Effects of protamine sulphate on spontaneous and calcium-induced contractile activity in the rat uterus are potassium channels-mediated

Zorana Oreščanin-Dušić[1], Slobodan Milovanović[2], Ratko Radojičić[1,3], Aleksandra Nikolić-Kokić[1], Isabella Appiah[1], Marija Slavić[1], Neđo Čutura[4], Stevan Trbojević[2], Mihajlo Spasić[1] and Duško Blagojević[1]

[1] *Department of Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia*
[2] *Faculty of Medicine, University of Eastern Sarajevo, Foča, Bosnia and Herzegovina*
[3] *Faculty of Biology, University of Belgrade, Belgrade, Serbia*
[4] *Gynaecology and Obstetrics Clinic "Narodni Front", Belgrade, Serbia*

Abstract. Protamine sulphate (PS) effect on spontaneous and calcium-induced rhythmic contractions of isolated virgin rat uteri was studied. PS caused dose-dependent relaxation of both types of contractions (two-way ANOVA, significant dose effects). Pretreatment with NG-nitro-L-arginine methyl ester (L-NAME; 10^{-5} mol/l), methylene blue (MB; 0.9×10^{-6} mol/l) or propranolol (1.7×10^{-5} mol/l) enhanced PS-mediated uterine muscle relaxation of spontaneous contractions. Dose-dependent relaxation of spontaneous active isolated rat uterus with PS was lower in uteri pretreated with single dose of tetraethylammonium (TEA; 6×10^{-3} mol/l), glibenclamide (2×10^{-6} mol/l) and 4-aminopyridine (4-AP; 10^{-3} mol/l). Calcium-induced activity of the isolated rat uterus pretreated with the same concentration of L-NAME, MB, or propranolol modified the kinetic of PS-induced relaxation without changes in EC_{50} values. Pre-treatment with glibenclamide, TEA and 4-AP significantly reduce PS relaxing effect of calcium-induced activity and according to EC_{50} values the order of magnitude was glibenclamide > TEA > 4-AP.

PS is mixture of polyamines and may activate different signal-transduction pathways. Our results cleary demonstrate that in uterine smooth muscle PS act dominantly through potassium chanels and marginaly through β-adrenergic receptos or nitric oxide-dependent pathways.

Key words: Nitric oxide — Protamine sulphate — Potassium channels — Rat uterus

Abbreviations: PS, protamine sulphate; EDRF, endothelium-derived relaxing factor; BK_{Ca} channel, large conductance calcium-activated potassium channel; K_{ATP} channel, ATP-activated potassium channel; L-NAME, NG-nitro-L-arginine methyl ester; MB, methylene blue; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; NOS, nitric oxide synthase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PMCA, plasma membrane Ca^{2+} ATPase.

Introduction

Protamine sulphate (PS) is a mixture of polycationic amines used clinically to reverse heparin overdose. However, PS administration causes vasodilatation which leads to systemic hypotension. It was shown that PS-caused vasodilatation is mediated by endothelium-derived relaxing factor (EDRF) nitric oxide (NO) (Viaro et al. 2002). PS is rich in the amino acid L-arginine, a precursor of EDRF/NO, but there is little evidence to support PS-inducing EDRF/NO release by enhancing supply of its substrate L-arginine (Pearson et al. 1992). The endothelium-dependent relaxation induced by PS was inhibited by NG-monomethyl-L-arginine acetate demonstrating that PS stimulates the release of EDRF. Our recent results showed concentration-dependent PS-mediated

Correspondence to: Duško Blagojević, Department of Physiology, Institute for Biological Research "Siniša Stanković", Bul. Despota Stefana 142, 11000 Belgrade, Serbia
E-mail: dblagoje@ibiss.bg.ac.rs

relaxation of isolated mesenteric arteries without endothelium indicating that vascular smooth muscle plays a significant role in PS-mediated relaxation (Oreščanin-Dušić et al. 2008). There are several possible mechanisms that could cause the relaxation of smooth muscle *via* the action of different types of receptors or signalling processes: NO-mediated signalling, K^+ channels, Ca^{2+}-mediated effect, but to date there are no data of this kind available.

Uterus is rich in different kind of receptors, but the regulation of uterine relaxation is poorly understood as well as the contribution of different types of receptors and channels to the regulation of myometrial contractility. Research in myometrial tissue and other types of smooth muscle has defined a number of receptors, ion channels and regulatory proteins that are likely to be involved (Lopez-Bernal 2007). Myometrial ion channels are targets for a plethora of biological signals including hormones, peptides, pH fluctuations and stretch tension which have important consequences for myometrial function (Khan et al. 2001; Novaković et al. 2007). Large conductance calcium-activated potassium (BK_{Ca}) channel, β-2 adrenoceptor, and long-lasting type calcium channel are the main channels and receptor that are involved in the uterine contraction/relaxation process (Chanrachakul 2006). To date, several types of potassium channels have been identified in the myometrium. These include BK_{Ca} channels (Anwer et al. 1993) and three types of voltage-gated potassium currents (Knock et al. 1999). In addition, pharmacological and biochemical evidence for myometrial ATP-activated potassium (K_{ATP}) channels has also been presented (Morrison et al. 1993; Curley et al. 2002).

The aim of the study was to identify the main pathway of PS action on isolated rat uterus as model system.

Materials and Methods

Drugs and solutions

PS was supplied by Galenika a.d. (Belgrade, Serbia). Propranolol, methylene blue (MB), NG-nitro-L-arginine methyl ester (L-NAME), tetraethylammonium, glibenclamide and 4-aminopyridine (4-AP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in distilled water except for glibenclamide which was dissolved in polyethylene glycol. Salts for De Jalon's solution (g/l: NaCl 9.0, KCl 0.42, $NaHCO_3$ 0.5, $CaCl_2$ 0.06, glucose 0.5) were obtained from ZORKA Pharma (Sabac, Serbia), Merck (Darmstadt, Germany) and Centrohem d.o.o. (Stara Pazova, Serbia).

Tissue preparation and contractility recording

All protocols for handling the rats were approved by the Local Ethical Committee for Animal Experimentation that strictly follows international guidelines (the Institute for Biological Research, Belgrade, Serbia, approval No. 3/06). Isolated uteri of virgin Wistar rats (200–250 g) in oestrus state determined by examination of daily vaginal lavage were used in this study. Uterus was suspended in an isolated organ bath chamber (Experimetria, Budapest, Hungary) containing De Jalon's solution and aerated with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C. Isometric contractions were recorded using an isometric force transducer (Experimetria, Budapest, Hungary). The preload of the preparation was about 1 g.

Experimental procedures

After an equilibration period (about 30 min), when uteri achieved stable contractions (spontaneous or calcium-induced), the tissues were exposed to increasing concentrations of PS until total cessation of contractions took place. To explore the mechanism of PS action different antagonists were used as the pre-treatment of PS (L-NAME (10^{-5} mol/l), a NOS inhibitor; MB (0.9×10^{-6} mol/l), a cyclic guanosine monophosphate (cGMP) signalling pathway inhibitor; propranolol (1.7×10^{-5} mol/l), a non-selective β-adrenergic blocker; glibenclamide (2×10^{-6} mol/l), a selective ATP-sensitive potassium channel blocker; tetraethylammonium (TEA) (6×10^{-3} mol/l), non-specific inhibitor of BK_{Ca} and voltage-sensitive potassium channels; 4-AP (10^{-3} mol/l), a voltage-sensitive potassium channel blocker). Each substance was added to the De Jalon's solution 10 min before PS. PS-induced relaxation is expressed as the percent of maximal tension observed in the presence of L-NAME, MB, propranolol, glibenclamide, TEA or 4-AP.

Statistical analysis

Statistical analyses (descriptive statistics, analysis of variance – ANOVA, F-test) were performed according to protocols described by Manley (1986) using Statistical analysis software, version 9.1.3 (SAS Institute Inc., NC, USA). Effects of treatments on uterine contractions were calculated as the percentage of control, untreated, contractions. All data are expressed as the mean ± SEM. Differences between groups were tested by two-way ANOVA with treatment and dose as factors and were considered statistically significant when $p < 0.05$. Dose-response curves were fit sigmoid to a Boltzmann functions (the concentration axis was set linear) and PS concentration required for half-maximal effect (EC_{50}) was calculated. Sigmoid curves were compared using F-test. EC_{50} values were compared using one way-ANOVA followed by post hoc Newman-Keuls test for multiple comparisons (significance $p < 0.05$).

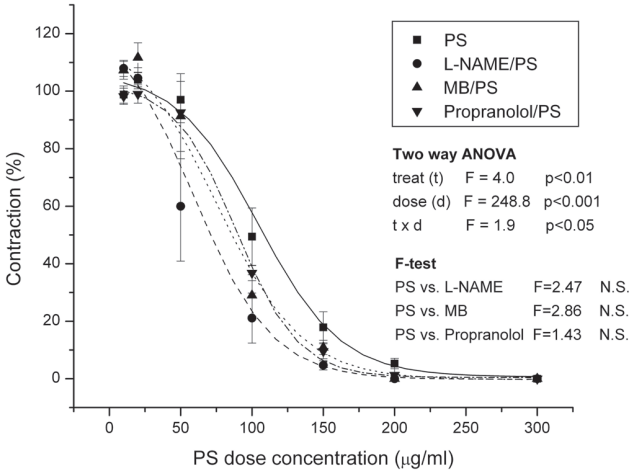

Figure 1. Dose-response sigmoid fit curves for PS-induced relaxation of spontaneous rhythmic activity of the isolated rat uterus pre-treated with L-NAME (10^{-5} mol/l), MB (0.9×10^{-6} mol/l), and propranolol (1.7×10^{-5} mol/l). Data are expressed as mean ± SE (the number of observations $n = 7$). Sigmoid fit was performed according to Boltzmann equation. Results of statistical analyses both two-way ANOVA (treatment – treat (t) and dose (d) as factors) and F-test are given (F factors and p values).

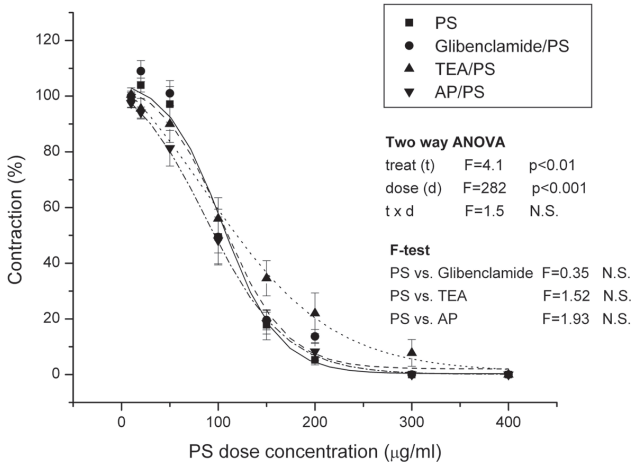

Figure 2. Dose-response sigmoid fit curves for PS-induced relaxation of spontaneous rhythmic activity of the isolated rat uterus pre-treated with TEA (6×10^{-3} mol/l), 4-AP (10^{-3} mol/l), and glibenclamide (2×10^{-6} mol/l). Data are expressed as mean ± SE (the number of observations $n = 7$). Sigmoid fit was performed according to Boltzmann equation. Results of statistical analyses both two-way ANOVA (treatment – treat (t) and dose (d) as factors) and F-test are given (F factors and p values).

Results

PS (μg/ml: 10, 20, 50, 100, 150, 200, 300, 400, 500, 600) relaxes both spontaneous active and calcium-induced rat uteri in a dose-dependant manner (two-way ANOVA, significant dose effects, Figs. 1–4). Pre-treatment of spontaneous active uteri with L-NAME (10^{-5} mol/l), MB (0.9×10^{-6} mol/l) or propranolol (1.7×10^{-5} mol/l) enhanced PS-mediated uterine muscle relaxation (two-way ANOVA, significant treatment t, and interaction treatment t × dose d effects, Fig. 1), without statistically significant effects on sigmoid fit shapes (non-significant F-test, Fig. 1). Dose-dependent relaxation of spontaneous active isolated rat uterus by PS was lower in uteri pre-treated with single dose of glibenclamide (2×10^{-6} mol/l), TEA (6×10^{-3} mol/l) and 4-AP (10^{-3} mol/l) (two-way ANOVA, significant dose d effect and treatment t effects without significant interaction effect, Fig. 2), without significant effect on sigmoid fit shapes (non-significant F-test, Fig. 2). EC_{50} values were similar in all analysed groups (one-way ANOVA, Table 1).

PS (μg/ml: 10, 20, 50, 100, 150, 200, 300, 400, 500, 600) caused dose-dependent relaxation of calcium-induced activity of the isolated rat uterus despite the presence of L-NAME, MB and propranolol (two-way ANOVA, significant dose effect, Fig. 3). The presence of used antagonists modified the kinetics of PS induced relaxation (significant differences obtained by F-test, Fig. 3), without changes in EC_{50} values (one-way ANOVA, Table 1). However, treatment with glibenclamide, TEA and 4-AP significantly reduced PS relaxing effects (two-way ANOVA, significant treatment t effect, $p < 0.001$, Fig. 4) and the kinetics of PS relaxation (significant F-test, Fig. 4). The extent of the reduction was treatment dependent (two-way ANOVA, interaction treatment t × dose d effect, $p < 0.001$). According to the EC_{50} values, the order of magnitude was glibenclamide > TEA > AP (one-way

Table 1. EC50 values for protamine sulphate doses calculated from sigmoid fit curves for different treatments

Treatment	Ca^{2+}-induced EC_{50}	Spontaneous EC_{50}
PS	149 ± 13	98 ± 1
L-NAME/PS	168 ± 20	58 ± 14
MB/PS	133 ± 10	76 ± 9
Propranolol/PS	141 ± 5	94 ± 3
Glibenclamide/PS	241 ± 15*	94 ± 4
TEA/PS	211 ± 10*	96 ± 16
AP/PS	187 ± 15	93 ± 12
ANOVA	$p < 0.001$	N.S.

Results are presented as mean ± SE, number of examinations $n = 7$. Results were tested by one-way ANOVA. $p < 0.05$ was considered as significant) and post hoc compared by Newman-Keuls test. * statistical significant difference comparing to PS; N.S., non significant.

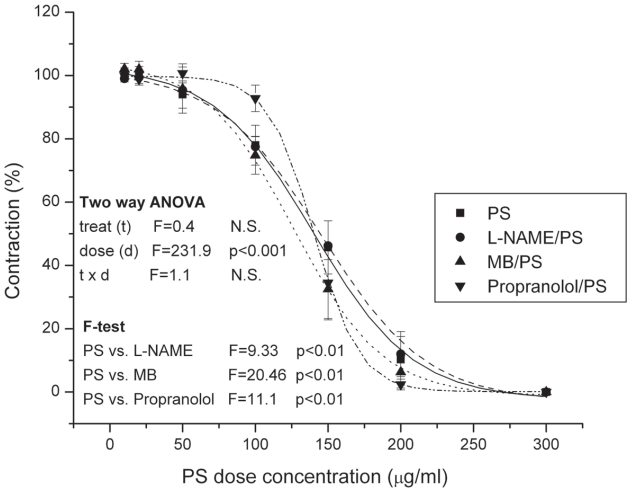

Figure 3. Dose-response sigmoid fit curves for PS-induced relaxation of calcium-induced rhythmic activity of the isolated rat uterus pre-treated with L-NAME (10^{-5} mol/l), MB (0.9×10^{-6} mol/l), and propranolol (1.7×10^{-5} mol/l). Data are expressed as mean ± SE (the number of observations $n = 7$). Sigmoid fit was performed according to Boltzmann equation. Results of statistical analyses both two-way ANOVA (treatment – treat (t) and dose (d) as factors) and F-test are given (F factors and p values).

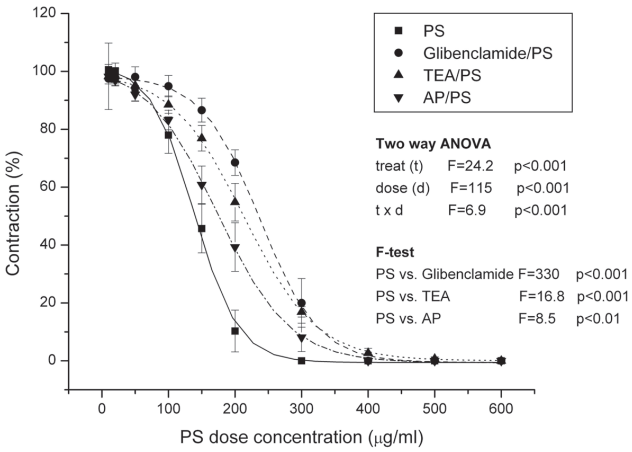

Figure 4. Dose-response sigmoid fit curves for PS-induced relaxation of calcium-induced rhythmic activity of the isolated rat uterus pre-treated with TEA (6×10^{-3} mol/l), 4-AP (10^{-3} mol/l), and glibenclamide (2×10^{-6} mol/l). Data are expressed as mean ± SE (the number of observations $n = 7$). Sigmoid fit was performed according to Boltzmann equation. Results of statistical analyses both two-way ANOVA (treatment – treat (t) and dose (d) as factors) and F-test are given (F factors and p values).

ANOVA, $p < 0.01$, post hoc comparison by Newman-Keuls test, Table 1).

Discussion

PS is a mixture of polyamines and it may have action through different pathways. Our results clearly demonstrate (according to EC_{50} values and maximum relaxation dose) that its action on uterine smooth muscle is dominantly through potassium channels. Processes through β-adrenergic receptor and by NO dependent pathway play a role in PS-mediated relaxation (treatment with L-NAME, MB and propranolol had effect on PS-mediated relaxation, Fig. 1), but its significance is far less than through potassium channels according to maximum relaxation dose (up to 300 μg/ml vs. up to 600 μg/ml in uteri pretreated with potassium channel antagonists: TEA, glibenclamide and 4-AP). Although basic mechanisms of PS-mediated relaxation of spontaneous and calcium-induced contractile activity seem to be similar, their extent is different.

TEA is widely used as an inhibitor of BK_{Ca} channels (Gil-Longo et al. 2005; Rogers et al. 2007; Rosenfeld et al. 2008; Chen et al. 2009; Lloyd et al. 2009) but it is also known that suppress some types of voltage-gated potassium channel currents (Aaronson et al. 2006). In rat myometrium smooth muscle cells BK_{Ca} current coexists with three types of voltage-gated K^+ currents. These include 4-AP-sensitive, rapidly inactivating A-type current and two delayed rectifier K^+ currents that could be suppressed by TEA and 4-AP (Knock et al. 1999). Although there are indications that BK_{Ca} channels play little or no part in controlling basal rhythmicity in rat myometrium (Aaronson et al. 2006) and that voltage-gated potassium channels play a crucial role in this regard, human myometrial contractility was affected by TEA suggesting that the large conductance BK_{Ca} channels may actively participate in control of human myometrial cell membrane potential (Anwer et al. 1993). That may suggest that TEA inhibition of PS effects on spontaneous contractile activity indicate important role of BK_{Ca} and two delayed rectifier K^+ currents in control of spontaneous contractile activity in our experimental conditions.

A large number of previously conducted studies support NO's involvement in PS-mediated relaxation of blood vessels (Pearson et al. 1992; Raikar et al. 1996; Cable et al. 1999; Milovanović et al. 2004; Oreščanin et al. 2007) and other smooth muscles (Li et al. 1999; Chuang et al. 2003). That mechanism by which PS mediates its effects and the effects of free haemoglobin on PS-induced responses in endothelium-denuded and intact human internal thoracic artery rings pre-contracted with phenylephrine or high KCl suggests that PS-induced relaxation responses is not NO- or endothelium-dependent but appears to depend on interactions between PS and calcium ion influxes and/or calcium

ion release from intracellular stores (Golbasi et al. 2003). Our current study showed that L-NAME (a NOS inhibitor), propranolol (a non-selective β-adrenergic blocker) and MB (a cGMP inhibitor), enhanced PS's relaxing effect on spontaneous activity. These results indicate that PS's relaxation capacity is downstream of adrenoceptors. Castresana and colleagues (1995) showed that PS does not alter the levels of cGMP and cAMP in response to the sodium nitroprusside, atrial natriuretic peptide, isoproterenol and forskolin (all vasodilators). David et al. (2001) found that although PS may act at several sites downstream of the adrenoceptor, one of its main sites of action is situated downstream from cAMP-mediated protein phosphorylation. Since PS is a mixture of different amines the other metabolic pathways and subsequent resulting relaxation (or changes in relaxing signalling) can not be excluded.

When contractile activity is extracellular calcium-induced, TEA, glibenclamide and 4-AP decreased PS-mediated uterine muscle relaxation and increased PS dose (EC_{50}) that relax calcium-dependant uterine activity. This implies potassium channels as important mediators of PS induced uterine relaxation. On the other hand, there were no effects of pre-treatment with L-NAME, MB or propranolol on PS dose response (Fig. 3, no dose effect). However, pre-treatment changed the shape of sigmoid curves, according to inherent receptors characteristics and signalling kinetics.

The calcium-mediated initiation spike of the tissue level action potential in myometrium is terminated relatively quickly by a partial repolarization driven by both voltage-sensitive (IK1, IK2 and IK, A) (Knock et al. 1999; Parkington et al. 1999) and calcium-sensitive (BK_{Ca} [Maxi K] and SK3) (Khan et al. 2001) potassium currents. The main function of potassium channels is to dampen cellular excitability by maintaining the cell membrane potential close to the reversal potential of potassium ions. Therefore, depolarizing stimuli are blocked by the generation of outward potassium current which causes hyperpolarization or repolarization thereby terminating action potential generation and ultimately rendering contraction less likely (Khan et al. 2001). The predominant source of calcium for a contraction triggered by an action potential is voltage-gated calcium entry from the extracellular space. The relaxation of uterine smooth muscle is initiated by the intracellular Ca^{2+} decrease following the removal of contractile stimuli (agonists or depolarization). For a Ca^{2+} efflux, the two plasma membrane routes of calcium extrusion, Na-Ca exchange and plasma membrane Ca^{2+} ATPase (PMCA) are required, with PMCA playing the major role in uterine preparations (Taggart et al. 1997; Shmigol et al. 1998; Matthew et al. 2004).

In conclusion, our results demonstrated that PS relaxation effect on the smooth muscle of isolated rat uteri is dominantly potassium channel-dependent. In spontaneous contractile activity BK_{Ca} and two delayed rectifier K^+ currents play significant role in PS-induced relaxation. When contractions are calcium-induced we found that all three types of potassium channels play a crucial role in PS-induced relaxation on this type of smooth muscle and according to EC_{50} values the order of magnitude was glibenclamide > TEA > 4-AP (far less significant). Models of spontaneous and calcium-induced contractile activity are significant for elucidation mechanisms of PS action, indicating PS interactions with all types of potassium channels.

Acknowledgement. This work was supported by the grant from the Ministry of Science of the Republic of Serbia, project No. 143034B.

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