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Vasodilatory effects of a variety of positive allosteric modulators of $GABA_A$ receptors on rat thoracic aorta

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ABSTRACT

Different subtypes of $GABA_A$ (gamma-aminobutyric acid A) receptors, through their specific regional and cellular localization, are involved in the manifestation of various functions, both at the central and peripheral levels. We hypothesized that various non-neuronal $GABA_A$ receptors are expressed on blood vessels, through which positive allosteric modulators of $GABA_A$ receptors exhibit vasodilatory effects.

This study involved two parts: one to determine the presence of α1-6 subunit $GABA_A$ receptor mRNAs in the rat thoracic aorta, and the other to determine the vasoactivity of the various selective and non-selective positive $GABA_A$ receptor modulators: zolpidem (α1-selective), XHe–III–074 (α4-selective), MP–III–022 (α5-selective), DK-I-56-1 (α6-selective), SH-I-048A and diazepam (non-selective).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis data demonstrated for the first time the expression of α1, α2, α3, α4 and α5 subunits in the rat thoracic aorta tissue. Tissue bath assays on isolated rat aortic rings revealed significant vasodilatory effects of diazepam, SH-I-048A, XHe–III–074, MP–III–022 and DK-I-56-1, all in terms of achieved relaxations (over 50% of relative tension decrease), as well as in terms of preventive effects on phenylephrine (PE) contraction. Diazepam was the most efficient ligand in the present study, while zolpidem showed the weakest vascular effects. In addition, diazepam-induced relaxations in the presence of antagonists PK11195 or bicuculline were significantly reduced ($P < 0.001$ and $P < 0.05$, respectively) at lower concentrations of diazepam (10^{-7} M and 3×10^{-7} M).

The present work suggests that the observed vasoactivity is due to modulation of "vascular" $GABA_A$ receptors, which after further detailed research may provide a therapeutic target.

1. Introduction

In addition to its function of the main inhibitory neurotransmitter, GABA is important in a diversity of tissues outside the brain and spinal cord (Erdö and Wolff, 1990; Watanabe et al., 2002). Different combinations of a total of nineteen $GABA_A$ receptor subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) are manifested in functionally and pharmacologically diverse receptor subtypes recognized in the mammalian nervous system (Olsen and Sieghart, 2009).

$GABA_A$ receptor heterogeneity depends highly on the existence of six α subunits, whereas usually one of them participates, in duplicate, in formation of a single pentameric ligand-gated Cl-channel (Olsen and Sieghart, 2008). PCR and Western blot analyses revealed expression of various α subunits in human non-neural tissues, including pancreatic β cells (Korol et al., 2018), monocytes and T lymphocytes (Alam et al., 2006; Mendu et al., 2012), and trachea (Mizuta et al., 2008). In peripheral blood vessels, there is still no clear evidence on functional $GABA_A$ receptors or specific subunits, other than findings on the expression of an unspecified $GABA_A$ receptor protein in the mouse aorta (Tyagi et al., 2007), and expression of β subunit-containing $GABA_A$ receptors in the rat aorta (El Idrissi et al., 2013). The first studies with isolated cerebral blood vessels suggested that $GABA_A$ receptors exist in

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vascular smooth muscle, so that GABA or $GABA_A$ receptor agonists produced a dose-dependent dilatation of cerebral, but not the peripheral arteries (Fujiwara and Muramatsu, 1975; Edvinsson and Krause, 1979; Anwar and Mason, 1982). It was more recently reported that cultured human aortic and umbilical vein endothelial cells express the machinery to produce GABA (Sen et al., 2016).

It has long been known that benzodiazepines (BZs) cause relaxing effects on the vascular smooth muscle (French et al., 1989; Chang et al., 1994; Yamaguchi et al., 1997; Klockgether-Radke et al., 2005; Colussi et al., 2011; Kagota et al., 2021), but so far mechanism of vasoactivity is not elucidated. The functional studies on isolated blood vessels indicated that vasodilatory effect of BZs does not involve a peripheral BZ receptor (TSPO) (French et al., 1989; Galindo et al., 2001; Park et al., 2006; Colussi et al., 2011), and that a "vascular" $GABA_A$-like receptor might be involved in the direct vasodilatory effects of GABA and BZs (Edvinsson and Krause, 1979; Shirakawa et al., 1989; Jacob and White, 2000; El Idrissi et al., 2013).

The present study, firstly, assessed the presence of α1 − α6 subunits of $GABA_A$ receptor in the rat thoracic aorta, and, secondly, investigated the vasodilatory effects of a series of PAMs on precontracted rat aortic rings. We tested ligands active at extrasynaptic α4, α5 and α6 subunit-containing $GABA_A$ receptors, with code names XHe–III–074, MP–III–022 and DK-I-56-1, respectively (Sieghart and Savić, 2018), as well as SH-I-048A, as a non-selective "super-PAM" (Obradović et al., 2014). Diazepam and zolpidem, as standard non-selective and α1-subtype selective PAM, respectively, were used as reference ligands. We aimed to correlate specific α subunit selectivity and vascular activity of tested ligands, in hope to assess implications of the "vascular" $GABA_A$ receptors and potential vasodilatory relevance of tested PAMs.

2. Materials and methods

2.1. Tissue preparation

The experiments were conducted within the project approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, project approval No. OI175076. Wistar rats, weighing 250–350 g each, were anesthetized by the intraperitoneal administration of ketamine hydrochloride (90 mg/kg, Ketamidor, Richter Pharma AG, Wels, Austria) and xylazine hydrochloride (10 mg/kg, Xylased, Bioveta, A. S., Ivanovice na Hane, Czech Republic), or were euthanized with carbon dioxide for RT-PCR analysis only.

The descending thoracic aorta was rapidly but gently removed to avoid stretching or damaging and was placed in Petri dish containing chilled (4 °C) modified Krebs-bicarbonate solution of the following composition: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose (Rizvić et al., 2017). After the surrounding adipose and connective tissue were removed carefully, the aortic rings (circulatory cut rings of approximately 3 mm length) were rapidly placed for measurement of isometric contraction or were prepared for RT-PCR analysis.

2.2. Qualitative RT-PCR

After rapid dissection, thoracic aorta was snap frozen in liquid nitrogen until RNA isolation. Total RNA isolation was performed using TRI Reagent solution (Ambion, Foster City, CA) according to manufacturer's instructions. RNA quantification was done on NanoDrop™ 1000 (Thermo Fisher Scientific, USA). RNA extracts of aortas from 5 animals were pooled and, after the DNase treatment (Thermo Scientific, Vilnius, Lithuania), 1 μg of total RNA was reverse-trascribed to cDNA using Hight Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania), according to the manufacturer's recommendations.

PCR reactions were performed using 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) in 10 μl reaction volume containing 5 μl Power SYBR® Green PCR Master Mix (Life Technologies, Warringtone, UK), 300 nM of each forward and reverse primer and cDNA corresponding to 100 ng of total RNA equivalent. The cycling conditions were: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A melting curve was generated for every PCR product to ensure the specificity of the reaction. Previously characterized primers were used for *gabra 1–6* (Mendu et al., 2012) and *actb* (β-actin) (Faseleh et al., 2017) (Supplementary Table 1). The predicted sizes of all the amplified products (base pairs) were as follows: 113 bp for α1, 134 bp for α2, 174 bp for α3, 135 bp for α4, 178 bp for α5, 221 bp for α6 and 290 bp for β-actin. All amplicons were shown to be of expected size in the control rat brain tissue. Ten μl of PCR products were analyzed by electrophoresis in 1.8% agarose gel stained with ethidium bromide, and visualized on a UV transilluminator.

2.3. Measurement of isometric contraction

Smooth muscle contractility was isometrically measured and digitally recorded using MLT0201 force-displacement transducer (Panlab, Spain), PowerLab/4SP data acquisition system (AD Instruments, Castle Hill, Australia) and LabChart 7 Pro software (AD Instruments), as described previously (Rizvić et al., 2017). Briefly, aortic rings were mounted and secured between two stainless steel triangles and then were suspended in 15 ml temperature-controlled baths (37 °C) containing Krebs-bicarbonate solution, continuously bubbled with 5% CO_2 and 95% O_2. One end of the aortic ring was connected to an isometric force transducer, whereas the other one was fixed to the organ bath wall. The rings were placed under an optimal passive stretching tension of 2.0 g, after which the tissue was allowed to adapt and spontaneously reduce the set tension. Then the tension was increased by another 2.0 g in relation to the achieved plateau value, during which time the bathing solution was changed every 10 min (Jespersen et al., 2015).

After 1 h equilibration, each aortic ring was passed through the initial challenge with phenylephrine (PE) and/or potassium chloride (KCl) (final concentration in the bath was 3×10^{-7} M and 6×10^{-2} M, respectively), achieving approximately 60–70% of the maximal contraction. The tissues were then left to re-equilibrate for 40–50 min, with wash-out periods every 10 min, and when the tone reached the baseline rings were used for the appropriate protocol procedures.

Endothelium-denuded rings were prepared by rubbing the intimal surface with stainless wire. Successful removal of endothelium was confirmed by the loss of the relaxation in response to 10 μM acetylcholine (aortic rings, submaximal pre-contracted, and responding with less than 30% relaxations, were considered to be endothelium-denuded). Four to six rings taken from the same aorta were studied in parallel according to the protocol, but for each type of experiment only one ring was used from each animal.

2.4. Experimental protocol for measurement of isometric contraction

2.4.1. The first protocol

The first series of experiments aimed to evaluate the concentration-dependent vascular effects of GABA, diazepam, zolpidem and other PAMs (SH–I-048A, XHe–III–074, MP–III–022 and DK-I-56-1) on PE- (3×10^{-7} M) and KCl- (6×10^{-2} M) precontracted aortic rings with intact endothelium.

After the precontraction had reached a steady state and stability (for approximately 15 min), PAM concentration-response curves (from 10^{-8} M to 10^{-5} M) were obtained by the cumulative application in half-log increments, with each new dose added after reaching a steady state from the preceding dose (duration usually 20 min or longer). As the time-control experiments showed that PE- or KCl-induced tone was maintained throughout the procedure lasting for 5–6 h, conditions adequate for measuring the slower vasoactivity of the tested ligands were satisfied.

The control curve, obtained from the preparations treated with the

vehicle additions only, showed a reduction in tone of 15–20% at the end of the experiments. Relaxation responses of PAMs were reported as the percentage of decrease in relative tension induced by PE (3×10^{-7} M) or KCl (6×10^{-2} M).

2.4.2. The second protocol

The second series of experiments investigated the involvement of the endothelium in the diazepam-induced relaxation of PE-precontracted aortic rings. Concentration-response curves for diazepam (10^{-8}-10^{-5} M) were obtained from precontracted denuded preparations and vasodilatory effects of diazepam were assessed by comparing the response in the presence or absence of endothelium.

2.4.3. The third protocol

The third series of experiments examined the effects of diazepam, zolpidem and other tested PAMs (SH–I-048A, XHe–III–074, MP–III–022 and DK-I-56-1) on the contractile response induced by the PE, in the endothelium-intact aortic rings.

At the beginning of the protocol, to obtain a reference contraction, the contractile response induced by KCl (6×10^{-2} M) was measured. After the preparation had been washed-out several times and the tone had reached the baseline, concentration-response curve of PE (control curve) was generated (10^{-9}-10^{-4} M). Aortic ring had been washed-out again and diazepam ie other PAMs had been added directly to the organ bath, 60 min before another PE-induced contraction was obtained. The effects of tested compound on the PE contraction were assessed by comparing the contractile response in the presence or absence of compound (each at concentration 10^{-7} M, 10^{-6} M and 10^{-5} M). Results were expressed with reference to the contraction reached by the same ring precontracted with KCl (6×10^{-2} M).

2.4.4. The fourth protocol

The fourth series of experiments aimed to evaluate the concentration-dependent vascular effects of competitive $GABA_A$ receptor antagonist, bicuculline and specific antagonist of the peripheral BZ receptors, PK11195, on PE- (3×10^{-7} M) precontracted aortic rings with intact endothelium.

The concentration-response curves for antagonists were obtained at concentration range 10^{-7}- 3×10^{-5} M. Namely, in a preliminary series with tested ligands, concentration 10^{-4} M demonstrated non-selectivity in response (over 200% of decrease in relative tension), while concentration 10^{-8} M exerted negligible vascular effects. The control curve was obtained by the cumulative addition of the mixture of distilled water and solvent DMSO.

Relaxation responses of antagonists were reported as the percentage of decrease in relative tension induced by PE (3×10^{-7} M), and they were compared with relaxation response of diazepam under the same organ bath conditions.

2.4.5. The fifth protocol

The fifth series of experiments examined the effects of diazepam on the PE-induced contraction in the endothelium-intact aortic rings, in the absence and presence of antagonist bicuculline or PK11195. After the preparation was submaximally contracted (stable response induced by 3×10^{-7} M PE) an antagonist bicuculline (10^{-5} M) or PK11195 (10^{-5} M) had been added directly to the organ bath, 30 min before achieving concentration-response curve for diazepam (10^{-7} – 3×10^{-5} M). The vasoactivity of diazepam was assessed by comparing the relaxation response in the presence or absence of the specific antagonist.

2.5. Drugs and solutions

Commercially available chemicals used in these studies were: γ-aminobutyric acid, phenylephrine hydrochloride, acetylcholine iodide, bicuculline, PK11195 (Sigma-Aldrich, St. Louis, USA), zolpidem (Toronto Chemical Research, North York, Canada), and diazepam (Galenika, Belgrade, Serbia).

The ligands SH-I-048A ((S)-7-bromo-5-(2-fluorophenyl)-3-methyl-1H-benzo[e][1,4]diazepin-2(3H)-one), XHe–III–074 (S)-tert-Butyl-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c] pyrrolo[1,2-a][1,4]diazepine-1-carboxylate), MP–III–022 ((R)-8-ethynyl-6-(2-fluorophenyl)-N, 4-dimethyl-4H-benzo[f]imidazo[1,5-a][1,4] diazepine-3-carboxamide) and DK-I-56-1 (7-methoxy5-(4-methoxy-d3) phenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one) were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin – Milwaukee, USA.

All drugs were prepared as concentrated 10^{-1} M stock solutions in 100% ethyl alcohol, with the exception for PE, acetylcholine and GABA, as well as bicuculline and PK11195, the stocks of which were prepared in distilled water and DMSO, respectively. The subsequent dilutions were carried out in a mixture of the respective solvent and distilled water, so that the final concentration of solvent (ethanol or DMSO) never exceeded 0.33% in the organ bath.

2.6. Statistical analysis

Statistical analysis and graphs were prepared using LabChart 7 Pro software (AD Instruments), SigmaPlot 11 (Systat Software Inc.) or, only for RT-PCR results, GraphPad Prism 5.01 (GraphPad Software). Results were summarized as the mean ± standard error of n replicates, where n is the number of aortic rings tested in one protocol, each obtained from a separate animal.

Statistical comparisons were performed using Student's paired t-test and differences were considered statistically significant at P values less than 0.05. The pEC_{50} values (negative logarithm of the ligand concentration eliciting 50% of the maximum response) were obtained by the LabChart 7 Pro Dose response module analysis software. Non-linear regression analysis used a sigmoidal four-parameter model, with equation Y = Bottom + (Top - Bottom)/(1 + 10^((LogEC$_{50}$-X)*Hill slope)), where Top represents the maximum contraction, and Bottom is the response value in the absence of ligand.

3. Results

3.1. Detection of mRNAs for $GABA_A$ receptor subunits in the rat thoracic aorta

RT-PCR was applied in order to investigate the presence of $GABA_A$ receptor subunits (α1-6) at the mRNA level. As shown in Fig. 1A, we detected the presence of α1, α2, α3, α4 and α5 subunits in rat thoracic aorta. When resolved on a 1.8% agarose gel, all PCR products showed the expected size. Subunits α1, α2 and α3 gave a clear, single band while subunits α4 and α5 gave single, but less visible band, indicating markedly lower level of expression compared to the α1−3. Subunit α6 was undetectable under the applied conditions in the tested aortic samples. Rat brain RNA was used as a positive control for the presence of all subunits (α1-6) mRNAs (Fig. 1B). Representative real-time PCR measurement of the $GABA_A$ receptor subunits mRNA levels and housekeeping gene (*Actb*) in rat aorta are presented in Supplementary Fig. 1.

3.2. Vasodilatory effects of GABA and PAMs of $GABA_A$ receptors on PE- or KCl-precontracted rat aortic rings

We examined the vascular effects of GABA and PAMs (diazepam, zolpidem, SH-I-048A, XHe–III–074, MP–III–022 and DK-I-56-1) on rat aortic rings precontracted with PE or KCl.

The studied ligands (with the exception of GABA) induced dose-dependent vasodilatory effect, with a maximum extent (in the range 20–80% of the observed relaxation) varying substantially in dependence on the α−subtype selectivity and type of precontraction stimulus (Figs. 2–7. A1, A2). However, there was no statistically significant difference in relaxation produced with GABA (Fig. 8B) and zolpidem

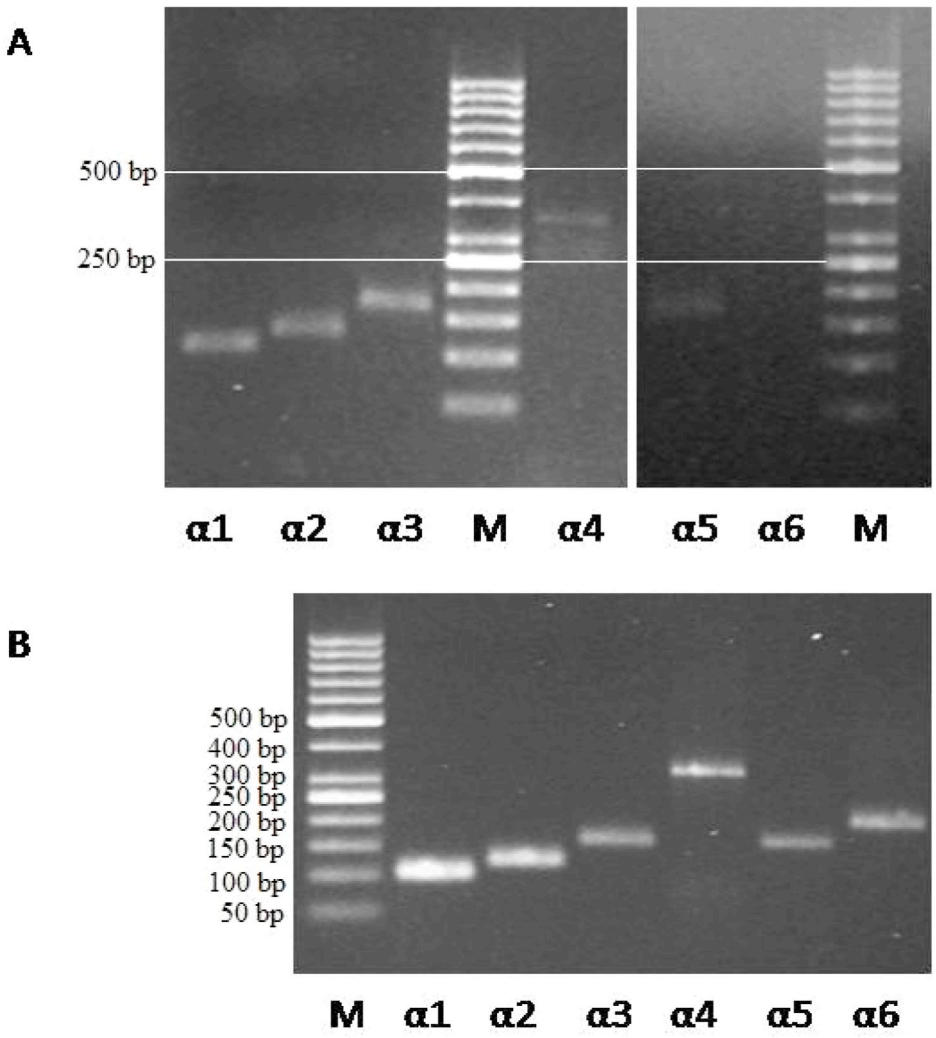

Fig. 1. $GABA_A$ receptor subunits expressed in rat thoracic aorta. (A) Representative RT-PCR gels of $GABA_A$ receptor subunits mRNAs detected in rat aorta. Ethidium bromide stained 1.8% agarose gels containing cDNA amplified using primers for $GABA_A$ receptor subunits α1–6. The aortic cDNA input into PCR reactions corresponded to 100 ng of total RNA equivalent. cDNA bands of the predicted size were obtained for all the detected subunits α1–α5. Subunit α6 was not detected in the tested aortic samples. (B) Rat brain RNA was used as positive control. The brain cDNA input into PCR reactions corresponded to 10 ng of total RNA equivalent. The predicted sizes (in base pairs) of all the amplified products were as follows: α1, 113 bp; α2, 134 bp; α3, 174 bp; α4, 345 bp; α5, 178 bp and α6, 221 bp. A 50-bp DNA ladder (M) was included.

(Fig. 4A1) in PE-precontracted rings in comparison to vehicle response.

The maximal relaxant effect was obtained at ligand concentration of 10^{-5} M (Supplementary Table 2). For preparations precontracted with PE, maximal relaxations were: for diazepam, 87% (Fig. 2A1), for XHe–III–074, 81% (Fig. 5A1), for MP–III–022, 73% (Fig. 6A1), for SH-I-048A, 62% (Fig. 3A1), for DK-I-56-1, 59% (Fig. 7A1), and for zolpidem, 30% (Fig. 4A1), whereas the maximal relaxant effects on KCl-precontracted preparations were: for MP–III–022, 55% (Fig. 6A2), for SH-I-048A, 50% (Fig. 3A2), for XHe–III–074, 45% (Fig. 5A2), for diazepam, 44% (Fig. 2A2), for DK-I-56-1, 37% (Fig. 7A2), and for zolpidem, 28% (Fig. 4A2). Also, pEC_{50} values were greater for concentration-response curves of preparations precontracted with PE than with KCl (except for XHe–III–074), suggesting that PAMs exhibit greater potency for PE-precontracted preparations (Supplementary Table 2).

Noticeably, the concentration-response curves for diazepam and others PAMs showed that the receptors were not saturated at concentration 10^{-5} M. However, a number of experiments using additional concentrations of PAMs (3×10^{-5} and 10^{-4} M) revealed that response was far above 100% of relaxation (Supplementary Fig. 2).

3.3. Role of endothelium in the diazepam-induced relaxation of PE-precontracted rat aortic rings

We examined the involvement of the endothelium in the diazepam-induced relaxation of PE-precontracted preparations. In the endothelium-intact aortic rings, diazepam (10^{-8}-10^{-5} M) induced a concentration-dependent relaxation with E_{max} of 87% (n = 8), whereas, after endothelial denudation, E_{max} of diazepam was 49% (n = 12) (Fig. 8A). Although the efficacy of diazepam was higher in the presence of endothelium, the pEC_{50} value was similar in both groups of preparations (pEC_{50} (endo+) 5.57 ± 0.12 vs. pEC_{50} (endo-) 6.05 ± 0.20) indicating that endothelial removal does not affect diazepam potency (Supplementary Table 3). Considering that the results for diazepam showed that endothelial cells are important for the vasodilatory effect, the assumption is that the same applies to other tested PAMs.

Taken together with the above-described results, these findings provide further support that the studied PAMs induce vasodilation in dose-, precontraction- and partially endothelium-dependent manner.

3.4. Effects of PAMs of $GABA_A$ receptors on the PE-induced vasoconstriction in rat aortic rings

The effects of diazepam and other PAMs on PE-induced contractions were investigated (Supplementary Table 4).

In the endothelium-intact rings, diazepam (10^{-7} M) decreased ($P < 0.01$) the pEC_{50} value for PE (no diazepam: 7.90 ± 0.25; diazepam 10^{-7} M: 6.86 ± 0.16) compared with the rings not treated with diazepam, but had no effect on PE-induced maximal contraction (except at 10^{-8} M concentration of PE) (Fig. 2B1).

At concentration of 10^{-6} M, diazepam also produced a significant rightward shift ($P < 0.05$) in the PE concentration-response curve (pEC_{50} value: no diazepam, 7.93 ± 0.26; diazepam 10^{-6} M, 7.07 ± 0.24)

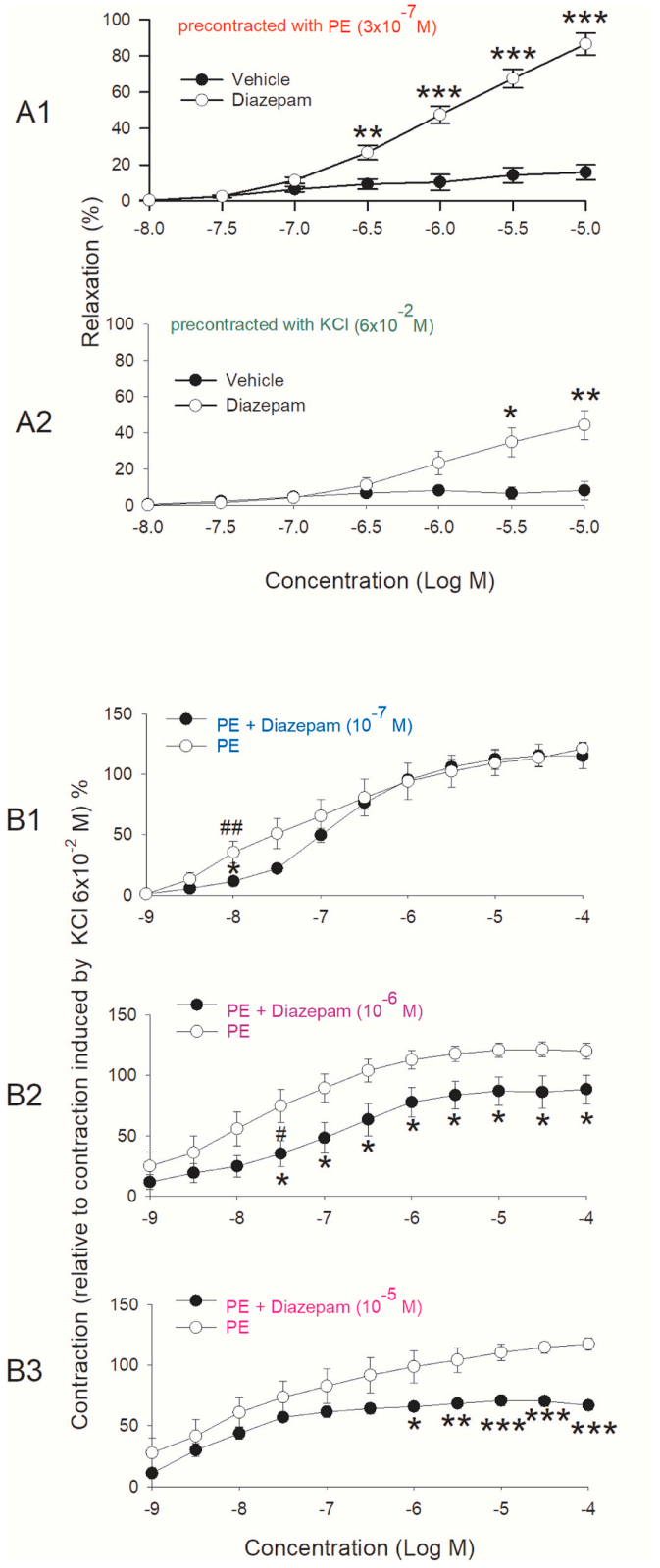

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Fig. 2. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for diazepam (n = 8) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for diazepam (n = 8) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (A2). Effect of diazepam on the PE concentration-response curve, when aortic rings were pre-incubated with diazepam at concentrations: (B1) 10^{-7} M (n = 5), (B2) 10^{-6} M (n = 9) and (B3) 10^{-5} M (n = 6). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. *P < 0.05; **P < 0.01; ***P < 0.001; significantly different E_{max} values. #P < 0.05; ##P < 0.01; significantly different pEC_{50} values.

and also decreased the maximal contraction (P < 0.05) (Fig. 2B2). However, a high concentration of diazepam (10^{-5} M) produced a significant attenuation (P < 0.001) of maximal contractile response of PE (no diazepam: 117.24 ± 5.30%; diazepam 10^{-5} M: 66.62 ± 3.71%), but had no effect on the pEC_{50} value for PE (Fig. 2B3).

When aortic rings were pre-treated with SH-I-048A, zolpidem, XHe–III–074 or MP–III–022 (at concentration 10^{-7} M or 10^{-6} M), a significant effect of these ligands on the PE-induced contraction has not been established (Fig. 3B1, B2; Fig. 4B1, B2; Fig. 5B1, B2 and Fig. 6B1, B2, respectively).

Ligand SH-I-048A (at concentration 10^{-5} M) significantly decreased (P < 0.05) the pEC_{50} value (no SH-I-048A: 8.00 ± 0.64; 10^{-5} M SH-I-048A: 5.96 ± 0.32), producing rightward shifting for PE curve, and also significantly decreased (P < 0.05) the PE-induced maximal contraction (Fig. 3B3).

Pre-treatment with zolpidem at the highest tested concentration (10^{-5} M) had not caused significant effect on responses induced by PE (10^{-9}-10^{-4} M) (Fig. 4B3).

XHe–III–074 and MP–III–022 produced strong attenuation of PE maximal contraction (P < 0.001, P < 0.05, respectively), when applied at 10^{-5} M concentration (no pre-treatment: 133.59 ± 5.72%, 124.21 ± 6.72%; pre-treatment with ligand at 10^{-5} M: 96.33 ± 6.76%, 89.05 ± 10.05%, respectively) (Fig. 5B3, 6B3).

Similar to diazepam, ligand DK-I-56-1 showed significant attenuation of PE maximal contraction (P < 0.05) at all tested concentrations (10^{-7} M, 10^{-6} M and 10^{-5} M).

Also, at 10^{-6} M concentration, DK-I-56-1 decreased (P < 0.05) the pEC_{50} value for PE (no DK-I-56-1: 7.25 ± 0.26; 10^{-6} M DK-I-56-1: 6.11 ± 0.33) (Fig. 7B1-B3).

When tested PAMs were applied at 10^{-4} M concentration, PE-induced contractions were inhibited completely (Supplementary Fig. 3).

3.5. Vasodilatory effects of antagonists bicuculline and PK11195 on PE-precontracted rat aortic rings

We first investigated the vascular effects of antagonists bicuculline and PK11195 on the endothelium-intact aortic rings, previously contracted with PE (3×10^{-7} M), and then compared them with the effects of diazepam, under the same organ bath conditions.

The results indicated that relaxant effect could be attributed to PK11195 only, while bicuculline was devoid of vasoactivity, since maximal relaxant effect for bicuculline was not significantly different compared to vehicle response (Fig. 9A).

PK11195 induced a significantly smaller relaxation in comparison to diazepam at lower concentrations (10^{-7} M, 3×10^{-7} M, 10^{-6} M, 3×10^{-6} M), although the maximal relaxant effect (observed at concentration 3×10^{-5} M) was insignificantly greater for PK11195 (E_{max} PK11195: 91.86 ± 4.32% vs. E_{max} diazepam: 85.20 ± 9.35%) (Fig. 9B).

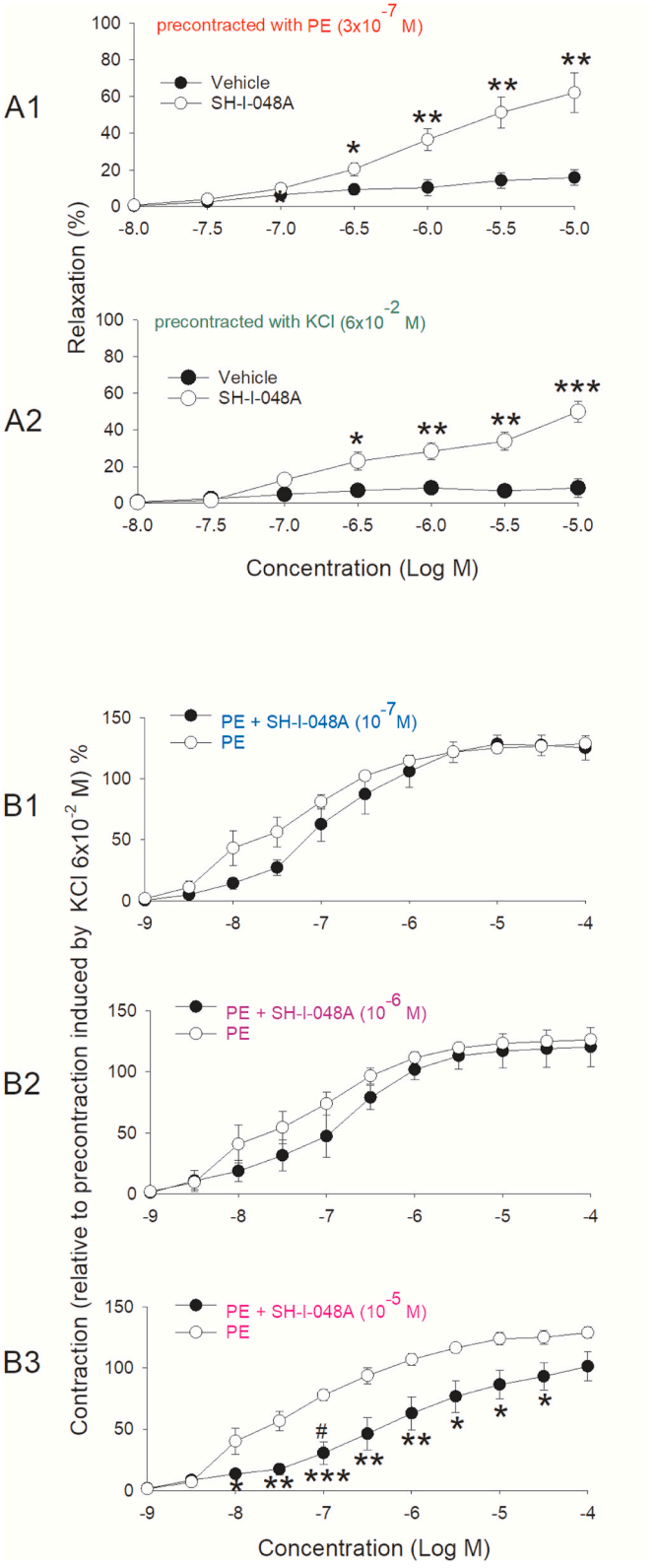

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Fig. 3. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for SH-I-048A (n = 6) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for SH-I-048A (n = 8) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (A2). Effect of SH-I-048A on the PE concentration-response curve when aortic rings were pre-incubated with SH-I-048A at concentrations: (B1) 10^{-7} M (n = 5), (B2) 10^{-6} M (n = 5) and (B3) 10^{-5} M (n = 7). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. *P < 0.05; **P < 0.01; ***P < 0.001; significantly different E_{max} values. #P < 0.05; significantly different pEC_{50} values.

3.6. *Effects of bicuculline and PK11195 on diazepam–induced relaxation of the rat aortic rings*

In order to examine the influence of antagonists on the vasodilatory effect of diazepam, as well as the possible mechanism of the observed vasoactivity, we conducted quantitative comparative studies in the presence and absence of bicuculline and PK11195, both at concentration of 10^{-5} M.

By comparison with rings not treated with an antagonist, rings in presence of PK11195 or bicuculline showed significantly lower relaxations ($P < 0.001$ and $P < 0.05$, respectively) induced by diazepam at concentration of 10^{-7} M and 3×10^{-7} M.

At higher concentrations of diazepam (10-6 M; 3 × 10-6 M; 10-5 M; 3 × 10-5 M) there was no significant difference in the observed vasoactivity in the presence of either of two antagonists (Fig. 9C) (Supplementary Table 5).

4. Discussion

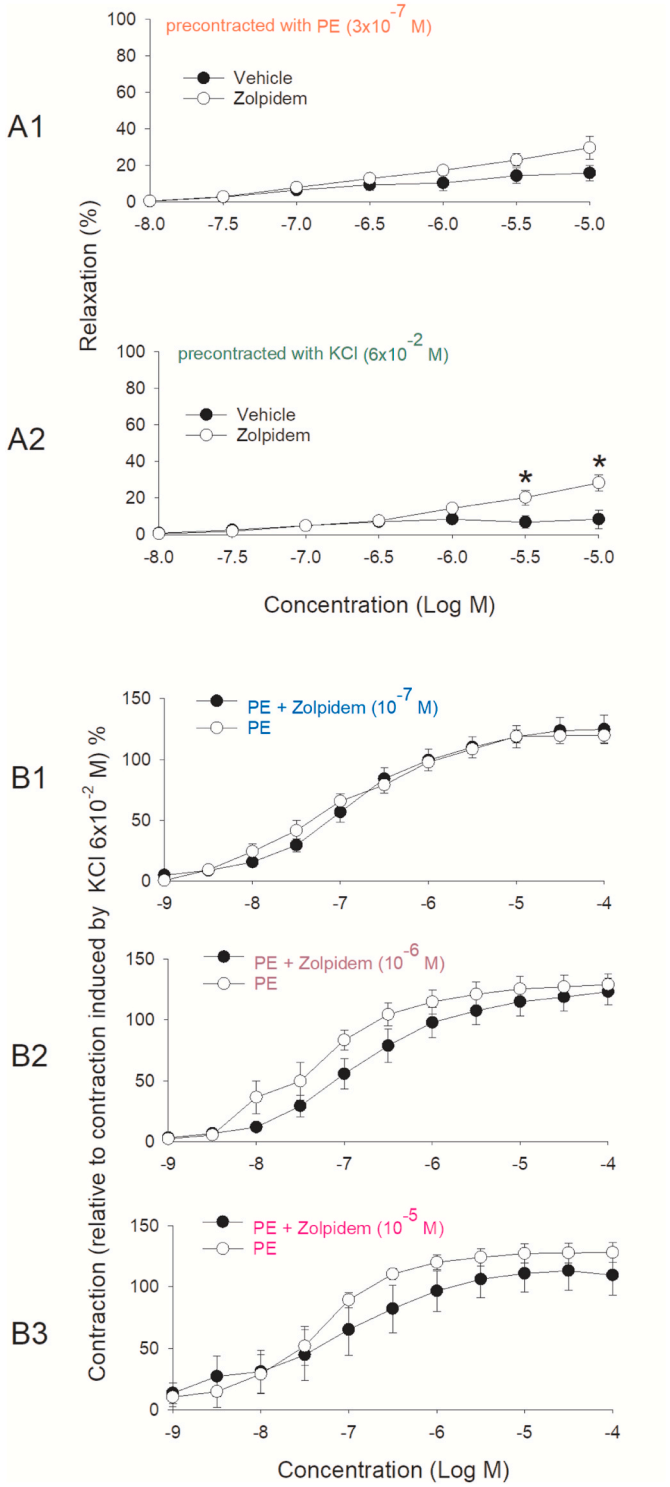
Vasodilatory effects of GABA and $GABA_A$ receptor ligands, as well as hypotension induced by parenteral administration of the blood-brain barrier-impermeable GABA (Elliott and Hobbiger, 1959; Hayakawa et al., 2002), suggested the existence of "vascular" $GABA_A$ receptors on peripheral blood vessels (Jacob and White, 2000; El Idrissi et al., 2013), the evidence of which is still limited. The Western blot analysis revealed that a non-specified $GABA_A$ receptor protein of 57 kD is expressed in mouse aorta (Tyagi et al., 2007), while immunohistochemical analysis found that β subunit-containing $GABA_A$ receptors are expressed on the smooth muscle cells of rat aorta (El Idrissi et al., 2013). The present RT-PCR data demonstrate for the first time the expression of α1, α2, α3, α4 and α5 subunits in the rat thoracic aorta.

The literature is not consistent on GABA activity on vascular tone. A study showed that GABA does not relax cerebral arteries of rat, rabbit, and monkey (Lai et al., 1988), while the others revealed that GABA relaxes the rat mesenteric bed (Farsi et al., 2011; Kharazmi et al., 2015). In line with previous reports for the rat aorta (Perusquía et al., 1996; Colussi et al., 2011), our results indicated that GABA does not affect it.

The other tested ligands, with the exception of zolpidem and bicuculline, exerted a significant vasodilatory effect on the isolated rat thoracic aorta, suggesting the involvement of "vascular" $GABA_A$ receptors. A concept that GABA, synthesized in endothelial cells (Sen et al., 2016), acts as an autacoid and instructs neighboring vascular smooth muscle cells, which express $GABA_A$ receptors, has already been proposed (Brandes, 2016). These "vascular" $GABA_A$ receptors are believed to have extrasynaptic properties, and to be continuously activated by low, ambient levels of GABA (100 nM/L, Petty et al., 1994).

The endothelial denudation in the present study did not abolish diazepam-induced relaxation. Nonetheless, our and previous reports (Chang et al., 1994; Colussi et al., 2011) indicate some involvement of endothelium in the relaxant effect of BZs, since response was significantly reduced in endothelium-denuded preparations.

Diazepam, a positive $GABA_A$ receptor modulator with high affinity



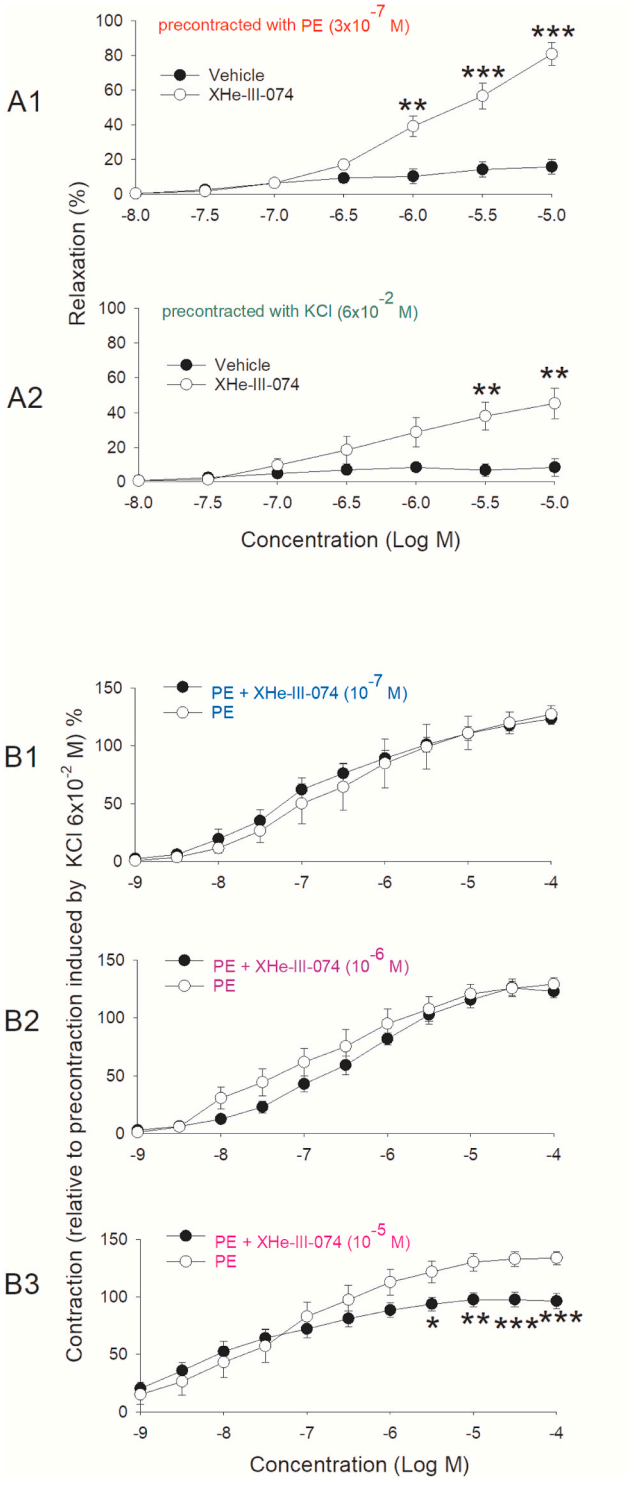


Fig. 4. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for zolpidem (n = 6) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for zolpidem (n = 8) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (A2). Effect of zolpidem on the PE concentration-response curve when aortic rings were pre-incubated with zolpidem at concentrations: (B1) 10^{-7} M (n = 6), (B2) 10^{-6} M (n = 5) and (B3) 10^{-5} M (n = 5). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. *P < 0.05; significantly different from the respective control.

Fig. 5. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for XHe–III–074 (n = 7) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for XHe–III–074 (n = 6) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (A2). Effect of XHe–III–074 on the PE concentration-response curve when aortic rings were pre-incubated with XHe–III–074 at concentrations: (B1) 10^{-7} M (n = 5), (B2) 10^{-6} M (n = 6) and (B3) 10^{-5} M (n = 11). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. **P < 0.01; ***P < 0.001; significantly different from the respective control.

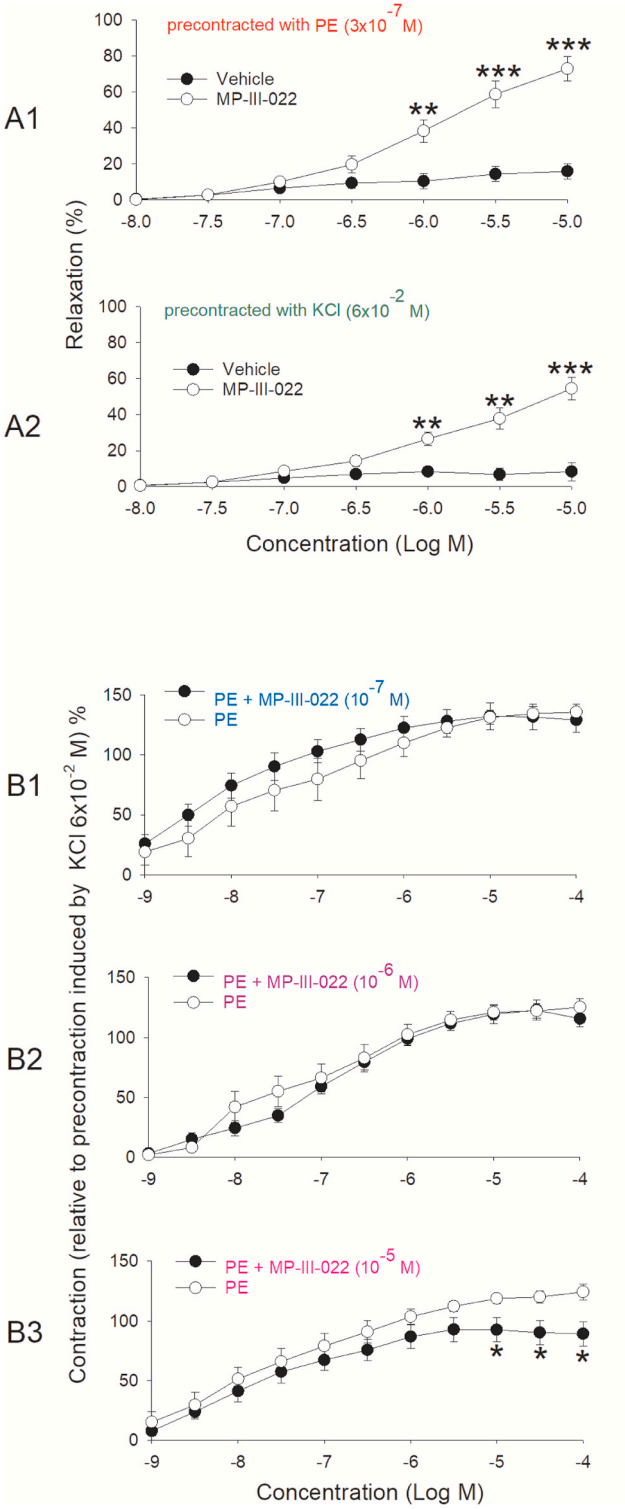


Fig. 6. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for MP–III–022 (n = 6) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for MP–III–022 (n = 7) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (A2). Effect of MP–III–022 on the PE concentration-response curve when aortic rings were pre-incubated with MP–III–022 at concentrations: (B1) 10^{-7} M (n = 8), (B2) 10^{-6} M (n = 7) and (B3) 10^{-5} M (n = 7). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. *P < 0.05. **P < 0.01; ***P < 0.001; significantly different E_{max} values.

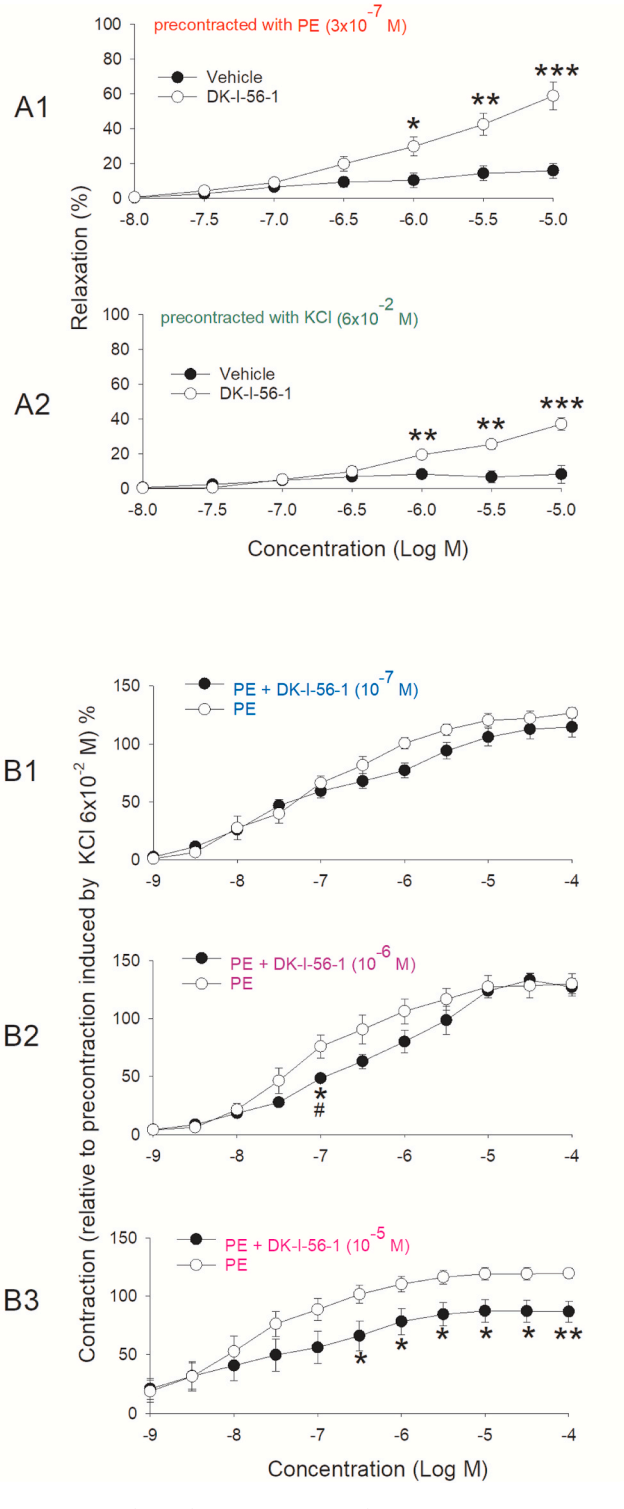


Fig. 7. (A1) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for DK-I-56-1 (n = 8) and vehicle (n = 6). (A2) Cumulative log concentration-relaxation curves in aortic rings precontracted with KCl (6×10^{-2} M) for DK-I-56-1 (n = 7) and vehicle (n = 5). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by 3×10^{-7} M PE (A1), or 6×10^{-2} M KCl (B2). Effect of DK-I-56-1 on the PE concentration-response curve when aortic rings were pre-incubated with DK-I-56-1 at concentrations: (B1) 10^{-7} M (n = 7), (B2) 10^{-6} M (n = 5) and (B3) 10^{-5} M (n = 7). Results (mean ± S.E.M.) are expressed with reference to the contraction reached by the same ring contracted with KCl (6×10^{-2} M). Parentheses indicate the number of preparations studied. *P < 0.05; **P < 0.01; ***P < 0.001; significantly different E_{max} values.

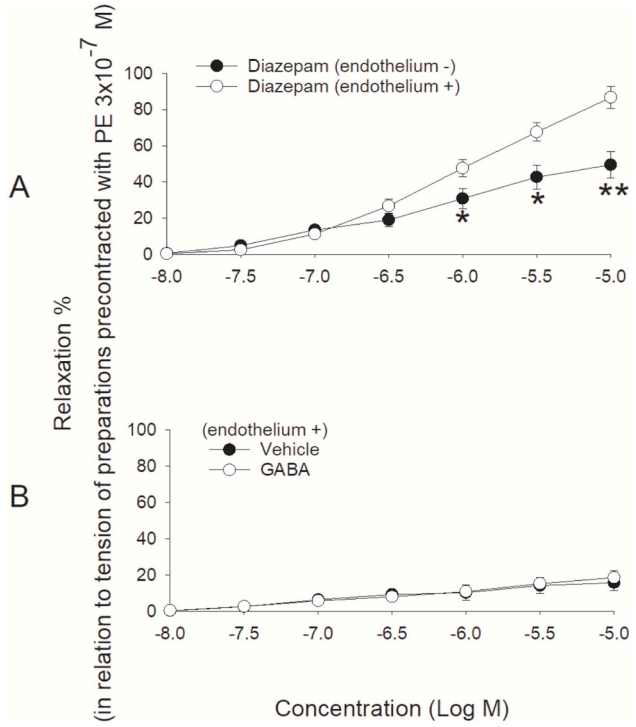


Fig. 8. (A) Comparison of cumulative log concentration-relaxation curves for diazepam in endothelium-intact (n = 8) and endothelium-denuded (n = 12) aortic rings precontracted with PE (3×10^{-7} M). (B) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for GABA (n = 9) and vehicle (n = 6). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by PE (3×10^{-7} M). Parentheses indicate the number of preparations studied. *P < 0.05; **P < 0.01; significantly different from the respective control.

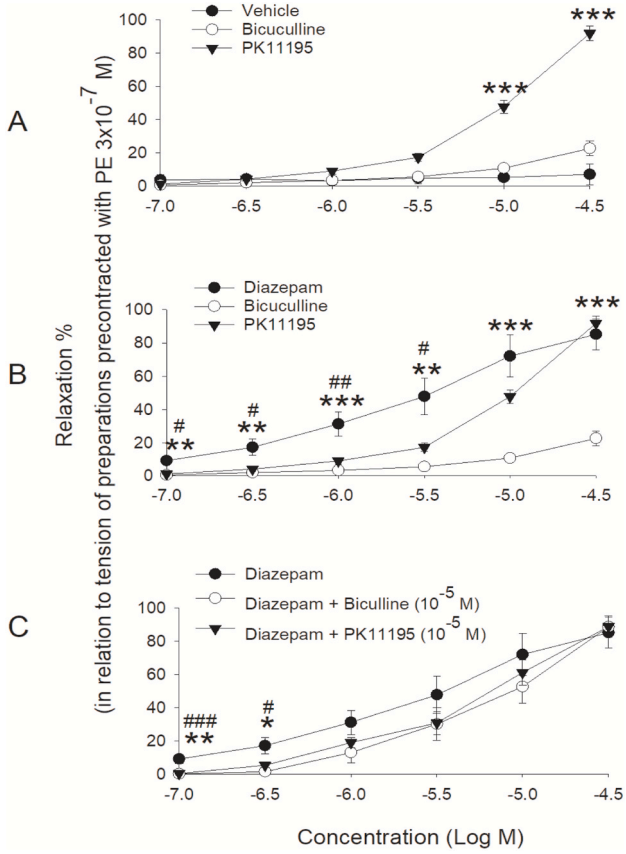


Fig. 9. (A) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for: bicuculline (n = 7), PK11195 (n = 7) and vehicle (n = 3). (B) Cumulative log concentration-relaxation curves in aortic rings precontracted with PE (3×10^{-7} M) for: bicuculline (n = 7), PK11195 (n = 7) and diazepam (n = 5). (C) Effects of diazepam on the PE-induced contraction in the aortic rings in the absence (n = 5) and presence of 10^{-5} M bicuculline (n = 7) or 10^{-5} M PK11195 (n = 9). Results (mean ± S.E.M.) are expressed as the percentage of decrease in relative tension induced by PE (3×10^{-7} M). Parentheses indicate the number of preparations studied. *P < 0.05; **P < 0.01; ***P < 0.001; significantly different from respective control - presence of bicuculline (10^{-5} M). #P < 0.05; ##P < 0.01; ###P < 0.001; significantly different from respective control - presence of PK11195 (10^{-5} M).

for the α1,2,3,5 $GABA_A$ receptors, was the most efficient ligand in the present study, both in terms of achieved relaxation and preventive effects on PE-induced contraction. Apparently paradoxically, diazepam at 10^{-6} M, but not at 10^{-5} M, produced the significant rightward shift in the PE concentration-response curve. An antagonist, if competitive, will produce shifts to the right of the agonist dose-response curve, but if noncompetitive, will not shift the curve to the right but rather depress it. The present results may be interpreted as if at higher doses diazepam tends to behave more like a noncompetitive, rather than a competitive antagonist, not necessarily based on the interaction with the same substrate structure(s). However, the underlying nature of receptor and molecular interactions of diazepam with $GABA_A$ receptors, and possible other targets, in blood vessels still needs to be elucidated.

Results indicate that the vascular effects of diazepam are more pronounced than effects of SH-I-048A, although the latter was characterized as a stronger α1/2/3/5β3γ2 positive modulator, with considerably higher affinity and efficacy than diazepam at all four $GABA_A$ receptor subtypes (Obradović et al., 2014) (Supplementary Table 6). This could be partly explained by previously reported discrepancies in *in vivo* profiles and relative differences in the approximated receptor activity of diazepam and SH-I-048A (Obradović et al., 2014).

Zolpidem, a highly effective modulator of α1−containing $GABA_A$ receptors, showed the lowest relaxation of precontracted preparations. A possible explanation for this poor vasoactivity of zolpidem, compared to other BZ site ligands of $GABA_A$ receptor tested here, might be related to an absence of its efficacy or weak efficacy at $GABA_A$ receptors containing other α subunits (Savić et al., 2010) (Supplementary Table 7). Namely, if the α1 subunit in blood vessels is predominantly co-expressed with another α subunit in the same receptor aggregate, the α1 subunit would not participate in the BZ binding site (del Río et al., 2001), and zolpidem would not exert the expected effects.

Remarkably, the positive modulator of α4−containing $GABA_A$ receptors, XHe–III–074, exhibited a strong vasodilatory effect, comparable to the effects of diazepam, whereas positive modulator of α5−containing $GABA_A$ receptors, MP–III–022, showed even more pronounced relaxation than diazepam. The observed vasoactivity of these ligands could be explained by the expression of α4 and α5 subunit mRNAs in the rat aortic tissue, which indicates potential modulation of the respective receptors. Despite lower affinity (Ki value for diazepam at α5β3γ2 $GABA_A$ receptors is 5-fold lower than the Ki value for MP–III–022) (Stamenić et al., 2016) (Supplementary Table 8), MP–III–022 showed higher percentages of relaxation than diazepam in both types of precontractions. The clinical significance of other α4 and α5 selective $GABA_A$ receptor modulators has been already proposed, as their bronchodilator effect was associated with modulation of α4-and α5-containing $GABA_A$ receptors expressed in airway smooth muscle cells (Forkuo et al., 2017).

DK-I-56-1 is the only PAM besides diazepam that suppressed PE-induced contraction at lower concentrations (10^{-7} M and 10^{-6} M). This vasoactivity is of particular interest, given its pronounced functional preference for α6-containing $GABA_A$ receptors (Knutson et al., 2018), while mRNA expression of the α6 subunit was not detected. The complexity of gene expression regulation, particularly alternative

splicing, may result in *gabra6* transcript variant/s in rat aortic tissue that are undetectable by the primer used. Namely, short and long spliced variants of the α6 subunit have been reported (Korpi et al., 1994). It is also possible that the expression level of α6 subunit mRNAs is much lower compared to other subunits, particularly if this subunit is expressed only in discrete cells of the thoracic aorta. As a comparison, the rest of the brain other than cerebellum exhibits smaller, very low, or no expression of *gabra6* (Lein et al., 2007). In addition, DK-I-56-1 is a high affinity antagonist at the classical benzodiazepine site of $GABA_A$ receptors (Knutson et al., 2018), which may somehow affect its overall influence on blood vessels or some of their structures. Further studies, including separation of the endothelium from the smooth muscle layer and immunohistochemical analyzes, are necessary to elucidate the expression and the exact localization of the expressed subunits within the aorta.

Our results indicate that vasoactivity of studied PAMs is influenced by the contractile agent used. Finding that the maximal response is greater in PE-precontracted than in KCl-precontracted preparations is in accordance with the previous *in vitro* observations for BZ site modulators (Chang et al., 1994; Yamaguchi et al., 1997; Colussi et al., 2011). The assumption is that the contractile agents exhibit different interactions with the mechanisms of tonic inhibition on the smooth muscle cell level. Contractions produced by PE involve α-adrenergic receptor-operated intracellular Ca^{2+} release and Ca^{2+} influx through receptor-operated Ca^{2+} channels, whereas those elicited by KCl are primarily due to extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels (Yamaguchi et al., 1997; Hussain and Marshall, 1997).

PK11195, a specific ligand for TSPO (translocator protein 18 kDa, formerly called the peripheral BZ receptor), induces vasodilation of vascular smooth muscle (French et al., 1989), which we have confirmed herein. It was suggested that diazepam-induced relaxation of rat aortic rings, precontracted with PE, is not dependent on either $GABA_A$ or TSPO receptors, since relaxation was not antagonized by flumazenil or PK11195 (Galindo et al., 2001). In the present study, flumazenil was not used as an antagonist at BZ binding site because it acts at the same time as a PAM at α4-and α6-containing $GABA_A$ receptors (Sieghart and Savić, 2018).

Our results showed that diazepam-induced relaxations, obtained at lower concentrations (10^{-7} M and 3×10^{-7} M), were significantly diminished in the presence of antagonists, but neither bicuculline nor PK11195 did significantly rightward shift or reduced the maximum effect of diazepam. Putative engagement of additional target substrates, and/or change of the quality of the diazepam interaction with the primary "vascular" binding site(s), as hypothesized above, may explain, at least in part, the fact that bicuculline and PK11195 did not affect the vasodilation induced by diazepam at concentration of 10^{-6} M or higher. Moreover, one cannot exclude intricate interactions of "vascular" $GABA_A$ receptors with cell pathways reportedly involved in BZ-induced relaxation, such as elevation of cyclic AMP (Collado et al., 1998; Galindo et al., 2001), blockade of Ca^{2+} influx (Chang et al., 1994; Perusquía et al., 1996), or activation of K^+ channels (Jacob and White, 2000; Klockgether-Radke et al., 2005).

Micromolar concentrations of BZs have achieved vasodilatory effects in *ex vivo* studies (French et al., 1989; Galindo et al., 2001; Park et al., 2006; Colussi et al., 2011). Here, PE-induced contraction was affected only by diazepam when applied at 10^{-7} M concentration, which is seven times lower than its concentration present in clinical settings with 96.8% protein binding (Klotz et al., 1976). However, when the tested PAMs were applied at concentration of 10^{-5} M, which approximately corresponds to the supraclinical concentration of diazepam (Park et al., 2006), all modulators, except zolpidem, significantly suppressed PE-induced contraction. Such vascular effects may still be significant in emergencies, such as hypertensive crises, overdose, or abuse (Colussi et al., 2011).

5. Conclusion

The present study used two methods: RT-PCR analysis which determined the presence of α1, α2, α3, α4 and α5 subunits of $GABA_A$ receptor in rat thoracic aorta, and tissue bath studies, which demonstrated vasodilatory effects on isolated rat aortic rings of five positive $GABA_A$ receptor modulators with different profiles of the BZ site affinity and efficacy at various $GABA_A$ receptors: diazepam, SH-I-048A, XHe–III–074, MP–III–022 and DK-I-56-1. The present work suggests that modulation of "vascular" $GABA_A$ receptors is responsible for the observed vasodilatory effects, so further research will be required to determine the full relevance and applicability of these findings.

CRediT authorship contribution statement

Milica Gajić Bojić: Methodology, Formal analysis, Investigation, Writing – original draft, preparation. **Lidija Todorović:** Methodology, Investigation, Writing – original draft, preparation. **Anja Santrač:** Methodology, Formal analysis, Investigation. **Md Yeunus Mian:** Methodology, Formal analysis, (synthesis of compounds). **Dishary Sharmin:** Methodology, Formal analysis, (synthesis of compounds). **James M. Cook:** Supervision, Writing – review & editing, Funding acquisition. **Miroslav M. Savić:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174023.

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