberiotoxin, P<0.05, n=5) of amplitude of SRC and it produced a significant suppression of the maximal response of resveratrol (93.64±2.53% in the absence versus 76.20±5.66% in the presence of iberiotoxin, P<0.05, n=5, Fig. 2A).

A predominant blocker of Kv channels, 4-AP (1 mM) produced a significant rightward shift of the concentration-response curve of resveratrol (pD2 values: 4.53±0.20 in the absence versus 4.41±0.50 in the presence of 4-AP, P<0.05, n=9). However, 4-AP failed to produce a suppression of the maximal response of resveratrol on SRC (93.64±2.53 % in the absence versus 93.22±6.47 % in the presence of 4-AP, P>0.05, n=9, Fig. 2A).

Iberiotoxin (100 nM) and TEA (1 mM) significantly affected resveratrol-induced inhibition of frequency of SRC (pD2 values: 4.66±0.28 in the absence versus 4.34±0.66 in the presence of iberiotoxin, n=5; versus 4.01±0.37 in the presence of TEA, respectively, n=6 P<0.05). Glibenclamide (10 µM, n=7) and 4-AP (1 mM, n=9) failed to antagonized the reduction of frequency of SRC by resveratrol (pD2 values were 4.37±0.42 and 4.36±0.30, respectively).

Effects of resveratrol on phasic contractions provoked by oxytocin

Application of low nanomolar concentration of oxytocin (0.2 nM) to bath medium produced phasic contractions of constant amplitude 2.95±0.70 g and frequency 11.59±1.08 (n=12, Fig. 1B). Resveratrol (1–100 µM) significantly inhibited amplitude of oxytocin-induced phasic contractions in a concentration-dependent manner with pD2 value of 4.66±0.28, maximal responses: 93.67±3.00%, n=12. (Fig. 2B). The frequency of oxytocin phasic contractions was inhibited by resveratrol also (pD2 value was 4.69±0.38, n=12, Fig. 2B).

Fig. 2. Antagonism of the inhibitory effect of resveratrol by K+-channel blockers on SRC and contractions provoked by oxytocin (phasic and tonic) on the isolated non-pregnant rat uterus. (A) Concentration-response curves for resveratrol on the SRC, (B) the phasic contractions provoked by oxytocin (0.2 nM), and (C) the tonic contractions provoked by oxytocin (20 nM), in the absence (circle) and presence of glibenclamide (10 µM, square), 4-AP (1 mM, down triangle), TEA (1 mM, diamond) and iberiotoxin (100 nM, up triangle). The points are the means and the vertical lines show the S.E.M. (n=5–12). *P<0.05. SRC, spontaneous rhythmic contractions; GLB, glibenclamide; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; Ibtx, iberiotoxin.
Glibenclamide (10 µM) produced a significant rightward shift of the concentration-response curve of resveratrol (pD₂ values: 4.66±0.28 in the absence versus 4.26±0.73 in the presence of glibenclamide, P<0.05, n=5) and suppression of the maximal response (93.67±3.00% in the absence versus 85.40±5.19% in the presence of glibenclamide, P<0.05, n=5, Fig. 2B).

TEA, used in concentration of 1 mM produced a significant rightward shift of the concentration-response curve of resveratrol (pD₂ values: 4.66±0.28 in the absence versus 4.19±0.72 in the presence of TEA, P<0.05, n=5). TEA produced suppression of the maximal response (93.67±3.00% in the absence versus 74.60±7.44% in the presence of TEA, P<0.05, n=5, Fig. 2B).

Iberiotoxin (100 nM) produced a significant rightward shift of the concentration-response curve of resveratrol (pD₂ values: 4.66±0.28 in the absence versus 4.30±0.21 in the presence of iberiotoxin, P<0.05, n=5) without a suppression of the maximal response (93.67±3.00% in the absence versus 94.56±1.68% in the presence of iberiotoxin P<0.05, n=5, Fig. 2B).

Application of 4-AP (1 mM) produced a significant rightward shift of the concentration-response curve of resveratrol (pD₂ values: 4.66±0.28 in the absence versus 4.34±0.55 in the presence of 4-AP, P<0.05, n=7) with a significant suppression of the maximal response (93.67±3.00% in the absence versus 78.00±6.00 % in the presence of 4-AP, P<0.05, n=7, Fig. 2B).

Iberiotoxin (100 nM) and TEA (1 mM) significantly affected resveratrol-induced inhibition of frequency (pD₂ values: 4.69±0.38 in the absence versus 4.30±0.62 in the presence of iberiotoxin; versus 4.35±0.71 in the presence of TEA, P<0.05, n=5, both), too. Glibenclamide (10 µM, n=5) and 4-AP (1 mM, n=7) failed to produce statistically significant suppression of the reduction of frequency of phasic contractions provoked by oxytocin (pD₂ values were 4.67±0.83 and 4.60±0.59).

Effects of resveratrol and potassium channel openers on tonic contractions provoked by oxytocin

Application of high concentration of oxytocin (20 nM) to bath medium produced tonic contraction with or without phasic contractions in addition (Fig. 1C). Resveratrol inhibited tonic contractions induced by oxytocin in the concentration-dependent manner with pD₂ value of 4.06±0.29 and maximal responses of 54.78±4.52%, n=9. The all used K⁺ channel blockers, glibenclamide (10 µM, n=5), TEA (1 mM, n=5), iberiotoxin (100 nM, n=5) and 4-AP (1 mM, n=5) failed to produce statistically significant suppression of the reduction of frequency of phasic contractions provoked by oxytocin (pD₂ values were 4.67±0.83 and 4.60±0.59).

In order to compare the effect of resveratrol on tonic contractions induced by oxytocin with the effect of potassium channel openers, NS 1619, a highly specific BKCa channels opener and pinacidil, a predominant opener of KATP channels were used (n=5 both). NS 1619 (10 nM–100 µM) inhibited tonic contractions induced by oxytocin in the concentration-dependent manner with pD₂ value of 6.00±0.34 and maximal responses of 98.82±1.02%, n=9. The all used K⁺ channel blockers, glibenclamide (10 µM, n=5), TEA (1 mM, n=5), iberiotoxin (100 nM, n=5) and 4-AP (1 mM, n=5) failed to produce a rightward shift of the concentration-response curve of resveratrol and suppression of the maximal response on tonic oxytocin-induced contractions (Fig. 2C).

Fig. 3. The effects of K⁺ channels openers NS 1619 and pinacidil with resveratrol on the tonic contractions induced by oxytocin (20 nM). (A) Concentration-response curve for NS 1619, a highly specific BKCa channels opener in the absence (circle) and presence (up triangle) of iberiotoxin 100 nM; (B) concentration-response curve for pinacidil, a predominant opener of KATP channels in the absence (down triangle) and presence (square) of glibenclamide 10 µM; (C) The order of potency: concentration-response curve for NS 1619 (circle), pinacidil (down triangle) and resveratrol (diamond). The points are the means and the vertical lines show the S.E.M. (n=5–12). *P<0.05. GLB, glibenclamide; Ibtx, iberiotoxin.
presence of glibenclamide was 4.61±0.71, suppression of the maximal response to pinacidil was 74.84±2.96, P<0.05 both, n=5, Fig 3B). There are statistically significant difference between pD2 values of resveratrol, pinacidil and NS 1619 (4.06±0.29 > 5.29±0.20 > 6.00±0.34, respectively, P<0.05 all). The order of potency was shown in the Fig 3C.

The all used K+ channel blockers did not alter the resting tone of the uterus and they did not modify the amplitude of SRC and contractions evoked by oxytocin (n= 2 all, data not shown).

Effects of resveratrol on the contraction provoked by oxytocin in K+-rich, Ca2+-free solutions

In a K+ -rich, Ca2+-free solution, the SRC and the phasic contraction provoked by low concentration of oxytocin (0.2 nM) were abolished (n=4 all, data not show), but an application of 20 nM oxytocin induced tonic contraction. Resveratrol in a concentrations <30 µM did not affect the amplitude of contractions induced by 20 nM oxytocin (% of contraction: 100% without resveratrol versus 100% with 1 µM, 3 µM or 10 µM of resveratrol, n=15 all, P>0.05, Figs. 4A and 4C). However, higher concentrations of resveratrol (30 µM and 100 µM) significantly reduced tonic contraction (% of contraction: 100% without resveratrol versus 65±2% with 30 µM of resveratrol and 47%±2% with 100 µM of resveratrol, respectively, P<0.05, n=10 both, Figs. 4B and 4C).

Western blot analysis

Western blotting analysis detected Kir6.1 and Kir6.2 subunit of KATP channels, α- subunits Kv2.1 and Kv4.2 subtype of Kv channels and KCa1.1 subunit of BKCa channels in non-pregnant rat uterus (n=5, each, Fig. 5A). Results were normalized to β-actin expression. To quantify the relative abundance of level expression proteins of Kir6.1, Kir6.2, Kv2.1, Kv4.2 and KCa1.1 densitometry was used. Comparison by densitometry revealed a significant lower expression level of the Kir6.2 protein than protein of the others K+ channels subtypes. There were no
significant differences in the density of the expression levels among the others protein subtypes (Fig. 5B).

**Table 1.** The effects of resveratrol on the frequency of SRC and phasic contractions provoked by oxytocin (0.2 nM) in the absence and in the presence of K+ channels blockers.

<table>
<thead>
<tr>
<th>Type of contractions</th>
<th>pD2</th>
<th>n</th>
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<tbody>
<tr>
<td>SRC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.34±0.66 *</td>
<td>12</td>
</tr>
<tr>
<td>GLB</td>
<td>4.37±0.42</td>
<td>7</td>
</tr>
<tr>
<td>4-AP</td>
<td>4.36±0.30</td>
<td>9</td>
</tr>
<tr>
<td>TEA</td>
<td>4.01±0.37 *</td>
<td>6</td>
</tr>
<tr>
<td>Ibtx</td>
<td>3.47±0.41 *</td>
<td>5</td>
</tr>
<tr>
<td>Oxytocin provoked phasic contractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.69±0.38 *</td>
<td>12</td>
</tr>
<tr>
<td>GLB</td>
<td>4.67±0.83</td>
<td>5</td>
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<tr>
<td>4-AP</td>
<td>4.60±0.59</td>
<td>7</td>
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<tr>
<td>TEA</td>
<td>4.35±0.71 *</td>
<td>5</td>
</tr>
<tr>
<td>Ibtx</td>
<td>4.30±0.62 *</td>
<td>5</td>
</tr>
</tbody>
</table>

Each pD2 value is the mean ± S.E.M. for n experiments. *P<0.05 versus pD2 values in the absence and in the presence of potassium channels blockers. SRC, spontaneous rhythmic contractions; GLB = glibenclamide, 4-AP, 4-aminopyridine; TEA, tetraethylammonium; Ibtx, iberiotoxin.

**Fig. 5.** (A) The expression of Kir6.1 and Kir6.2 subunit of KATP channels, α-subunits Kv2.1 and Kv4.2 subtype of Kv channels and KCa1.1 subunit of BKCa channels protein relative to β-actin detected by Western blot in non-pregnant rat uterus. (B) Quantitation of Western blot signals of K+ channels bands expressed as mean values ± S.E.M. densitometric units for each K+ subtypes shown as a histogram (n=5, each). * P<0.05 when comparing Kir6.2 subunits protein expression with Kir6.1, Kv2.1 and Kv4.2 and KCa1.1 subunit protein expression.

**DISCUSSION**

It has been found that resveratrol significantly decreased the amplitude and frequency of phasic contractions induced by low concentration of oxytocin (0.2 nM); its sensitivity to resveratrol was comparable with the sensitivity of SRCs (EC50 were 29 µM and 23 µM, respectively) to the same agent. Hsia et al. (13) have shown that 100 µM of resveratrol induced only 40% of inhibition of oxytocin-induced contractions of rat non-pregnant uterus. However, in their experiment they used higher concentrations of oxytocin in order to induced contractions (1 µM versus 0.2 nM used in our study) (13). The tonic component of oxytocin contractions had lower sensitivity to resveratrol (EC50=87 µM) than both types of phasic contractions. Also, Kawamata et al. (15) have published that phasic component of oxytocin-induced contractions of non-pregnant mouse uterus had higher sensitivity to verapamil than its tonic component. This is in line with previous findings where authors have shown that phasic contractions of guinea pig gallbladder were more sensitive to resveratrol than its basal tension (25). On the other hand, we have shown previously that resveratrol inhibited tonic contractions of rat aorta (7) and mesenteric artery (8) with higher potency (EC50 were 10 µM, both) than oxytocin-induced tonic contraction investigated here. But, the sensitivity of tonic contraction of human internal mammary artery...
(EC<sub>50</sub>=30 µM) to resveratrol was comparable with results obtained in this paper (9). It is plausible to conclude that there are apparent differences in sensitivity to resveratrol among various types of contractions (phasic versus tonic), species and tissues.

In order to analyze the contribution of K<sup>+</sup> channels to the inhibition of uterine contractions produced by resveratrol, we used agents that are known to possess a K<sup>+</sup> channel-blocking activity.

According to the results presented here, it seems that BK<sub>Ca</sub> channels contributed to the resveratrol inhibition of frequency of both types of phasic contractions. It has been showed that iberiotoxin initiated SRC in human myometrium (26) and enhanced the frequency, whereas Taggart and Wray (27) showed that same substance had no obvious effect on frequency. Moreover, neither TEA nor 4-AP affected the contraction interval of SRC, suggesting that these drugs were not affecting the frequency (28). However, it has been showed that the changes in frequency of phasic contractions induced by oxiotoxin, are mediated by the sarcoplasmic reticulum Ca<sup>2+</sup> release (29). Anwer et al. also showed that when applied to cultured smooth muscle cell isolated from human myometrium, iberiotoxin caused depolarization and a rise in [Ca<sup>2+</sup>]<sub>i</sub> (30). By repolarizing the cell membrane, opening of BK<sub>Ca</sub> channels may decrease the entry of extracellular Ca<sup>2+</sup> thereby, influence the intracellular Ca<sup>2+</sup> transient and contractile property. Since resveratrol decrease [Ca<sup>2+</sup>]<sub>i</sub>, the inactivation BK<sub>Ca</sub> channels by iberiotoxin and TEA may constitute a feedback repolarizing system.

Functional studies indicated that the K<sub>ATP</sub> channels have an important role in regulation of uterus contractility (19).

Stimulation of these channels relaxed the smooth muscle of uterus (17, 18). Structurally, K<sub>ATP</sub> channels are composed of pore-forming inward rectifiers channels, Kir6.1 or Kir6.2, and regulatory subunits, SUR1, SUR2A or SUR2B that assemble to form an octameric complex (31). The properties of these channels are different in various tissues due to the combinations of the subunits forming the channel. It is generally accepted that glibenclamide is a selective blocker of K<sub>ATP</sub> channels (32). In the present study, glibenclamide antagonized the effect of resveratrol on the SRC and of phasic oxytocin-induced concentration indicating involvement of K<sub>ATP</sub> channels in resveratrol mechanism of action. Indeed, in the rat heart it was reported that resveratrol depressed cardiac muscle contraction and shortened action potential duration due to the activation of K<sub>ATP</sub> channels (33). But, glibenclamide failed to antagonize the effect of resveratrol on the tonic oxytocin-induced contractions.

Also, our previous studies have shown that the relaxant effect of resveratrol on the tonic contraction of rat mesenteric and human internal mammary artery was not blocked by glibenclamide (7, 8). Accordingly, it seems that K<sub>ATP</sub> channels are not involved in the pathway by which resveratrol induces a relaxation of the tonic contractions. To our knowledge, this is the first study which reveals the protein expression of Kir6.1 and Kir6.2 subunits of K<sub>ATP</sub> channels in rat non-pregnant myometrium. We obtained that the protein expression level of Kir6.1 subunit was higher than Kir6.2 subunit when they normalized to β-actin expression. Earlier papers have showed similar expression ratio of the two types of Kir channels (Kir6.1/SUR2B and Kir6.2/SUR1) in the smooth muscle of non-pregnant rat and human uterus but only at the transcription level (34, 35).

Recently, one study found the protein expression of Kir6.1 and Kir6.2 subunits in human pregnant myometrium; however the expression level of Kir6.1 was less compared with Kir6.2 (36).

The expression of K<sub>ATP</sub> channels depends on the gestational stage and the presence of labor contractions (37). Considering the fact that Kir6.1/SUR2B is down regulated late in pregnancy (36) different ratio Kir6.1/Kir6.2 in non-pregnant and pregnant myometrium is not surprising.

It has previously been shown that potassium channel blockers produce relaxation of different blood vessels via activation of BK<sub>Ca</sub> channels in the smooth muscle (38). Interestingly, Malinowsky et al. conclude that perivascular adipose tissue of human internal mammary artery releases relaxing factor that seems to act with the involvement of smooth muscle BK<sub>Ca</sub> channels (39). Previous pharmacological data have shown that BK<sub>Ca</sub> channels are abundant in myometrium and they are involved in mediating relaxation of uterine smooth muscle (20). Their activity are triggered by depolarization and enhanced by an increase in [Ca<sup>2+</sup>]<sub>i</sub>, providing a link between the metabolic and electrical state of cells. In the different experimental model, Chen et al. have suggested that resveratrol could activate BK<sub>Ca</sub> channels in the β cells through an increase of intracellular Ca<sup>2+</sup>-independent mechanism (40). To analyze the possibility that the inhibitory effect of resveratrol on the myometrium is mediated via BK<sub>Ca</sub> channels, iberiotoxin and TEA was tested. Iberiotoxin is highly specific BK<sub>Ca</sub> channels blocker. TEA selectively blocks the BK<sub>Ca</sub> channels with the IC<sub>50</sub> value around 2 mM. But used in a higher concentrations (up to 10 mM), TEA blocks other types of K<sup>+</sup> channels, too (28). The concentrations of TEA used in our study were sufficient to inhibit BK<sub>Ca</sub> channels. According to the results obtained with iberiotoxin, TEA and resveratrol on the SRC and on the phasic oxytocin-induced contractions, it is reasonable to conclude that BK<sub>Ca</sub> channels are involved in the resveratrol mechanism of action on the rat uterus. However, the effects of resveratrol on the tonic oxytocin-induced contractions were not sensitive to iberiotoxin and TEA indicating that BK<sub>Ca</sub> channels are not involved in this resveratrol action. By Western blot analyses we confirmed the presence of BK<sub>Ca</sub> channels in the smooth muscle of rat uterus. Basically, BK<sub>Ca</sub> channels consist of 4 α-subunits that form the channel pore and up to 4 regulatory β-subunits that modify phenotypic and functional diversity (20). The α-subunits is derived from a single gene, but may exist as several protein species varying from 83 to 110 kDa, demonstrating the occurrence of post-translational modification (41, 42). We obtained the expression of the ~83 kDa protein α-subunit for non-pregnant rat myometrium.

We observed that 4-AP (1 mM), a predominant blocker of Kv channels, antagonized the effect of resveratrol on the SRC and phasic oxytocin-induced contractions. Previously, in a model of SRC of non-pregnant rat uterus, it has been shown that Kv channels were inhibited by 4-AP (1–5 mM) (19). However, the relaxant effect of resveratrol on the tonic oxytocin-induced contractions was not blocked by 4-AP. In the same way, 4-AP did not alter relaxation of tonic contraction of the retinal arterioles by resveratrol (43). In contrast, a few studies have confirmed that Kv channels participate in the resveratrol effects on tonic contractions of the different blood vessels (7-9).

Furthermore, it has been shown that resveratrol inhibits the Kv channels on the β cells (12). These discrepancies in antagonism of resveratrol and 4-AP in different tissues may be consequences of different expression/function of various subtypes of Kv channels. For example, it has been demonstrated that uterus smooth muscle cells contain at least three types of Kv current: 4-AP insensitive, 4-AP sensitive rapidly inactivating current, 4-AP sensitive slowly inactivating current (44). Phenotypically, two main subfamily groups of Kv currents have been observed using electrophysiological methods which differ in their pharmacological sensitivity-delayed rectifiers (Kv2.x) and rapidly inactivating current(Kv4.x) (19). Therefore, we sought to
determine whether Kv4.2 and Kv2.1 channels protein are present in non-pregnant rat uterus. Indeed, the Western blot analysis confirmed protein expression of both types in similar ratio. Expression of several Kv1.x and Kv4.x subtypes have been demonstrated in mouse myometrium cells at the gene expression and protein level (44). However, the number of expressed subunits appears to be much larger than the number of apparent Kv current components. Smith and coworkers (44) suggest that Kv4.2 subtype could be molecular target for 4-AP in mouse myometrium. From another side, Kv2.1 is a delayed rectifier potassium channel ubiquitously expressed in the heart, brain and neurons (45, 46). But to date there is no evidence for expression of Kv2.1 channels in myometrial tissue. The data obtained on the vascular smooth muscle indicates that Kv2.1 current is 4-AP sensitive (47). According to this, it seems that effect of resveratrol on phasic contractions appears to be mediated by Kv4.2 and Kv2.1 subtype of Kv channels present in non-pregnant rat myometrium.

According to our results, it seems that K<sub>KATP</sub>, iberiotoxin, TEA- and 4-AP-sensitive channels are involved in the inhibition of SRC and phasic oxytocin-induced contraction of rat uterus produced by resveratrol. Similar results were obtained for potassium channel opener, pinacidil on the human pregnant myometrium and human radial artery (48, 49). The resistance of potassium channel opener, pinacidil on the human pregnant uterus resveratrol does not behave as a classical potassium channel opener. The all tested K<sub>+</sub> channels blockers indicated that resveratrol partly exerts its inhibition of SRC and phasic oxytocin-induced contraction by acting on multiple sites. Similarly, K<sup>+</sup>-channel independent effects of high concentrations of resveratrol were obtained in the rat aorta and uterus (7, 13). Moreover, in the model of oxytocin-induced tonic contractions of rat non-pregnant uterus resveratrol does not behave as a classical potassium channel opener. The all tested K<sub>ATP</sub> channels blockers did not antagonize the resveratrol’s effect on this type of contractions. In contrast, NS 1619, a highly specific K<sub>KATP</sub> channels opener and pinacidil, a predominant opener of K<sub>ATP</sub> channels produced more potent inhibition of tonic oxytocin-induced contractions than resveratrol. According to the affinity of iberiotoxin and glibenclamide, it seems that the effect of NS 1619 and pinacidil on the tonic oxytocin-induced contractions was mediated by K<sub>KATP</sub> and K<sub>KATP</sub> channels, respectively.

Thus, apart from different states of the smooth muscle, the differences between effects of resveratrol on contractions induced by high and low concentrations of oxytocin might be explained with different nature and sources of initiation and maintenance of phasic and tonic contractions, too. Hence, oxytocin, at low concentrations, produces phasic contraction of the isolated uterus of the non-pregnant rat which can be explained by Ca<sup>2+</sup> influx via L-types Ca<sup>2+</sup> channels. The oxytocin, at high concentrations produces tonic contraction resulting from another mechanism which does not appear to involve Ca<sup>2+</sup> influx only (50, 51). Therefore, in order to investigate whether resveratrol could influence contraction induces by oxytocin’s intracellular signal pathways, the series of experiments in the K<sup>+</sup>-rich, Ca<sup>2+</sup>-free Kreb’s solution (the absence of Ca<sup>2+</sup> influx and K<sup>+</sup> efflux) were done. Ca<sup>2+</sup>-free Kreb’s solution prevent Ca<sup>2+</sup> influx in the absence of extracellular Ca<sup>2+</sup> and high K<sup>+</sup> abolished the effect of K<sup>+</sup>-channels opening. Under this condition, SRC and phasic oxytocin-induced contractions were abolished, but an application of 20 nM oxytocin induced tonic contraction which was not sensitive to resveratrol used in concentrations lower than 30 µM. These findings provide support for the hypothesis that ≥30 µM of resveratrol can inhibit contractions of uterus through influence on Ca<sup>2+</sup>- and K<sup>+</sup>- channels independent intracellular signaling pathways. According to our observations, it seems that resveratrol may produce relaxation of non-pregnant uterus by activation of different smooth muscle K’ channel and/or by inhibition of the signaling pathways involved in the tonic contraction of oxytocin. This assumption is in line with the research on the guinea pig gallbladder smooth muscle (26). However, the additional experiments are necessary to define closely the role of the intracellular mechanisms of action of resveratrol on the tonic oxytocin-induced contractions of rat uterus. Having in mind that Choi et al. recently suggested that Ca<sup>2+</sup> transport genes may play roles in the uterus for Ca<sup>2+</sup> transport and reproductive function, it might be of interest to investigate whether resveratrol interact with these molecular structures (52).

The present study clearly demonstrates that resveratrol exert a potent inhibitory effect on the rat non-pregnant uterine contractility. The different way of initiation of contractions of the myometrium influenced differently its response to resveratrol. Based on K<sup>+</sup>-channels blockers affinity, it appears that the inhibitory response of resveratrol on the SRC and phasic contractions involves different myometrial K<sup>+</sup>-channels. But when applied in high concentrations, resveratrol has an additional K<sup>+</sup>-channels independent mechanism(s) of action. The suggestion that effect of resveratrol on the tonic contractions induced by high concentration of oxytocin is mainly K<sup>+</sup> channels independent, has to be investigated further.

The present study demonstrated, for the first time, the protein expression ratio of K<sub>KATP</sub>, Kv and BK<sub>K</sub> channels in myometrium of non-pregnant rat. Also, this is the first study which reveals the protein expression of Kv2.1 and Kir6.1 and Kir6.2 subunits of K<sub>K</sub> channels in rat non-pregnant myometrium.

Based on the results presented here it is plausible to conclude that resveratrol has potential to be used in prevention and treatment of uterine abnormal contractility. The concentration of resveratrol used in our study corresponds to a reasonable dose for human use (13).

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Author’s address: Professor Ljiljana Gojkovic-Bukarica, Institute of Pharmacology, Clinical Pharmacology and Toxicology, Medical Faculty, University of Belgrade, Belgrade 11129, Serbia.
E-mail: l.g.bukarica@sezampro.rs