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Vasorelaxant Effect of Alkaloid *N*-deacetyllappaconitine on Vascular Smooth Muscle

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Abstract

In the pathogenesis of cardiovascular diseases, disruption of Ca^{2+} homeostasis in vascular smooth muscle cells and the dysregulation of vasoregulatory mechanisms are considered decisive factors. In particular, dysfunction of the Ca^{2+} transport systems in the plasma membrane and the sarcoplasmic reticulum, and the resulting imbalance of endothelium-dependent vasoactive and regulatory factors, play a crucial role. The aim of this study is to comprehensively assess the mechanisms of the vasorelaxant effects of the alkaloid *N*-deacetyllappaconitine on rat aortic rings. The isometric contraction force of the aortic rings was recorded using a Grass-Telefactor FT.03 myograph and analysed with the PowerLab data-acquisition software (ADInstruments). The results showed that the *N*-deacetyllappaconitine alkaloid exerts a dose-dependent vasorelaxant effect on KCl- and PE-induced aortic contractions. *N*-deacetyllappaconitine alkaloids reduce intracellular Ca^{2+} levels by inhibiting L-type Ca^{2+} channels in the plasma membrane and by attenuating IP_3 receptor activity. Furthermore, by partially activating K_{ATP} channels, it causes membrane hyperpolarisation and, by inhibiting the reverse mode of the Na^+/Ca^{2+} exchange system, slows the influx of Ca^{2+} ions into the cell. Thus, the relaxant effect of the alkaloid *N*-deacetyllappaconitine on aortic rings is realised through a number of complex mechanisms.

Keywords: Alkaloid, Aorta, Endothelium, Ion channels, Smooth muscle, Vasorelaxant

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide [1,2]. Blood vessel dysfunction is the main pathogenic factor in the development and progression of hypertension, atherosclerosis and their associated complications [3,4]. The disruption of vascular tone is a pathophysiological process characterized by endothelial dysfunction and the derailment of the coordinated actions of smooth muscle ion channels and intracellular signaling pathways [5]. Disruption of vasorelaxation mechanisms, particularly endothelium-dependent responses, is a characteristic feature of many cardiovascular pathologies [6,7]. Therefore, identifying new drug compounds capable of modulating vascular reactivity is of great therapeutic importance [8,9].

Over the years, it has been widely recognized that bioactive compounds obtained from natural sources have significant therapeutic potential in the prevention and treatment of cardiovascular diseases [10-12]. Among them, alkaloids, which are extracted from medicinal plants, are distinguished by their chemical

diversity and broad biological activity [13]. Information is provided on the pharmacological effects of many alkaloids on the cardiovascular system, including antihypertensive, antiarrhythmic and vasodilator effects [14-16]. The main mechanisms of these effects often involve modulation of endothelial nitric oxide (NO) production, blockade of Ca^{2+} flux through voltage- or receptor-regulated Ca^{2+} channels, activation of K^+ channels or intracellular Ca^{2+} mobilization in vascular smooth muscle cells [17,18]. Moreover, despite the growing body of evidence on the various effects of alkaloids on vascular functional activity, the mechanisms of vasorelaxant action of many alkaloid compounds remain insufficiently characterized [19,20].

According to cardiologists, allapinin has been used as a first-line drug for the treatment of certain types of cardiac arrhythmia due to its unique properties [21]. Diterpene alkaloids isolated from plants of the genera Aconitum and Delphinium have been reported to exhibit a wide range of biological activities, including antiarrhythmic, curare-like, arrhythmogenic, psychostimulant, neurotoxic, ganglion-blocking, local anaesthetic and hypotensive effects [22].

However, despite the well-documented cardiovascular activities of Aconitum-derived diterpene alkaloids, the precise molecular mechanisms underlying the vasorelaxant effects of N-deacetyllappaconitine remain insufficiently elucidated. In particular, there is very little information on its role in regulating intracellular Ca^{2+} homeostasis through voltage-dependent and receptor-gated Ca^{2+} transport pathways, as well as its possible modulatory effects on K^+ channels in vascular smooth muscle cells. Therefore, further investigation of these calcium-dependent signaling mechanisms may provide important insights into the pharmacological basis of its vasorelaxant activity and its potential therapeutic significance in vascular disorders. From this perspective, the aim of this study is to investigate the mechanisms of the vasorelaxant effects of the alkaloid *N*-deacetyllappaconitine (N-DEZ) on rat aortic rings pre-contracted with vasoactive agents.

Materials and methods

Animals and preparation of aortic rings

In this study, male white rats (200 - 250 g) were used, anaesthetised with sodium pentobarbital, and then the chest cavity was opened and the aorta was isolated. The surrounding connective tissue and adipose layer were carefully trimmed away with scissors and cut into cylindrical rings approximately 3 - 4 mm in length [23]. The isolated aortic rings were immediately transferred to a 5 mL organ bath system containing the following composition (in mM) of Krebs-Henseleit saline: NaCl 120.4, KCl 5.0, $NaHCO_3$ 15.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, and glucose 11.5, supplemented with HEPES buffer and adjusted to pH 7.4 [24]. The solution temperature was maintained at 37 ± 0.5 °C using a thermostat (LK LAB KOREA), thereby maintaining physiological conditions throughout the experiment. The organ bath solution was continuously aerated with carbon dioxide gas consisting of 95% O_2 and 5% CO_2 to ensure adequate oxygenation and stable acid-base balance. Each aortic ring was horizontally mounted between 2 stainless steel hooks in the organ bath chamber. One hook was fixed, and the other was connected to an isometric force transducer for continuous recording of vascular tension. Before the experiment, the preparations were equilibrated under a force of 1.0 g for 45 - 60 min, and washed with solution to prevent metabolite accumulation and maintain tissue viability. This equilibration procedure allowed the vascular preparations to achieve stable baseline contractile activity before the initiation of pharmacological interventions. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and complied with the ARRIVE guidelines. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of IBB at the National University of Uzbekistan (Protocol No. 7, dated 04/07/2022).

Measurement of isometric tension

The isometric contraction force of the aortic rings was recorded on a computer using a Grass FT.03 (Grass-Telefactor, USA) mechanograph connected to a signal amplification system. Data collection and analysis were carried out using PowerLab software (ADInstruments). Initially, each aortic ring was subjected to a resting tension of 1 g (9.8 mN) and allowed to equilibrate for 60 min. Each experiment used one aortic ring from a single mouse. After the equilibration period, experiments on vascular reactivity were conducted to assess the vasorelaxant properties of the alkaloid N-DEZ.

Chemical experiment

The IR spectra were recorded using an FT-IR/NIT Spectrum N-DEZ spectrometer equipped with an ATR system. Mass spectra were recorded on a Waters ACQUITY® QDa™ spectrometer. NMR spectra were recorded for ^{1}H on a JNM-ECZ600R (JEOL, Japan) at 600 MHz, respectively, in $CDCl_3$ solutions. TMS (0 ppm) was used as an internal standard in ^{1}H NMR spectra. In ^{13}C NMR spectra, the solvent shift ($CDCl_3$, 77.16 ppm relative to TMS) is designated as the internal standard. NMR spectra were processed with MestReNova 14.2.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). The progress of the reaction and the purity of the compounds obtained were monitored on a Sigma-Aldrich Silufol L/W 10×20 cm, 254 nm high-performance thin-layer plate (Germany), using a system of benzene:chloroform:triethylamine (4:1:0.3). An iodine camera and Dragendorff reagent were used for decolourisation. The melting points of all synthesised compounds were determined using a BOETIUS apparatus.

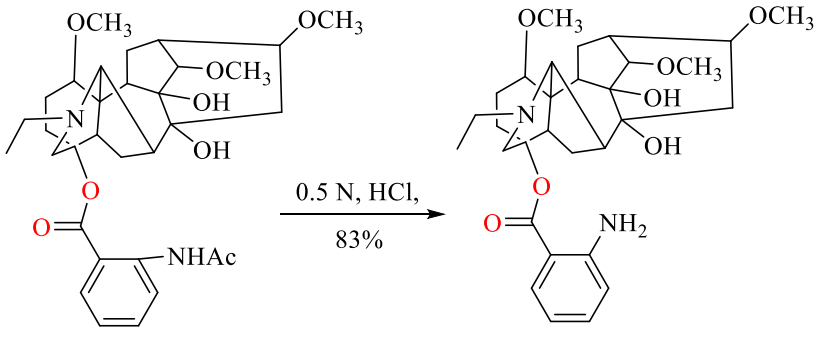
2-Aminobenzoate of ethyl 7a,11a-dihydroxy-6,8,10-trimethoxy-dodecahydro-2H-3,6a,12-(epitan[1,1,2]tryl)-7,9-methanonaphth[2,3-b]azocycl-3(4H)-yl (N-DEZ)

To a 9.4 mL solution of lappaconitine (**1**) in distilled water (2.0 g, 3.4 mmol), 0.884 ml of 1 N HCl solution (30.7 mmol) was added. The reaction was carried out in a water bath at 98 - 100 °C for 2 h. The reaction was monitored using the TLC method. After the reaction was completed, the reaction mixture was neutralized with a saturated $NaHCO_3$ solution (12 g of $NaHCO_3$ dissolved in 100 mL of distilled water) until the pH reached 8 (monitored using a pH meter). The obtained N-DEZ was extracted with chloroform, dried over Na_2SO_4, and then the chloroform was evaporated. The precipitate obtained was recrystallised from methanol. 1.545 g of N-DEZ was obtained in 83% yield. $C_{30}H_{42}N_2O_7$: colourless crystals, melting point 215 - 217 °C (methanol), Rf 0.45 (system benzene: chloroform: triethylamine, 4:1:0.3).

IR spectrum (cm^{-1}): 3,539, 3,376, 2,927, 2,821, 1,684, 1,616, 1,558, 1,245, 1,105, 1,073, 750 and 526. Mass spectrum (ESI-MS) m/z 543.5137 $[M–H]^+$, calculated 543.6738, $C_{30}H_{43}N_2O_7$.

The isolation of N-DEZ

By hydrolysis of alkaloid lappaconitine (**1**) under acidic conditions, N-DEZ was obtained (**Scheme 1**). The structure of the product was confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy.



lappaconitine (1) **N-deacetyllappaconitine (N-DEZ)**

Scheme 1 Hydrolysis of lappaconitine (**1**) alkaloid under acidic conditions to obtain N-DEZ. The structure of N-DEZ, obtained from the acidic hydrolysis of lappaconitine, was confirmed by mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy.

The IR spectrum of N-DEZ showed the (OH) group's valence vibrations in the 3,539 - 3,376 cm^{-1} region and the valence and deformation vibrations of the carbonyl group (C=O) at 1,684 cm^{-1}. In addition, the

valence and deformation vibrations of the methylene and methyl groups were observed in the 1,073 - 750 cm^{-1} region. Furthermore, the symmetric vibrational modes of the $–CH_2$ and $–CH_3$ groups have been determined. The remaining valence and deformation vibrations correspond to other functional groups in the molecule and were observed in the appropriate regions of the IR spectrum. The mass spectrometric signals of N-DEZ demonstrated that the ESI-MS m/z 543.5137 $[M+H]^+$ ion is a stable molecular ion.

The structure of N-DEZ was also confirmed by analysis of its ^1H and ^{13}C NMR spectra and by HSQC and HMBC experiments. In the ^1H NMR spectrum, in the strong-field region (1.10 ppm), a triplet signal with J = 7.2 is observed, which corresponds to the methyl group protons in the molecule. Between 1.62 - 3.17 ppm, doublets and multiplets characteristic of cyclic aliphatic protons were observed. In addition, in the medium-frequency region of the spectrum (3.28 - 3.40 ppm), 3 singlet signals corresponding to 3 methoxy groups (1–OCH_3, 16–OCH_3 and 14–OCH_3) were observed. In the region of 6.59 - 7.75 ppm, signals attributable to aromatic protons were observed, appearing as a doublet with spin-spin coupling constants J = 8.1 and 1.6 (H–6′). In the ^{13}C NMR spectrum, in the low-field region, a signal for the carbon atom of the carbonyl group was observed at δ 167.5 (C=O), and a signal for the aromatic sp^2 carbon atom at δ 150.5 (C–2′). The signals in the region of δ 58.0 - 61.7 ppm correspond to the carbon atoms of the 3 methoxy groups (16–OCH_3, 1–OCH_3 and 14–OCH_3). In addition, signals of the carbon atoms of the methylene, methyl and methine groups, as well as other aliphatic carbon atoms, were observed at δ 47.6 (C–13), δ 36.4 (C–3) and δ 55.7 (C–19). The positions of the 3 methoxy and one methyl group in the molecule were determined by HMBC experiments. In the HMBC spectrum, correlations were observed for 1–OCH_3/C–1, 14–OCH_3/C–14, 16–OCH_3/C–16 and H–19(H–18)/C–18, H–1′/C=O correlations were observed in the HMBC spectrum, confirming that the methoxy groups are located at C–1, C–14 and C–16, and the methyl group at C–18.

Table 1 ^1H and ^{13}C NMR data of the N-DEZ compound ($CDCl_3$, ^1H: 600 MHz; ^{13}C: 150 MHz).

№	δC	δH	№	δC	δH
1	84.51	2.69 (ddd, 12.20, 5.04, 2.80)	19	55.75	2.64 (dd, 15.41, 7.43 H_a) 2.58 - 2.43 (m, H_b)
2	26.34	2.03 - 1.90 (m, H_a) 1.63 (dd, 15.5, 8.8, H_b)	1-OCH_3	61.73	3.40 (s)
3	36.41	2.27 (m, H_a) 2.15 (m, H_b)	14-OCH_3	58.02	3.28 (s)
4	83.00	-	16-OCH_3	56.63	3.30 (s)
5	49.94	2.58 - 2.43 (m)	OCO	167.48	-
6	26.92	2.03 - 1.90 (m, H_a) 1.63 (dd,15.5, 8.8, H_b)	1'	112.05	-
7	49.11	2.58 - 2.43 (m)	2'	150.5	-
8	78.67	-	3'	116.36	6.61-6.57 (m)
9	75.79	-	4'	133.92	7.21 (ddd, 8.3, 7.1, 1.7)
10	32.07	2.58 - 2.43 (m)	5'	116.74	6.61 - 6.57 (m)
11	50.94	-	6'	131.66	7.75 (dd, 8.1, 1.6)
12	24.16	2.08 (dd, 12.6, 4.8, H_a) 1.74 (m, H_b),	8-OH	-	2.98 (s)
13	47.64	2.42 - 2.31 (m)	9-OH	-	3.54 (s)
14	90.28	3.60 (d,11.5)	$N\underline{CH_2}CH_3$	48.92	2.42-2.31 (m, NCH_aCH_3) 2.27 (m, NCH_bCH_3)
15	44.96	2.42 - 2.31 (m, H_a) 2.15 (m, H_b)	$NCH_2\underline{CH_3}$	13.66	1.10 (t, 7.2)
16	83.04	3.43 (d, 4.9)	NH_2	-	5.63 (s)
17	56.22	3.17 (m)			

Experimental design

The contraction responses of aortic vascular smooth muscle were induced by potassium chloride (KCl) or the α1-adrenoceptor agonist phenylephrine (PE). The maximum contraction produced by KCl or PE was taken as 100% as the control [25]. After the contractions induced by KCl (50 mM) or PE (1 μM) had reached a steady state, the dose-dependent relaxant effect of the alkaloid N-DEZ (1 - 175 μM) was examined. To investigate the contribution of the endothelium to the observed vasorelaxation, additional experiments were conducted on aortic rings in which the endothelial layer had been mechanically removed. The endothelial layer of the aortic rings was removed using cotton swabs. The removal of the endothelial layer was confirmed by acetylcholine (0.1 μM). In these aortic rings, when acetylcholine was added to contractions induced by KCl or phenylephrine, no relaxation (less than 10%) occurred, and the dose-dependent effect of the alkaloid on these contractions was examined. In addition, further experiments were carried out in the presence of the NO synthase inhibitor L-NAME.

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Assessment of calcium channel involvement

To assess the contribution of L-type Ca^{2+} channels to the vasorelaxant effect of the alkaloid N-DEZ, the preparation was incubated with the channel-selective blocker verapamil prior to contraction induction. A dose of verapamil that reduced contractile force by 50% (IC_{50} = 0.1 μM) was used. Once the contraction had stabilised, the relaxing effect of the N-DEZ alkaloid (IC_{50} = 43.8 μM) was examined. At the same time, the role of Ca^{2+} channels was further determined in additional experiments by varying the $CaCl_2$ concentration (0.5 - 2.5 mM) in the Krebs-Henseleit solution. The role of voltage-dependent Ca^{2+} channels in the mechanism of action of this alkaloid was assessed by altering the extracellular Ca^{2+} concentration. The maximal concentrations of the alkaloid (175 μM) and verapamil (1 μM) were used. The resulting relaxant effect was compared with the control.

Assessing SR Ca^{2+} transport function

A calcium-free Krebs solution was used to determine the effect of the N-DEZ alkaloid on the release of Ca^{2+} ions from the sarcoplasmic reticulum's Ca^{2+} transport systems. Contractile responses were stimulated with PE or caffeine to promote IP_3R- and RyR-mediated Ca^{2+} release. Aortic rings were incubated with the maximum concentration of N-DEZ for 20 min prior to stimulation. In addition, the IP3R blocker heparin and the RyR blocker ryanodine red were used. Cyclopiazonic acid (CPA) was used to determine the role of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) in the vasorelaxant effect of the N-DEZ alkaloid.. In experiments, under CPA incubation conditions, the vasorelaxant effect of N-DEZ was examined in relation to the contraction force induced by PE.

Investigation of potassium channel participation

The role of various K^+ channels in N-DEZ - induced vasorelaxation was assessed using selective blockers: 4-AP (voltage-dependent K^+ channels), glibenclamide (ATP-sensitive K^+ channels), tetraethylammonium (Ca^{2+}-activating K^+ channels) and $BaCl_2$ (inward-rectifying K^+ channels). Aortic rings were incubated with K^+ channel blockers for 20 min before PE induction, and the effect of N-DEZ was studied in a dose-dependent manner.

Chemicals

All chemical reagents were of analytical grade and purchased from commercial sources. Verapamil acetylcholine, EGTA, L-NAME, indomethacin, phenylephrine, heparin, ruthenium red, CPA, 4-aminopyridine (4-AP), glibenclamide, tetraethylammonium (TEA) and $BaCl_2$ were obtained from Sigma Ltd Co., (St. Louis, MO, USA). Alkaloid N-DEZ (**Figure 1**) was prepared by researchers at the Institute of the Chemistry of Plant Compounds, Uzbek Academy of Sciences.

Statistical analysis

In the experiments, the values of smooth muscle contraction force were expressed as a percentage of the maximum response. IC_{50} values were determined as the concentration causing a 50% reduction in the maximal contractile response. The results of experimental studies (n replicates) are presented as M ± m, where M is the arithmetic mean and m is the standard error of the mean (SEM). Prior to statistical analysis, the data were tested

for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. The results confirmed that the data met the assumptions of normal distribution and homogeneity of variances ($p > 0.05$). Therefore, parametric statistical methods were applied. For comparisons between multiple groups, one-way ANOVA was used, followed by Tukey's post hoc test. No non-parametric tests were required, as all datasets met the assumptions for parametric analysis. Statistical significance was considered at $*p < 0.05$ and $**p < 0.01$. All analyses were performed using OriginPro 8.6 (OriginLab Corporation, USA).

Results and discussion

The vasorelaxant effect of the alkaloid N-DEZ

In the initial experiments, the dose-dependent vasorelaxant effect of the N-DEZ alkaloid on hyperkalaemic-induced contractions of rat aortic smooth muscle was assessed. The results showed that the alkaloid N-DEZ reduced 50 mM KCl-induced aortic contraction by 4.8 ± 2.3% at a concentration of 5 µM and by 79.8 ± 3.9% at the maximum concentration of 175 µM compared with the control group. Under these conditions, the half-maximal inhibitory concentration (IC_{50}) of the alkaloid N-DEZ was 43.8 µM (**Figure 2(A)**). In such experiments, the alkaloid N-DEZ exhibited a dose-dependent relaxing effect on PE-induced aortic contractions. In particular, at a concentration of 5 µM the contraction force decreased by 3.8 ± 2.6%, whereas at 150 µM it decreased by 85.2 ± 4.1%. In this case, the IC_{50} value of the alkaloid N-DEZ was 38.3 µM (**Figure 2(B)**).

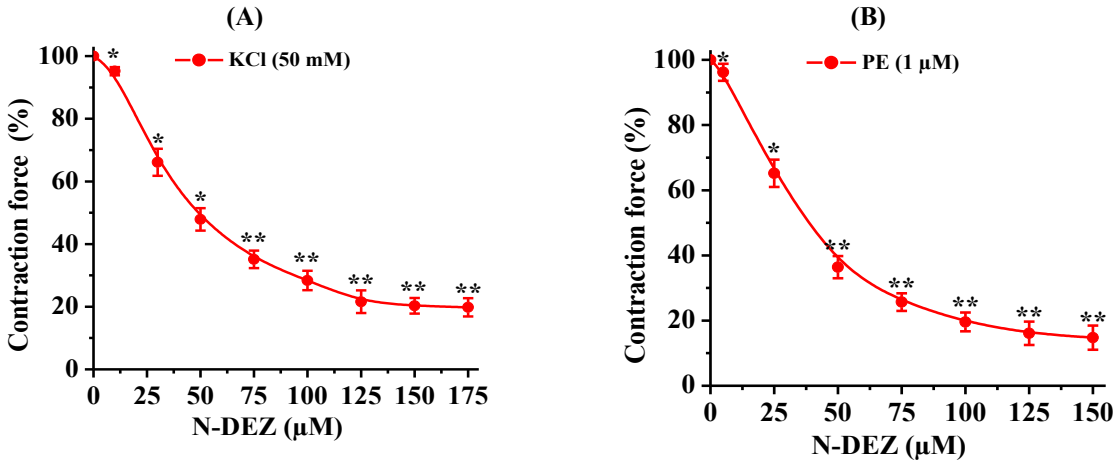


Figure 2 Dose-dependent relaxant effect of alkaloid N-DEZ on the induced aortic ring contraction activity by 50 mM KCl (A) and 1 µM PE (B). Data are expressed as a percentage of the contraction induced by PE (1 µM) or KCl (50 mM). Values are mean ± SEM (n = 6). $*p < 0.05$, $**p < 0.01$, compared with control.

Based on the obtained results, it can be concluded that the vasorelaxant effect of the alkaloid N-DEZ is relatively stronger than that of PE-induced contractions. In these results, it was found that the alkaloid N-DEZ has a potent vasorelaxant effect on KCl- and PE-induced aortic contractions.

The role of the endothelial layer in the vasorelaxant effect of the alkaloid N-DEZ

Experiments were conducted on aortic rings with the endothelial layer removed to determine the potential involvement of the endothelium in the vasorelaxant effect of the alkaloid N-DEZ. In aortic rings with the endothelial layer removed, the effect of N-DEZ alkaloid on KCl- and PE-induced contractions was reduced from 79.8 ± 3.9% to 69.0 ± 4.0% and from 85.2 ± 4.1% to 68.5 ± 3.1%, respectively, compared to control (**Figures 3(A)** and **3(B)**). The results showed that the involvement of the endothelial layer in the aortic vasorelaxant effect of the alkaloid N-DEZ is very minimal. To clarify this, we conducted experiments using the nitric oxide synthase inhibitor L-NAME (Nω-nitro-L-arginine methyl ester). Blood vessel endothelial cells produce a number of vasoactive factors, among which nitric oxide

(NO) plays a key role in the relaxation of smooth muscles [26]. In experiments, pretreatment of aortic rings with L-NAME (100 µM) did not produce significant changes in the vasorelaxant effect of the alkaloid N-DEZ (**Figures 3(A)** and **3(B)**).

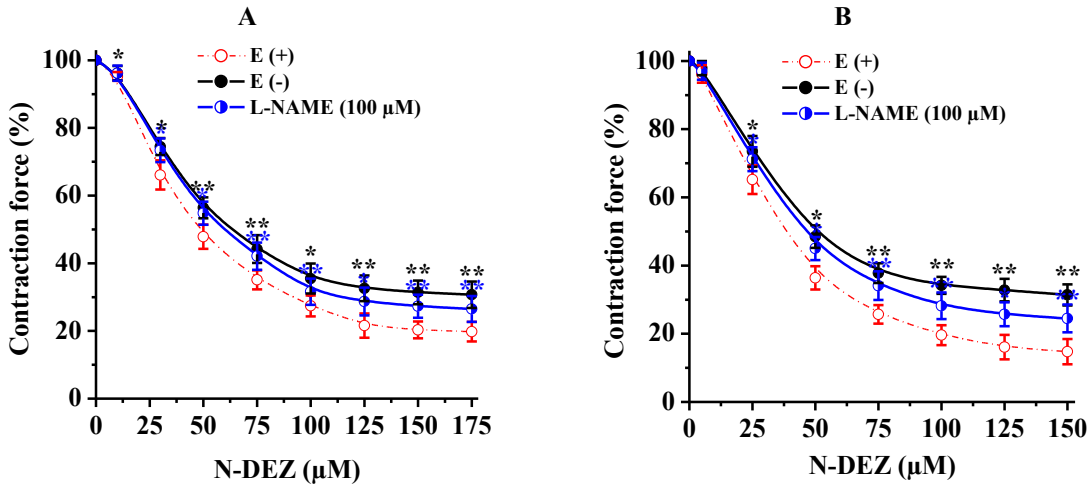


Figure 3 Effect of alkaloid N-DEZ on the force of contraction induced by KCl (A) and PE (B) in aortic rings with the endothelial layer removed, incubated with L-NAME (100 µM). Data are expressed as a percentage of the contraction induced by PE (1µM) or KCl (50 mM). Values are mean ± SEM (n = 5). One-way ANOVA followed by Dunnett's post hoc test, *$p < 0.05$, **$p < 0.01$ vs. control group.

The results of the above experimental series show that the vasorelaxant effect of the alkaloid N-DEZ is not endothelium-dependent, as it retains its effect even in endothelium-denuded preparations. Therefore, this indicates that endothelial cells and NO are not the primary relaxing factors under the vasorelaxant effect of this alkaloid.

The role of L-type Ca^{2+} channels in the vasorelaxant effect of the alkaloid N-DEZ

In experiments, the effect of varying the $CaCl_2$ concentration on alkaloid N-DEZ in aortic rings was evaluated. In a Krebs solution devoid of Ca^{2+} ions and incubated with KCl (50 mM), the cumulative addition of $CaCl_2$ to aortic rings caused a concentration-dependent increase in the contraction response [27]. Under these experimental conditions, incubation of the alkaloid N-DEZ was found to significantly attenuate the concentration-dependent contractile force of aortic rings in the presence of $CaCl_2$ (**Figure 4(A)**). That is, in aorta rings preincubated with 175 µM alkaloid N-DEZ, the amplitude of the contraction elicited by the maximal concentration of $CaCl_2$ was reduced by 70.7 ± 3.9% compared to the control group. This effect of alkaloid N-DEZ likely indicates inhibition of voltage-dependent L-type Ca^{2+} channels. To clarify this hypothesis, the effect of the alkaloid N-DEZ was compared with that of verapamil, an L-type Ca^{2+} channel blocker, on Ca^{2+}-dependent aortic ring contraction induced by $CaCl_2$. Verapamil (1 µM) reduced $CaCl_2$-dependent aortic ring contraction by 92.6 ± 3.9% compared with control, and this inhibitory effect was observed to be more potent than that of the alkaloid N-DEZ. In addition, the vasorelaxant effect of the alkaloid N-DEZ in combination with verapamil, a specific blocker of the L-type Ca^{2+} channel, was studied. Incubation with verapamil (IC_{50} = 0.1 µM) attenuated KCl-induced aortic contraction by 50 ± 3.9% compared to control. Under these conditions, the alkaloid N-DEZ (IC_{50} = 43.8 µM) was found to further reduce contraction by 21.4 ± 3.6% (**Figure 4(B)**).

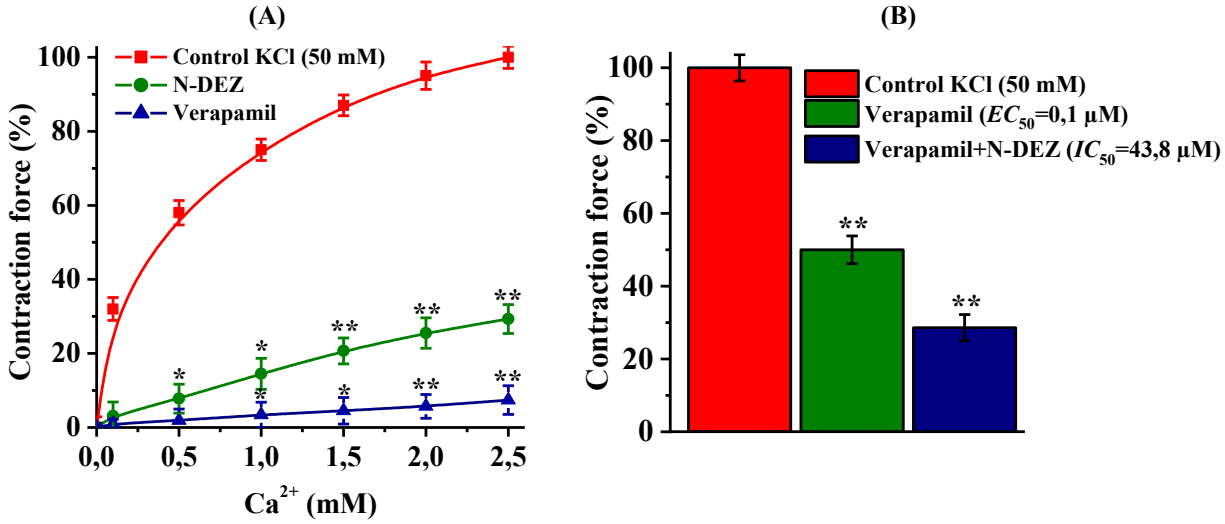


Figure 4 Effect of alkaloid N-DEZ and verapamil on $CaCl_2$ concentration–dependent contractions in aortic rings (A) and in the presence of preincubation with verapamil (0.1 µM) (B) Relaxant effect on KCl (50 mM)-induced contractions. **(**A) Cumulative concentration-response curves for $CaCl_2$ (0 - 2.5 mM) were constructed in aortic rings that has been pre incubated in Ca^{2+}-free Krebs solution supplemented with 50 mM KCl. (B) Effect of verapamil (0.1 µM) on vasorelaxation induced by N-DEZ. Values are mean ± SEM (n = 5). One-way ANOVA followed by Dunnett's post hoc test, *$p < 0.05$, **$p < 0.01$ vs. control group.

These results indicate that the vasorelaxant effect of the alkaloid N-DEZ involves the active participation of voltage-dependent L-type Ca^{2+} channels. However, the additional relaxant effect of the N-DEZ alkaloid under verapamil-blocked conditions suggests that it depends on other ion transport systems or messengers.

The role of Na^+/Ca^{2+} exchange in the vasorelaxant effect of the N-DEZ alkaloid

In experiments, the effect of the alkaloid N-DEZ on Na^+/Ca^{2+}-exchange activity was assessed by examining slow, spontaneously occurring contractions in Krebs solution without Na^+ ions. A decrease in external Na^+ concentration causes the Na^+/Ca^{2+} exchange system to operate in reverse, transporting Ca^{2+} ions into the cell, and a slowly developing contraction is observed [28,29]. Under these conditions, the alkaloid N-DEZ (150 µM) was found to attenuate aortic ring contraction by 55.8 ± 4.1% (**Figure 5(A)**). This result can be explained by the fact that the vasorelaxant effect of the alkaloid N-DEZ inhibits the reverse-mode operating function of the Na^+/Ca^{2+} exchange system. To further clarify this, studies were conducted to investigate the effect of the alkaloid N-DEZ on the Na^+/Ca^{2+} exchange system by modulating the Na^+/K^+-ATPase. Owabain (10 µM) attenuates the functional activity of the Na^+/K^+-ATPase, leading to an increase in intracellular Na^+ concentration and causing the Na^+/Ca^{2+} exchange system to operate in reverse [30]. The alkaloid N-DEZ (150 µM) reduced the ouabain-assisted slowly developed contraction force by 59.5 ± 3.9% compared with control (**Figure 5(B)**).

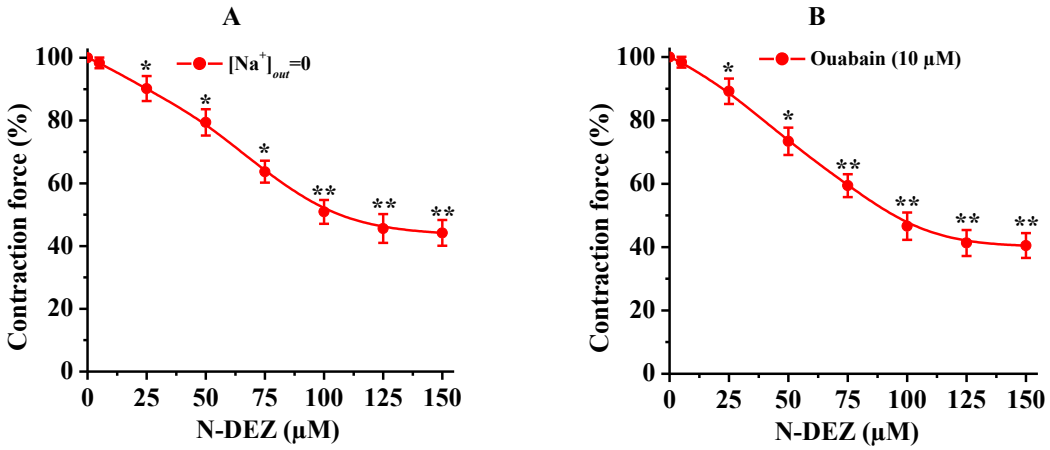


Figure 5 Effect of alkaloid N-DEZ on contractions induced by Krebs solution (A) and ouabain (B) in the absence of Na^+ ions. The contraction force of the formed aortic rings was taken as 100%. Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$, compared with control.

The results suggest that the N-DEZ alkaloid can reduce Ca^{2+} ion concentrations in smooth muscle cells by inhibiting Na^+/Ca^{2+} exchange system activity and thereby induce cellular relaxation.

The role of IP_3R in the vasorelaxant effect of the alkaloid N-DEZ

The transient contraction of aortic rings induced by PE (1 µM) in EGTA-supplemented Krebs solution (EGTA 1 mM) in the absence of Ca^{2+} ions is explained by the release of Ca^{2+} ions from the sarcoplasmic reticulum via IP_3 receptors [31]. The observed contraction was 67.2 ± 3.9% compared with the PE (1 µM)-induced aortic ring contraction in normal Krebs physiological saline (control). The PE (1 µM)-induced contraction force of aortic rings in Krebs solution decreased from 67.2 ± 3.9% to 21.7 ± 4.1% when incubated with N-DEZ (100 µM) (**Figure 6**).

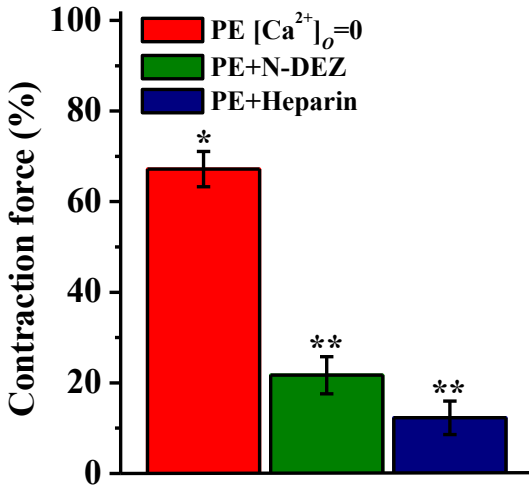


Figure 6 Effect of alkaloid N-DEZ and heparin on PE-induced contractions of rat aortic preparations in Ca^{2+}-free Krebs solution. Data are expressed as a percentage of the contraction induced by PE (1µM). Values are mean ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$, compared with control.

In our subsequent experiments, the inhibitory effect of the alkaloid N-DEZ on IP_3R was compared with that of the IP_3R inhibitor heparin. In a Krebs solution without Ca^{2+}, heparin (10 mg/mL) reduced the PE-induced aortic ring contraction force from 67.2 ± 3.9% to 12.3 ± 3.7% (**Figure 6**). Therefore, under these

conditions, the vasorelaxant effect of the alkaloid N-DEZ is similar to that of heparin, indicating the presence of IP_3R inhibitory properties.

The role of RyR in the vasorelaxant effect of the alkaloid N-DEZ

It is known that caffeine is a SR RyR activator and is widely used in studying the mechanisms of action of biologically active compounds [32]. In Ca^{2+}-free Krebs solution, caffeine (10 mM) induced a contraction that was 41.2 ± 3.6% of the contraction normally produced by PE (1 μM). This aortic SMC contraction partially reflects the release of Ca^{2+} ions via RyR. In the experiments performed, incubation of aortic rings with the N-DEZ (100 μM) alkaloid reduced caffeine-induced contraction from 41.2 ± 3.6% to 29.3 ± 4.2% (**Figure 7**).

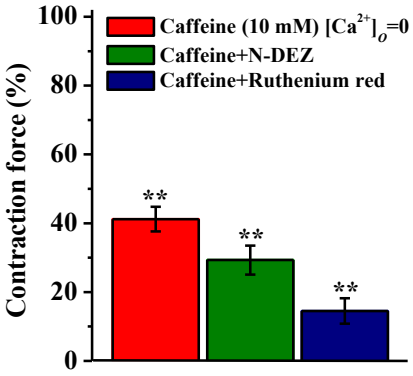


Figure 7 Effects of the alkaloid N-DEZ and ruthenium red on caffeine-induced contractions of rat aortic preparations in Krebs solution without Ca^{2+} ions. Data are expressed as a percentage of the contraction induced by Caffeine (10 mM). Values are mean ± SEM (n = 4). *$p < 0.05$, **$p < 0.01$, compared with control.

Based on the results, we can say that the reduction in caffeine-induced contraction force under N-DEZ incubation conditions is due to inhibition of Ca^{2+} release through RyR. To clarify this, additional experiments were conducted using ruthenium red. Ruthenium red (10 μM) reduced caffeine-induced aortic contraction from 41.2 ± 3.6% to 14.5 ± 3.7% (**Figure 7**). The vasorelaxant effect of N-DEZ was half as great as that of ruthenium red. Based on this, we can say that the N-DEZ alkaloid has a weak inhibitory effect on RyR.

The role of SERCA in the vasorelaxant effect of the alkaloid N-DEZ

Experiments were conducted in the presence of CPA to study the effect of the alkaloid on SERCA. The vasorelaxant effect of the alkaloid N-DEZ was significantly reduced in the presence of PE-induced aortic contraction under conditions of prior incubation with CPA (10 μM), It was found to decrease from 85.2 ± 4.1% to 75.9 ± 4.3% (**Figure 8(A)**). In subsequent experiments, aortic rings were incubated with verapamil (1 μM), and 10 min later, when CPA (10 μM) was added, a transient contraction occurred. This contraction was 37.8 ± 4.1% of the contraction force elicited by Ca^{2+} (1 μM). Under these conditions, the alkaloid N-DEZ was observed to reduce the transient reduction of the aortic ring induced by CPA by 26.5 ± 3.8% (**Figure 8(B)**).

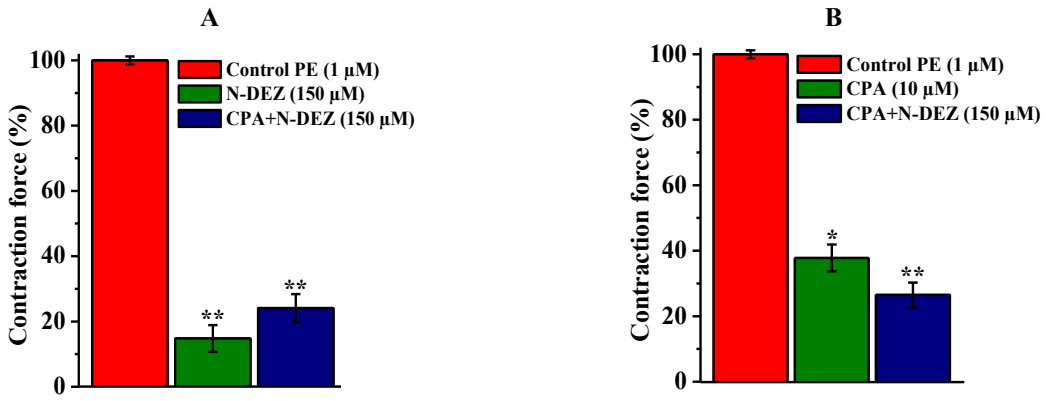


Figure 8 Effect of the alkaloid N-DEZ on PE (A)- and CPA (B)-induced rat aortic contraction under CPA incubation conditions. Data are expressed as a percentage of the contraction induced by PE (1 µM). Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$, compared with control.

The results obtained show that under conditions of SERCA inhibition by CPA, the vasorelaxant effect of the alkaloid N-DEZ is significantly attenuated, indicating that SERCA activity is involved in its mechanism of action.

The role of K^+ channels in the vasorelaxant effect of the alkaloid N-DEZ

To determine the involvement of K^+ channels in the vasorelaxant effect of the alkaloid N-DEZ, selective potassium channel inhibitors, including, Experiments were conducted using glibenclamide for K_{ATP} channels, TEA for K_{Ca} channels, 4-AP for K_V channels and $BaCl_2$ for K_{ir} channels [33]. In the experiments, when incubation with 50 µM glibenclamide was carried out before the FE-induced contraction, the vasorelaxant effect of the alkaloid N-DEZ decreased from 85.3 ± 3.7% to 59.0 ± 4.2%. When incubated with 4-AP (1 mM), the vasorelaxant effect of the N-DEZ alkaloid decreased from 85.3 ± 3.7% to 67.8 ± 3.9%, whereas TEA in experiments with TEA (1 mM) and $BaCl_2$ (0.1 mM), the vasorelaxant effect N-DEZ was found to decrease from 85.3 ± 3.7% to 75.9 ± 3.5% and from 85.3 ± 3.7% to 79.9 ± 3.8%, respectively (**Figure 9**).

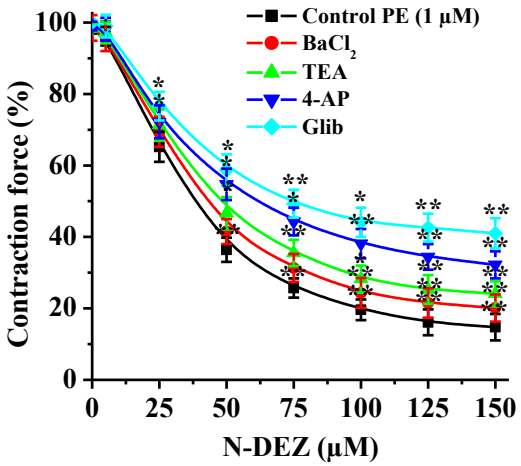


Figure 9 Effect of alkaloid N-DEZ K-channel inhibitors on PE (1 µM)-induced contractions of rat aorta under incubated conditions. Data are expressed as a percentage of the contraction induced by 1 µM PE Values are mean ± SEM (n = 5). One-way ANOVA followed by Dunnett's post hoc test, *$p < 0.05$, **$p < 0.01$, vs. control group.

The results obtained showed that in the vasorelaxant effect of the alkaloid N-DEZ, K_{ATP} and K_v channels play a partial role, while K_{Ca} and K_{ir} channels are not involved. Therefore, the vasorelaxant mechanism of action of N-DEZ may involve membrane hyperpolarisation through activation of K_{ATP} and K_v

channels, and a reduction in Ca^{2+} influx in smooth muscle.

Discussion

Alkaloids play an important role in correcting the functional activity of vascular smooth muscle cells by affecting their ion channels and calcium homeostasis. [34,35]. Therefore, studies have described the vasorelaxant mechanisms of action of the alkaloid N-DEZ. In preliminary experiments, the dose-dependent relaxant effect of the N-DEZ on KCl- and PE-induced aortic ring contractions was investigated. The attenuation of KCl-induced contraction may be due to the alkaloid's inhibition of Ca^{2+} ion entry into the cell via voltage-dependent Ca^{2+} channels. At the same time, it is not unlikely that the relaxation observed during PE-induced contractions is mediated via α_1-adrenoceptor-linked signalling pathways or by inhibition of Ca^{2+} release from the sarcoplasmic reticulum. The slightly lower IC_{50} value observed for phenylephrine-induced contraction relative to KCl-induced contraction may indicate that the alkaloid exhibits greater efficacy in modulating receptor-operated Ca^{2+} signaling pathways than voltage-dependent L-type Ca^{2+} channels, thereby contributing more prominently to its vasorelaxant effect.

The endothelial layer in blood vessels has important physiological significance in regulating vascular tone. Endothelial cells produce a range of vasoactive factors, such as nitric oxide (NO), and ensure vasodilation by regulating intracellular Ca^{2+} ion homeostasis [36]. Moreover, the endothelial layer is an important experimental factor in assessing the vasorelaxant mechanisms of action of alkaloids [37]. In experiments, the vasorelaxant effect of the alkaloid N-DEZ was found to be preserved even in aortic rings lacking an endothelial layer. The relatively preserved degree of relaxation in the absence of endothelium suggests that the alkaloid N-DEZ primarily acts directly on vascular smooth muscle, this suggests that the alkaloid N-DEZ may inhibit Ca^{2+} influx or intracellular Ca^{2+} signalling pathways. At the same time, the fact that the effect is more pronounced in the presence of endothelium does not rule out the possibility that it is partially amplified by endothelium-dependent mechanisms. Moreover, in experiments involving the NO synthase inhibitor L-NAME, the absence of any change in the relaxing effect of the alkaloid N-DEZ confirms that it is not mediated via the NO/NO synthase signalling pathway. It is well known that endothelium-derived NO plays a key role in the relaxation of smooth muscles [38], but in this case, it was found that this mechanism was not involved. Overall, the results suggest that the vasorelaxant effect of the alkaloid N-DEZ may be associated with a direct regulation of Ca^{2+} homeostasis.

The contraction and relaxation of blood vessel SMCs are directly dependent on the intracellular Ca^{2+} concentration and play a crucial role in the regulation of vascular tone [39]. This process is maintained through Ca^{2+} homeostasis, that is, the balance between the entry of Ca^{2+} ions into the cell via the plasma membrane, their release from the sarcoplasmic reticulum, their reuptake and their efflux from the cell [40]. L-type Ca^{2+} channels in the plasma membrane, in response to membrane depolarisation, increase the intracellular $[Ca^{2+}]_i$ concentration by releasing Ca^{2+} ions, thereby stimulating contraction processes [41]. Initially, the alkaloid N-DEZ reduced the KCl-induced contraction of aortic rings. This suggested that its relaxant effect may be due to a reduction in Ca^{2+} ion influx into the cell via blockade of plasma-membrane L-type Ca^{2+} channels. The reduction of contraction induced by cumulative addition of $CaCl_2$ in the absence of Ca^{2+} ions by the alkaloid N-DEZ indicates inhibition of its voltage-dependent L-type Ca^{2+} channels. However, the additional vasorelaxant effect of N-DEZ under conditions of verapamil-blocked L-type Ca^{2+}-channel blockade suggests the involvement of other ion transport systems or intracellular signalling pathways.

The Na^+/Ca^{2+} exchanger plays an important role in regulating intracellular Ca^{2+} concentration [42]. It typically ensures relaxation by extruding Ca^{2+} ions from the cell, and under certain conditions it regulates the influx of Ca^{2+} ions into the cell depending on the membrane potential [43,44]. Experiments showed that the alkaloid N-DEZ reduces the contraction force generated in Krebs solution in the absence of Na^+ ions. The contraction under these conditions is explained by the Na^+/Ca^{2+} exchanger operating in reverse due to the decrease in external Na^+ concentration and by the influx of Ca^{2+} ions into the cell. The results obtained indicate that the alkaloid N-DEZ has the ability to inhibit the Na^+/Ca^{2+} antiporter in reverse mode, thereby limiting Ca^{2+} influx. Additional experiments carried out by

modulating the activity of the Na^+/K^+-ATPase also support this idea. As a result of Na^+/K^+-ATPase inhibition, intracellular Na^+ ions accumulate, thereby enhancing the reverse operation of the Na^+/Ca^{2+} exchanger [45]. Studies have shown that the N-DEZ alkaloid ouabain reduces the contractions that occur in aortic rings under incubated conditions. Overall, it may be due to the N-DEZ alkaloid inhibiting the reverse mode of the Na^+/Ca^{2+} exchange system, thereby reducing the intracellular Ca^{2+} concentration.

While the release of Ca^{2+} ions from the SR via RyR and IP_3R raises the intracellular [Ca^{2+}] concentration and drives contraction, the reuptake of Ca^{2+} ions via SERCA ensures the relaxation process [46,47]. In skeletal muscle, Ca^{2+} release via IP_3Rs is one of the primary mechanisms that maintain intracellular Ca^{2+} homeostasis and drive the contraction response [48]. In experiments, the alkaloid N-DEZ was found to affect PE-induced contractions of aortic rings in the absence of Ca^{2+} ions. It is known that in the absence of Ca^{2+} ions, the PE-induced contraction of aortic rings arises not from an influx of Ca^{2+} into the cell but from the release of Ca^{2+} from the sarcoplasmic reticulum via IP_3 receptors [49]. N-DEZ suggested that this effect may be associated with the inhibition of IP_3R-mediated Ca^{2+} mobilisation. It was found that the IP_3R inhibitor has a profile similar to that of heparin's relaxant effect, indicating that its mechanism of action is related to IP_3R inhibition [50,51]. Based on these results, the alkaloid N-DEZ reduces Ca^{2+} release from the sarcoplasmic reticulum by inhibiting IP_3 receptors.

RyR primarily functions as an amplifying mechanism for intracellular Ca^{2+} signalling by facilitating the release of Ca^{2+} ions from the sarcoplasmic reticulum [52]. In experiments, caffeine was used to investigate the involvement of RyR in the vasorelaxant effect of the alkaloid N-DEZ. It is known that stimulation with caffeine causes activation of the RyR located in the sarcolemmal membrane of skeletal muscle fibres, which manifests as an increase in cytosolic Ca^{2+} ion concentration [53]. The observed attenuation of caffeine-induced aortic contraction in the presence of N-DEZ suggests a possible modulation of RyR-associated Ca^{2+} release. However, this effect was less pronounced compared to that produced by ruthenium red, a well-established RyR inhibitor. Therefore, although the alkaloid N-DEZ inhibits Ca^{2+} release via RyR, its primary vasorelaxant mechanism is more likely to be related to other Ca^{2+} pathways or ion transport systems.

SERCA plays an important role in maintaining Ca^{2+} homeostasis and ensuring the relaxation process in smooth muscle cells [54]. In research, SERCA plays a central role in describing the vasorelaxant mechanisms of action of alkaloids [55,56]. In investigating the effect of the alkaloid N-DEZ on SERCA, the selective blocker of this pump, CPA, was used [57]. Under conditions of SERCA inhibition by CPA, the vasorelaxant effect of the N-DEZ was markedly reduced in the presence of PE (1 μM)-induced contraction. This may be associated with a reduction in the uptake of Ca^{2+} ions into the sarcoplasmic reticulum via SERCA. In subsequent experiments, the effect of the alkaloid N-DEZ on the transient contractions that occurred in aortic rings incubated with CPA was examined. SERCA inhibits CPA, restricting the influx of Ca^{2+} ions into the sarcoplasmic reticulum, resulting in an increase in intracellular $[Ca^{2+}]_{in}$ concentration and the onset of a transient contraction [58]. Although the quantitative contribution of SERCA to the overall relaxation response appears to be relatively small under these experimental conditions, this does not negate its physiological importance, as SERCA-mediated Ca^{2+} reuptake works in concert with other Ca^{2+} transport mechanisms in vascular smooth muscle cells. The results of the study showed that the alkaloid N-DEZ attenuated the contractions induced by CPA. In this case, the alkaloid N-DEZ can activate SERCA, thereby reducing the cytosolic Ca^{2+} concentration.

Importantly, the vasorelaxant mechanisms associated with SERCA activation and inhibition of reverse-mode Na^+/Ca^{2+} exchange should not be considered as independent processes, but rather as functionally interconnected components of intracellular Ca^{2+} homeostasis. Inhibition of the Na^+/Ca^{2+} exchanger inverse mode in vascular smooth muscle cells reduces extracellular Ca^{2+} influx, thereby limiting the increase in cytosolic Ca^{2+} concentration, while SERCA promotes the re-entry of cytosolic Ca^{2+} into the sarcoplasmic reticulum, which contributes to the restoration of intracellular Ca^{2+} homeostasis and smooth muscle relaxation. This coordinated regulation may represent a hierarchically integrated protective mechanism, whereby suppression of Ca^{2+} influx through reverse-

mode Na^+/Ca^{2+} exchanger reduces cytosolic Ca^{2+} loading and thereby facilitates more effective SERCA-mediated sequestration of Ca^{2+} into the sarcoplasmic reticulum. Consequently, the combined modulation of these transport systems by N-DEZ may synergistically contribute to the sustained reduction of intracellular Ca^{2+} concentration and enhancement of vascular smooth muscle relaxation.

In VSMC, K^+ channels play an important role in regulating membrane potential and thereby controlling cell function [59] There are several types of these channels, which play an important role in regulating blood vessel tone [60]. Therefore, to assess the involvement of K^+ channels in the relaxant effect of the alkaloid N-DEZ, experiments were conducted using the selective K_{ATP} channel blocker glibenclamide and the K_{Ca} channel blocker TEA, Experiments were conducted in the presence of the K_{ir}-channel blocker $BaCl_2$ and the K_v-channel blocker 4-AP. Under conditions of prior incubation with glibenclamide and 4-AP, the vasorelaxant effect of the alkaloid N-DEZ on PE-induced aortic contractility is partially reduced. In contrast, the vasorelaxant effect of the alkaloid N-DEZ in the presence of TEA and $BaCl_2$ did not differ from control. K_{ATP} channels and K_v-channels are major regulators of membrane potential in vascular smooth muscle cells. In this study, inhibition of these channels partially attenuated the vasorelaxant effect of N-DEZ, suggesting their involvement in membrane hyperpolarization and subsequent reduction in Ca^{2+} influx. In contrast, blockade of K_{Ca} channels and K_{ir}-channels had no significant effect on the response, suggesting that these pathways are not major contributors in the present setting. In general, The results indicate that the vasorelaxant effect of the alkaloid N-DEZ is partly associated with activation of K_{ATP} and K_v channels, leading to membrane hyperpolarisation and reduced Ca^{2+} influx into smooth muscle cells.

Conclusions

It has been demonstrated that the relaxant effect of the alkaloid N-DEZ on rat aortic rings is mediated through several complex mechanisms. The vasorelaxant effect of the alkaloid N-DEZ inhibits L-type Ca^{2+} channels and IP_3R activity in the plasma membrane, independent of the endothelium, as well as SERCA and, to a partial extent, K_{ATP}, It achieves this by activating Kv channels, thereby causing membrane hyperpolarisation. Furthermore, it reduces Ca^{2+} influx by inhibiting the Na^+/Ca^{2+} exchange system from operating in reverse mode. Overall, the alkaloid N-DEZ is important in vascular regulation by multifaceted regulation of Ca^{2+} homeostasis in smooth muscle cells and by exerting a vasorelaxant effect, and may be considered a potential therapeutic approach.

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Conflict of interest

The authors declare that there are no commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

This statement does not apply to this article.

Ethics approval

All procedures involving the laboratory animals adhered to the established guidelines and mandates concerning their ethical care, as stipulated by the Ethical Commission of the IBB at the National University of Uzbekistan. (This is documented under Protocol No. 7, dated 04/07/2022, regarding the utilization of research subjects). Segments of the aorta were isolated and prepared employing a previously described technique.

Informed consent statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

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