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REVIEW ARTICLE

Molecular Mechanisms and Experimental Protocols in the Study of Vasorelaxant Activity in Aortic Smooth Muscle Cells

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Abstract

Vasorelaxation is a key process in cardiovascular regulation and a promising target for treating hypertension and vascular dysfunctions. This study outlines a comprehensive experimental framework for evaluating vasorelaxant mechanisms in isolated rat aortic rings using isometric tension recording. A combination of pharmacological tools and ionic modulation was used to explore the roles of voltage-dependent calcium channels, GPCR signaling, intracellular calcium stores (SERCA and ryanodine receptors), ion exchangers (NCX, Na^+/K^+-ATPase) and endothelium-derived factors. Computational docking complemented experimental data by identifying ligand-binding sites on key ion channels. Findings revealed that vasorelaxation involves multifaceted modulation of calcium influx, receptor-mediated signaling, and intracellular calcium cycling, with both endothelium-dependent and -independent pathways contributing. This integrative approach provides novel insights into vascular smooth muscle regulation and supports the development of targeted cardiovascular therapies.

Keywords: Vasorelaxation, Calcium channels, SERCA, Ryanodine receptors, Na^+/Ca^{2+} exchanger

Introduction

Vasorelaxation, the process by which blood vessels decrease their tone and widen, plays a fundamental role in the regulation of blood pressure, tissue perfusion, and vascular homeostasis. It is a tightly controlled physiological response governed by a complex interplay of neural, humoral and local signals that act on the vascular smooth muscle [1]. Therapeutically, enhancing vasorelaxation is central to the management of hypertension, ischemic heart disease and other cardiovascular disorders. Agents that promote smooth muscle relaxation—either by interfering with calcium signaling, modulating ion channels, or activating endothelial-derived pathways—form the pharmacological backbone of antihypertensive and vasoprotective therapies [2,3].

Among the various vascular segments, the aorta stands out as a crucial structure in the maintenance of systemic hemodynamics. As the largest elastic artery, the aorta buffers pulsatile cardiac output and ensures continuous blood flow during diastole [4]. The smooth muscle cells (SMCs) embedded within its medial layer not only provide structural integrity but also respond dynamically to vasoactive stimuli. Aortic SMCs

regulate vascular tone via calcium influx, intracellular calcium mobilization and actomyosin cross-bridge cycling—processes that are highly sensitive to pharmacological agents and pathological stimuli. Importantly, alterations in aortic SMC reactivity are strongly associated with the pathogenesis of hypertension, arterial stiffness, and cardiovascular remodeling [5].

Due to their well-characterized contractile behavior and accessibility in ex vivo setups, aortic smooth muscle cells (particularly in isolated aortic rings) have become a widely accepted experimental model for studying vasorelaxation [6]. This model allows for reproducible investigation of vascular responses under controlled physiological conditions, enabling the dissection of both endothelium-dependent and -independent mechanisms [7].

To unravel the intricate molecular mechanisms underlying vasorelaxation, structured experimental protocols are essential. These include protocols designed to isolate specific ion channel activities (e.g., L-type Ca^{2+} channels, store-operated calcium entry, Na^{+}/Ca^{2+} exchangers), investigate receptor-mediated signaling pathways and study intracellular calcium handling via SERCA pumps and ryanodine receptors [8,9]. Combining pharmacological tools with functional assays in the aorta not only aids in identifying molecular targets of candidate vasorelaxant compounds but also provides a translational platform for developing next-generation antihypertensive drugs [10].

In this review, we aim to present a detailed overview of the molecular mechanisms that govern aortic smooth muscle tone, alongside the experimental strategies commonly employed to evaluate them. Emphasis is placed on ion transport systems, receptor-mediated signaling, and intracellular calcium dynamics, with a view toward understanding how these pathways can be modulated to promote vascular relaxation under physiological and pathological conditions.

Aortic smooth muscle cell structure and functional mechanisms

Aortic smooth muscle cells (ASMCs) are elongated, spindle-shaped, non-striated cells organized in concentric layers within the tunica media of the aorta. Unlike skeletal and cardiac muscle fibers, these cells do not form sarcomeres and are instead embedded in a complex extracellular matrix composed predominantly of elastin, collagen and glycosaminoglycans [11]. This matrix not only provides mechanical support and elasticity but also plays a crucial role in mechanotransduction and cellular signaling. The cells are interconnected via gap junctions, allowing for coordinated contraction across the vascular wall [12].

Each smooth muscle cell contains a single, centrally located nucleus, surrounded by abundant contractile filaments composed of actin and myosin. These filaments are anchored to dense bodies and dense bands—cytoplasmic and membrane-associated structures, respectively—that serve a function analogous to Z-lines in striated muscle [13]. The spatial arrangement of these filaments allows for a unique contractile behavior characterized by slow, sustained contractions and high energy efficiency, critical for maintaining vascular tone over prolonged periods [14].

Aortic smooth muscle cells also exhibit significant phenotypic plasticity, transitioning between a "contractile" state—marked by high expression of contractile proteins such as α-smooth muscle actin, myosin heavy chain and calponin—and a “synthetic” state, in which the cells show increased proliferative, migratory, and secretory activity [15]. This transition plays a key role in vascular development, remodeling, and disease progression, particularly in conditions such as atherosclerosis and hypertension [16] (**Figure 1**).

The functional activity of ASMCs is primarily governed by complex ion transport systems and intracellular signaling cascades that control the excitation-contraction coupling process. Intracellular calcium (Ca^{2+}) serves as the central second messenger regulating contraction [17]. Under physiological conditions, contraction is initiated when an external stimulus—mechanical stretch, neurotransmitter, or hormone—activates membrane-bound receptors or causes membrane depolarization. This leads to the opening of L-type voltage-dependent calcium channels, allowing extracellular Ca^{2+} influx into the cytoplasm [18].

In addition to voltage-gated channels, receptor-operated calcium channels (ROCCs) are activated by ligands such as norepinephrine or angiotensin II, which bind to G protein-coupled receptors (GPCRs) on the cell membrane [19]. Activation of GPCRs stimulates phospholipase C (PLC), leading to the generation of

inositol trisphosphate (IP_3), which diffuses to the sarcoplasmic reticulum (SR) and triggers Ca^{2+} release through IP_3 receptors (IP_3Rs). Parallelly, ryanodine receptors (RyRs) on the SR membrane can also release stored Ca^{2+}, particularly through calcium-induced calcium release mechanisms [20].

As intracellular Ca^{2+} levels rise, Ca^{2+} binds to calmodulin, forming a Ca^{2+}–calmodulin complex that activates myosin light chain kinase (MLCK). MLCK phosphorylates the regulatory light chains of myosin, enabling cross-bridge formation with actin filaments and initiating contraction. Relaxation occurs when intracellular Ca^{2+} levels fall, either through active reuptake into the SR via SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) or extrusion across the plasma membrane by plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchangers (NCX) [21].

Membrane potential dynamics are also critical in regulating vascular tone. Potassium channels—such as BK_Ca (large-conductance Ca^{2+}-activated K^+ channels), voltage-gated K^+ (KV) channels and ATP-sensitive K^+ (K_ATP) channels—mediate membrane hyperpolarization, thereby inhibiting voltage-gated calcium channels and promoting relaxation [22]. Conversely, closure of these K^+ channels leads to depolarization and contraction. Chloride channels and non-selective cation channels may further modulate membrane excitability and ion fluxes [23].

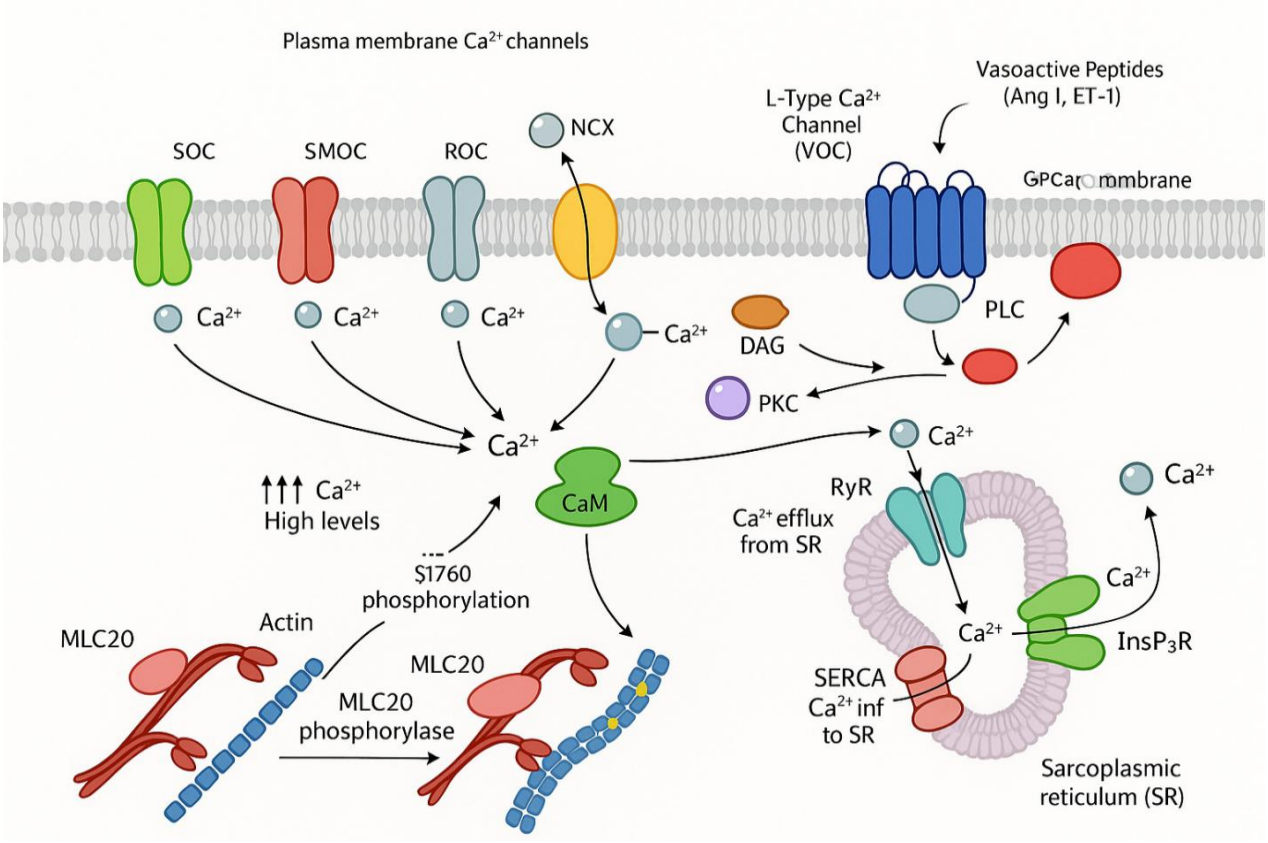

Figure 1 Mechanisms of intracellular Ca^{2+} regulation and smooth muscle contraction in vascular smooth muscle cells. Activation of G protein-coupled receptors (GPCRs) leading to myosin light chain phosphorylation and smooth muscle contraction.

Importantly, smooth muscle contractility is not solely determined by $[Ca^{2+}]_i$, but also by Ca^{2+} sensitization mechanisms, such as the inhibition of MLCP via RhoA/ROCK and PKC pathways. These pathways enhance contractility without altering intracellular calcium concentration, contributing to sustained vascular tone often seen in pathological states [24].

The coordination between ion channel activity, receptor signaling, calcium handling, and cytoskeletal dynamics defines the contractile behavior of aortic smooth muscle. Dysregulation of any of these

components can contribute to vascular dysfunction, increased arterial stiffness, and elevated blood pressure. Thus, understanding the structural and functional basis of ASMC physiology is vital for developing targeted therapeutic interventions in cardiovascular diseases [25].

Experimental setup and required equipment

Tissue preparation and tension recording

Functional studies of vascular smooth muscle, particularly for assessing vasorelaxant or vasoconstrictor responses, are typically conducted using isolated aortic ring preparations mounted in organ bath systems. This classic ex vivo approach allows for precise control of the extracellular environment and facilitates the investigation of direct effects of pharmacological agents on vascular tone under isometric conditions [26].

Freshly excised thoracic aortae from adult rodents (commonly rats) are carefully cleaned of connective tissue and fat under a stereomicroscope in cold, oxygenated physiological saline solution. The aorta is then sectioned into 3 - 5 mm wide ring segments (**Figure 2**). In some experiments, the endothelium is mechanically removed by gently rubbing the luminal surface with a stainless steel wire, which enables separate analysis of endothelium-dependent and -independent mechanisms [27].

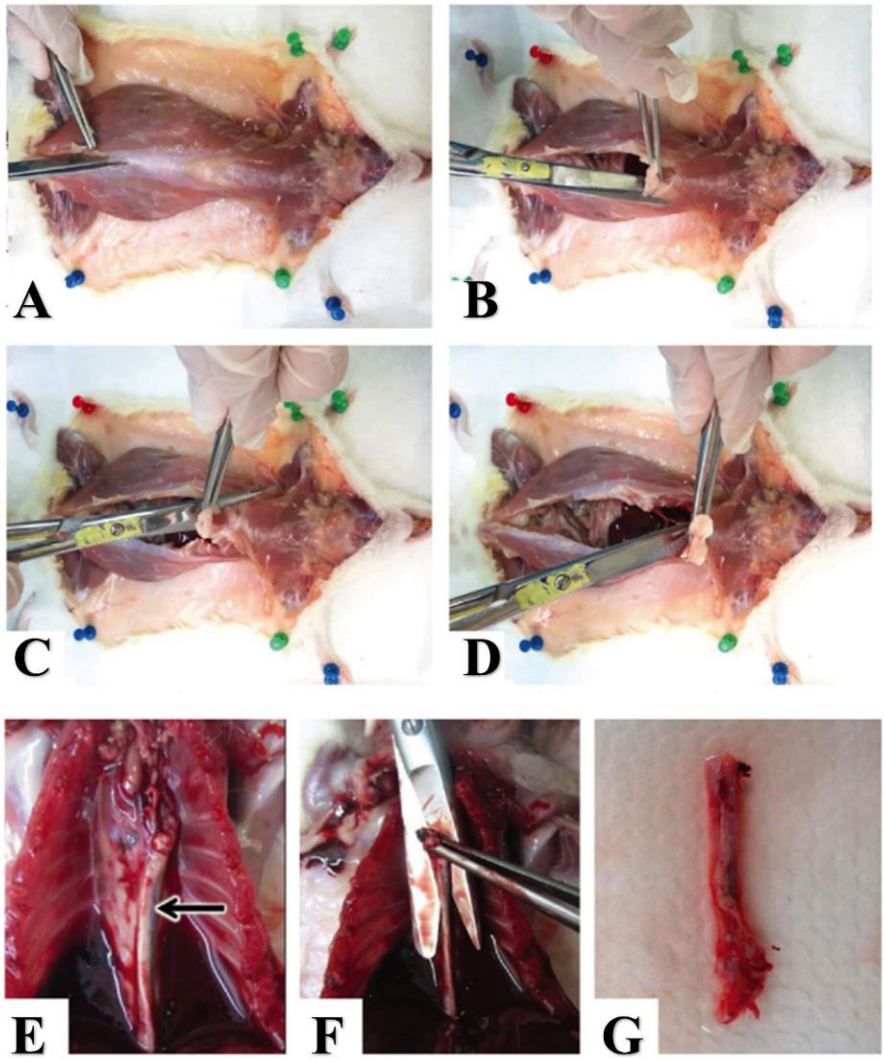

Figure 2 Dissection and isolation of the thoracic aorta and spinal cord in a rodent model (A - D).

Sequential steps of thoracic aorta isolation: (A) The chest cavity is opened and the heart is exposed; (B - D) The thoracic aorta is carefully separated from surrounding connective tissue and excised using micro

scissors and forceps. (E - F) Spinal cord exposure: (E) The vertebral column is exposed by removing overlying muscle tissue (black arrow indicates spinal cord); (F) Laminectomy is performed to isolate the spinal cord. (G) The excised thoracic spinal cord segment placed on a clean surface for further analysis.

The aortic rings are suspended between two stainless steel hooks: One fixed to the bottom of the organ chamber and the other connected to a highly sensitive isometric force transducer (e.g., ADInstruments MLT0202 or Grass FT03), allowing continuous recording of changes in vascular tone (**Figure 3**). Each organ bath contains Krebs-Henseleit buffer solution (composed of NaCl 118.0 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25.0 mM and glucose 11.0 mM) maintained at 37 ± 0.5 °C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 to maintain physiological pH (7.4). Before initiating experiments, aortic rings are allowed to equilibrate under an optimal resting tension of 1 - 2 g for at least 45 - 60 min, during which the buffer is replaced every 15 min [28].

Standardized protocols include precontraction of the tissue using high-potassium depolarizing solution (e.g., 60 mM KCl) or agonists such as phenylephrine (PE, 1 μM), followed by cumulative or single-dose administration of the test compound. Isometric tension changes are continuously recorded using data acquisition software (e.g., LabChart, PowerLab) for analysis of relaxation responses expressed as percentage of initial contraction [29].

This model system remains a gold standard in vascular pharmacology, enabling mechanistic dissection of ion channel modulation, second messenger involvement and endothelial contribution in the vasorelaxant or vasoconstrictor activity of bioactive compounds [30].

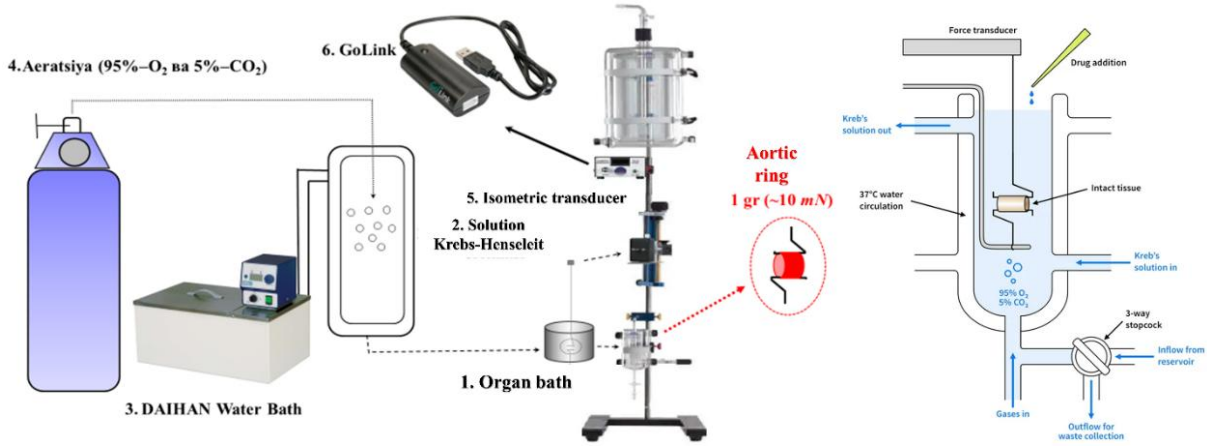

Figure 3 Schematic representation of the organ bath system used for isometric tension recording of isolated rat aortic rings. The setup includes: (1) organ bath chamber maintained at 37°C by (3) a circulating water bath (DAIHAN), (2) filled with Krebs-Henseleit solution, (4) continuously aerated with a 95% O_2 and 5% CO_2 gas mixture, and (5) connected to an isometric force transducer to detect contractile responses. The data is acquired through (6) a GoLink interface. The aortic ring (~1 g tension ≈ 10 mN) is suspended between two hooks within the chamber (right), allowing for precise drug addition and real-time measurement of smooth muscle responses under controlled physiological conditions.

Reagents and solutions

Krebs–Henseleit (sources?) buffer (KHB) was used as the physiological solution to maintain the viability and contractile responsiveness of isolated aortic rings during ex vivo studies. The standard composition of KHB (in mM) was as follows: NaCl 118.0, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.0. The buffer was freshly prepared each day using analytical-grade reagents dissolved in double-distilled water. The solution was continuously gassed with a carbogen mixture (95% O_2 and 5% CO_2) to maintain a stable physiological pH of 7.4 and kept at 37 ± 0.5 °C throughout the experiments [31,32].

Solvent systems for dissolving test compounds included dimethyl sulfoxide (DMSO) and methanol (MeOH), depending on the compound's solubility profile. To avoid solvent-induced artifacts or tissue toxicity, the final concentration of organic solvents in the organ bath was strictly limited to ≤ 0.1% (v/v). All stock solutions were prepared at high concentrations (e.g., 10 - 100 mM) and stored at −20 °C in airtight amber vials to minimize degradation and photo-oxidation. Immediately prior to application, stock solutions were diluted into KHB to achieve working concentrations [33].

During pre-incubation, isolated aortic rings were allowed to equilibrate in KHB for at least 45 - 60 min under a resting passive tension of 1.0 - 2.0 g. This period ensures stabilization of basal tone and allows recovery from mechanical stress induced during dissection and mounting. During equilibration, the buffer was replaced every 15 - 20 min to maintain optimal ionic composition and oxygenation. In some protocols, a pre-treatment with inhibitors or vehicle control was performed during the last 15 - 30 min of the equilibration phase to assess modulatory effects on contractility or relaxation responses [34].

This rigor in buffer composition, solvent handling, and pre-experimental conditioning is essential to maintain tissue viability and ensure reproducibility of pharmacological responses in vascular reactivity studies.

Voltage-dependent ca^{2+} channels: Membrane influx mechanism

Depolarization-induced contraction in high K^+ medium

Aortic smooth muscle contraction is initiated by the activation of voltage-dependent L-type calcium (Ca^{2+}) channels. When vascular smooth muscle cells are placed in a high potassium (K^+) environment—typically around 60 to 80 mM—the concentration gradient of K^+ across the membrane is disrupted, leading to membrane depolarization. This electrical change opens L-type Ca^{2+} channels located in the plasma membrane, allowing extracellular Ca^{2+} to enter the cytoplasm [35].

The influx of Ca^{2+} serves as a key second messenger that binds to calmodulin, activating myosin light chain kinase (MLCK). MLCK then phosphorylates the regulatory light chains of myosin, enabling interaction with actin filaments and initiating contraction. As Ca^{2+} levels decline—either by reuptake into the SR or extrusion from the cell—relaxation occurs, mediated by MLCP, which dephosphorylates myosin and stops cross-bridge cycling [36].

Experimental procedure: Aortic ring contraction in high K^+ medium

After the thoracic aorta is carefully isolated from the animal and cleaned of connective tissue, it is cut into rings and mounted in an organ bath filled with KHB. The tissue is then incubated for approximately 60 min at 37 °C under oxygenated conditions (typically 95% O_2 and 5% CO_2) to allow equilibration [37].

Following this incubation, a high-potassium medium is introduced into the bath to induce depolarization. The elevated K^+ concentration triggers the opening of L-type Ca^{2+} channels, leading to a rapid influx of Ca^{2+} into smooth muscle cells. As a result, the aortic ring begins to contract (**Figure 4**). This contraction is recorded in real time using the GoLink system, which displays a rising tension curve on the screen. The onset of contraction is characterized by a sharp upward movement of the trace, eventually reaching a plateau phase [38].

Once the maximum contraction is achieved, test compounds are added directly to the bath in a dose-dependent manner. If the substance being tested has calcium channel-blocking properties, it will begin to reduce the tension of the contraction gradually. This is seen as a downward trend in the recorded trace, indicating that the L-type Ca^{2+} channels are being inhibited and that the compound is inducing relaxation [39].

This method is widely used to evaluate the vasorelaxant potential of natural or synthetic agents by determining their capacity to block calcium influx and modulate vascular tone.

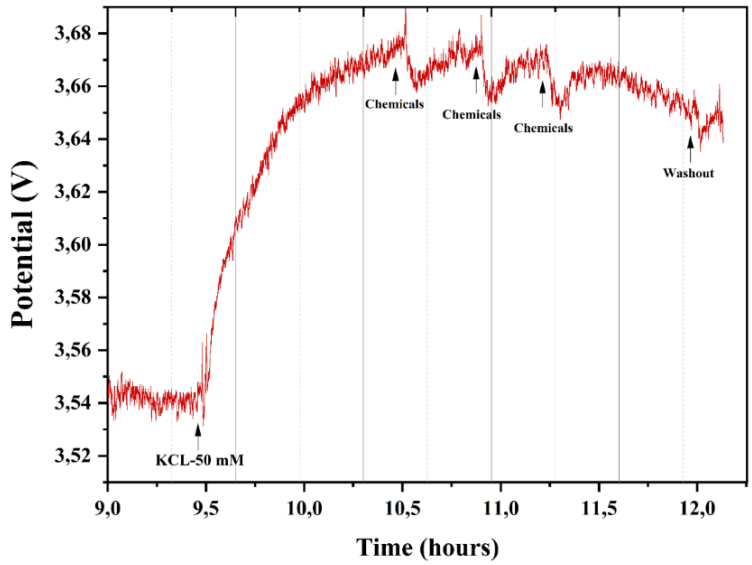

Figure 4 Representative potential change recorded over a 3-hour period in response to sequential chemical applications following membrane depolarization by 50 mM KCl. The initial sharp rise in potential at ~9.5 h corresponds to KCl-induced depolarization. Subsequent additions of test chemicals (indicated by arrows) elicited modulating effects on membrane potential, followed by partial recovery upon washout. The graph demonstrates the dynamic electrophysiological response of the system to chemical interventions.

Use of Ca^{2+} channel blockers

Mechanism: Competitive inhibition of L-Type Ca^{2+} channels

To determine whether the test compounds exert their vasomodulatory effects through L-type Ca^{2+} channel inhibition, they are evaluated alongside established calcium channel blockers such as verapamil, nifedipine, and diltiazem. These blockers inhibit voltage-dependent Ca^{2+} influx by binding to specific sites on the α_1 subunit of the L-type channels, thereby preventing depolarization-induced contraction in vascular smooth muscle [40].

If a test compound demonstrates an ability to reduce contractile force in depolarized aortic rings, especially in the presence of known blockers, this may indicate competitive or non-competitive interaction with L-type Ca^{2+} channels — or possibly effects on additional ion transport systems [41].

Experimental protocol: IC_{50}-based comparative assay design

After determining the IC_{50} value of the test compound, a comparative assessment is conducted with known calcium channel blockers such as verapamil. In this experimental design, the aortic ring is first incubated with verapamil at its IC_{50} concentration (0.1 µM) for 15 min to allow complete interaction with L-type Ca^{2+} channels. Following this pre-incubation, the tissue is exposed to the test compound at its own IC_{50} dose for an additional 15 min. After both agents have interacted with the tissue, a depolarizing concentration of KCl is added to induce contraction through L-type Ca^{2+} channel activation [42,43].

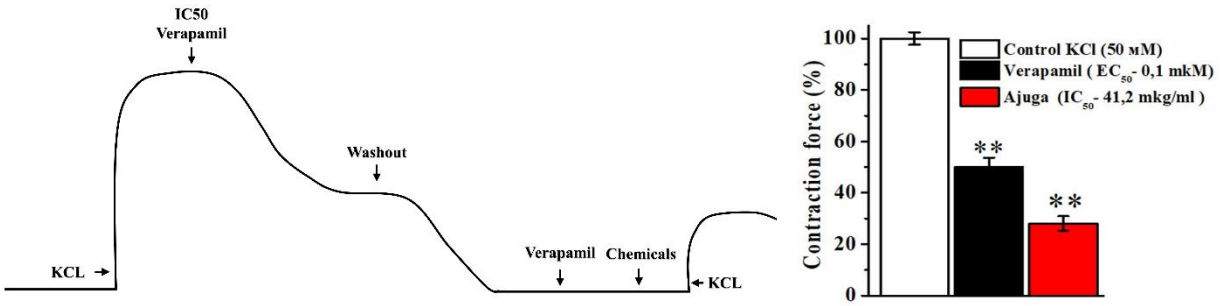

Figure 5 Left: Schematic representation of the experimental design for assessing the vasorelaxant effect of Ajuga extract using isolated aortic rings precontracted with 50 mM KCl. Verapamil (IC_{50} = 0.1 μM) was used as a reference calcium channel blocker. Following washout, the combined effect of verapamil and test chemicals (Ajuga extract) was evaluated. Right: Quantitative comparison of contraction force (%) in rat aortic rings after treatment with KCl (control), verapamil, and Ajuga extract (IC_{50} = 41.2 μg/mL). Data are presented as mean ± SEM; *p-value* < 0.01 compared to control.

The resulting contractile response is monitored via GoLink software. If the contraction amplitude appears lower than that induced by verapamil alone, this suggests that the test compound competitively inhibits calcium influx more effectively, potentially through additional ion channels beyond L-type Ca^{2+} channels. Conversely, if the response does not decrease compared to the IC_{50} of verapamil, it indicates that the compound may not exhibit significant activity on these or related channels (**Figure 5**).

This approach not only helps confirm the mechanism of action of the test compound but also provides insight into possible broader channel-blocking properties, guiding further pharmacological exploration.

Extracellular Ca^{2+} dependence: Cumulative calcium reintroduction protocol

To determine whether the contractile or relaxant effects of the test compounds are truly dependent on extracellular calcium influx, a cumulative calcium reintroduction protocol is employed using Ca^{2+}-free conditions. This experimental model helps reveal the involvement of membrane-bound voltage-operated calcium channels (VOCCs), particularly the L-type channels, in mediating smooth muscle contraction, and evaluates how the test compounds influence this process [44].

The molecular mechanism underlying this protocol is based on the essential role of extracellular calcium in smooth muscle contraction. In vascular tissues, calcium influx through plasma membrane calcium channels is the primary trigger for myofilament activation and force generation. By eliminating extracellular Ca^{2+} from the KHB, typically using 0.5 - 1 mM EGTA as a chelating agent, extracellular calcium stores are depleted and intracellular calcium flux is minimized. Under these Ca^{2+}-free conditions, contraction cannot occur unless Ca^{2+} is reintroduced, making this a powerful tool to directly assess the role of extracellular calcium in the pharmacodynamics of the test compound [45].

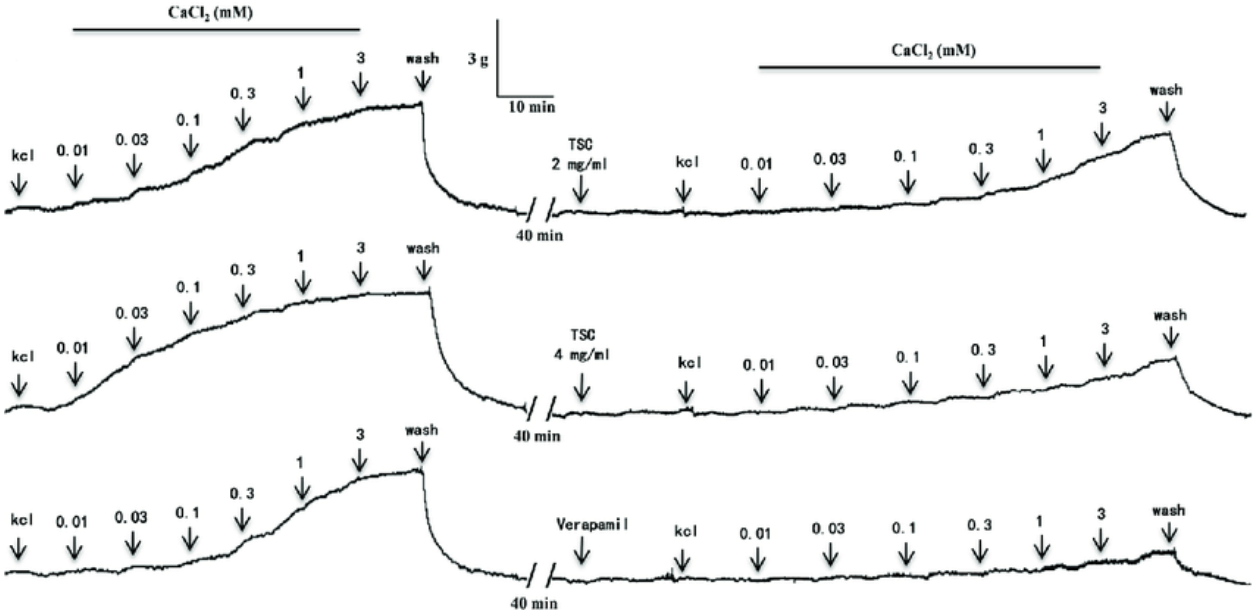

Figure 6 Representative tracings illustrating the cumulative concentration–response curves to extracellular calcium ($CaCl_2$; 0.01 - 3 mM) in endothelium-denuded rat aortic rings pretreated with high K^+ (50 mM) to induce depolarization. The contractile response was recorded in the absence (control) and presence of TSC (test sample compound) at 2 mg/mL and 4 mg/mL, as well as with the reference Ca^{2+} channel blocker verapamil. All treatments were followed by washout. Each arrow indicates a stepwise addition of $CaCl_2$.

The figure demonstrates the calcium entry blockade potential of TSC, as evidenced by the reduced contractile force compared to control. After an initial equilibration period in Ca^{2+}-free Krebs solution, the tissue is subjected to cumulative addition of $CaCl_2$ in increasing concentrations, typically ranging from 0.1 mM to 2.5 mM. This stepwise increase allows for the generation of a concentration–response curve, reflecting the tissue's sensitivity to calcium and its capacity to restore contraction in a dose-dependent manner (**Figure 6**).

When test compounds are added prior to or during calcium reintroduction, their ability to inhibit or modulate calcium-dependent contraction can be quantitatively assessed. A significant rightward shift in the $CaCl_2$ response curve, or a reduction in maximal contraction, suggests that the compound interferes with calcium influx, possibly via VOCC blockade or calcium-sensing receptor modulation.

The reason for examining calcium concentrations from as low as 0.1 mM up to physiological levels (~2.5 mM) lies in the need to determine not only the potency but also the threshold and saturation effects of calcium-mediated responses. Low concentrations help evaluate high-affinity interactions and initial channel activation, while higher concentrations reflect full physiological activation of contractile machinery. This gradient-based approach also aids in distinguishing between partial antagonism and full inhibition of calcium channels [46].

Altogether, this protocol enables a precise evaluation of whether the pharmacological activity of a compound is directly related to calcium influx mechanisms, and it helps elucidate the molecular targets involved in smooth muscle contractility.

G-Protein coupled receptor (GPCR)-mediated calcium entry

Stimulation via adrenergic receptors

GPCR-mediated calcium entry is an essential signaling mechanism involved in smooth muscle contraction. When adrenergic receptors—especially α_1-adrenoceptors—are stimulated by agonists such as phenylephrine or adrenaline, a cascade of intracellular signaling events is triggered [47].

The activation of the α_1-adrenergic receptor, a Gq-protein-coupled receptor, initiates the stimulation of phospholipase C (PLC). PLC hydrolyzes membrane phospholipid PIP_2 (phosphatidylinositol 4,5-

bisphosphate) into two secondary messengers: IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP_3 then binds to its receptors on the endoplasmic or sarcoplasmic reticulum, causing the release of stored Ca^{2+} into the cytoplasm, thereby promoting contraction. This form of calcium release is rapid and independent of extracellular calcium influx [48].

This mechanism contrasts with voltage-dependent calcium channels, where Ca^{2+} influx is driven by membrane depolarization. The GPCR–PLC–IP_3 axis provides a rapid, receptor-controlled way of elevating intracellular Ca^{2+} concentration without initial involvement of voltage changes.

Experimental approach

To study GPCR-mediated calcium signaling, classic adrenergic agonists such as adrenaline (a non-selective agonist) and phenylephrine hydrochloride (a selective α_1-agonist) are used.

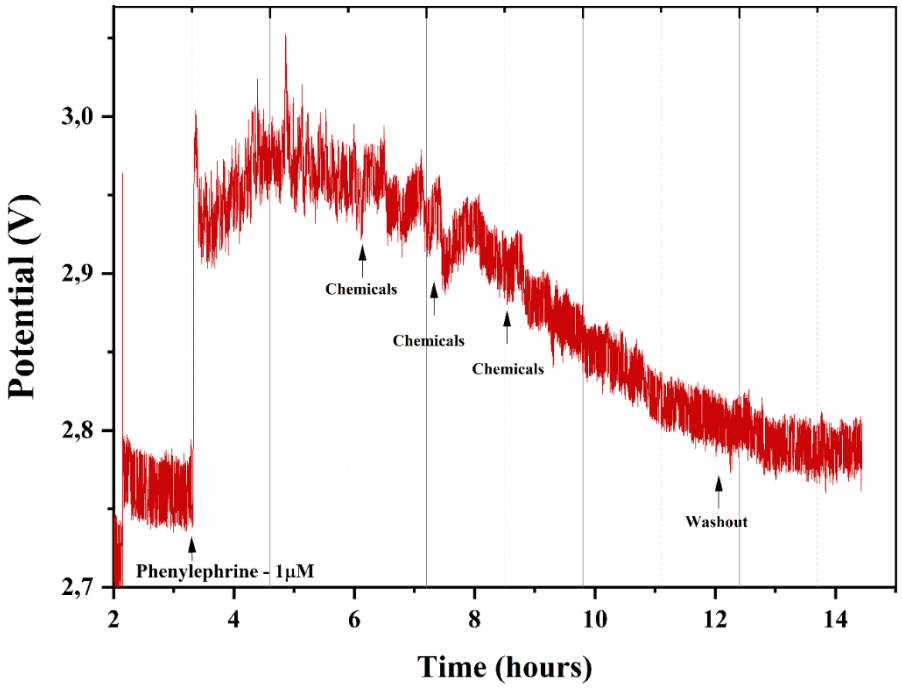

Figure 7 Real-time potentiometric recording of membrane potential in rat aortic smooth muscle following stimulation with phenylephrine (1 μM), showing the vasoconstrictive response followed by sequential application of test compounds ("Chemicals") and subsequent washout. The gradual decline in potential indicates a dose-dependent vasorelaxant effect of the tested agents. Each upward arrow marks the time point of chemical administration. Washout leads to partial restoration of baseline potential. This assay provides insight into the modulatory action of test compounds on α_1-adrenergic receptor–mediated vascular tone.

In this experiment, phenylephrine at a concentration of 1 μM is added to isolated smooth muscle tissue, such as aortic rings. The addition leads to a rapid and strong contraction, which occurs faster than contractions induced by voltage-dependent calcium channel activation. This quick contractile response highlights the efficacy of receptor-mediated intracellular calcium mobilization (**Figure 7**).

Once the contraction stabilizes (plateau phase), the influence of test compounds is assessed in a dose-dependent manner. This allows researchers to determine whether the test substance modulates GPCR-mediated calcium signaling—either by interfering with receptor binding, PLC activation, IP_3 formation, or calcium release from internal stores.

Use of adrenergic receptor blockers

Receptor-operated calcium entry (ROCE) represents a critical calcium influx pathway that is initiated by G protein-coupled receptor (GPCR) activation but operates independently of membrane depolarization. In vascular smooth muscle cells

(VSMCs), α_1-adrenergic receptors (α_1-ARs) are predominantly coupled to the Gq/11 protein, which, upon agonist binding (e.g., phenylephrine), activates phospholipase C (PLC). PLC hydrolyzes membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers: Diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3).

IP_3 subsequently binds to IP_3 receptors on the sarcoplasmic reticulum (SR), triggering the release of stored Ca^{2+} into the cytoplasm. Concurrently, DAG activates transient receptor potential canonical (TRPC) channels, which facilitate Ca^{2+} entry from the extracellular space, contributing to sustained vasoconstriction. This mechanism is distinct from voltage-dependent calcium channels (VDCCs) and thus can be selectively interrogated using specific receptor antagonists [49].

To determine whether a test compound modulates ROCE via adrenergic pathways, pharmacological inhibition using non-selective α-adrenergic blockers like phentolamine and β-adrenergic blockers such as propranolol is employed. These agents competitively inhibit receptor binding, thereby suppressing downstream Ca^{2+} mobilization and contraction. If the test compound retains its activity despite receptor blockade, its mechanism may be independent of receptor-operated pathways or act downstream of receptor activation.

Experimental protocol

The test is conducted using isolated aortic rings or vascular smooth muscle tissue, maintained in KHB at physiological temperature and gas conditions (**Figure 8**). Following initial equilibration, tissues are incubated with phentolamine at a concentration of 10 μM for 15 min to achieve complete α-adrenergic receptor blockade. Without removing the blocker, the test compound is then introduced and allowed to incubate for an additional 15 min, providing sufficient time for any interaction with downstream signaling elements or ion channels.

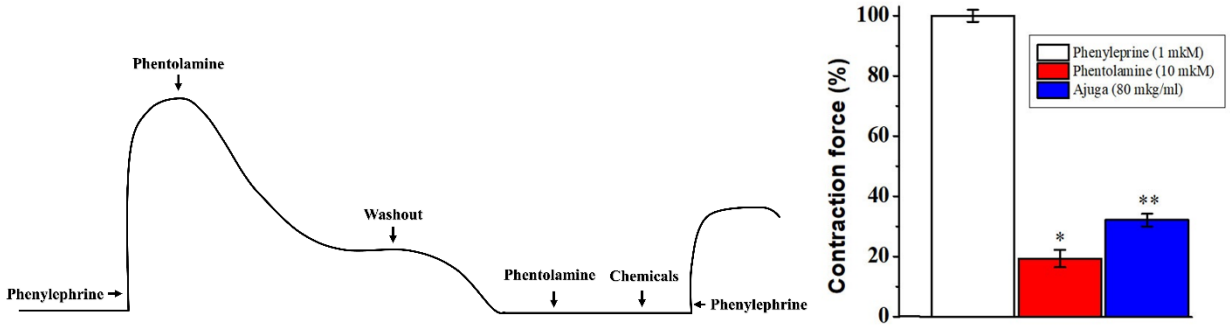

Figure 8 Assessment of the vasorelaxant effect of test compounds on α_1-adrenergic receptor–mediated contraction in isolated rat aortic rings. The left panel illustrates the experimental protocol: Phenylephrine (1 μM) induced sustained contraction, followed by the addition of the selective α_1-blocker phentolamine (10 μM), washout and subsequent co-application of phentolamine and the test compound (Ajuga extract).

The right panel quantifies the contraction force (%) relative to the phenylephrine control. Both phentolamine and Ajuga (80 μg/mL) significantly reduced vascular tone (*p-value < 0.05, **p-value < 0.01), indicating potential α_1-receptor antagonistic activity of Ajuga.

Once the incubation period is complete, phenylephrine (1 μM) is administered to provoke receptor-mediated contraction. The amplitude of the contractile response is recorded in real time and compared to control traces obtained in the absence of phentolamine, as well as to traces with phentolamine alone.

If the presence of the test compound further suppresses contraction beyond the effect of phentolamine, this suggests the compound may interfere with calcium entry downstream of receptor activation, possibly by modulating TRPC channels or intracellular calcium signaling. Conversely, if the contractile response is greater than that seen with phentolamine alone, the compound may partially reverse α-blockade or engage alternative signaling pathways.

This approach provides critical insight into whether test compounds operate via classical receptor pathways or exert effects on calcium handling through alternative or non-receptor-mediated mechanisms, as established in modern vascular pharmacology research.

Intracellular Ca^{2+} signaling assessment under Ca^{2+}-free conditions

To specifically dissect the intracellular calcium mobilization pathway (independent of extracellular Ca^{2+} influx), a parallel experimental series is conducted in Ca^{2+}-free Krebs solution (with $CaCl_2$ omitted and 0.5 mM EGTA added). In this configuration, extracellular calcium influx is blocked, allowing the selective investigation of IP_3-mediated intracellular Ca^{2+} release, primarily from the sarcoplasmic reticulum [50].

Tissues are incubated in Ca^{2+}-free medium for 30 min, followed by the addition of

- the test compound (at max effective concentration), and
- phenylephrine (1 μM), which, via Gq/PLC/IP_3 signaling, induces intracellular Ca^{2+} release [51].

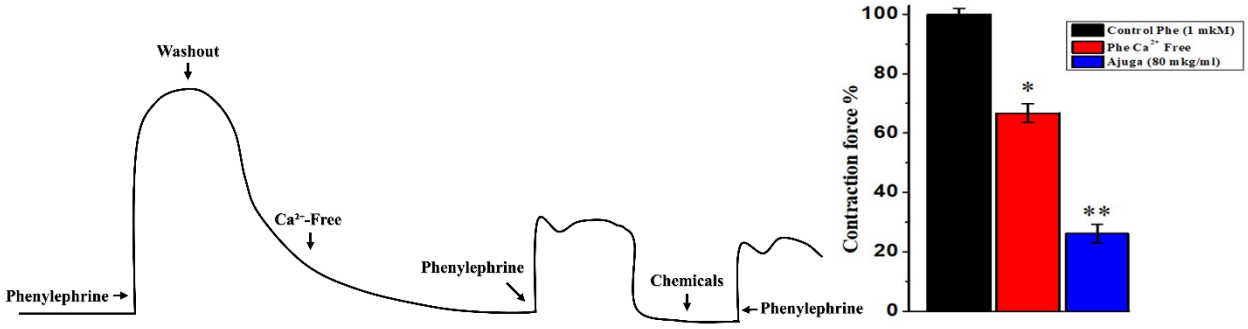

Figure 9 Evaluation of the role of extracellular Ca^{2+} in phenylephrine (Phe)-induced vasoconstriction and the inhibitory effect of Ajuga extract. The experimental protocol (left) involved contraction with phenylephrine (1 μM), followed by washout and incubation in Ca^{2+}-free solution to assess Ca^{2+}-dependent tone. Re-application of phenylephrine and treatment with Ajuga (80 μg/mL) were then performed.

The bar graph (right) shows that Ca^{2+}-free conditions significantly reduced contractile force (*p-value < 0.05) and Ajuga extract further inhibited contraction to a greater extent (**p-value < 0.01), suggesting that Ajuga interferes with Ca^{2+}-dependent vasoconstriction mechanisms.

The contractile amplitude in Ca^{2+}-free conditions is compared with the response observed in standard 2.5 mM Ca^{2+} Krebs solution. A significant contraction in Ca^{2+}-free medium suggests that the compound affects intracellular Ca^{2+} stores or modulates IP_3 receptor signaling. A diminished response in this setting relative to the normal Ca^{2+} condition also helps distinguish between ROCC-mediated influx and intracellular release mechanisms (**Figure 9**).

Intracellular Ca^{2+} handling: SERCA and RyR

SERCA (Sarco/endoplasmic reticulum Ca^{2+} ATPase) function

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) plays a pivotal role in vascular smooth muscle by actively pumping cytosolic Ca^{2+} back into the sarcoplasmic reticulum (SR), thereby terminating contraction and maintaining intracellular Ca^{2+} homeostasis. Dysregulation of SERCA activity contributes to sustained vasoconstriction and pathological vascular tone, making it a key target for pharmacological investigation [52].

Molecular Mechanism

SERCA functions by hydrolyzing ATP to sequester cytosolic Ca^{2+} into the SR, opposing the action of IP_3 receptor–mediated Ca^{2+} release. Inhibition of SERCA leads to depletion of the SR Ca^{2+} pool, accumulation of cytosolic Ca^{2+} and prolonged contractile responses. The prototypical inhibitor

thapsigargin, a sesquiterpene lactone, selectively and irreversibly binds to SERCA pumps, rendering them inactive without directly affecting plasma membrane Ca^{2+} channels or other ATPases [53].

Experimental approach

To assess SERCA function in vascular smooth muscle, isometric tension studies can be conducted using isolated rat aortic rings in a tissue bath system. Tissues are equilibrated in Ca^{2+}-containing KHB buffer (2.5 mM Ca^{2+}) and precontracted with 1 μM phenylephrine (PE) to activate α_1-adrenergic receptors and initiate intracellular Ca^{2+} mobilization [54].

Stepwise Protocol

Baseline Control: Following stabilization, a control contraction trace is recorded with PE alone to establish normal contractile kinetics.

SERCA Inhibition: Tissues are preincubated with thapsigargin (1 μM) for 15 - 30 min to irreversibly inhibit SERCA-mediated Ca^{2+} reuptake into the SR. This leads to accumulation of cytosolic Ca^{2+} and depletion of SR Ca^{2+} stores.

Re-stimulation with PE: After thapsigargin preincubation, PE is reapplied and the contractile profile is monitored. Compared to control traces, prolonged, elevated, or slowly relaxing contractions indicate successful SERCA inhibition.

Testing the compound of interest: To assess the interaction of test compounds with intracellular Ca^{2+} regulation, they can be added in the presence or absence of thapsigargin. If the test compound attenuates the thapsigargin-prolonged contraction, it may promote alternative Ca^{2+} clearance mechanisms (e.g., PMCA activation, mitochondrial uptake), or modulate SR release channels (IP_3R/RyR).

Additional considerations

To differentiate between SR Ca^{2+} release and extracellular Ca^{2+} influx, parallel experiments should be performed in Ca^{2+}-free buffer supplemented with EGTA (0.5 mM) to chelate residual Ca^{2+}.

Simultaneous application of caffeine or IP_3 analogs may be used to evoke SR Ca^{2+} release, enabling the quantification of remaining SR stores post-thapsigargin treatment.

Interpretation

Increased contraction duration **or** lack of relaxation following thapsigargin treatment confirms SERCA blockade.

If the test compound alters this profile, it may influence intracellular Ca^{2+} recycling**,** Ca^{2+} buffering**, or** non-SERCA-dependent Ca^{2+} efflux pathways.

Ryanodine receptors (RyR)

Ryanodine receptors (RyRs) are intracellular calcium release channels primarily located on the sarcoplasmic reticulum (SR) membrane in muscle cells, including vascular smooth muscle. They play a pivotal role in excitation–contraction coupling by mediating the release of Ca^{2+} from the SR into the cytosol, thereby triggering contractile responses. RyRs can be activated pharmacologically by agents such as caffeine, which sensitizes the receptors and promotes calcium-induced calcium release (CICR), or modulated by ryanodine itself, which exhibits a biphasic effect—low concentrations activate, whereas high concentrations inhibit RyR function [55].

Experimental protocol (Ryanodine receptor-mediated Ca^{2+} release)

To assess the contribution of RyR-dependent intracellular Ca^{2+} release in vascular smooth muscle contractility, isolated aortic rings are used under physiologically relevant conditions. The preparations are maintained in KHB at 37 °C, gassed continuously with a 95% O_2 / 5% CO_2 mixture. Tissues are initially equilibrated in standard Ca^{2+}-containing buffer (2.5 mM $CaCl_2$) to establish baseline contractile responses [56].

Following stabilization, caffeine is applied at an effective concentration (e.g., 10 mM) to evoke contraction via direct activation of RyRs, facilitating the release of Ca^{2+} from the sarcoplasmic reticulum (**Figure 10**). The response to caffeine in normal Ca^{2+}-containing conditions is recorded. In parallel, tissues are then washed and transferred into a Ca^{2+}-free Krebs solution (nominally 0 mM Ca^{2+}, with 0.1 mM EGTA) to eliminate extracellular calcium influx and isolate intracellular sources [57].

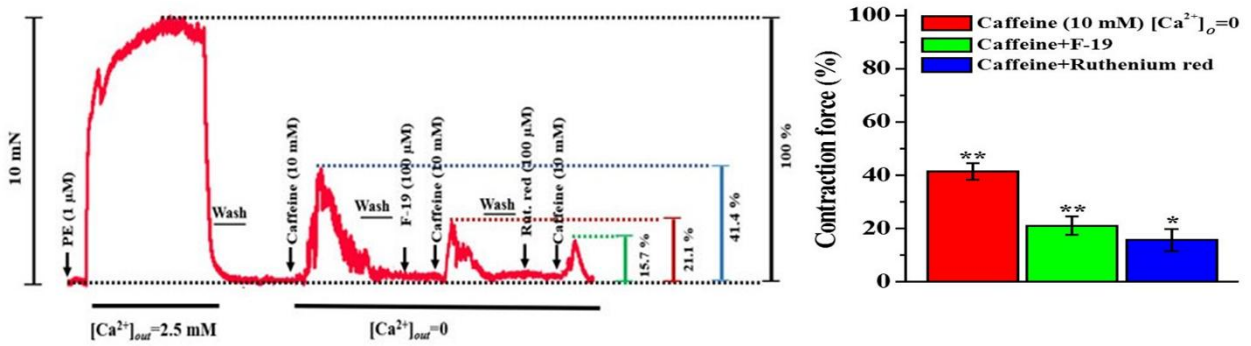

Figure 10 Effect of caffeine and intracellular calcium-release inhibitors on aortic smooth muscle contraction under Ca^{2+}-free conditions. The representative tracing (left) shows phenylephrine (PE, 1 μM)-induced contraction in the presence of extracellular Ca^{2+} (2.5 mM), followed by washout and caffeine (10 mM) stimulation in Ca^{2+}-free medium. The application of F-19 and ruthenium red after caffeine indicates inhibition of caffeine-induced contraction. The bar graph (right) shows percentage contraction force relative to the PE-induced maximum. Caffeine-induced contraction was significantly reduced in the presence of F-19 and ruthenium red, suggesting that Ajuga's effect involves modulation of intracellular Ca^{2+} release from ryanodine-sensitive stores (**p-value < 0.01, *p-value < 0.05).

Caffeine is re-applied under Ca^{2+}-free conditions, and the resulting contractile response is recorded as an indicator of RyR-mediated calcium release from intracellular stores. The maximal concentration of the test compound is introduced 15 min prior to caffeine application in separate tissue baths to evaluate its influence on RyR function. Contractions are compared to control responses in the absence of the test compound under both Ca^{2+}-containing and Ca^{2+}-free conditions [58].

A significant reduction in caffeine-induced contraction in the presence of the test compound, particularly under Ca^{2+}-free conditions, suggests an inhibitory effect on RyR-mediated Ca^{2+} release. If no change is observed, the compound may not interact directly with RyRs or associated intracellular calcium-handling mechanisms.

This protocol enables the differentiation of extracellular versus intracellular Ca^{2+} sources in vascular contraction and provides mechanistic insight into whether a test substance modulates intracellular Ca^{2+} dynamics through RyR pathways.

Ion exchange mechanisms: NCX and Na^+/K^+ ATPase

Na^+/Ca^{2+} Exchanger (NCX)

Molecular mechanism

The Na^+/Ca^{2+} exchanger (NCX) is a critical bidirectional membrane transport protein involved in cellular calcium homeostasis. Under physiological conditions, NCX operates predominantly in its forward mode, expelling one intracellular Ca^{2+} ion in exchange for the influx of three extracellular Na^+ ions. However, when the extracellular Na^+ concentration is reduced or intracellular Na^+ is elevated, the exchanger can operate in reverse mode, allowing Ca^{2+} influx into the cell in exchange for Na^+ efflux. This reverse mode plays a significant role in vascular smooth muscle contraction, especially under pathophysiological conditions like ischemia or oxidative stress [59].

Experimental protocol

To assess the role of NCX in Ca^{2+} influx, NaCl is removed from the extracellular solution and replaced with choline chloride or LiCl, which cannot be transported by NCX (**Figure 11**). This manipulation reduces extracellular Na^+ levels, thereby favoring the reverse operation of NCX, resulting in Ca^{2+} entry into the cell and a slow, sustained contraction of the smooth muscle [60].

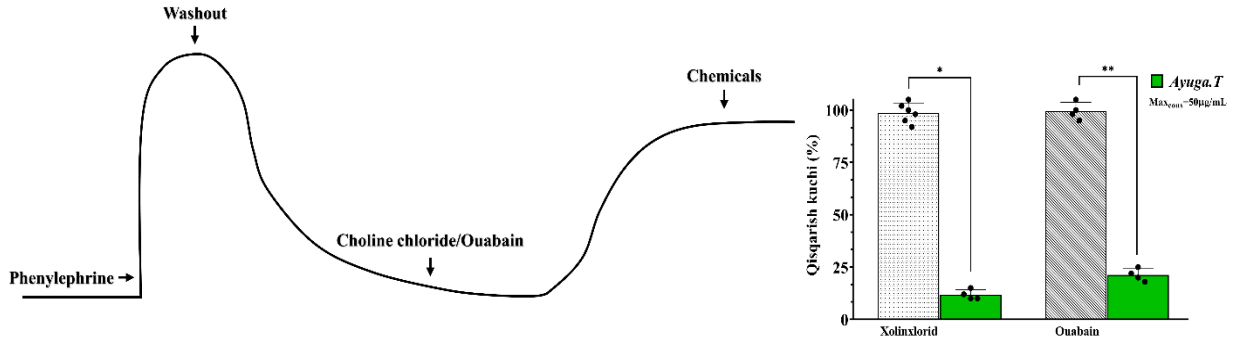

Figure 11 Involvement of Na^+/K^+-ATPase and Na^+/Ca^{2+} exchange in the vasorelaxant mechanism of Ajuga turkestanica extract. Representative tracing (left) shows phenylephrine-induced contraction followed by washout and incubation with choline chloride or ouabain (Na^+/K^+-ATPase inhibitors), then application of the extract. Bar graph (right) indicates a significant reduction in contraction force after extract treatment in both choline chloride and ouabain conditions, suggesting possible interference with Na^+-dependent Ca^{2+} handling. Data are presented as mean ± SEM (n=5); **p-value* < 0.05, ***p-value* < 0.01 vs control.

Initially, the tissue is incubated in a Na^+-free solution containing choline chloride until a stable tonic contraction is observed. This contraction is mediated primarily by NCX working in reverse mode. Once the contraction reaches a plateau, the test compound is added to evaluate its potential effect on Ca^{2+} entry via NCX. The observed contraction is compared to a control contraction induced by phenylephrine in a normal Na^+-containing physiological solution to quantify relative strength.

To confirm the involvement of NCX, the selective NCX blocker KB-R7943 is introduced at a concentration of 25 µM, followed by a 15-min incubation. After this incubation, the test compound is added again, and its effect is compared to the pre-blocker response. This allows for clear differentiation between NCX-mediated and non–NCX-mediated effects of the test substance.

Na^+/K^+ ATPase Activity

Molecular mechanism

The Na^+/K^+-ATPase plays a fundamental role in maintaining the electrochemical gradients of Na^+ and K^+ across the plasma membrane, which are critical for cellular excitability and secondary transport processes, including the Na^+/Ca^{2+} exchanger (NCX). In vascular smooth muscle cells, inhibition of Na^+/K^+-ATPase using ouabain leads to intracellular accumulation of Na^+. This disrupts the driving force for NCX, favoring its reverse mode—promoting Ca^{2+} influx in exchange for Na^+ efflux. The rise in intracellular Ca^{2+} subsequently enhances vascular tone, leading to contraction. This interplay highlights the indirect role of Na^+/K^+-ATPase in modulating intracellular Ca^{2+} homeostasis and smooth muscle contractility through its coupling with NCX [61].

Experimental approach

Isolated rat thoracic aortic rings are mounted in an organ bath system filled with KHB solution, continuously gassed with 95% O_2 and 5% CO_2 at 37°C. After equilibration, a stable contraction is induced by the addition of 1 µM phenylephrine. The maximal contraction obtained with phenylephrine is taken as 100% reference [62].

To assess Na^+/K^+-ATPase function, ouabain is added to the bath at a concentration of 20 µM. The resulting increase in contractile force, due to intracellular Na^+ accumulation and subsequent activation of NCX in reverse mode, is measured. For example, ouabain alone may induce a contractile response equivalent to ~76.4% of the phenylephrine-induced maximum.

Once the contraction stabilizes under ouabain treatment, the test compound is introduced into the organ bath, and its effect on the pre-contracted aortic ring is recorded over a 15-min period. The extent of relaxation or additional contraction is expressed as a

percentage relative to the ouabain-induced contraction level.

This protocol allows for the evaluation of test substances in the context of Na^+/K^+-ATPase–NCX interplay and their influence on calcium handling in vascular smooth muscle [63].

Endothelium-dependent mechanisms

The vascular endothelium plays a critical role in regulating vascular tone through the synthesis and release of various vasoactive substances, including nitric oxide (NO), prostacyclin (PGI_2) and endothelin. Many pharmacological agents exert their vascular effects either through endothelium-derived factors or via direct action on vascular smooth muscle ion channels. Therefore, assessing whether a compound acts in an endothelium-dependent or -independent manner is essential for elucidating its mechanism of action [64].

If a compound induces vasorelaxation even after endothelial removal, it suggests a direct interaction with vascular smooth muscle components, such as ion channels and indicates that the endothelium is not essential for its effect. In contrast, a loss or significant reduction of the compound's effect in endothelium-denuded vessels implies that the endothelium plays a necessary role in mediating its activity—most likely through the production of endothelial mediators like nitric oxide.

Moreover, the endothelial nitric oxide synthase (eNOS) enzyme is a key source of NO under physiological conditions. To specifically confirm NO involvement, pharmacological inhibitors such as L-NAME (a non-selective NOS inhibitor) and ODQ (a soluble guanylate cyclase inhibitor) can be used to block NO production or downstream signaling, respectively. Comparing vascular responses in the presence and absence of these inhibitors helps determine whether the NO pathway contributes to the compound's mechanism of action [65].

Experimental protocol

Mechanical removal of endothelium

Isolated vascular rings (e.g., aortic rings) are divided into two groups: Endothelium-intact and endothelium-denuded (mechanically removed by gentle rubbing of the luminal surface).

The contractile responses of both groups are compared in the presence of the test compound.

A significant difference in relaxation between the two indicates endothelium involvement.

Pharmacological inhibition of the NO pathway

Endothelium-intact rings are preincubated with L-NAME (100 μM) to inhibit NOS or ODQ (10 μM) to block guanylate cyclase.

After pretreatment, the relaxation response to the test compound is recorded.

A decrease in vasorelaxation following inhibitor application supports the role of the NO signaling pathway.

Validation with known agents

Acetylcholine is used as a positive control to confirm the presence of functional endothelium (induces NO-dependent relaxation).

Sodium nitroprusside (SNP) is used to assess the smooth muscle's sensitivity to NO donors, independent of endothelium.

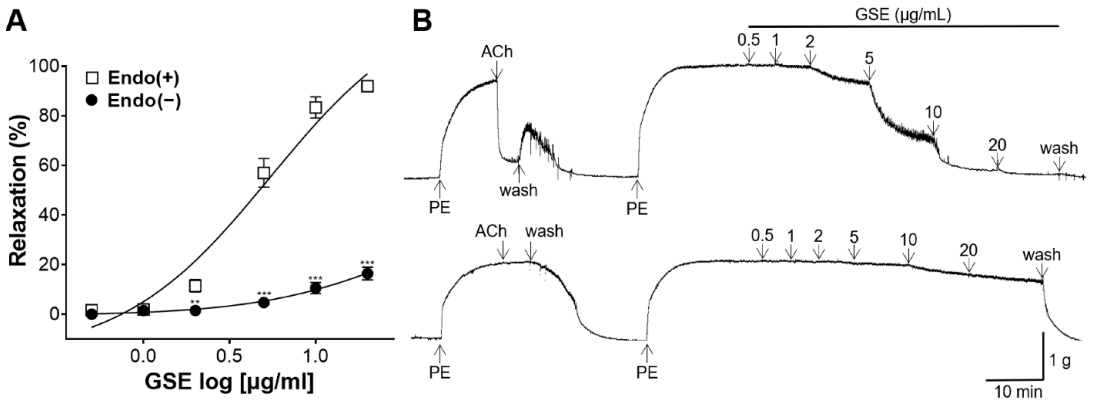

Figure 12 Endothelium-dependent vasorelaxant effects of grape seed extract (GSE) in phenylephrine (PE)-precontracted rat aortic rings. (A) Concentration–response curve showing GSE-induced relaxation in endothelium-intact [Endo(+), open squares] and endothelium-denuded [Endo(–), filled circles] aortic rings. GSE produced significantly greater relaxation in Endo(+) rings, indicating endothelium-dependent effects. (B) Representative isometric tension recordings of aortic rings exposed to increasing concentrations of GSE (0.5 - 20 µg/mL), with precontraction induced by PE. The relaxation was more pronounced in endothelium-intact tissues. ACh = acetylcholine. Data are expressed as mean ± SEM; ****p-value* < 0.001 vs. Endo(–).

By integrating these experimental approaches, the endothelium-dependence of the compound's vascular effects can be clearly characterized, distinguishing between NO-mediated and endothelium-independent mechanisms.

Computational modeling of phytochemical–protein interactions

To elucidate the molecular mechanisms underlying the observed vasorelaxant effects of the investigated phytochemical compound, advanced *in silico* approaches, including molecular docking and molecular dynamics (MD) simulations, were employed (**Figure 13**). These computational strategies are critical for predicting the binding behavior, affinity, and stability of ligand–target interactions at the atomic level, thus offering a complementary and mechanistic perspective to experimental findings [66].

Rationale for computational modeling

Experimental data, while providing functional and physiological outcomes, often lack atomic-scale resolution of the interaction between bioactive molecules and their protein targets. Computational modeling bridges this gap by simulating molecular recognition processes and conformational changes that may occur upon ligand binding. Specifically, molecular docking predicts the most favorable binding pose of a ligand within a protein's active site and estimates the binding affinity through scoring functions. Molecular dynamics simulations, in turn, allow the study of the time-dependent behavior of the ligand–protein complex under near-physiological conditions, evaluating structural stability, flexibility, and key intermolecular interactions over time [67].

Such integrative modeling not only supports the identification of pharmacophoric features and potential binding residues but also aids in rational drug design, structure–activity relationship (SAR) exploration and in some cases, can replace time-consuming or technically challenging *in vitro* assays. For ion channels and transporters, which often involve dynamic conformational states, computational modeling is especially valuable for probing allosteric regulation and ion gating mechanisms.

Workflow overview

Ligand preparation

The chemical structure of the selected phytochemical was retrieved from the PubChem database in SDF or SMILES format. Using ChemDraw and Avogadro, the 2D structure was redrawn, converted into a 3D format and energy-minimized using a force field (MMFF94 or UFF). The molecule's protonation state at physiological pH (7.4) was adjusted using Open Babel to reflect its biologically active form

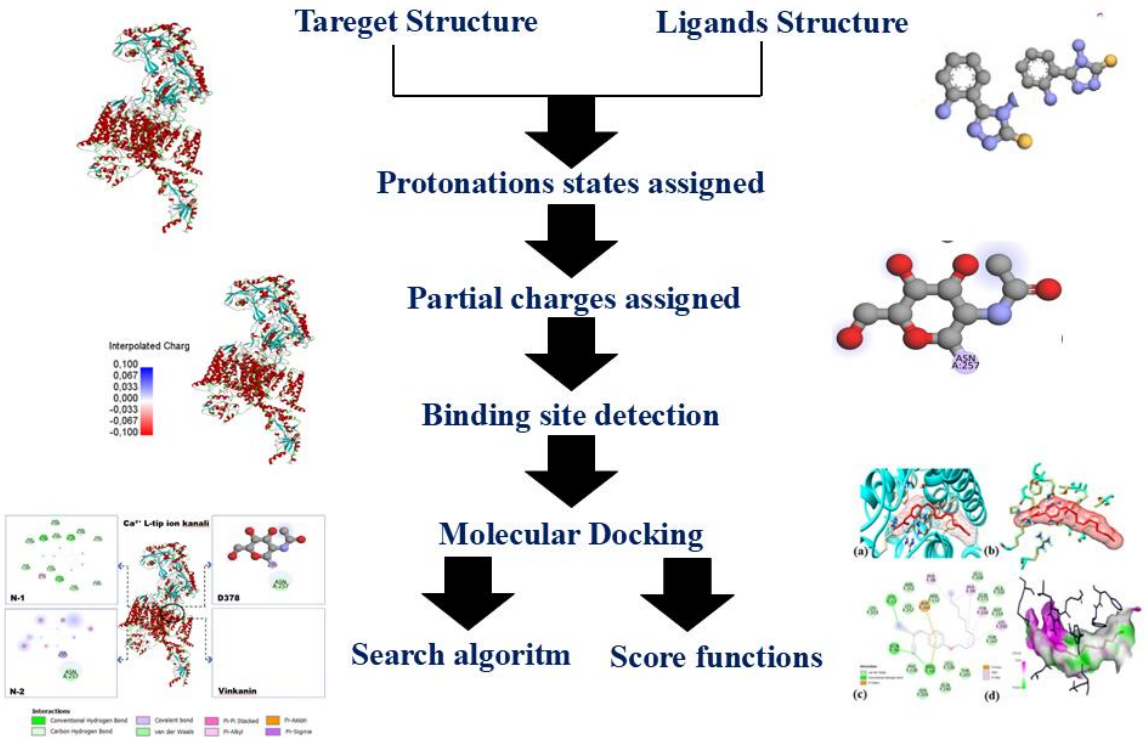

Figure 13 Schematic workflow of molecular docking analysis between protein targets and ligands. The process begins with the preparation of the target structure (e.g., Ca^{2+} L-type ion channel) and ligand molecules, followed by assignment of protonation states and partial charges. Binding site detection is then performed to identify potential interaction pockets. Molecular docking is carried out using specific search algorithms and scoring functions to predict optimal binding conformations. The final output includes interaction maps, docking poses and binding energy evaluations.

Target protein selection and preparation

High-resolution 3D structures of vascular ion channel proteins were obtained from the Protein Data Bank (PDB)

- L-type Ca^{2+} channel (PDB ID: 6JP5)
- R-type Ca^{2+} channel (PDB ID: 7MIY)
- Na^{+}/Ca^{2+} exchanger (NCX) (PDB ID: 3V5U)

Preprocessing was performed using PyMOL and Discovery Studio Visualizer: Water molecules, co-crystallized ligands and non-essential heteroatoms or chains were removed. Hydrogen atoms were added and Gasteiger charges were assigned via AutoDockTools [68].

Molecular docking

Docking simulations were conducted using AutoDock Vina. The grid box was centered on the known or predicted active/binding site of each protein, determined by prior literature or surface pocket analysis (e.g., using CASTp). Docking results were evaluated based on

- Binding affinity (kcal/mol)
- Orientation and conformation of the ligand
- Interaction residues (e.g., hydrogen bonds, π–π stacking, hydrophobic contacts)

Molecular dynamics simulations

To assess the stability and dynamics of the docked complexes, MD simulations were carried out using GROMACS or Desmond

- The protein–ligand complex was solvated in a TIP3P water box with Na^{+}/Cl^{-} ions for neutralization.
- Energy minimization followed by equilibration (NVT and NPT ensembles) was performed.
- A 50 - 100 ns production run was executed at 300 K and 1 atm.

- Analysis included RMSD, RMSF, radius of gyration (Rg) and hydrogen bonding profiles.

Significance

This comprehensive modeling approach provides valuable mechanistic insights into how the phytochemical may regulate vascular tone by directly interacting with membrane-bound ion channels and exchangers. The docking results identify potential binding residues and energetics, while MD simulations confirm the stability, flexibility and durability of these interactions under dynamic conditions. Together, these findings support the hypothesis that modulation of intracellular Ca^{2+} dynamics—critical for smooth muscle contraction—is mediated, at least in part, by specific ligand–channel interactions [69].

Moreover, this *in silico* strategy lays the groundwork for future structure-guided optimization of phytochemical scaffolds and opens avenues for developing more potent and selective cardiovascular agents derived from natural products [70].

Discussion (Add several references on these discussion session)

Integrative analysis of vasorelaxant mechanisms in aortic smooth muscle

The present study employed a comprehensive experimental framework to elucidate the molecular mechanisms underlying vasorelaxant activity in isolated aortic smooth muscle preparations. Through systematic investigation of multiple calcium handling pathways, ion transport systems and receptor-mediated signaling cascades, we have gained valuable insights into the complex regulatory networks that govern vascular tone and the potential therapeutic targets for cardiovascular intervention.

Voltage-dependent calcium channel modulation

The depolarization-induced contraction experiments using high K^+ medium provided fundamental evidence for the involvement of L-type voltage-dependent calcium channels (VDCCs) in mediating the vasorelaxant effects observed. The ability of test compounds to attenuate KCl-induced contractions suggests direct or indirect modulation of calcium influx through these channels, which represent the primary pathway for excitation-contraction coupling in vascular smooth muscle.

The comparative analysis with established calcium channel blockers such as verapamil offered critical mechanistic insights. When test compounds demonstrated enhanced inhibitory effects beyond those observed with verapamil alone, this indicated potential multi-target activity involving additional ion channels or downstream signaling pathways. This finding is particularly significant given that many clinically effective antihypertensive agents exhibit pleiotropic effects on multiple calcium handling mechanisms, contributing to their therapeutic efficacy. While verapamil and nifedipine serve as reliable blockers in this study, their use as sole controls may have limitations in clinical applications, such as differing pharmacokinetics or side effects in long-term use. Future studies should consider alternative calcium channel blockers or multimodal therapeutic agents for a more comprehensive

The cumulative calcium reintroduction protocol under Ca^{2+}-free conditions further validated the extracellular calcium dependence of the observed effects. The rightward shift in $CaCl_2$ concentration-response curves in the presence of test compounds confirmed their ability to interfere with calcium influx mechanisms, providing quantitative evidence for VOCC modulation. This approach effectively distinguished between calcium channel blockade and other potential mechanisms such as intracellular calcium mobilization or myofilament sensitivity modulation.

GPCR-mediated signaling and receptor-operated channels

The investigation of phenylephrine-induced contractions revealed important insights into G protein-coupled receptor (GPCR)-mediated calcium signaling pathways. The rapid onset and sustained nature of α_1-adrenergic receptor-mediated contractions highlighted the efficiency of the Gq/PLC/IP_3 signaling cascade in mobilizing intracellular calcium stores. The ability of test compounds to modulate these responses suggests potential interactions with ROCCs or downstream signaling components.

The use of adrenergic receptor blockers, particularly phentolamine, provided mechanistic clarity by distinguishing between receptor-dependent and

receptor-independent effects. When test compounds retained activity in the presence of α-adrenergic blockade, this indicated direct effects on smooth muscle ion channels or intracellular signaling pathways, independent of receptor activation. Conversely, compounds that lost activity following receptor blockade likely exerted their effects through modulation of receptor-mediated pathways.

The parallel experiments conducted under Ca^{2+}-free conditions were particularly informative in dissecting the relative contributions of extracellular calcium influx versus intracellular calcium mobilization. The preservation of contractile responses to phenylephrine in the absence of extracellular calcium confirmed the integrity of IP_3-mediated calcium release from sarcoplasmic reticulum stores, while the differential effects of test compounds under these conditions provided insights into their specific targets within the calcium signaling cascade.

Intracellular calcium handling mechanisms

The investigation of SERCA pump function using thapsigargin revealed critical aspects of intracellular calcium homeostasis in vascular smooth muscle. The prolonged contractile responses observed following SERCA inhibition emphasized the importance of calcium reuptake mechanisms in terminating smooth muscle contraction. Test compounds that could attenuate thapsigargin-induced contractile prolongation suggested their ability to enhance alternative calcium clearance mechanisms or modulate calcium release from intracellular stores.

The ryanodine receptor (RyR) studies using caffeine as a pharmacological tool provided insights into calcium-induced calcium release mechanisms. The differential responses observed under Ca^{2+}-containing versus Ca^{2+}-free conditions helped distinguish between effects on calcium influx and intracellular calcium mobilization. Compounds that specifically modulated caffeine-induced responses in Ca^{2+}-free conditions demonstrated direct effects on RyR function or associated calcium handling proteins.

These findings highlight the complexity of intracellular calcium dynamics in vascular smooth muscle and suggest that effective vasorelaxant agents may need to target multiple components of the calcium handling machinery to achieve optimal therapeutic effects. The ability to modulate both calcium influx and intracellular calcium cycling may contribute to the sustained vasorelaxant effects observed with certain natural compounds.

Ion exchange and transport mechanisms

The investigation of Na^+/Ca^{2+} exchanger (NCX) function revealed an important secondary mechanism for calcium regulation in vascular smooth muscle. The use of Na^+-free solutions to promote reverse NCX operation demonstrated the potential for this transporter to contribute to calcium influx under pathophysiological conditions. Test compounds that modulated NCX-mediated contractions suggested their ability to influence this critical calcium transport mechanism.

The Na^+/K^+-ATPase studies using ouabain provided insights into the indirect regulation of calcium homeostasis through sodium gradient manipulation. The enhanced contractile responses observed following Na^+/K^+-ATPase inhibition highlighted the importance of maintaining proper electrochemical gradients for optimal NCX function. Compounds that could counteract ouabain-induced effects demonstrated their potential to restore normal ion transport function.

These findings emphasize the interconnected nature of ion transport systems in vascular smooth muscle and suggest that therapeutic interventions targeting these pathways may offer novel approaches for cardiovascular disease management. The ability to modulate multiple ion transport mechanisms simultaneously may contribute to the polypharmacological effects observed with certain natural products.

Endothelium-dependent versus independent mechanisms

The comparative studies using endothelium-intact and endothelium-denuded preparations provided crucial insights into the site of action of vasorelaxant compounds. The preservation of vasorelaxant activity following endothelial removal indicated direct effects on vascular smooth muscle, while compounds that lost activity suggested endothelium-dependent mechanisms involving nitric oxide or other endothelial mediators.

The use of NOS inhibitors such as L-NAME and guanylate cyclase inhibitors like ODQ provided mechanistic clarity regarding the involvement of the

NO-cGMP pathway. Compounds that retained activity in the presence of these inhibitors demonstrated endothelium-independent mechanisms, while those that lost activity confirmed their dependence on endothelial NO production.

These findings are particularly relevant for understanding the therapeutic potential of vasorelaxant compounds, as endothelium-independent mechanisms may be more effective in pathological conditions characterized by endothelial dysfunction, such as hypertension, diabetes and atherosclerosis.

Computational modeling insights

The integration of molecular docking and molecular dynamics simulations provided valuable atomic-level insights into the potential binding interactions between test compounds and their protein targets. The identification of specific binding residues and interaction patterns offered mechanistic explanations for the observed pharmacological effects and suggested potential sites for structure-activity relationship optimization.

The computational predictions regarding binding affinity and complex stability correlated well with experimental IC_{50} values and duration of action, validating the utility of *in silico* approaches in natural product drug discovery. The identification of multiple potential binding sites on different ion channels supported the hypothesis of multi-target activity, which may contribute to the overall therapeutic efficacy of natural vasorelaxant compounds.

Clinical implications and therapeutic potential

The comprehensive mechanistic characterization presented in this study has important implications for the development of novel cardiovascular therapeutics. The identification of multiple molecular targets suggests that natural vasorelaxant compounds may offer advantages over single-target synthetic drugs through their ability to modulate multiple aspects of vascular function simultaneously.

The demonstration of both calcium channel blocking activity and intracellular calcium handling modulation suggests potential applications in conditions requiring sustained vasodilation, such as hypertension and coronary artery disease. The endothelium-independent mechanisms identified may be particularly valuable in pathological conditions characterized by endothelial dysfunction.

Limitations and future directions

While the present study provides comprehensive mechanistic insights, certain limitations should be acknowledged. The use of isolated tissue preparations, while providing controlled experimental conditions, may not fully recapitulate the complex *in vivo* environment. Future studies should include *in vivo* validation of the identified mechanisms and assessment of potential drug interactions.

The computational modeling, while providing valuable structural insights, relies on static protein structures that may not fully represent the dynamic conformational changes occurring during ion channel gating. Advanced simulation techniques incorporating channel dynamics may provide more accurate predictions of ligand-channel interactions.

Conclusions

This comprehensive mechanistic study has successfully identified multiple molecular targets and pathways involved in vasorelaxant activity in aortic smooth muscle. The integration of functional pharmacology, molecular biology and computational approaches has provided a detailed understanding of the complex mechanisms governing vascular tone regulation. These findings lay the foundation for rational drug design approaches and support the continued investigation of natural products as sources of novel cardiovascular therapeutics. The multi-target nature of the identified mechanisms suggests that natural vasorelaxant compounds may offer therapeutic advantages through their ability to modulate multiple aspects of vascular function simultaneously, potentially leading to more effective and better-tolerated cardiovascular medications.

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Declaration of generative AI in scientific writing

OpenAI is used for improving grammar of the manuscript

CRediT author statement

Sirojiddin Omonturdiev: Conceptualization, Supervision, **Izzatullo Abdullaev**: Writing - Original Draft Preparation, **Alikhon Khasanov, Azizbek Abdullaev** - Data Visualization, Graph Preparation, **Ulugbek Gayibov, Kuzijon Baratov** - Project Idea, Conceptual Input, **Sabina Gayibova, Inomjonov Dolimjon, Mirtaza Allamuratov** - Supervision, Review & Editing, **Yuludzkon Mirzaeva**, **Pulat Usmonov** - Supervision, Methodological Oversight, **Takhir Aripov** - Supervision, Scientific Guidance.

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