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The role of potassium channels and calcium in the relaxation mechanism of magnesium sulfate on the isolated rat uterus

Dragana Sokolović[1], Dragana Drakul[1], Zorana Oreščanin Dušić[2], Nikola Tatalović[2], Milica Pecelj[3,4,5], Slobodan Milovanović[1] and Duško Blagojević[2,*]

[1] *Department of Pharmacology, Faculty of Medicine at Foča, University of East Sarajevo*, East Sarajevo, 73301 Foča, Republic of Srpska, Bosnia and Herzegovina

[2] *Department for Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade*, 11000 Belgrade, Serbia

[3] *Faculty of Philosophy, University of East Sarajevo*, Pale, Republic of Srpska, Bosnia and Herzegovina

[4] *Geographical Institute "Jovan Cvijić" of the Serbian Academy of Sciences and Arts*, 11000 Belgrade, Serbia

[5] *Institute of Sports, Tourism and Service, South Ural State University*, Chelyabinsk, Russia

***Corresponding author**: dblagoje@ibiss.bg.ac.rs

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Abstract: $MgSO_4$ is used as a tocolytic agent. It is considered to be a calcium channel antagonist, but a different mechanism of its action might be involved. The aim of this study was to examine the contribution of calcium concentrations and potassium channels in the mechanism of $MgSO_4$-mediated uterine relaxation. Isolated uteri from female Wister rats were treated with increasing $MgSO_4$ concentrations (0.1-30 mM). $MgSO_4$ induced dose-dependent inhibition of spontaneous activity. Addition of Ca^{2+} (6 mM and 12 mM) stimulated uterine contractile activity and attenuated the inhibitory activity of $MgSO_4$. In order to analyze the role of different subtypes of potassium channels, Ca^{2+}-stimulated uteri were pretreated with glibenclamide (Glib), a selective ATP-sensitive potassium channel inhibitor (K_{ATP}), tetraethylammonium (TEA), a non-specific inhibitor of large conductance calcium-activated potassium channels (BK_{Ca}), and 4-aminopyridine (4-AP), a voltage-sensitive potassium channel inhibitor (Kv), at concentrations that had no effect *per se*. Pretreatment with 4-AP had no effect on $MgSO_4$-mediated relaxation of Ca^{2+}-stimulated uteri. The relaxing effect of $MgSO_4$ was potentiated by pretreatment with glibenclamide. Pretreatment with TEA attenuated the $MgSO_4$-mediated decrease in frequency. Our results suggest that $MgSO_4$ acts as a general calcium antagonist that influences Ca^{2+}-mediated potassium channels. Furthermore, it seems that $MgSO_4$ uterine relaxation activity is partially mediated by selective ATP-sensitive potassium channels, suggesting an ATP-dependent role.

Keywords: $MgSO_4$; uterus; K^+ channels; Ca^{2+} channels; tocolytic

INTRODUCTION

Magnesium sulfate ($MgSO_4$, mineral salt, soluble in water) is used as a laxative, tocolytic agent and it is known as a functional blocker of calcium channels [1,2]. Despite the long-standing experience of its application, the use of $MgSO_4$ in gynecology has been a source of controversy for years. $MgSO_4$ was first used in 1906 to prevent eclamptic attacks by Horn in Germany, when administered intrathecally [3]. Its intramuscular use was first performed in 1926 to prevent repeated attacks in women with eclampsia [4], while the first intravenous administration was in 1933 to women with eclampsia and preeclampsia [5].

The tocolytic effects of $MgSO_4$ were originally described by Hall et al. in 1959 [6]. Stallworth et. al. (1981) found a slight decrease in the incidence of uterine contractions, but no significant change in the intensity of contractions during $MgSO_4$ administration [7]. Meta-analysis has shown that that magnesium reduces the risk of birth within 48 h by 15%, but it is considered not significant [8]. However, a combination of a betamimetic agonist and $MgSO_4$ has been introduced, and studies have shown its efficiency in prolonging gestation [9-11]. On the other hand, tocolytic efficacy was not improved and side effects were increased [12]. Although there are many studies

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dealing with the tocolytic effect of $MgSO_4$, the exact mechanism of its action is still unknown.

Different types of β2-adrenergic agonists, Ca^{2+} channel blocker, oxytocin receptor antagonist and nonsteroidal antiinflammatory drugs are also used as tocolytics, but their insufficient effectiveness and side effects compromise their preliminary use [13,14]. Therefore, agents with potential tocolytic characteristics are still needed, and they could include calcium antagonists, potassium channel openers and other vasodilators [15]. Potassium channels are abundant and active in the smooth muscle of the uterus [16-18]. Based on structure and function, the channels are categorized in different groups (Kv channels, BK_{Ca} channels, ATP-sensitive potassium channels), and each group contains many subtypes and isoforms [19]. Large conductance calcium-activated potassium channels (BK_{Ca}) are dominant and active in uterine smooth muscles, especially during gestation [20]. ATP-dependent potassium channels (K_{ATP}) in the smooth muscles of the uterus form the connection between the metabolic state of the cell and uterine contractility [20,21,22]. Therefore, studying the influence of potassium channel modulators on uterine tissue has been suggested as important for finding new therapeutic concepts in the treatment of uterine contractility disturbances [15,23].

Although wide-scale examinations of drugs as modulators of contractility have been performed, a final therapeutic preference has been omitted [17,23,24]. $MgSO_4$ is considered a general calcium antagonist, but the potential site of $MgSO_4$ cellular physiological activity can also be at the level of potassium channels. Potassium channels are widespread in all living cells and very important for regulating cell membrane excitability [25]. Therefore, the aim of our study was to explore the effect of $MgSO_4$ on uterine contractility with regard to the role of calcium concentrations and potassium channels.

MATERIALS AND METHODS

Experimental system

All animals were treated according to directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and experiments were approved by the Ethical Committee for the Use of Laboratory Animals of the Faculty of Medicine Foča, University of East Sarajevo, Decision No. 01-3-88. Animals were kept at 22°C, housed 3 per cage and fed *ad libitum*. Uteri from intact Wistar rats (250-300 g) in the estrus phase of the estrus cycle, determined by examination of a daily vaginal lavage [26], were used.

Reagents

$MgSO_4$ was supplied by Galenika a.d. (Belgrade, Serbia). Tetraethylammonium, glibenclamide and 4-aminopyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Salts for De Jalon's solution were obtained from Zorka Pharma (Šabac, Serbia), Merck (New Jersey, USA) and Centrohem d.o.o. (Stara Pazova, Serbia).

Isolated organ bath studies

All rats were killed by rapid decapitation. The uterine horns were rapidly excised, carefully cleaned of surrounding connective tissue and mounted vertically in a 10-mL-volume organ bath containing De Jalon's solution (NaCl 154 mM, KCl 5.6 mM, $CaCl_2 \times 2H_2O$ 0.41 mM, $NaHCO_3$ 5.9 mM and glucose 2.8 mM), under 1 g tension, aerated with 95% oxygen and 5% CO_2 at 37°C. Experiments were performed after an equilibration period of about 30 min. The effect of $MgSO_4$ was examined on a spontaneously active uterus (incubated for 30 min in an organ bath in De Jalon's solution at 37°C, oxygenated with 95% of O_2 and 5% CO_2), as well as on calcium-stimulated (6 and 12 mM Ca^{2+}, the latter was referred to as double Ca) uteri. In order to analyze the possible role of different subtypes of potassium channels, Ca^{2+}-stimulated uteri (with 6 mM Ca^{2+}) were pretreated individually with Glib (10^{-5} M), TEA (10^{-3} M), or 4-aminopyridine (4-AP, 10^{-3} M). After 10 min, increasing concentrations of $MgSO_4$ (0.1-30 mM) were added. Myometrial tension was recorded isometrically with a TSZ-04-E isolated organ bath and transducer (Experimetria, Budapest, Hungary) and an Ugo Basile isolated organ bath and a transducer (Gemonio, Italy).

Data analysis and statistical procedures

Statistical analyses (descriptive statistics, analysis of variance (ANOVA), *post hoc* tests, F-test and Student's t test) were performed according to the protocols de-

scribed by Hinkle et al. [27] and Manley [28]. Effects of the treatments on uterine contractions were calculated as the percentages of untreated control contractions. Each value is expressed as the mean±SD. Differences between groups were analyzed by two-way ANOVA on a logarithmic transformed data row, using concentrations and pretreatments as factors (ANOVA was considered statistically significant when $p<0.05$), and *post hoc* tested by Tukey's HSD t-test. Since concentration-response curves were sigmoidal in shape, they were fitted according to Boltzmann functions (the concentration axis was logarithmic) and the EC_{50} were calculated. Sigmoid curves were compared using the F-test. EC_{50} values were compared using Student's t test (significance: $p<0.05$).

RESULTS

MgSO4 relaxed spontaneous uterine activity in a concentration-dependent manner with regard to frequency (Fig. 1A). There were no significant changes in amplitude until complete cessation of contractions occurred (at the highest used $MgSO_4$ concentration of 30 mM). The addition of Ca^{2+} (6 and 12 mM) caused intensive contractile activity, and these types of uterine activity were referred to as Ca^{2+}- or double Ca^{2+}-stimulated, according to the concentration of Ca^{2+} used. $MgSO_4$ also relaxed both Ca^{2+}- and double Ca^{2+}-stimulated active uteri in a concentration-dependent manner (Fig. 1B and C; ANOVA significant concentration effect, $p<0.001$), but the concentration of $MgSO_4$ necessary for relaxation of the Ca^{2+}- and double Ca^{2+}-stimulated active uteri was significantly higher (significant difference in EC_{50} values, ANOVA significant type and interaction effect, $p<0.001$, significant *post hoc* Tukey t-test; Fig. 1D). EC_{50} was 5 times higher for double Ca^{2+}-stimulated uteri than for the spontaneously active uterus. The addition of both single and double Ca^{2+} extended the $MgSO_4$ -induced relaxation effect and shifted the sigmoid shape for the frequency toward higher concentrations (significant F-test effect, $p<0.05$; Fig. 1D).

Pretreatment with 4-AP had no effect on $MgSO_4$-mediated relaxation of Ca^{2+}-stimulated uteri (no significant ANOVA pretreatment effect, no difference between either the sigmoidal fit curve shape or EC_{50}; Fig. 2A and B). On the other hand, a relaxing effect

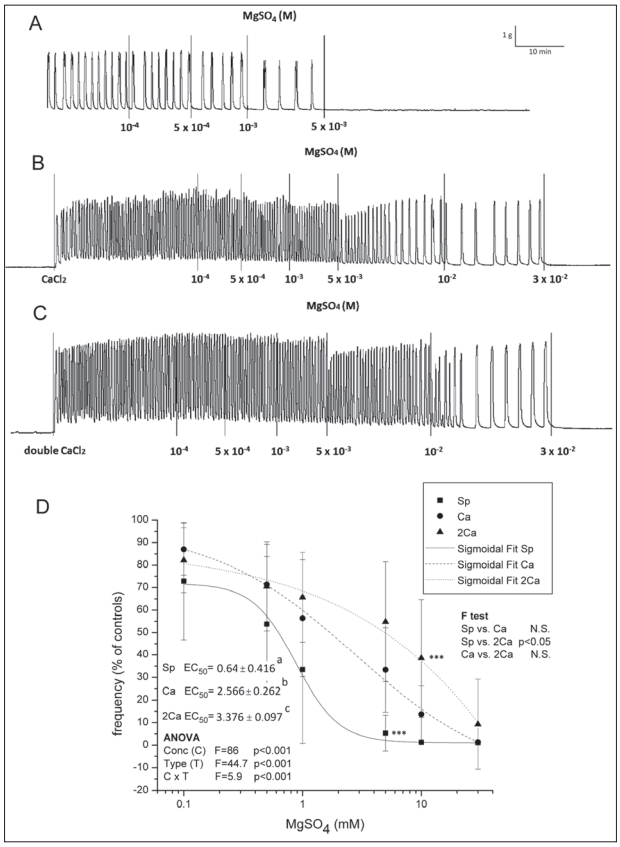

Fig. 1. The effect of $MgSO_4$ on uterine contractile activity under different Ca^{2+} concentrations. Graphs show original traces for **A** – spontaneously active, **B** – Ca^{2+}-stimulated active, and **C** – double Ca^{2+}-stimulated active. Results are presented as the means±SD (n=8) for frequency measurements (**D**). The results were compared by two-way ANOVA for the concentration of $MgSO_4$ (C) and the type of contractile activity (T) as factors, and F values are presented. From the curves, the EC_{50} values were calculated, expressed as the mean±SD and compared by one-way ANOVA and *post hoc* Tukey's t-test. Differences in the shape of curves were tested by the F-test.

of $MgSO_4$ was potentiated by pretreatment with glibenclamide (Fig. 3A and B). Pretreatment with glibenclamide significantly deepened relaxation by decreasing frequency (significant ANOVA pretreatment effect, $p<0.05$). Pretreatment with glibenclamide lowered the concentrations of $MgSO_4$ that were needed for relaxation (*post hoc* Tukey difference between degrees of relaxation by single equivalent concentration of 5 and 10 mM). There was no difference between the $MgSO_4$-induced relaxing effect of Ca^{2+}-stimulated and glibenclamide pretreated uteri as regards the amplitude.

Pretreatment with TEA led to the elevation of frequency (significant ANOVA pretreatment effect,

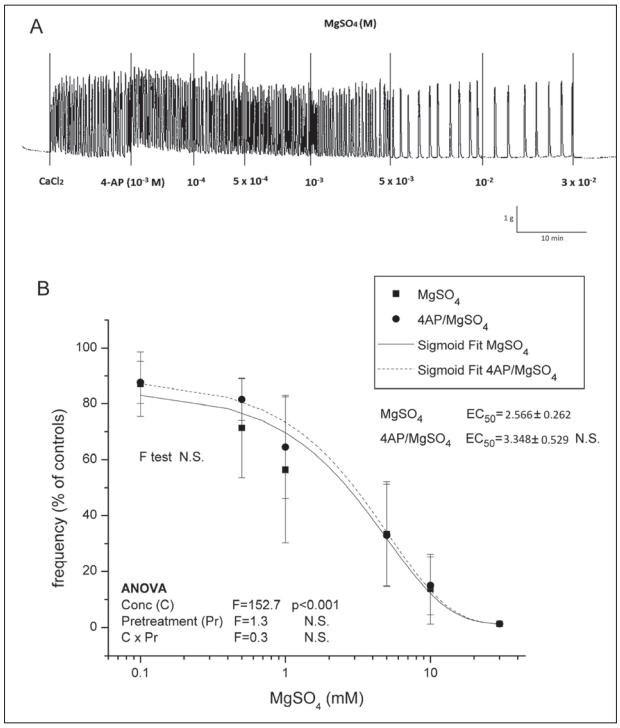

Fig. 2. The effect of $MgSO_4$ on contractile activity of uteri pretreated with 4-AP. Graphs show original traces for: **A** – Ca^{2+}-stimulated active uteri pretreated with 4-AP. The results are presented as the means±SD (n=8) for frequency (**B**) measurements. The results were compared by two-way ANOVA for the concentration of $MgSO_4$ (**C**) and the type of contractile activity (T) as factors, and F values are presented. From the curves, EC_{50} values were calculated, expressed as the mean±SD and compared by one-way ANOVA and *post hoc* Tukey's t-test. Differences in the shape of curves were tested by the F-test.

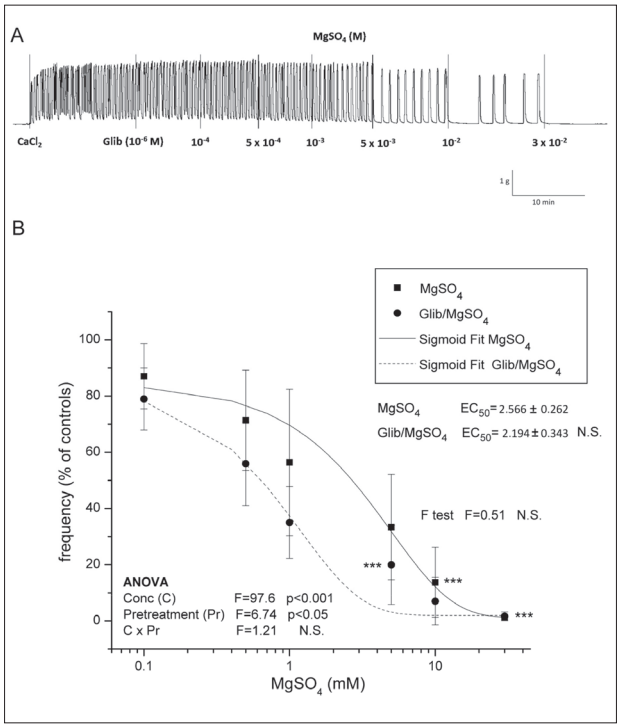

Fig. 3. The effect of $MgSO_4$ on contractile activity of uteri pretreated with glib. Graphs show original traces for: **A** – Ca^{2+}-stimulated active uteri pretreated with Glib. The results are presented as the means±SD (n=8) for frequency (**B**) measurements. The results were compared by two-way ANOVA for the concentration of $MgSO_4$ (**C**) and the type of contractile activity (T) as factors, and F values are presented. From the curves, EC_{50} values were calculated, expressed as the mean±SD and compared by one-way ANOVA and *post hoc* Tukey's t-test. Differences in the shape of curves were tested by the F-test.

p<0.001; Fig. 4A and 4B) after application of $MgSO_4$ concentrations above 1 mM (*post hoc* Tukey t test, p<0.001). However, there was no statistically significant difference between EC_{50} values for frequency since there were large distributions of data in the middle part of the TEA curve and therefore the SD was high. There was no difference between the $MgSO_4$-relaxing effect of Ca^{2+}-stimulated and TEA pretreated uteri regarding amplitude.

DISCUSSION

Our results showed that $MgSO_4$ inhibited spontaneous uterine activity in a concentration-dependent manner, and that therefore it could be considered as a uterine relaxant. This effect was in the range from 1-30 mM in the *ex vivo* extra uterine fluid (the EC_{50} for frequency was 2.6 mM). These external $MgSO_4$ concentrations are not toxic, but pharmacologically they are selective with regard to the dosage, i.e. the therapeutic window is rather narrow. Magnesium ion concentrations in the plasma and extracellular fluid are approximately 1.2-1.4 mM. One-third is bound by albumin or other proteins and biochemical moieties [29]. This means that small increases in Mg^{2+} in the extra uterine fluid can slow down the spontaneous frequency, but for complete relaxation a 3-fold higher concentration is required. Literature data indicate that increasing human serum Mg^{2+} concentration by 4-6 mEq/L (2-3 mmol/L) decreases uterine activity in preterm labor [30]. However, our results with pretreatment with glibenclamide and TEA indicated that the effective $MgSO_4$ concentration range might be under the influence of applied drugs that can shift its therapeutic potential.

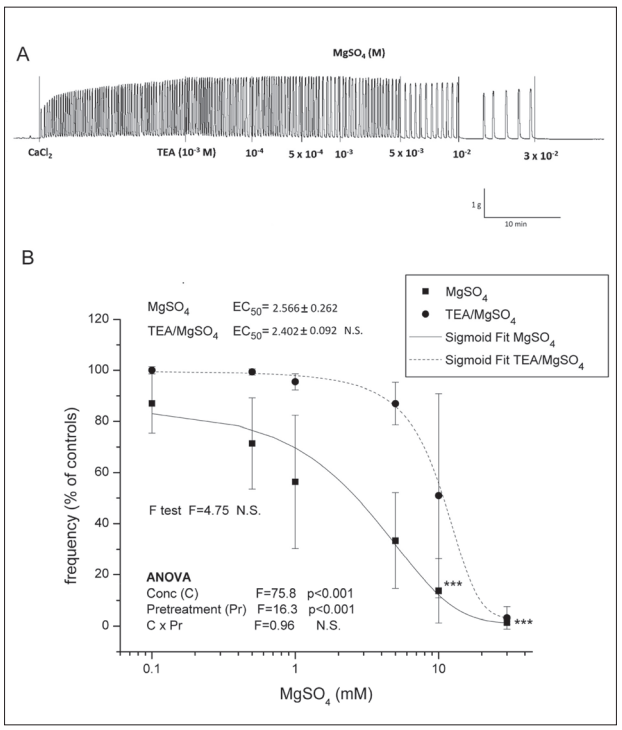

Fig. 4. The effect of $MgSO_4$ on contractile activity of uteri pretreated with TEA. Graphs show original traces for: **A** – Ca^{2+}-stimulated active uteri pretreated with TEA. The results are presented as the means±SD (n=8) for frequency (**B**) measurements. The results were compared by two-way ANOVA for the concentration of $MgSO_4$ (**C**) and the type of contractile activity (T) as factors, and F values are presented. From the curves, EC_{50} values were calculated, expressed as the mean±SD and compared by one-way ANOVA and *post hoc* Tukey's t-test. Differences in the shape of curves were tested by the F-test.

Our study showed that the addition of Ca^{2+} to the isolated organ bath prior to $MgSO_4$ significantly attenuated the relaxing effect of $MgSO_4$. Addition of the single Ca^{2+} concentration provoked an increase of the force of spontaneous contractions by elevating both amplitude and frequency. This increase was additionally elevated by the double concentration Ca^{2+}. In both cases, $MgSO_4$ inhibited contractile activity, suggesting its physiological role as a general Ca^{2+} antagonist. It is known that Mg^{2+} inhibits the ryanodine receptor (RyR) Ca^{2+}-release channels by competing with Ca^{2+} at the cytosolic activation sites of the channel in the mM range [31-33] and influences the fidelity of coupling between L-type Ca^{2+}channels and RyRs [34]. On the other hand, cytosolic levels of H^+, Ca^{2+}, adenine nucleotides and Mg^{2+} during fatigue influence the gating properties of the SR Ca^{2+} channel [35], and the functional roles of the three main intracellular ions, Na^+, Ca^{2+} and Mg^{2+}, are modulated by calmodulin connected voltage-gated Na^+ channels [36].

Since increasing concentrations of Ca^{2+} inhibited the relaxing effect of $MgSO_4$ only in part, this indicated the existence of additional cellular signaling pathways that Mg^{2+} could operate through. Therefore, we partially inhibited potassium channels by different inhibitors prior to the addition of $MgSO_4$. Our results showed that the voltage-gated K_V subfamilies of potassium channels K_V1-K_V4 were not involved in the inhibitory action of Mg^{2+}, since pretreatment with 4-AP had no effect on concentration-dependent Mg^{2+}-promoted uterine relaxation. On the other hand, pretreatment with TEA modified the relaxing activity of $MgSO_4$. TEA is a potent inhibitor of voltage-gated K_V1-K_V4 subfamilies of potassium channels, but it also inhibits K_V7 (KCQN) as well as BK_{Ca} potassium channel subfamilies, suggesting that KCQN as well as BK_{Ca} channels operated during the $MgSO_4$-induced relaxant effect. In our experiment, pretreatment with TEA attenuated the relaxing effect as regards the frequency. Given that XE991, a KCNQ channel inhibitor, elevated the frequency of the murine myometrium [37], it seems that the partial blockade of the potassium channels' pore by TEA in our experiment contributed to the elevated frequency, and that $MgSO_4$ operated as a mild KCQN channel inhibitor. It is known that intracellular Mg^{2+} enhances the function of BK_{Ca} potassium channels [38] through distinct binding sites and the activation is not directly affected either by voltage or Ca^{2+}. However, TEA is also an efficient blocker of this type of channel, and its suppression contributed to other mechanisms of Mg^{2+}-induced inhibition of uterine contractility. Moreover, BK_{Ca} channels are in neuronal cells colocalized with voltage-dependent Ca^{2+} channels [39,40,41] or RYR [42], and these functional couples seem to be responsive to the Ca^{2+} entering into the cytosol and to the control Ca^{2+} concentration [40,42,43]. Shi and Cui [38] demonstrated that the competitive inhibition of Ca^{2+}-dependent activation of BK_{Ca} channels by Mg^{2+} results in a significant reduction of the Mg^{2+}-dependent activation at $[Ca^{2+}]i$ of ~0.1-100 μM. Taken together, our results suggest that the overall interplay between Ca^{2+} and Mg^{2+} is concentration-dependent. Blocking BK_{Ca} requires more Ca^{2+} that is prevented by the presence of Mg^{2+}. On the other hand, if the Ca^{2+} concentration is

in physiological balance with Mg^{2+}, after the addition of Mg^{2+}, its Ca^{2+} antagonist role is potentiated.

Magnesium interacts with organic phosphates such as ATP and PIP2. Intracellular Mg^{2+} depresses KCNQ currents by binding to PIP2 electrostatically, thus reducing the availability of PIP2 for direct interaction with the channels [44]. Most of cytoplasmic Mg^{2+} is in the form of a complex with ATP, phosphonucleotides and phosphometabolites and Mg^{2+}, with ATP constituting the largest metabolic pool capable of binding Mg^{2+} within the cytoplasm and the mitochondrial matrix as well [45,46]. Our results showed that a partial blockade of K_{ATP} channels by glibenclamide stimulated the relaxing activity of $MgSO_4$, which points to its ATP-dependent role.

The results presented in our study show that $MgSO_4$ acts only partially as a general calcium antagonist. Moreover, a part of its physiological pathway is through BK_{Ca} channels since the blocking of BK_{Ca} channels with TEA led to the stimulation of frequency. Since relaxing activity was predominantly achieved by the reduction of frequency, it seems that $MgSO_4$ is a direct K^+ channel inhibitor, but it also affects Ca^{2+} availability. Furthermore, the $MgSO_4$ uterine relaxing activity is influenced by selective ATP-sensitive potassium channels suggesting also an ATP-dependent role.

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Author contributions: Duško Blagojević, Slobodan Milovanović conceptualized and defined the research idea and created the research design; Zorana Oreščanin-Dušić and Nikola Tatalović searched the literature data; Duško Blagojević selected the statistical tests; Dragana Jokanović, Dragana Drakul, Milica Pecelj, Zorana Oreščanin-Dušić collected and prepared the experimental data; Nikola Tatalović and Duško Blagojević performed the statistical analyses; Dragana Drakul, Milica Pecelj and Dragana Jokanović wrote the first draft of the manuscript; Duško Blagojević, Slobodan Milovanović and Zorana Oreščanin-Dušić wrote the second draft of the manuscript; Nikola Tatalović and Duško Blagojević edited the manuscript.

Conflict of interest disclosure: The authors declare that there is no conflict of interests.

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