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ORIGINAL ARTICLE

The Vasorelaxant Properties of Novel Benzodiazepine-like Ligands on Isolated rat Thoracic Aorta

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Abstract

Background/Aim: In addition to well-established central effects, benzodiazepines, but also some other allosteric modulators of gamma-amino-butyric acid (GABA) receptor exhibit significant vascular effects. However, there are currently no elucidated mechanisms for manifested vasodilatory properties and very little is known about GABA gamma-amino-butyric acid function and $GABA_A$ receptor expression within peripheral blood vessels.
Methods: In the present study, we demonstrated the vasorelaxant properties of diazepam, GABA and novel imidazobenzodiazepine amide ligands GL-II-73 and GL-II-74, which are characterized as positive allosteric modulators of α5- containing $GABA_A$ receptor. Using isometric organ bath system, we examined the vascular responses to phenylephrine, in the presence and absence of various ligands, in the rat thoracic aorta.
Results: The observed significant and strong attenuation of the maximal contractile response of phenylephrine indicates a non-competitive antagonism of diazepam, GL-II-73 and GL-II-74 ($p < 0.001$), whereas GABA does not affect phenylephrine contraction.
Since the strongest inhibitory effect was observed with compound GL-II-74, that, compared to other tested ligands, exhibited a higher potentiation at α5 $GABA_A$Rs, it could be assumed that the α5 subunit plays a significant role in the structure of putatively present "vascular" $GABA_A$Rs.
Conclusion: This work emphasizes the importance of $GABA_A$Rs research in the periphery and also points to the possibility of using α5 selective $GABA_A$R modulators as potential therapeutic targets for novel vasodilators.

Key words: $GABA_A$ receptor, positive allosteric modulators, vasodilatation, rat thoracic aorta.

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Introduction

In addition to being a major inhibitory neurotransmitter in the central nervous system (CNS), GABA has a functional importance in many peripheral tissues. Peripheral GABA regulation of cardiovascular function has long been known,[1, 2] but to date no distinct roles or exact mechanisms have been established.

The first studies with isolated cerebral blood vessels had suggested that $GABA_A$ receptors ($GABA_A$Rs) exist in vascular smooth muscle, where GABA or GABA-agonists produced a dilatation of cerebral arteries.[3, 4] Even though $GABA_A$R subunit mRNA expression has been demonstrated in various rat peripheral organs, such as kidneys, adrenal gland, ovary, testis, uterus and ileum[5, 6] very lit-

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tle is known about $GABA_AR$ expression and GABA function within the peripheral vascular smooth muscle.

GABA levels in the peripheral vessels and activity of GABA-related enzymes, especially glutamic acid decarboxylase (GAD) and gamma-aminobutyric acid-transaminase (GABA-T), have been found to be up to 1 % of those in the brain[7] and such a modest expression can be regarded as insufficient to directly elicit vasoactivity of GABA. However, the finding that cultured human aortic and umbilical vein endothelial cells synthesize GABA, which further exhibits direct effects on endothelial cell metabolism[8] indicates the potential role of GABA as an autocoid for neighbouring smooth muscle cells.

Benzodiazepines (BZs) as positive modulators of $GABA_ARs$ have a wide range of acute effects, such as anxiolytic, sedative, hypnotic, skeletal muscle relaxant, anticonvulsant, anterograde amnesic and ataxic action. In addition to well-established central role, BZ's also exhibit vasodilatory properties.[9,10,11] However, there are currently no elucidated mechanisms of BZ`s vasoactivity and propensity to reduce the intracellular influx of calcium into the smooth muscle cell.

Vascular effects similar to those of diazepam are also exhibited by other $GABA_AR$ allosteric modulators, such as endogenous neurosteroids.[12] Considering that the peripheral benzodiazepine receptor (officially known as translocator protein, TSPO) has no role in regulating smooth muscle contractility,[10] the published results suggest that activation or positive modulation of $GABA_ARs$, such as that effected by diazepam, result in vascular dilation.[12] However, the receptor subtype substrate of that action is totally unknown.

Herein, the vasorelaxant properties of novel ligands with imidazobenzodiazepine (IBZD) amide structure GL-II-73 and GL-II-74 were demonstrated, which are characterised as positive allosteric modulators (PAMs) of $GABA_AR$ with preferential potentiation at α5 subunit-containing receptors.[13] In order to examine their possible vasoactivity, isometric organ bath study of vascular responses to phenylephrine was conducted. Diazepam and GABA were used in the same protocols, and in this way the manifested effects were compared and thus the possible mechanisms of vasoactivity were assessed.

Methods

Vessel preparation

Wistar rats were obtained from the Military Medical Academy and housed in vivarium facilities of the Faculty of Pharmacy, University of Belgrade (Belgrade, Serbia) under normal housing conditions (temperature: 22 ± 1 °C, relative humidity: 40-70 %, 12/12 h light/dark period). As a part of a wider national project led by the senior author, the experiments were 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. Male rats were anaesthetised with combination 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). The descending thoracic aortas were dissected and cleared of surrounding adipose and connective tissue.

Aortic rings of approximately 3 mm length were obtained from isolated blood vessels bathed in Petri dish containing chilled (4 °C) modified Krebs-bicarbonate solution (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).[14] The aortic rings were rapidly placed for measurement of isometric contraction.

Experiments with isolated vascular rings

The aortic rings were suspended between two wire hooks in organ bath chambers filled with 15 mL modified Krebs-bicarbonate solution (37° C, pH 7.40) aerated with mixture of 95 % oxygen/5 % carbon dioxide. The upper hook was connected to the MLT0201 force displacement transducer (Panlab, Spain), and changes in isometric force were recorded using PowerLab/4SP data acquisition system (AD Instruments, Castle Hill, Australia) and software LabChart 7 Pro (AD Instruments). Experiments were performed on four organ baths in parallel.

The rings were placed under the optimal passive stretching tension of 4.0 g, defined previously.[15] The equilibration period of the preparation lasted 60 min and during that time the bathing solution was changed every 10 min. Each aortic ring was subjected first to the initial challenging contraction with potassium chloride (6×10^{-2} M) to assess the viability of preparations. The rings

was then left to re-equilibrate for 40-50 min, before the appropriate protocol procedures were used.

Experimental protocol: experiments were aimed to investigate the effects of diazepam, GABA and novel imidazobenzodiazepine (IBZD) amide ligands (GL-II-73 and GL-II-74) on the contractile response induced by the α1 adrenoreceptor agonist phenylephrine (PE), in the endothelium-intact aortic rings.

At the beginning of the protocol, to obtain a reference contraction, the contractile response induced by potassium (6 x 10^{-2} M) was measured. After preparations were washed-out several times until tone returned to baseline, concentration-response curve of PE (control curve) was generated (10^{-9}-10^{-4} M). Aortic ring had been washed-out again and test compound (each at concentration 10^{-4} M and 10^{-5} M, except for diazepam with the applied concentration of 10^{-5} M) were added individually to the organ bath, 60 min before another PE-induced contraction was obtained. The effects of the test compound on the PE contraction were assessed by comparing the contractile response in the presence or absence of compound. Results were expressed in relation to the contraction achieved by the same ring previously contracted with isotonic potassium.

Drugs and solutions

Phenylephrine hydrochloride and GABA were purchased from Sigma-Aldrich (St. Louis, USA). Diazepam was generously supplied by Galenika (Belgrade, Serbia).

The ligands GL-II-73 ((R)-8-Ethynyl-6-(2-fluorophenyl)-N,N,4-trimethyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxamide) and GL-II-74 ((R)-N-Ethyl-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxamide) were synthesised at the Department of Chemistry and Biochemistry, University of Wisconsin, Milwaukee, USA.

All drugs were prepared as concentrated stock solutions 10^{-1} M in 100 % ethyl alcohol, with exception for PE and GABA, the stocks of which were prepared in distilled water. The subsequent dilutions were carried out in mixture of solvent and distilled water, so that the final solvent concentration was never higher than 0.3 % in the 15 mL-organ bath.

Statistical analysis

Statistical analysis and graphs were prepared using LabChart 7 Pro software (AD Instruments) and SigmaPlot 11 (Systat Software Inc.) Results were summarised 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. The negative logarithm of the ligand concentration (pEC_{50}) producing 50% of the maximum response was calculated in LabChart 7 Pro software. Statistical analyses were performed using Student's paired t-test (p values less than 0.05 were considered statistically significant).

Results

Diazepam (10^{-5} M) produced a significant attenuation ($p < 0.001$) of the maximal contractile response of PE (117.24 ± 5.30 % vs 66.62 ± 3.71 %), while it did not affect the pEC_{50} value of PE (Figure 1).

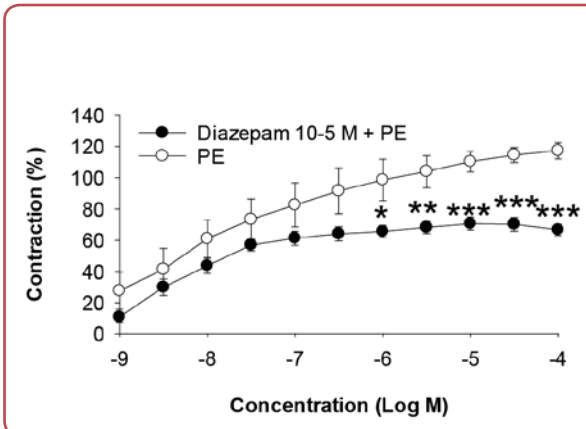

*Figure 1. Effect of diazepam on the phenylephrine concentration-response curve when aortic rings were pre-incubated with diazepam (10^{-5} M, n = 6). Results (mean ± SEM) are expressed with reference to the contraction induced by potassium (6 x 10^{-2} M). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (significantly different E_{max} values). Parentheses indicate the number of preparations studied, each obtained from a separate animal.*

Although applied at a very high concentration (10^{-4} M), GABA did not shift the PE concentration-response curve or affect the PE-induced maximal contraction (Figure 2A). GABA used at concentration of 10^{-5} M also did not affect the PE contraction (data not shown).

The ligand GL-II-73 (10^{-5} M) significantly decreased ($p < 0.05$) the maximal contractile re-

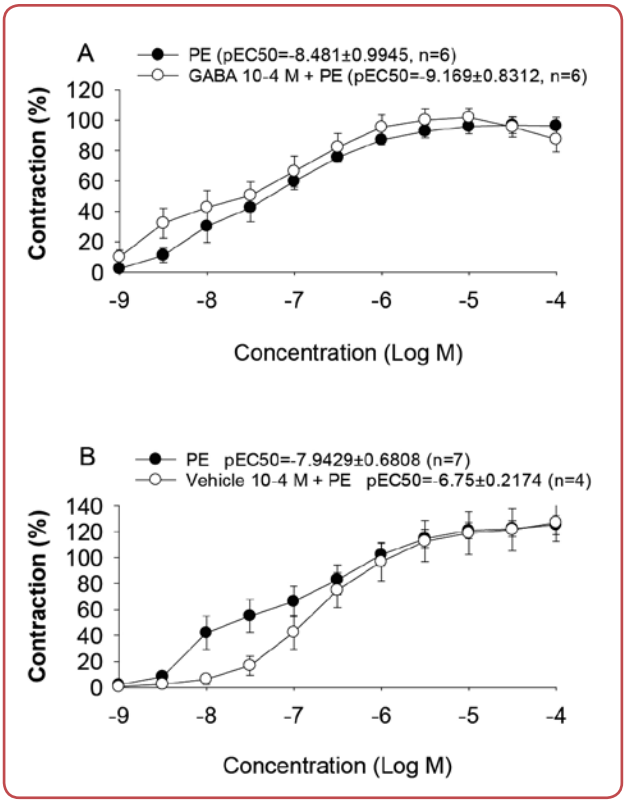

Figure 2. Cumulative log concentration-relaxation curves for pheny-lephrine (PE) in the absence and presence of A) GABA 10^{-4} M (n = 6) vehicle 10^{-4} M (n = 4). Results (mean ± SEM) are expressed with reference to the contraction induced by potassium (6 x 10^{-2} M). Parentheses indicate the number of preparations studied, each obtained from a separate animal.

sponse to PE (82.36 ± 16.41 % vs 125.17 ± 7.45 %), but had no effect on the PE potency (there was no significant differences between pEC_{50} values). Pre-treatment with GL-II-73 at a ten-fold higher concentration (10^{-4} M) strongly attenuated ($p < 0.001$) the maximal PE contraction (35.03 ± 9.60 % vs 125.17 ± 7.45 %), whereas had no significant effect on the pEC_{50} value of PE (10^{-9}-10^{-4} M) (Figure 3A, B).

The ligand GL-II-74 used at the higher concentration (10^{-4} M) strongly decreased ($p < 0.001$) the PE-induced maximal contraction, compared with untreated rings (18.08 ± 4.48 % vs 125.17 ± 7.45 %). When aortic rings were pre-treated with a lower concentration of GL-II-74 (10^{-5} M) there also was no effect on the pEC_{50} value for PE, while the maximal contractile response was significantly ($p < 0.001$) decreased (42.99 ± 11.63 % vs 125.17 ± 7.45 %) (Figure 3C, D).

The influence of vehicle was obtained when the same volume, as for ligand additions at concentration (10^{-4} M), was added in pre-incubation period. There was no altered pharmacological activity on aortic rings in presence of vehicle (Figure 2B).

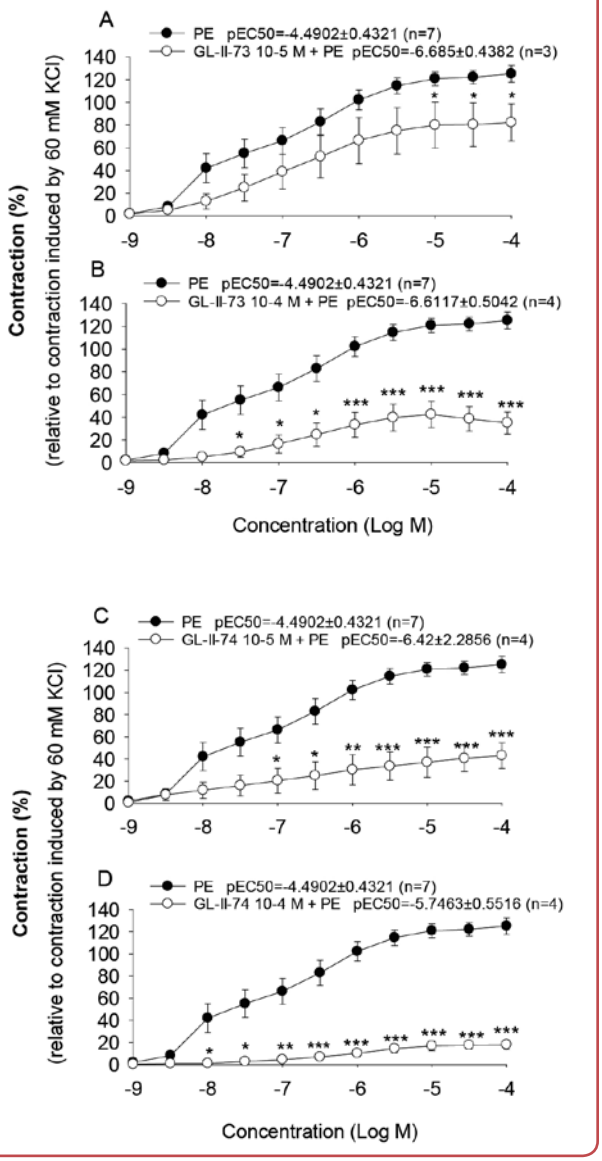

Figure 3. Cumulative log concentration-relaxation curves for phenyl-ephrine (PE) in the absence and presence of A) GL-II-73 10^{-5} M (n = 3); B) GL-II-73 10^{-4} M (n = 4); C) GL-II-74 10^{-5} M (n=4); D) GL-II-74 10^{-4} M (n=4). Results (mean ± SEM) are expressed with reference to the contraction induced by potassium (6 x 10^{-2} M).
**** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (significantly different E_{max} values). Parentheses indicate the number of preparations studied, each obtained from a separate animal.*

Discussion

The differential expression of total of nineteen $GABA_AR$ subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) has been demonstrated in various peripheral organs, indicating that $GABA_AR$ subunits are expressed in a tissue-specific manner.[6, 16] Immunohistochemical analyses, western blotting and real time reverse transcription polymerase chain

reaction (RT-PCR), had revealed the presence of functional $GABA_A$Rs within the gastrointestinal tract,[16] airway smooth muscle of trachea,[17,18] pancreatic β cells.[19] However, there is still no clear evidence for the expression of functional $GABA_A$Rs on vascular smooth muscle cells. This study was based on the hypothesis that positive allosteric modulation of $GABA_A$Rs that contain the α5 subunit contributes to vasodilating effects of BZs. The inhibiting influence of diazepam on the contractile activity of phenylephrine in isolated rat aorta was demonstrated, thus confirming the previous *in vitro* studies, where diazepam inhibited PE- induced calcium oscillations,[20] attenuated the PE- induced contractions in the rat aorta[10] and produced vasodilation in the PE-precontracted rat aortic rings.[9] The observed significant and strong attenuation of the maximal contractile response of PE indicates a non-competitive antagonism of diazepam, in terms of signalling mechanisms of contraction in vascular smooth muscle cells.

Concentration of GABA in the systemic circulation of humans was found to be between 0.5 to 3 μM.[8] It has been suggested that apart from GABA produced by the pancreatic beta cells, adrenal gland and certain immune cells, an important source of GABA in circulation may be that related to endothelial cells of blood vessels.[8] The examination of the effect of GABA on vascular response to PE in isolated rat aorta indicated that GABA did not affect PE contraction, even when applied in high concentration (100 μM). Findings of GABA indifference on contracted aortic rings found in this study may correlate with earlier data that no vasodilating effects on peripheral blood vessels have been reported for GABA.[8,11,21] Nevertheless, the results from other studies with isolated blood vessels have shown that GABA has relaxatory effect on rat mesenteric bed.[22,23]

Diazepam, a standard non-selective PAM of $GABA_A$Rs, was used as the reference ligand, in order to investigate the vasorelaxant properties of GL-II-73 and GL-II-74. Previously performed electrophysiological and binding studies showed that ligands GL-II-73 and GL-II-74 acted as PAMs with primary efficacy and affinity at α5-containing $GABA_A$Rs,[13] whereas diazepam modulates $GABA_A$R activity as a non-selective PAM, with high affinity and efficacy at α1, α2, α3 or α5–containing $GABA_A$Rs.[24] Both ligands (GL-II-73 and GL-II-74) reduced the maximum contraction induced by PE, compared to the untreated rings, indicating similarity to the effects of diazepam in the same protocol.

It was also shown that the vascular responses to PE in the isolated aortic rings vary significantly, depending on concentrations of GL-II-73 and GL-II-74 used during incubation. When aortic rings were pre-treated with a higher concentration (10^{-4} M), the maximal contractile response was approximately 20-30 % of the corresponding control maximal contraction ie (without the presence of ligand), while at lower concentrations of tested ligand, the inhibitory effects were weaker (approximately 50 % reduction in contraction). This clearly indicates a concentration-dependent inhibitory effects of the tested IBZDs.

Concentrations of compounds used in this study were in accordance with those in studies of vascular effects of BZs on isolated blood vessels. Although these concentrations are too high to correspond with the clinical use of BZs, they can still be reached in cases of overdose or other abuse.[11] In this regard, their vascular effects should not be neglected. Interestingly, a stronger inhibitory effect on the PE concentration-response curve was observed with compound GL-II-74 than with GL-II-73. This might be explained by the observed differences in their modulatory properties, taking into account that GL-II-74 exhibited a higher potentiation at α5 $GABA_A$Rs than GL-II-73.[13] Accordingly, it could be assumed that the presence of the α5 subunit in the structure of putatively present "vascular" $GABA_A$Rs may play a substantial role in the overall observed vasoactivity.

Conclusion

The present work highlights the importance of $GABA_A$Rs research in the periphery and also opens the possibility of using α5 selective $GABA_A$R modulators as potential therapeutic targets for novel vasodilators.

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Conflict of interest

None.

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