



The turmeric polyphenol calebin A relaxes rat aorta through $\text{Ca}_v1.2$ channel inhibition with evidence for $\text{K}_{\text{Ca}1.1}$ channel involvement

Francesca De Rosa^a, Francesco Pettini^b, Ottavia Spiga^{a,c,*}, Atanas G. Atanasov^{d,e,f,g}, Fabio Fusi^{a,*}

^a Department of Biotechnology, Chemistry, and Pharmacy, University of Siena, Via A. Moro 2, 53100 Siena, Italy

^b School of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy

^c Centro della Scienza e della Tecnica Laboratorio Congiunto, Fondazione Polo Universitario Grossetano and University of Siena, Italy

^d Patient Safety & Digital Health (PaDiH) Group, Danube Private University, Fakultät Medizin/Zahnmedizin, Steiner Landstraße 124, Krems-Stein 3500, Austria

^e Department of Biochemistry, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, India

^f Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzebiec, 05-552 Magdalenka, Poland

^g Ludwig Boltzmann Institute Digital Health and Patient Safety, Medical University of Vienna, Spitalgasse 23, 1090 Vienna, Austria

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ABSTRACT

Calebin A, a naturally occurring polyphenol isolated from *Curcuma longa*, has attracted attention for its diverse pharmacological activities, although its vascular effects remain poorly defined. The present study investigated the vasorelaxant properties of calebin A in isolated rat blood vessels and explored the underlying molecular mechanisms using electrophysiological, functional, and computational approaches. Whole-cell patch-clamp recordings in rat tail artery smooth muscle cells showed that calebin A reduced Ba^{2+} currents through $\text{Ca}_v1.2$ channels in a concentration-dependent manner, accelerating current decay without significantly affecting voltage-dependent activation. Under experimental conditions favouring $\text{K}_{\text{Ca}1.1}$ current recording, calebin A also enhanced TEA-sensitive outward currents and modified spontaneous transient $\text{K}_{\text{Ca}1.1}$ current activity. Computational docking analyses identified plausible binding sites for calebin A on both $\text{Ca}_v1.2$ and $\text{K}_{\text{Ca}1.1}$ channels. Functional studies demonstrated concentration-dependent, endothelium-independent relaxation of phenylephrine-contracted thoracic aorta rings. Vasorelaxation was reduced under high extracellular K^+ conditions, consistent with a contribution of K^+ channel-dependent mechanisms. Moreover, calebin A significantly attenuated the contractile response elicited by restoration of extracellular Ca^{2+} influx under Ca^{2+} -free conditions, whereas it did not significantly modify the contraction attributed to phenylephrine-induced intracellular Ca^{2+} release. Together, these findings support inhibition of $\text{Ca}_v1.2$ channels as a major mechanism underlying calebin A-induced vasorelaxation and support a contributory role for $\text{K}_{\text{Ca}1.1}$ -dependent pathways, whose precise molecular basis requires further investigation. This dual pharmacological profile identifies calebin A as a promising template for the development of innovative vasorelaxant compounds acting through complementary mechanisms.

1. Introduction

Curcuma longa L. (turmeric), a member of the Zingiberaceae family, is extensively cultivated throughout tropical and subtropical areas of Asia, South America, and Australia. Besides its widespread use as a culinary spice and natural colouring agent, turmeric is recognised as an abundant source of bioactive phytochemicals. Its rhizome contains

numerous constituents, including curcuminoids, terpenoids, cinnamic acid derivatives, and other phenolic compounds that collectively account for many of its biological effects. Several investigations have attributed antioxidant, antimicrobial, anti-inflammatory, antitumour, antimutagenic, hypolipidaemic, and anti-ageing activities to turmeric extracts and isolated constituents [1]. Among these molecules, curcumin together with demethoxycurcumin and bisdemethoxycurcumin has

Abbreviations: $\text{I}_{\text{Ba}1.2}$, Ba^{2+} current through $\text{Ca}_v1.2$ channel; $\text{I}_{\text{KCa}1.1}$, K^+ current through $\text{K}_{\text{Ca}1.1}$ channel; KHS, Krebs-Henseleit solution; STOC, spontaneous transient $\text{I}_{\text{KCa}1.1}$; TEA, tetraethylammonium; V_h , holding potential; τ , time constant.

* Corresponding authors at: Department of Biotechnology, Chemistry, and Pharmacy, University of Siena, Via A. Moro 2, 53100 Siena, Italy.

E-mail addresses: ottavia.spiga@unisi.it (O. Spiga), fabio.fusi@unisi.it (F. Fusi).

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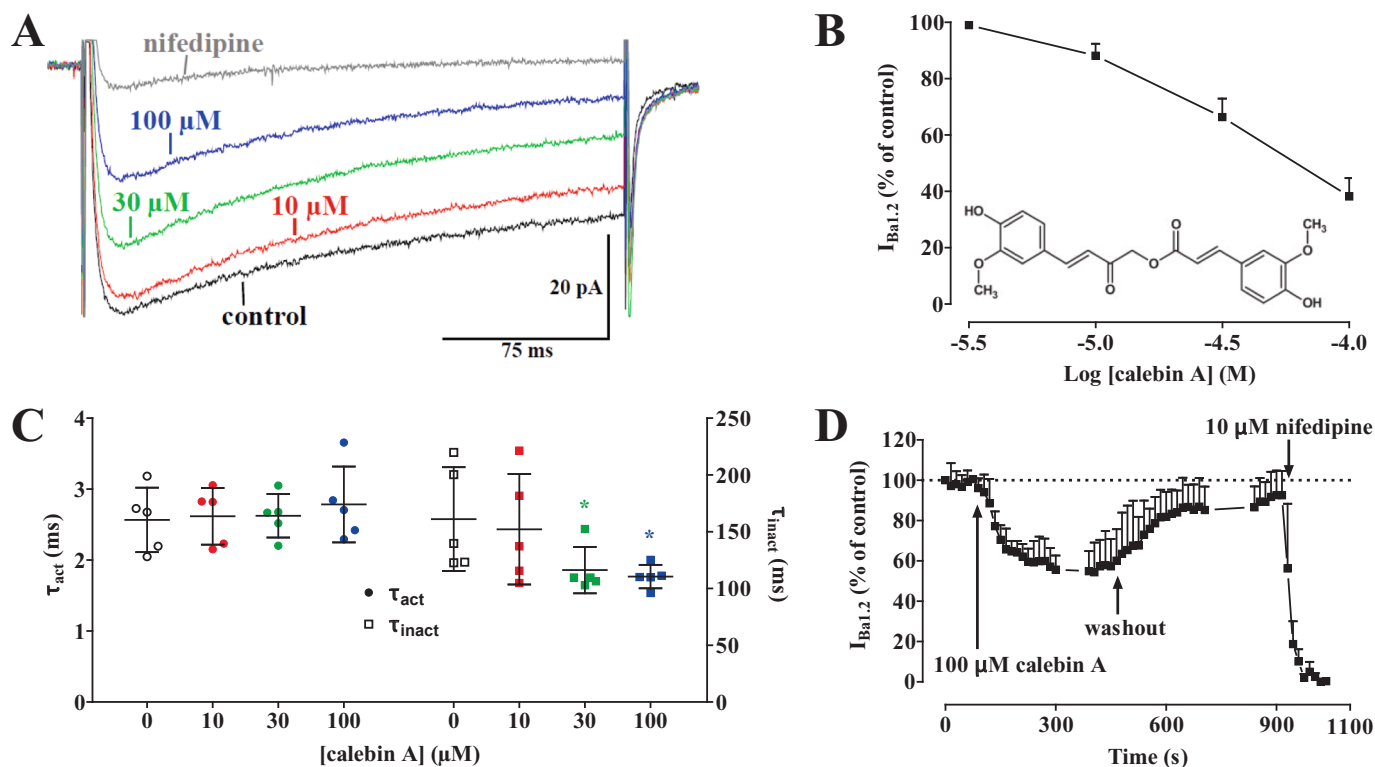


Fig. 1. Effects of calebin A on $I_{Ba1.2}$ in rat tail artery smooth muscle cells. (A) Representative averaged recordings ($n = 5$) of whole-cell $I_{Ba1.2}$ evoked by 250-ms depolarising pulses from a V_h of -50 mV to 10 mV under control conditions and after cumulative administration of calebin A. Inhibition produced by 10 μ M nifedipine is also illustrated: the remaining current represents the leak component. (B) Concentration-response relationship describing the inhibitory effect of calebin A on peak $I_{Ba1.2}$. The leak-subtracted current values are expressed as percentages of the corresponding control response. Data points represent the mean $\pm$ SD ($n = 5$). Inset shows the chemical structure of calebin A. (C) Influence of calebin A on activation (τ_{act}) and inactivation (τ_{inact}) kinetics of $I_{Ba1.2}$. Horizontal lines represent the mean $\pm$ SD ($n = 5$). * $P < 0.05$ vs control (0), repeated measures ANOVA and Dunnett post-test. (D) Representative time course of the effect induced by 100 μ M calebin A on $I_{Ba1.2}$. Currents were elicited every 15 s by depolarising pulses from -50 mV to 10 mV, applied every 15 s (0.067 Hz). The leak-subtracted current values were normalised to those recorded immediately before drug application. Recovery after washout and subsequent inhibition by 10 μ M nifedipine are also shown. Data points represent the mean $\pm$ SD ($n = 5$).

received particular attention because of their strong antioxidant and cytoprotective actions [2,3].

In addition to the major curcuminoids, turmeric also contains less abundant compounds with relevant pharmacological potential. One such molecule is calebin A, chemically identified as [(E)-4-(4-hydroxy-3-methoxyphenyl)-2-oxobut-3-enyl] (E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-en-1-ol. Previous studies indicate that calebin A shares several biological activities with curcumin. In particular, the compound has demonstrated antineoplastic properties in different experimental models by limiting cell proliferation and migration while promoting apoptotic pathways. Calebin A has also been shown to decrease tumour growth in xenograft models and to potentiate the efficacy of selected anticancer therapies [4,5].

The biological relevance of calebin A extends beyond oncology. Experimental evidence suggests anti-inflammatory, neuroprotective, anti-obesity, antidiabetic, and antioxidant effects [6,7]. The anti-inflammatory action appears to involve suppression of NF- κ B signalling together with inhibition of TNF- α production [5]. Neuroprotective activity has been associated with reduced β -amyloid accumulation in neuronal tissue, supporting the hypothesis that calebin A may represent a useful candidate for neurodegenerative disorders such as Alzheimer's disease [8]. Additional reports indicate beneficial effects on obesity-related metabolic disturbances and hepatic steatosis [9]. Importantly, oral administration of calebin A at 100 mg/kg/day did not produce significant toxicity, although its clinical development is limited by poor pharmacokinetic characteristics. Because the molecule is present only in trace amounts within turmeric rhizomes, large-scale production generally relies on chemical synthesis or enzymatic conversion from curcumin

[10].

Despite the growing interest in the pharmacology of calebin A, its effects on the cardiovascular system remain insufficiently explored. In particular, no detailed information is currently available regarding its direct influence on vascular smooth muscle ion channels, which are central regulators of arterial tone. Therefore, the present investigation aimed to characterise the vasorelaxant properties of calebin A and to identify the mechanisms underlying its vascular actions. To address this objective, we combined electrophysiological experiments, *ex vivo* vascular reactivity studies, and computational modelling approaches. Whole-cell patch-clamp recordings were carried out in rat tail artery smooth muscle cells to assess the effects of calebin A on $Ca_v1.2$ and $K_{Ca1.1}$ channels, while isolated rat thoracic aorta rings were used to evaluate vascular responses. Structural docking analyses were additionally employed to predict possible binding interactions between the compound and the ion channels under investigation. Collectively, the results demonstrate that calebin A is a vasodilator capable of suppressing Ca^{2+} entry through $Ca_v1.2$ channels in vascular smooth muscle; findings also support the involvement of $K_{Ca1.1}$ channel-dependent mechanisms.

2. Materials and methods

2.1. Animals

All experimental procedures complied with the European Union Guidelines for the Care and Use of Laboratory Animals (European Union Directive 2010/63/EU) and received approval from both the Animal

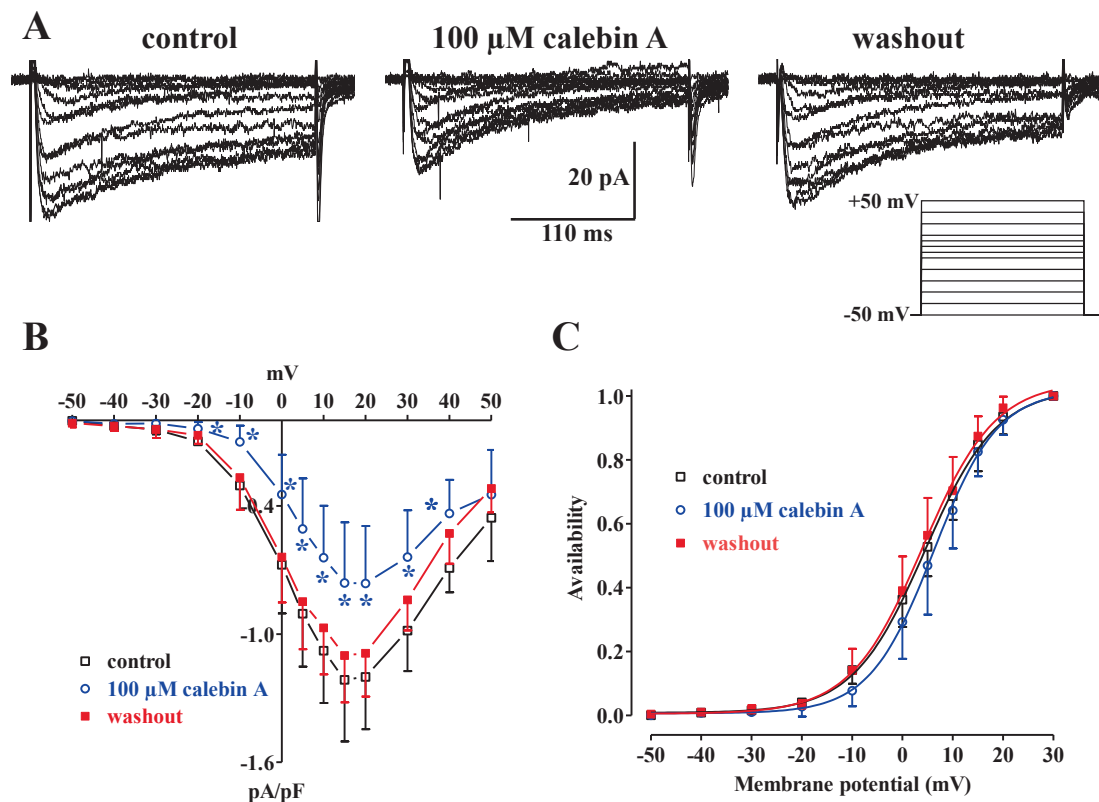


Fig. 2. Influence of calebin A on current-voltage relationships and activation properties of $I_{Ba1.2}$. (A) Recordings from one myocyte (representative of other four cells) of whole-cell $I_{Ba1.2}$ evoked by 250-ms depolarising pulses from a V_h of -50 mV to various voltages (increments 5–10 mV, see inset) under control conditions, in the presence of $100 \mu\text{M}$ calebin A, and after washout. (B) Current-voltage curves obtained from tail artery myocytes before exposure to calebin A, during application of $100 \mu\text{M}$ calebin A, and after washout. Membrane currents were elicited from a V_h of -50 mV. Data points represent the mean $\pm$ SD ($n = 5$). * $P < 0.05$ vs control, repeated measures ANOVA and Dunnett post-test. (C) Steady-state activation curves derived from the current-voltage relationships shown in panel B and fitted with the Boltzmann equation. Channel availability is expressed as fractional activation, where 1 corresponds to maximal channel opening. Data points represent the mean $\pm$ SD ($n = 5$).

Care and Ethics Committee of the University of Siena and the Italian Department of Health (7DF19.N.SJQ). Male Wistar rats weighing 250–350 g and aged 8–10 weeks were obtained from Charles River Italia (Calco, Italy). Male animals were selected to minimise variability associated with hormonal fluctuations during the oestrous cycle. Nevertheless, we recognise that future studies should include both sexes to evaluate potential sex-dependent differences. Animals were housed under controlled environmental conditions ($25 \pm 1^\circ\text{C}$; 12-h light/dark cycle) with unrestricted access to water and standard laboratory chow. Anaesthesia was induced with 4% isoflurane in oxygen using a Fluovac apparatus (Harvard Apparatus, Holliston, MA, USA). Following decapitation and exsanguination, the thoracic aorta and tail main artery were rapidly excised and transferred into modified Krebs-Henseleit solution (KHS) or external solution, respectively.

2.2. Isolation of vascular smooth muscle cells

Smooth muscle cells were enzymatically dissociated from rat tail main arteries at 37°C . Arterial segments were incubated for 40–45 min in 2 mL of external solution containing collagenase type XI (1.1 mg mL^{-1}), bovine serum albumin (1 mg mL^{-1}), and trypsin inhibitor (1 mg mL^{-1}). The external solution consisted of (mM): 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 MgCl_2 , and 5 Na-pyruvate, adjusted to pH 7.4 with NaOH. The digestion medium additionally contained taurine (20 mM) and Ca^{2+} (0.1 mM) and was moderately oxygenated with 95% O_2 and 5% CO_2 , according to previously reported procedures [11]. Following isolation, cells were maintained at 4°C in external solution supplemented with 0.05 mM Ca^{2+} , 20 mM taurine, and 0.5 mg mL^{-1}

bovine serum albumin under atmospheric air conditions. Experiments were performed within 48 h after cell isolation [12].

2.3. Recording of Ba^{2+} currents through $\text{Ca}_v1.2$ channels

Whole-cell patch-clamp recordings were carried out in freshly isolated tail artery myocytes to evaluate Ba^{2+} currents through $\text{Ca}_v1.2$ channels ($I_{Ba1.2}$). Patch electrodes fabricated from borosilicate glass (WPI, Berlin, Germany) exhibited resistances of 2–4 M Ω after filling with internal solution containing (mM): 100 CsCl, 10 HEPES, 11 EGTA, 1 CaCl_2 (pCa 8.4), 2 $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 5 Na-pyruvate, 5 succinic acid, 5 oxaloacetic acid, 3 $\text{Na}_2\text{-ATP}$, and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), which was also used for pulse generation, compensation of whole-cell capacitance and series resistance (70–75%), and cancellation of junction potentials. Signals were low-pass filtered at 1 kHz, digitised at 3 kHz, and stored for offline analysis. Cells were continuously superfused at room temperature ($20\text{--}22^\circ\text{C}$) with external solution containing 0.1 mM Ca^{2+} and 30 mM tetraethylammonium (TEA) delivered through a peristaltic pump (LKB 2132, Bromma, Sweden) at $400 \mu\text{L min}^{-1}$.

To record $I_{Ba1.2}$, extracellular Ca^{2+} was replaced with 5 mM Ba^{2+} in the presence of 30 mM TEA. Currents were elicited every 15 s (0.067 Hz) by 250-ms depolarising pulses to 10 mV from a holding potential (V_h) of -50 mV. Experimental protocols were initiated only after current stabilisation, which generally occurred 7–10 min after membrane rupture. Under these conditions, current amplitude remained stable for at least

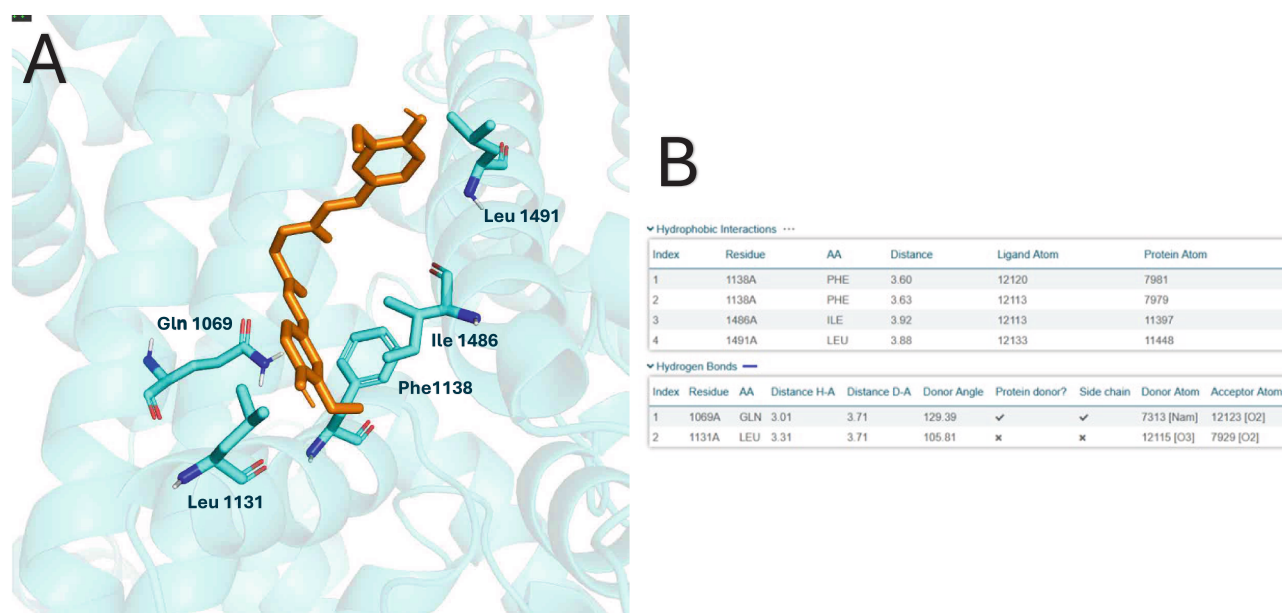


Fig. 3. Docking poses and interaction analysis of calebin A in Cav1.2 channels. (A) Predicted binding mode of calebin A (orange sticks) within the Cav1.2 channel binding pocket (protein shown as teal ribbons and sticks) obtained from HADDOCK docking calculations. The best-ranked complex displayed a moderately favourable HADDOCK score (-17.60). The ligand is accommodated within a predominantly hydrophobic pocket, establishing contacts with residues such as Phe1138, Ile1486, and Leu1491, while Gln1069 forms a stabilising hydrogen bond and Leu1131 contributes weaker polar interactions. (B) Interaction profiles of residues involved in ligand binding obtained from the best docking poses using the PLIP tool. In Cav1.2 channels, calebin A establishes mainly hydrophobic contacts with residues Phe1138, Ile1486, and Leu1491, indicating a predominantly hydrophobic binding pocket accommodating the ligand scaffold. Hydrogen-bond analysis reveals limited polar interactions, with Gln1069 forming a stabilising side-chain hydrogen bond and Leu1131 contributing a weaker interaction.

30–40 min [13].

Outward K^+ currents were blocked by including TEA in the extracellular solution and Cs^+ in the pipette solution. Residual leak and outward components were subtracted offline using 10 μM nifedipine, which completely abolished $I_{Ba1.2}$. Therefore, the Figures display only leak-subtracted currents.

A pulse duration equal to three times the time constant (τ) is ideal for evaluating complete steady-state inactivation. However, to minimise current rundown and preserve recording stability, a 250-ms pulse duration, routinely used in our laboratory and in previous studies (e.g., [14]), was applied. Therefore, the values calculated represent the apparent τ of inactivation (τ_{inact}), which nevertheless remains a sensitive indicator of the kinetic effects of the compound.

Steady-state activation curves were derived from the current–voltage relationships. Conductance (G) was calculated from the equation $G = I_{Ba1.2}/(E_m - E_{rev})$, where $I_{Ba1.2}$ is the peak current elicited by depolarising test pulses between -50 mV and 30 mV from a V_h of -50 mV; E_m is the membrane potential; and E_{rev} is the reversal potential (estimated from the extrapolated current–voltage curves in Fig. 2B). G_{max} is the maximal Ba^{2+} conductance (calculated at potentials ≤ 30 mV). The G/G_{max} ratio (availability, i.e., the number of channels available to open at fixed membrane potential values, with 1 indicating 100%) was plotted against the membrane potential and fitted to the Boltzmann equation [15].

2.4. Recording of K^+ currents through $K_{Ca1.1}$ channels

Whole-cell patch-clamp experiments were also performed to measure $K_{Ca1.1}$ channel-mediated K^+ currents ($I_{KCa1.1}$) in freshly isolated tail artery myocytes. The external solution contained (mM): 145 NaCl, 6 KCl, 10 glucose, 10 HEPES, 5 Na-pyruvate, 1.2 $MgCl_2$, 0.1 $CaCl_2$, 0.003 nifedipine; pH was adjusted to 7.4 with NaOH. The pipette solution consisted of (mM): 90 KCl, 10 NaCl, 10 HEPES, 10 EGTA, 1 $MgCl_2$, 6.41 $CaCl_2$ (pCa 7.0); pH was adjusted to 7.4 with KOH. Currents were recorded during 500-ms depolarising pulses from -40 mV to avoid activation of voltage-gated K^+ channels. Stable current amplitudes were

generally achieved within 6–10 min and remained unchanged for 20–30 min [16]. Residual leak and currents other than $I_{KCa1.1}$ components were subtracted offline using 1 mM TEA, a concentration widely recognised as selective towards $K_{Ca1.1}$ channels [17,18].

For spontaneous transient $I_{KCa1.1}$ (STOC) recordings, the internal solution contained (mM): 140 KCl, 10 HEPES, 0.1 EGTA, 1.9 $MgCl_2$, 2 Na_2ATP , 0.037 $CaCl_2$ (pCa 7.29); pH was adjusted to 7.3 with KOH.

2.5. Preparation of thoracic aorta rings

Thoracic aortae were dissected free of connective and adipose tissue and sectioned into rings approximately 3–4 mm in length. Rings were mounted in organ baths between two stainless-steel hooks, one fixed and the other connected to an isometric force transducer (BLPR, WPI, Berlin, Germany) [19]. Preparations were equilibrated for 60 min in KHS under a passive tension of 2 g, with replacement of the bathing solution every 15 min.

Changes in isometric force were monitored using a PowerLab 8/30 acquisition system (ADInstruments). Tissue viability was verified by exposure to 0.3 μM phenylephrine and 60 mM KCl. When required, endothelial cells were mechanically removed by gently rubbing the luminal surface with forceps. Successful removal of the endothelium was confirmed by testing the relaxant response to 10 μM acetylcholine during 0.3 μM phenylephrine-induced contraction. Relaxation $\geq 75\%$ indicated functional endothelium, whereas relaxation $< 15\%$ confirmed endothelial removal [19].

2.6. Evaluation of vasorelaxant responses induced by calebin A

Thoracic aorta rings were contracted either pharmacomechanically with 0.3 μM phenylephrine or electromechanically with 60 mM KCl. Phenylephrine experiments were conducted in both endothelium-intact and endothelium-denuded tissues, whereas KCl experiments were performed only in preparations lacking endothelium. After stable contraction was achieved, calebin A was cumulatively added to the organ bath,

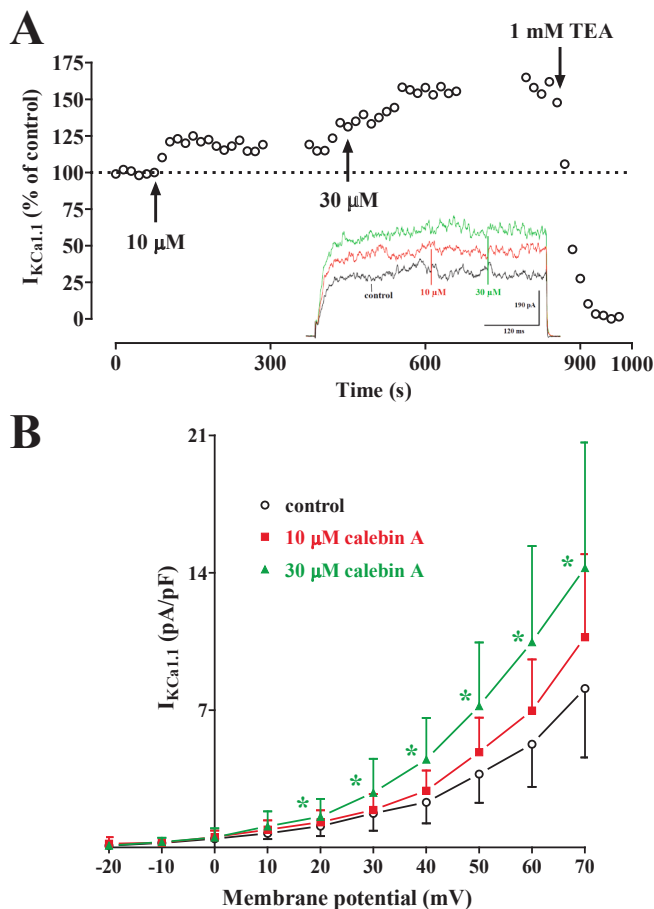


Fig. 4. Stimulatory effect of calebin A on TEA-sensitive outward currents in tail artery myocytes. (A) Representative time course (from one of eight cells analysed) showing the increase in TEA-sensitive outward currents induced by calebin A. Currents were evoked every 15 s by depolarising steps to 70 mV from a V_h of -40 mV. Current amplitudes are expressed relative to baseline values measured before compound application. The inhibitory effect of 1 mM TEA is also illustrated. Inset: representative current traces recorded under control conditions and after exposure to 10 μ M and 30 μ M calebin A. (B) Current-voltage relationships measured before and during cumulative application of calebin A. Current amplitudes are reported as current density (pA/pF). Data points are mean $\pm$ SD ($n = 9$). * P < 0.05 vs control, repeated measures ANOVA and Dunnett post-test.

and vasorelaxation was expressed as a percentage reversal of the initial contractile response.

In selected experiments, the same ring was exposed to two consecutive stimulations with either 25/30 mM or 60 mM KCl. The first stimulation was performed in the presence of vehicle alone, whereas the second was preceded by a 6-min incubation with calebin A [13].

At the conclusion of each experiment, maximal smooth muscle relaxation was verified by the addition of sodium nitroprusside (100 μ M). In KCl-contracted rings, nifedipine (1 μ M) was administered before sodium nitroprusside.

2.7. Assessment of intracellular Ca^{2+} release and extracellular Ca^{2+} entry

To distinguish between intracellular Ca^{2+} mobilisation and extracellular Ca^{2+} influx, endothelium-denuded rings were incubated with calebin A for 30 min in normal KHS, which, in the last 5 min, was replaced by a Ca^{2+} -free KHS containing 1 mM EGTA. Phenylephrine (10 μ M) was then added to evoke contraction attributable to IP_3 -induced Ca^{2+} release from intracellular stores. Reintroduction of 3.5 mM

extracellular Ca^{2+} generated a second contractile response corresponding to Ca^{2+} influx across the plasma membrane [20]. Because a higher phenylephrine concentration was required under Ca^{2+} -free conditions, responses were normalised to the reference contraction elicited by 60 mM KCl.

2.8. Drugs and reagents

The commercially available, chemically defined, synthetic calebin A (>99% purity) was kindly provided by Sabinsa Corporation (East Windsor, NJ, USA; <https://sabinsa.com/product/phytochemicals-for-nutritional-use>). Additional reagents included collagenase type XI, trypsin inhibitor, bovine serum albumin, TEA chloride, phenylephrine, acetylcholine, and nifedipine (Sigma Chimica, Milan, Italy), as well as sodium nitroprusside (Riedel-De Haën AG, Seelze-Hannover, Germany). All remaining chemicals were analytical grade. Phenylephrine stock solution was prepared in 0.1 M HCl. Nifedipine and calebin A were dissolved in ethanol and DMSO, respectively, and diluted at least 1:1000 before use. Control experiments demonstrated that the final solvent concentration (0.1% v/v) did not affect vascular responses.

2.9. Statistical analysis

Electrophysiological and functional data were analysed using pClamp 9.2.1.8 (Molecular Devices), LabChart 7.3.7 Pro (ADInstruments), and GraphPad Prism 5.04 (GraphPad Software Inc., Boston, MA, USA). Results are expressed as mean $\pm$ SD, with n representing the number of independent experiments. Data distribution was assessed before statistical testing, and comparisons were performed using the tests indicated in the corresponding Figure legends. Differences were considered statistically significant when P < 0.05. Drug potency (IC_{50}) and maximal effect (E_{max}) were determined by nonlinear regression using the “log inhibitor versus normalised response – variable slope” model without constraints.

2.10. Computational methods

The atomic structure of $Ca_v1.2$ and $K_{Ca1.1}$ channels was retrieved from the AlphaFold Database (ID: AF-P22002-F1) [21] and a previous study by Carullo et al. [22], respectively. The chemical structure of calebin A was retrieved from the PubChem Database (CID 637429) (<https://pubchem.ncbi.nlm.nih.gov/compound/637429#section=3D-Conformer>; [23]) and parameterised using UCSF Chimera v1.17.3 [24] with Amber14SB forcefield [25]. Docking calculations were performed using HADDOCK v2.4 [26,27] considering as active residues the residues identified as part of the binding sites for modulator compounds by Ahmed et al. [28] ($Ca_v1.2$ channel: Phe778B, Gln1069C, Ser1141C, Tyr1178C, Ile1179C, Ala1183C, Phe1191C, Phe1143C, and Tyr1489C; $K_{Ca1.1}$ channel: Lys640, Asn722, Glu1388, Lys1404, Phe1405, Phe1409, Thr1410, Val1412, Glu1413, and Phe1414). Default settings for small-molecule docking were used both for data production and clustering. The interaction profile of residues involved in the binding to the active site was investigated on the best pose of each channel using the PLIP tool [29].

3. Results

3.1. Calebin A inhibited $I_{Ba1.2}$ in vascular smooth muscle cells

The influence of calebin A on $Ca_v1.2$ channel activity was investigated in freshly isolated rat tail artery myocytes by recording $I_{Ba1.2}$ under whole-cell patch-clamp conditions. Depolarising pulses from -50 mV to 10 mV evoked inward Ba^{2+} currents that progressively declined after exposure to increasing concentrations of calebin A (Fig. 1). The inhibition developed in a concentration-dependent manner, yielding an estimated IC_{50} value of 64.5 ± 14.6 μ M ($n = 5$; Fig. 1B), with an E_{max}

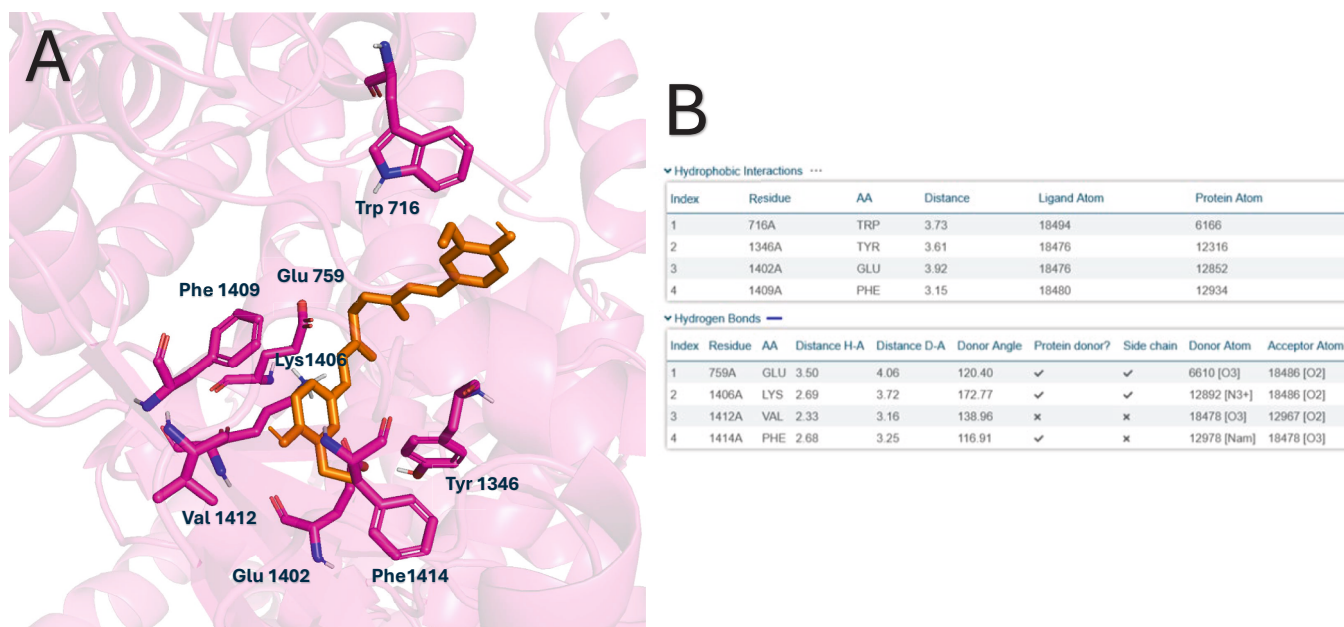


Fig. 5. Docking poses and interaction analysis of calebin A in $K_{Ca}1.1$ channels. (A) Docking pose of calebin A within the predicted modulatory site of the $K_{Ca}1.1$ channel. The best-ranked pose showed a markedly favourable HADDOCK score (-61.83). Calebin A interacts with hydrophobic residues lining the binding pocket, including Trp716, Tyr1346, Glu1402, and Phe1409, and forms additional polar contacts with residues such as Glu759, Lys1406, Val1412, and Phe1414. (B) In $K_{Ca}1.1$ channels, calebin A forms multiple hydrophobic interactions with residues lining the binding pocket, including Trp716, Tyr1346, Glu1402, and Phe1409 (interaction distances ~ 3.1 – 3.9 Å). Additional polar contacts involve Glu759, Lys1406, Val1412, and Phe1414, where Glu759 and Lys1406 act as hydrogen-bond donors with favourable geometries, while Val1412 and Phe1414 contribute weaker or non-canonical hydrogen-bond-like interactions.

value close to 62%.

Kinetic analysis demonstrated that calebin A selectively modified current inactivation. Specifically, the apparent τ_{inact} progressively decreased as the concentration of the compound increased, whereas activation kinetics remained essentially unchanged (Fig. 1C).

The temporal profile of the response showed that the inhibitory effect developed gradually after compound (100 μ M) application and stabilised within approximately 3–4 min (Fig. 1D). Removal of calebin A from the superfusion solution almost completely restored current amplitude. Subsequent administration of the Ca^{2+} antagonist nifedipine abolished the residual inward current. Interestingly, although washout reversed the reduction in current amplitude, the acceleration of current decay persisted after drug removal (apparent τ_{inact} control 173.99 ± 41.58 ms, 100 μ M calebin A 95.15 ± 5.82 ms, $n = 5$; $P < 0.05$, repeated measures ANOVA and Bonferroni post-test; washout 126.04 ± 15.39 ms, $P < 0.05$ vs. control and $P > 0.05$ vs. calebin A). No effects were recorded on the τ_{act} (control 3.38 ± 0.56 ms, 100 μ M calebin A 3.17 ± 0.86 ms, washout 3.10 ± 0.74 ; $P > 0.05$).

Current–voltage analysis revealed that 100 μ M calebin A significantly reduced inward current amplitude over a voltage range between -20 mV and $+40$ mV without appreciably altering either the activation threshold or the membrane potential corresponding to maximal current amplitude (Fig. 2A,B). Consistently, Boltzmann analysis of steady-state activation curves for $I_{Ba1.2}$, derived from the current–voltage relationships shown in Fig. 2B, demonstrated that neither the half-activation potential (control 4.74 ± 2.67 mV, calebin A 6.14 ± 3.61 mV, and washout 4.31 ± 3.02 mV, $n = 5$; $P = 0.0927$) nor the slope factor (7.18 ± 0.75 mV, 6.00 ± 0.74 mV, and 7.16 ± 1.31 mV, respectively; $P = 0.1819$) was significantly modified by the compound (Fig. 2C).

3.2. Docking simulations predicted stable interaction of calebin A with $Ca_v1.2$ channels

In the complex of $Ca_v1.2$ channel and calebin A, docking calculations produced a well-defined best-ranked complex characterised by a

moderately favourable HADDOCK score (-17.60), mainly driven by van der Waals interactions (-24.53) and supported by a negative desolvation contribution (-9.54) (Fig. 3A). In contrast, electrostatic interactions played a marginal role in complex stabilisation. The solution satisfied all applied ambiguous interaction restraints, showing no significant violations and a low AIR rms-dev (0.11), indicating good agreement with the docking constraints.

In $Ca_v1.2$ channels, hydrophobic contacts were mainly mediated by Phe1138, Ile1486, and Leu1491, indicating the involvement of a predominantly hydrophobic pocket accommodating the ligand scaffold (Fig. 3B). Hydrogen-bond analysis revealed fewer polar interactions than in $K_{Ca}1.1$ channels (see below), with Gln1069 forming a stabilising hydrogen bond via its side chain, while Leu1131 contributed a weaker interaction consistent with its nonpolar character.

3.3. Calebin A increased TEA-sensitive outward currents in rat tail artery myocytes

The effects of calebin A on vascular TEA-sensitive outward currents were subsequently examined. Under the experimental conditions employed, the outward current mainly corresponded to ibertoxin-sensitive $I_{KCa1.1}$ [16]. Exposure to 10 μ M and 30 μ M calebin A produced a gradual increase in current amplitude that became evident within a few minutes and reached a stable plateau after approximately 3–4 min (Fig. 4A). Addition of 1 mM TEA at the end of the experiments allowed the isolation of the $I_{KCa1.1}$ component of the outward current. The stimulatory action of the compound was concentration-dependent and occurred in the voltage range 20–70 mV (Fig. 4B).

3.4. Computational analysis identified a plausible interaction of calebin A with $K_{Ca}1.1$ channels

Docking of calebin A to $K_{Ca}1.1$ channel generated a well-defined best-ranked pose characterised by a favourable HADDOCK score (-61.83), driven by both van der Waals (-27.50) and electrostatic

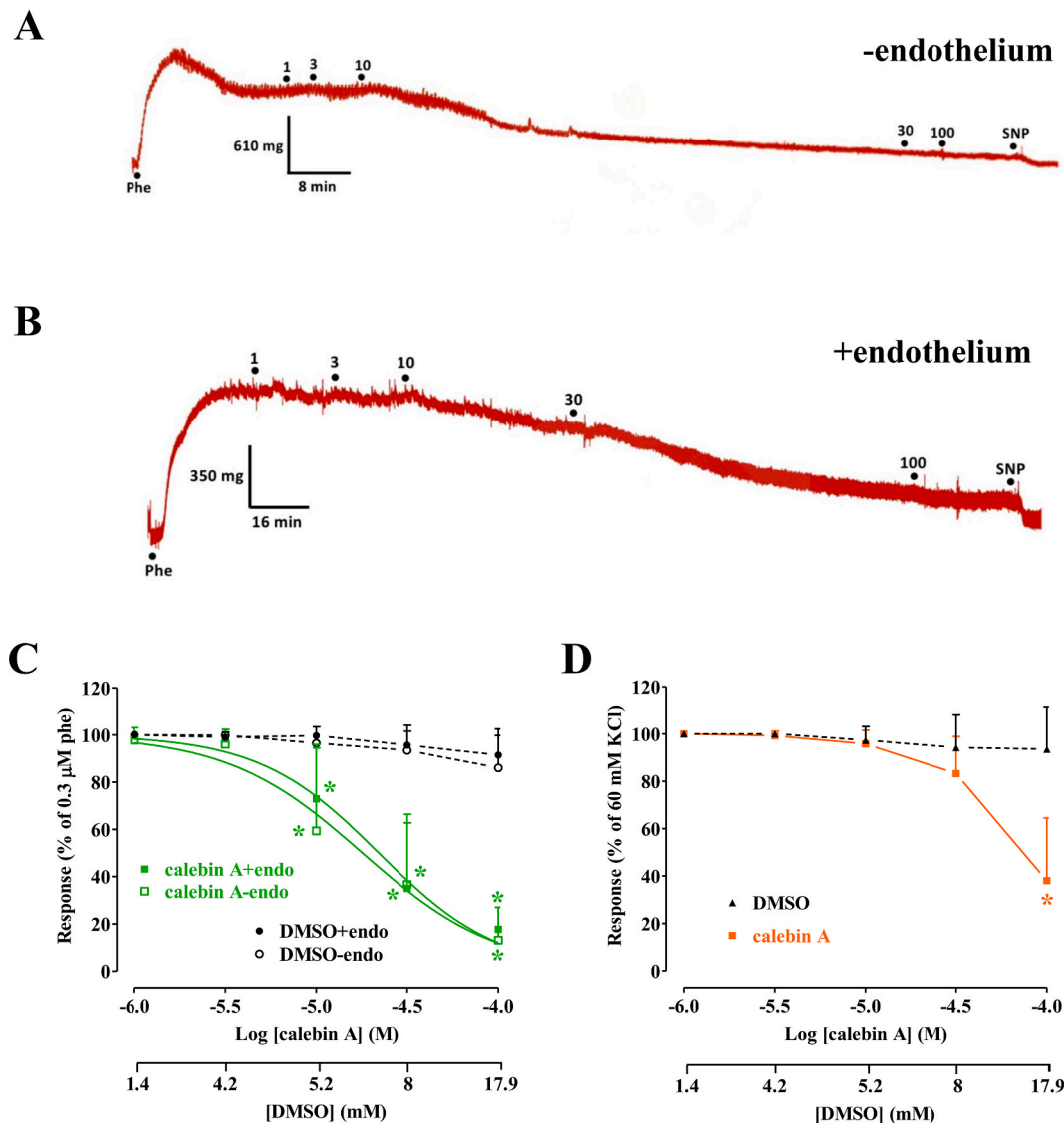


Fig. 6. Vasorelaxant effects of calebin A in thoracic aorta rings precontracted by phenylephrine or KCl. (A,B) Representative recordings obtained from thoracic aorta rings either (A) lacking endothelium or (B) with intact endothelium and precontracted by 0.3 μ M phenylephrine (Phe). Increasing concentrations of calebin A were cumulatively added after contraction reached a stable plateau. At the end of each experiment, 100 μ M sodium nitroprusside (SNP) was applied to restore basal tone. (C) Concentration-response curves describing relaxation induced by calebin A in endothelium-intact (+endo) and endothelium-denuded (–endo) preparations. Vehicle (DMSO) responses are also reported. Vehicle-treated rings were followed over the same experimental time course (approximately 90 min) as calebin A-treated preparations. Relaxation is expressed as percentage reduction of the initial phenylephrine-induced contraction. Data are presented as mean $\pm$ SD ($n = 5-6$). * $P < 0.05$ vs DMSO, two-way ANOVA followed by Bonferroni post-test. (D) Concentration-response relationship obtained in endothelium-denuded rings contracted by 60 mM KCl. The effect of the vehicle (DMSO) is also shown. Vehicle-treated rings were followed over the same experimental time course (approximately 90 min) as calebin A-treated preparations. Responses are expressed relative to the initial KCl-induced tone. Data points are mean $\pm$ SD ($n = 5-6$). * $P < 0.05$ vs DMSO, two-way ANOVA followed by Bonferroni post-test.

contributions (-47.69), and further supported by a negative desolvation term (-8.68) (Fig. 5A). The solution satisfied the applied ambiguous interaction restraints, showing no significant violations and a low AIR rms-dev (0.125). Overall, the scoring profile indicates a stable ligand accommodation within the targeted modulatory pocket, consistent with the interaction pattern observed in the contact analysis.

Hydrophobic interactions were primarily established with aromatic and nonpolar residues lining the binding pocket of $K_{Ca1.1}$ channels, including Trp716, Tyr1346, Glu1402, and Phe1409, with interaction distances ranging between approximately 3.1 Å and 3.9 Å (Fig. 5B). In addition, hydrogen bond analysis identified several polar contacts involving Glu759, Lys1406, Val1412, and Phe1414. Among these, Glu759 and Lys1406 acted as hydrogen-bond donors through side-chain atoms, displaying favourable donor–acceptor distances and geometries,

while Val1412 and Phe1414 contributed weaker or non-canonical hydrogen-bond-like interactions.

3.5. Calebin A relaxed phenylephrine-contracted thoracic aorta rings

The vasorelaxant activity of calebin A was evaluated in isolated thoracic aorta rings precontracted by the α_1 -adrenergic agonist phenylephrine. Cumulative addition of the compound induced progressive relaxation in endothelium-denuded preparations, with an estimated IC_{50} value of 22.3 ± 19.9 μ M (Fig. 6A). The presence of functional endothelium (Fig. 6B) did not significantly modify either the potency (IC_{50} 27.1 ± 23.6 μ M; $n = 6$; $P = 0.5501$) or the maximal efficacy of calebin A (E_{max} –endothelium $86.8 \pm 4.0\%$, +endothelium $82.3 \pm 9.3\%$; $P = 0.3031$; Fig. 6C).

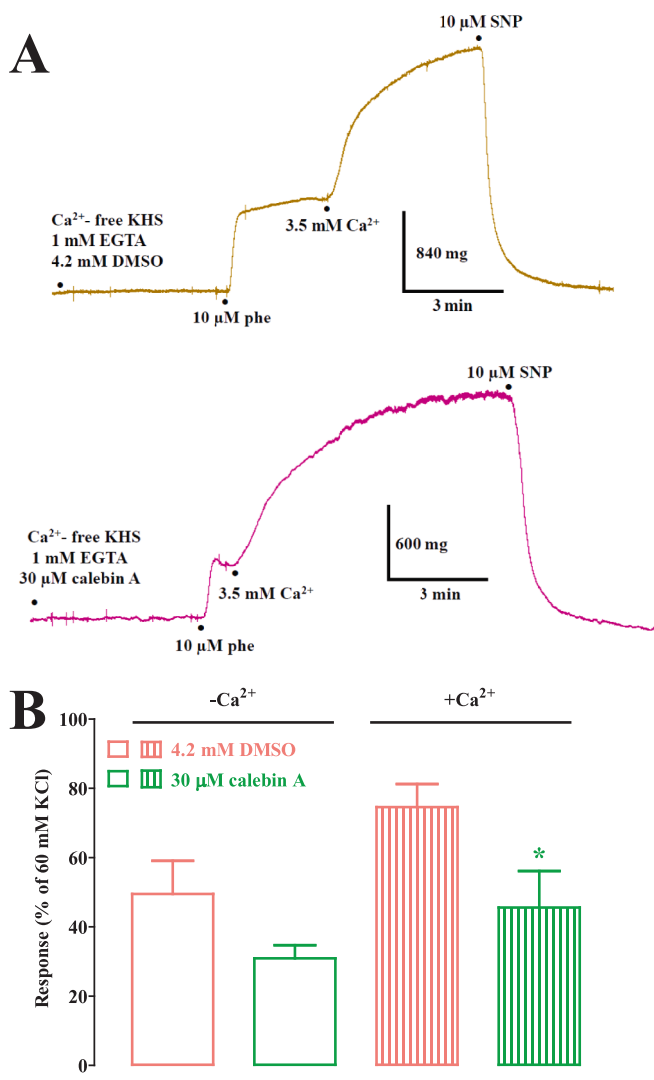


Fig. 7. Effects of calebin A on phenylephrine-induced contractions under Ca^{2+} -free and Ca^{2+} -containing conditions. (A) Endothelium-denuded thoracic aorta rings were incubated for 30 min with either 30 μM calebin A or vehicle (DMSO) before stimulation with 10 μM phenylephrine (phe) in the absence of extracellular Ca^{2+} . Then 3.5 mM Ca^{2+} was reintroduced into the organ bath. The effect of sodium nitroprusside (SNP) is also shown. (B) Quantitative analysis of the effects of calebin A on intracellular Ca^{2+} release ($-\text{Ca}^{2+}$) and extracellular Ca^{2+} influx ($+\text{Ca}^{2+}$) triggered by phenylephrine. Contractile responses are expressed as percentages of the reference contraction induced by KCl during functional testing. Columns are mean $\pm$ SD ($n = 8$). * $P < 0.05$ vs DMSO, Student's t test for unpaired samples.

3.6. Relaxation induced by calebin A was reduced in depolarised vessels

Additional experiments were conducted in endothelium-denuded aorta rings contracted by 60 mM KCl. Under these conditions, calebin A still produced concentration-dependent relaxation, although both potency (estimated IC_{50} of $87.4 \pm 24.9 \mu\text{M}$, $n = 6$; $P = 0.0005$) and maximal efficacy ($62.0 \pm 26.4\%$; $P = 0.0457$; Fig. 6D) were lower than those observed in phenylephrine-stimulated preparations.

3.7. Calebin A preferentially reduced the contractile response elicited by extracellular Ca^{2+} restoration

The effects of calebin A on intracellular Ca^{2+} mobilisation and extracellular Ca^{2+} entry triggered by phenylephrine were analysed in

endothelium-denuded thoracic aorta rings. During the 30-min incubation period in normal KHS, 30 μM calebin A did not evoke any measurable effect. Then, in Ca^{2+} -free conditions, 10 μM phenylephrine evoked a transient contraction attributable to IP_3 -induced Ca^{2+} release from intracellular stores. Calebin A reduced this response by approximately 38% (Fig. 7), although statistical significance was not achieved ($P = 0.0914$). By contrast, restoration of extracellular Ca^{2+} produced a sustained contractile response that was significantly attenuated by calebin A (about 39% inhibition; $P = 0.0354$).

3.8. Calebin A altered spontaneous transient $I_{\text{KCa1.1}}$ activity

STOCs were recorded to evaluate the influence of calebin A on local Ca^{2+} sparks-dependent $\text{K}_{\text{Ca1.1}}$ channel activity. Application of vehicle alone (0.06% v/v DMSO) did not significantly affect STOC frequency or amplitude (Fig. 8B,C). In contrast, exposure to 30 μM calebin A produced a biphasic response characterised by an initial increase in STOC frequency followed by a progressive reduction in both frequency and amplitude (Fig. 8A,D,E). Subsequent administration of 1 mM TEA abolished STOC activity.

3.9. Calebin A attenuated contractions induced by moderate and high extracellular KCl

The effect of calebin A on depolarisation-induced contraction was further examined using moderate (25 mM) and high (60 mM) extracellular KCl concentrations. Pre-incubation with 30 μM calebin A significantly reduced the contractile responses elicited under both experimental conditions (25 mM KCl, $P = 0.0031$, and 60 mM KCl, $P = 0.0420$) (Fig. 9). The magnitude of inhibition was comparable in preparations stimulated with either moderate or high KCl concentrations.

4. Discussion

The present study demonstrates that calebin A, a naturally occurring polyphenol from *Curcuma longa*, produces marked endothelium-independent vasorelaxation in rat thoracic aorta through direct modulation of vascular smooth muscle function. By integrating electrophysiological recordings, vascular reactivity studies, intracellular Ca^{2+} experiments and computational modelling, we identified inhibition of $\text{Ca}_v1.2$ channels as a major mechanism underlying the vasorelaxant effect of calebin A. In addition, several independent observations support the contribution of $\text{K}_{\text{Ca1.1}}$ channel-dependent mechanisms to its vascular activity, although the present data do not establish whether this reflects direct channel activation or indirect modulation secondary to changes in intracellular Ca^{2+} signalling.

$\text{Ca}_v1.2$ channels, the principal pathway for Ca^{2+} entry during membrane depolarisation in vascular smooth muscle, play a pivotal role in the regulation of arterial tone. Pharmacological inhibition of these channels reduces intracellular Ca^{2+} concentration, thereby promoting relaxation. Whole-cell patch-clamp experiments demonstrated that calebin A inhibited $\text{I}_{\text{Ba1.2}}$ in a concentration-dependent manner without significantly modifying voltage-dependent activation. Instead, calebin A accelerated current decay, suggesting preferential interaction with channel states associated with inactivation rather than with voltage sensing itself.

The characteristics of channel inhibition further support the hypothesis of an allosteric modulatory mechanism rather than direct pore occlusion. In fact, the relatively slow onset of action, together with the almost complete recovery of current amplitude after washout, differs from the rapid blocking behaviour classically observed with pore-targeting antagonists such as verapamil-like phenylalkylamines [30]. Moreover, the persistence of the reduction in the τ_{inact} after washout suggests that calebin A may interact with membrane-accessible regulatory regions involved in channel gating. Such behaviour is consistent with the lipophilic nature of many polyphenols, which can partition into

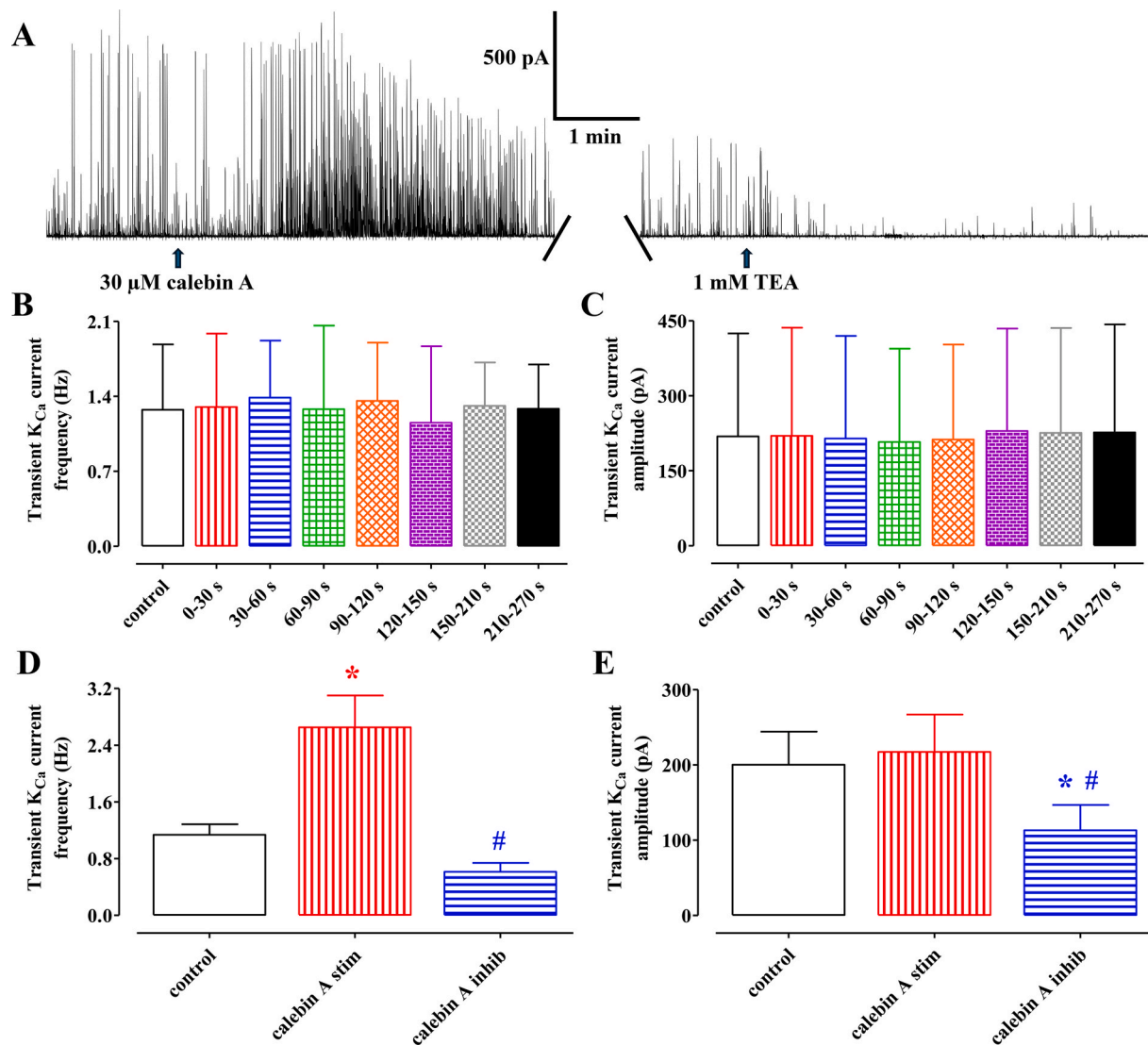


Fig. 8. Modulation of STOCs by calebin A in tail artery myocytes. (A) Representative STOC recordings obtained at a V_h of -20 mV before and after exposure to $30 \mu\text{M}$ calebin A and subsequent addition of 1 mM TEA. (B,C) Effects of vehicle alone (0.06% DMSO) on STOC (B) frequency and (C) amplitude analysed over consecutive 30 -s intervals. (D,E) Biphasic effects of calebin A on STOC activity, consisting of an initial stimulation followed by inhibition of STOC (D) frequency and (E) amplitude. Columns are mean $\pm$ SD ($n = 5$). * $P < 0.05$ vs control and # $P < 0.05$ vs calebin A stim, repeated measures ANOVA followed by Bonferroni post-test.

the membrane and exert prolonged modulatory effects on ion channel function by altering bilayer elasticity [31]. This raises the possibility of off-target actions on, e.g., Na_v channel isoforms. However, in rat tail artery myocytes, excitation–contraction coupling is dominated by Ca^{2+} entry through $\text{Ca}_v1.2$ channels, whereas, to the best of our knowledge, Na_v currents are notoriously absent or negligible during normal contractile homeostasis. Consequently, the downstream vasorelaxation observed in this study is best attributed to direct interactions with localised Ca^{2+} regulatory pathways rather than upstream Na_v channel blockade.

When compared to clinically used Ca^{2+} channel blockers, the weaker potency of calebin A emerges because, under similar experimental conditions (see [12]), nifedipine, verapamil, and diltiazem show IC_{50} values in the mid-nanomolar to low-micromolar range. However, they primarily target $\text{Ca}_v1.2$ channels, whereas calebin A also showed evidence of $\text{K}_{Ca}1.1$ -dependent modulation, suggesting a complementary mechanism that may enhance vasorelaxation through membrane hyperpolarisation. Thus, its multitarget profile and putative binding mode distinguish it from currently available drugs and may provide useful information for the development of novel vasorelaxant agents.

Calebin A produced different degrees of inhibition under the experimental conditions used. This apparent discrepancy may arise from differences in the experimental protocols used across Figs. 1 and 2. The time-course protocol (repetitive pulsing at a fixed potential) and the current–voltage protocol (sequential depolarisations to increasing potentials) may alter channel inactivation kinetics and steady-state drug binding relative to the concentration–response protocol, which employed a single test pulse. Therefore, the apparent discrepancy may reflect voltage- and state-dependent blocking actions of calebin A, which may account for the modest differences in fractional block observed between the different voltage-clamp protocols employed. However, an in-depth biophysical analysis is necessary to support this hypothesis.

The mechanism by which calebin A reduces $\text{I}_{\text{Ba}1.2}$ amplitude appears to be kinetically driven rather than a result of pore blockade or altered voltage sensitivity. This is supported by the fact that while the steady-state activation curves remained perfectly overlapping between control and $100 \mu\text{M}$ calebin A, the τ_{inact} was significantly reduced in a concentration-dependent manner. In biophysical terms, an accelerated inactivation rate outcompetes the activation process during the onset of the voltage step. This shifts a fraction of the channel population into a

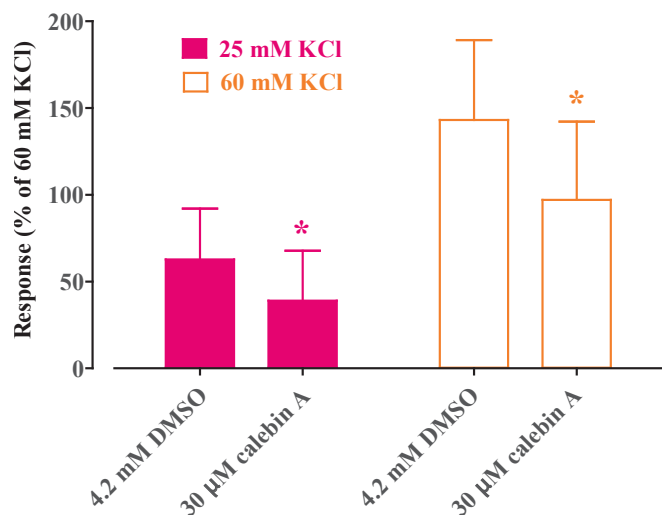


Fig. 9. Effect of calebin A on contractions elicited by moderate and strong membrane depolarisation. Endothelium-denuded thoracic aorta rings were pretreated for 6 min with 30 μ M calebin A or vehicle (DMSO) before stimulation with either 25 mM or 60 mM KCl. Contractile responses are expressed as percentages of the reference KCl-induced contraction recorded during viability testing. Columns are mean $\pm$ SD ($n = 6-7$). * $P < 0.05$ vs DMSO, Student's t test for paired samples.

fast-inactivated or refractory state before they can fully synchronise in the open state at the peak. Consequently, calebin A causes an operational, persistent loss of available conducting channels during depolarisation, which fully accounts for the reduction in peak current amplitude without requiring modifications to the unitary channel conductance.

The functional experiments carried out in isolated thoracic aorta rings closely paralleled the electrophysiological observations. Calebin A produced marked relaxation of either phenylephrine- or high extracellular K^+ -precontracted vessels, although both potency and efficacy were reduced under depolarising conditions. The vasorelaxant effect was maintained after endothelial removal, indicating that vascular smooth muscle represents the principal target of the compound. Moreover, calebin A significantly attenuated the contractile response elicited by the restoration of extracellular Ca^{2+} under Ca^{2+} -free conditions, whereas the contraction attributable to intracellular Ca^{2+} release was not significantly altered. However, because Ca^{2+} movements were not measured directly, these findings should be interpreted as indirect functional evidence rather than definitive proof of reduced Ca^{2+} entry, and they do not allow definitive conclusions regarding intracellular Ca^{2+} release. Taken together, these findings consistently identify inhibition of Ca^{2+} entry through plasma membrane channels as the predominant mechanism responsible for the vasorelaxant activity of calebin A. Though $Ca_v1.2$ channels are known to play a role in the contraction induced by α_1 receptor agonists [32], further experiments are necessary to rule out a possible involvement of receptor- and store-operated Ca^{2+} channels in the drug-induced spasmodic.

Docking simulations predicted a stable accommodation of calebin A within a previously identified modulatory pocket of the $Ca_v1.2$ channel [33] compatible with the functional inhibition observed experimentally. The molecule established interactions with key residues involved in channel modulation, such as Phe1138, Ile1486 and Leu1491. HADDOCK calculations produced well-defined docking clusters, from which the best-ranked poses were selected for interaction analysis. Interaction profiling indicated the presence of multiple stabilising contacts, including hydrophobic interactions with aromatic residues and polar contacts with surrounding amino acids, suggesting a favourable accommodation of the ligand within the binding site. The agreement between computational and electrophysiological findings strengthens the mechanistic interpretation without, however, constituting direct

evidence of ligand binding.

Besides inhibiting $Ca_v1.2$ channels, calebin A also increased TEA-sensitive outward currents recorded under experimental conditions previously shown to isolate predominantly $K_{Ca1.1}$ -mediated current in rat tail artery myocytes [16]. This observation is physiologically relevant because activation of $K_{Ca1.1}$ channels promotes membrane hyperpolarisation, thereby decreasing the probability of $Ca_v1.2$ channel opening and limiting Ca^{2+} entry into smooth muscle cells [34]. In addition, docking calculations predicted energetically favourable accommodation of calebin A within a regulatory pocket of $K_{Ca1.1}$ channels previously associated with small-molecule modulation [35]. Here, the molecule established a network of hydrophobic and electrostatic interactions involving residues such as Trp716, Glu759, and Lys1406, which contributed to complex stabilisation. The strong predicted interaction is consistent with the robust stimulatory effect observed during electrophysiological recordings. Furthermore, vascular relaxation was attenuated under conditions of sustained membrane depolarisation, in which the contribution of K^+ channel activation is expected to be reduced [34]. Collectively, these independent observations support the hypothesis that $K_{Ca1.1}$ channel-dependent mechanisms contribute to the vascular actions of calebin A.

The STOC experiments deserve consideration. Calebin A produced a biphasic response characterised by an early increase in STOC frequency followed by a gradual reduction in both frequency and amplitude. Because STOCs arise from the functional coupling between local Ca^{2+} sparks and $K_{Ca1.1}$ channels, these observations indicate that calebin A modifies this signalling pathway. However, STOC recordings alone cannot distinguish between direct modulation of $K_{Ca1.1}$ channels and indirect effects mediated by alterations in local Ca^{2+} release from the sarcoplasmic reticulum. Interestingly, our functional experiments did not reveal evidence of a major effect on intracellular Ca^{2+} mobilisation through IP_3 receptors [32], nor did calebin A evoke vasoconstriction during the 30-min incubation period in normal KHS. Therefore, one possible interpretation is that the initial increase in STOC frequency reflects the modulation of $K_{Ca1.1}$ channel activity, which is subsequently masked by moderate changes in sarcoplasmic reticulum Ca^{2+} content. Nevertheless, the precise molecular mechanism responsible for the biphasic STOC behaviour remains to be established.

The vascular actions of calebin A therefore appear to result from complementary mechanisms. Inhibition of $Ca_v1.2$ channels, measurable under strong depolarisation conditions, directly limits Ca^{2+} entry and contractile activation, whereas increased $K_{Ca1.1}$ -dependent outward current, measurable under moderate depolarising conditions, would be expected to promote membrane hyperpolarisation, thereby reducing the probability of $Ca_v1.2$ channel opening [34]. Such reciprocal interactions between Ca^{2+} and K^+ channels are well recognised in vascular smooth muscle and provide an efficient mechanism for controlling arterial tone.

From a pharmacological perspective, these findings expand current knowledge of the cardiovascular actions of calebin A. Previous investigations have mainly focused on its anti-inflammatory, antioxidant, and anticancer properties, whereas little information has been available regarding its direct effects on vascular smooth muscle. The identification of $Ca_v1.2$ inhibition together with evidence for $K_{Ca1.1}$ -dependent modulation suggests that calebin A may represent a promising scaffold for the development of novel vasorelaxant compounds capable of simultaneously targeting complementary determinants of vascular tone. This pharmacological profile, which resembles that reported for several other naturally derived polyphenols, including cardamomin, biochanin A, 3,3'-O-dimethylquercetin, and (2S)-sakuranetin, as well as for selected semisynthetic flavonoid derivatives [13,36–39], appears, therefore, to represent a recurrent and pharmacologically advantageous feature among vascularly active polyphenols.

Despite these promising findings, several limitations deserve consideration. First, an important consideration concerns the concentrations required to produce vascular effects in the present study. The only pharmacokinetic study published so far indicates that calebin A has

low oral bioavailability in rats (approximately 0.5%) and is rapidly eliminated from the plasma (serum half-life is about 1–3 h; [40]). Consequently, the micromolar concentrations required to modulate vascular ion channels in the present experiments are unlikely to be achieved after conventional oral administration. Therefore, the present study should primarily be regarded as defining the pharmacological profile of calebin A and identifying molecular targets that may prove valuable for future structural optimisation or alternative delivery strategies. Second, single-channel recordings were not performed, preventing definitive assessment of whether calebin A directly gates $K_{Ca1.1}$ channels. Third, although the electrophysiological recording conditions preferentially isolated $I_{KCa1.1}$ and were based on protocols previously validated in this preparation, the contribution of other outward K^+ conductances cannot be completely excluded. Fourth, docking simulations generate hypotheses regarding ligand-channel interactions but cannot replace direct biophysical demonstration of binding. Fifth, male animals were selected to minimise variability associated with hormonal fluctuations during the oestrous cycle. Nevertheless, we recognise that future studies should include both sexes to evaluate potential sex-dependent differences. Finally, the experiments were conducted in different vascular preparations, which may differ in channel expression and pharmacological responsiveness.

In conclusion, the present findings support $Ca_v1.2$ channel inhibition as the principal mechanism underlying the vasorelaxant effect of calebin A in rat arteries. The combined electrophysiological, functional and computational findings further support the involvement of $K_{Ca1.1}$ channel-dependent mechanisms, although their precise molecular basis remains to be clarified. These results provide new insight into the vascular pharmacology of calebin A and identify this turmeric-derived polyphenol as a promising lead structure for the development of agents targeting vascular dysfunction.

CRedit authorship contribution statement

Francesca De Rosa: Investigation, Formal analysis, Data curation. **Francesco Pettini:** Writing – original draft, Investigation, Formal analysis, Data curation. **Ottavia Spiga:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Atanas G. Atanasov:** Writing – review & editing, Resources. **Fabio Fusi:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

None.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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