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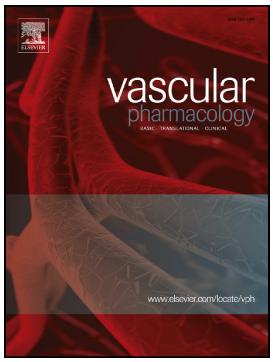
Angaben zur Veröffentlichung / Publication details:

Løgstrup Nielsen, Daniel, Josef Ali Khalid, Anna Giné Martínez, Elizaveta V. Melnikova, Benjamin Hørning Risager, Rudolf Schubert, Christian Staehr, and Vladimir V. Matchkov. 2026. "Interplay between Na+/K+-ATPase-dependent Src-mediated Ca2+ sensitization and BKCa channel negative feedback regulates rat mesenteric artery contraction." *Vascular Pharmacology*, 107676. https://doi.org/10.1016/j.vph.2026.107676.

Journal Pre-proof



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PII: S1537-1891(26)00096-0

DOI: https://doi.org/10.1016/j.vph.2026.107676

Reference: VPH 107676

To appear in: *Vascular Pharmacology*

Received date: 6 April 2026

Revised date: 1 July 2026

Accepted date: 2 July 2026

Please cite this article as: D.L. Nielsen, J.A. Khalid, A.G. Martínez, et al., Interplay between Na+/K+-ATPase-dependent Src-mediated Ca2+ sensitization and BKCa channel negative feedback regulates rat mesenteric artery contraction, *Vascular Pharmacology* (2026), https://doi.org/10.1016/j.vph.2026.107676

Interplay between Na^+/K^+-ATPase-dependent Src-mediated Ca^{2+} sensitization and BK_{Ca} channel negative feedback regulates rat mesenteric artery contraction.

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Abstract

The Na^+/K^+-ATPase α_2 isoform regulates vascular tone by modulating Na^+/Ca^{2+} exchange and Src kinase–dependent signaling within membrane microdomains. Inhibition of the Na^+/K^+-ATPase by cardiotonic steroids promotes vascular contraction through increased intracellular Ca^{2+} and enhanced Ca^{2+} sensitization of the contractile apparatus. This effect is counterbalanced by a negative feedback mechanism involving large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. Given inconsistent reports regarding ouabain-induced potentiation of agonist-evoked vasoconstriction, we hypothesized that the effects of ouabain depend on the agonist (thromboxane A_2 receptor agonist U46619, noradrenaline, and the α_1-adrenoceptor agonist methoxamine) used. We specifically assessed changes in intracellular Ca^{2+}, Ca^{2+} sensitization, and the contribution of BK_{Ca}-channel-mediated feedback. Rat mesenteric small arteries were studied using isometric myography. Intracellular Ca^{2+} was measured with the ratiometric dye FURA-2/AM, and signaling pathways were analysed by Western blotting. Micromolar ouabain enhanced U46619-induced contraction but had no statistically significant effect on responses to noradrenaline or methoxamine. BK_{Ca} channel inhibition with iberiotoxin increased sensitivity to U46619 via elevated intracellular Ca^{2+}. Combined ouabain and iberiotoxin treatment increased basal tone and augmented contractile responses to all agonists tested. Notably, ouabain enhanced Ca^{2+} sensitization during U46619 stimulation through Src–dependent MYPT1 phosphorylation. Ouabain also increased Src and MYPT1 phosphorylation during both U46619 and methoxamine stimulation. These findings identify the Na^+/K^+-ATPase as a signaling scaffold that promotes Src-mediated Ca^{2+} sensitization. This pro-contractile effect requires a sufficient intracellular Ca^{2+} level, partly regulated by BK_{Ca}-channel dependent negative feedback.

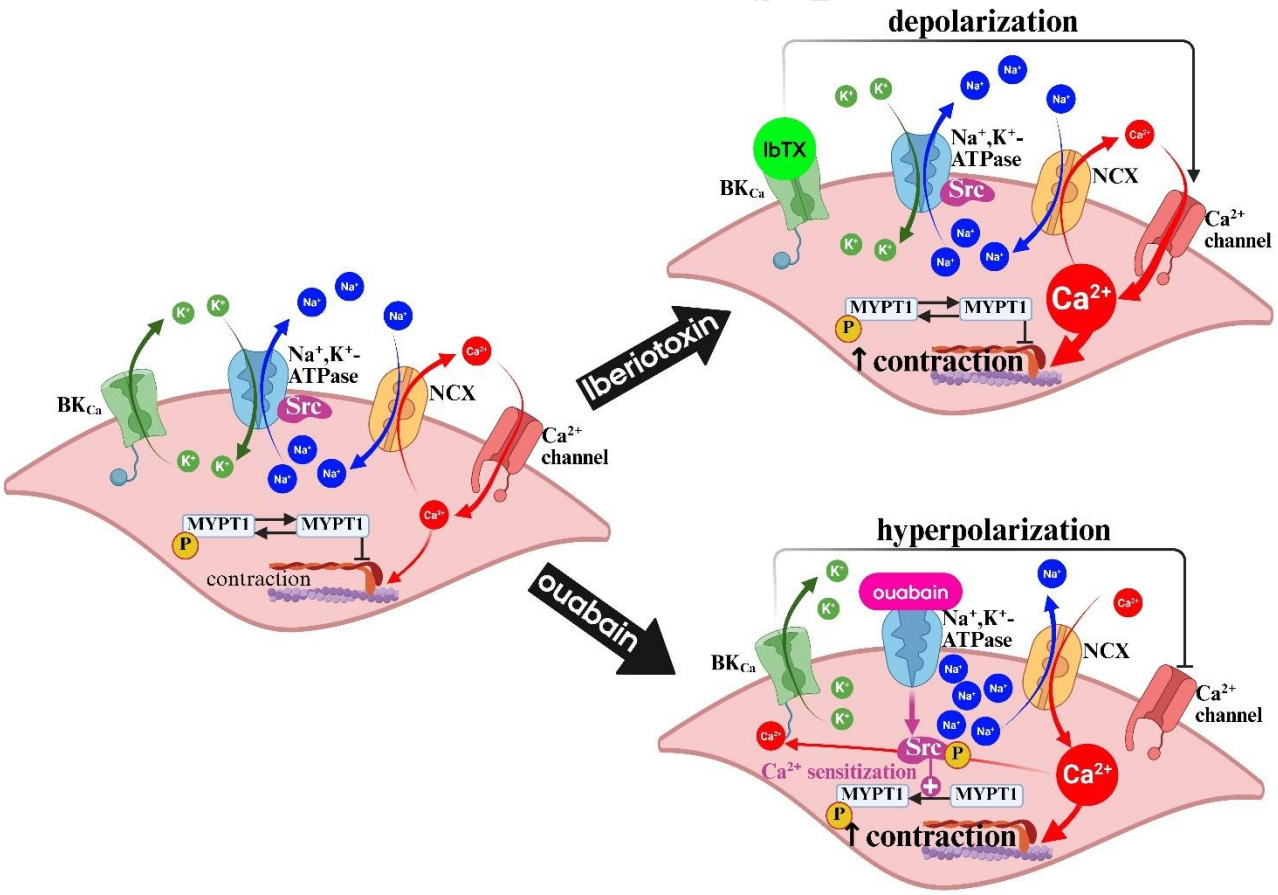
Keywords

Small artery, ouabain, iberiotoxin, thromboxane A_2 receptor, α_1-adrenoceptor, intracellular calcium, calcium sensitization

Highlights

- The Na^+/K^+-ATPase acts as a signaling scaffold that promotes Src-mediated Ca^{2+} sensitization and agonist-induced vascular contraction.
- Pro-contractile Ca^{2+} sensitization is only possible at a sufficient intracellular Ca^{2+} level in vascular smooth muscle cells.
- The large-conductance Ca^{2+}-activated K^+ channels serves as negative feedback for intracellular Ca^{2+} elevation that interferes with ouabain-induced potentiation of agonist-induced contraction.

Graphical abstract



1. Introduction

The Na/K-ATPase plays a major role in cellular biology by dynamically regulating membrane potential and ionic transmembrane homeostasis and by mediating intracellular signal pathways [1-3]. Structurally, the Na/K-ATPase is composed of three subunits, one ion transporting α subunit and two accessory subunits β and FXYD [4]. The catalytic α subunit contains the biding sites for cardiotonic steroids, such as digoxin and ouabain, which inhibit transmembrane ion translocation [5, 6]. The β subunit maintains enzyme stability and is important for membrane trafficking of the Na/K-ATPase. The FXYD subunit modulates the enzymatic activity [6]. Three α isoforms have been shown to be distributed throughout all somatic cells in the body. The α_1 isoform is normally considered housekeeping in nearly all cells in the body and is usually saturated at the physiological concentrations of Na^+ and K^+ [4]. In contrast, the α_2 and α_3 isoforms are expressed in a cell and tissue-dependent manner. Their transport can be activated by increasing concentrations of extracellular K^+, although this is strongly modulated by combination with an accessory β subunit type [7]. The vascular wall, consisting mainly of endothelial and smooth muscle cells, is characterized by co-expression of the Na/K-ATPase α_1 and α_2 isoforms [8], where the α_2 isoform is thought to play important modulatory functions [9].

During each ion translocation cycle, the Na/K-ATPase transports three Na^+-ions out and two K^+-ions into the cell, a process facilitated by ATP hydrolysis [4, 6, 10]. This transport provides the driving force for several secondary active ion transporters, including the Na^+/Ca^{2+} exchanger (NCX) [11-13]. The Na/K-ATPase α_2 isoform localizes in close proximity to the NCX within a membrane microdomain located over the peripheral extravagations of the sarcoplasmic reticulum [1, 14]. At resting membrane potentials, the NCX is driven by the transmembrane Na^+ gradient to extrude intracellular Ca^{2+}. Importantly, the polarity of this exchange is defined by the electrochemical driving force [9]. Under resting physiological transmembrane ion gradients, the reversal potential of the NCX operating in the 3 Na^+:1 Ca^{2+} exchange mode is estimated to be approximately -26 mV [9]. This transport stoichiometry has been confirmed experimentally [15, 16] and enables Ca^{2+} extrusion under resting conditions. However, even modest increases in intracellular Na^+ or membrane depolarization can reverse the direction of NCX transport, resulting in Ca^{2+} influx [17]. That is, the direction Ca^{2+} movement is defined by the combination of the membrane potential level and the cytosolic Na^+ and Ca^{2+} concentrations, where the Na^+ transmembrane gradient is modulated by the activity of the Na/K-ATPase, while intracellular Ca^{2+} is modified by membrane potential-dependent and -independent Ca^{2+} influx, as well as agonist-induced Ca^{2+} release [12]. Accordingly, inhibition of the Na/K-ATPase

increases cytosolic Na^+ and diminishes the Na^+ gradient, which will further limit Ca^{2+} extrusion by the NCX. This leads to intracellular Ca^{2+} increase and thus, potentiates constriction of vascular smooth muscle cells [18]. Further alterations in Na^+ or Ca^{2+} may reverse the NCX polarity towards Ca^{2+} influx [9, 12, 19]. Moreover, severe inhibition of the Na,K-ATPase depolarizes the membrane potential, thereby potentiating voltage-dependent Ca^{2+} influx in smooth muscle cells [9]. Membrane potential is rarely considerably affected by the Na/K-ATPase α_2 isoform but is depolarized with an inhibition of the housekeeping α_1 isoform [20-22].

The large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are one of the major hyperpolarizing membrane conductances in vascular smooth muscle cells, where K^+ flux is facilitated by the Na/K-ATPase maintained ion gradient. The BK_{Ca} channels serve as a negative feedback mechanism for depolarization-induced Ca^{2+} influx and subsequently vasoconstriction [11]. Their Ca^{2+}-dependent opening enables hyperpolarizing K^+ efflux, which, in turn, suppresses voltage-dependent Ca^{2+} influx, reducing intracellular Ca^{2+}, leading to vasorelaxation [23].

The Na/K-ATPase has been shown to initiate signal-transduction upon cardiotonic steroid, e.g., ouabain, binding [24]. This is, at least in part, mediated by the Na/K-ATPase-dependent Src-kinase autophosphorylation and activation [25, 26]. Activation of Src-kinase has been shown to facilitate smooth muscle contraction without major changes in intracellular Ca^{2+}, i.e., Ca^{2+} sensitization, through myosin phosphatase targeting subunit (MYPT) phosphorylation and, thus, myosin phosphatase inhibition [3, 14, 27]. Although the Na/K-ATPase α_1 isoform is the major controller of the Na/K-ATPase-dependent Src activity [28, 29], in vascular smooth muscles the α_2 subunit is also essential for this signaling [3, 14]. Downregulation of the Na/K-ATPase α2 subunit abolishes the pro-contractile effect of micromolar ouabain, although resting vascular tone under isometric conditions is not affected by micromolar ouabain or Na/K-ATPase downregulation [3, 14, 30].

A recent study suggested that the BK_{Ca} channels are an integral part of the Na,K-ATPase – NCX microdomain, which limit ouabain-induced potentiation of methoxamine-induced vascular contraction [31]. This BK_{Ca}-channel-dependent negative feedback is mediated by two independent pathways involving Src-kinase activation and modulation of NCX activity, i.e., uses the same pathways implicated in the pro-contractile action of ouabain [31]. That is, the Na,K-ATPase - NCX - BK_{Ca} channel microdomain provides a self-limiting circuit for cardiotonic steroid action in the vascular wall. Accordingly, micromolar concentrations of ouabain alone did not potentiate the methoxamine-induced contraction of rat mesenteric small arteries, while it did so in the presence of

the BK_{Ca} channel inhibitor, iberiotoxin (IbTX) [31]. This is in contrast with previous reports suggesting that micromolar concentrations of ouabain alone potentiated noradrenaline-induced contractions of rat mesenteric small arteries [3, 14] and thromboxane A_2 analog, U46619- and endothelin-induced contractions of mouse middle cerebral arteries [32]. The reason for this discrepancy is unclear but could include differences in downstream signaling of agonist receptors, different activation states of the vascular wall, or vascular bed and species specificity. In this study, we hypothesized that the potentiating effect of ouabain on rat mesenteric small artery depends on the agonist used for stimulations, the role of intracellular Ca^{2+} changes and Ca^{2+} sensitization mediating the effect of the agonist, and the contribution of the BK_{Ca}-channel-based negative feedback to these responses.

2. Methods

2.1. Animals

We conducted experiments on segments of mesenteric small arteries isolated from 10-14 weeks old male Wistar rats. The rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and were allowed to acclimatize upon arrival in the Animal Facility for at least a week prior being sacrificed with CO_2 inhalation for immediate mesentery dissection. The rats were fed ad libitum with a standard chow diet, had free access to water and housed in groups on a 12 h light/12 h dark cycle. Animal handling was approved by the University Animal Welfare Officer and complied with guidelines from the European Communities Council Directive (86/609/EEC) for the Protection of Animals used for Experimental and other Scientific Purposes.

2.2. Solutions and chemicals

Physiological salt solution (PSS) was composed of (in mM) NaCl 115.88 KCl 2.82, KH_2PO_4 1.18, $MgSO_4$ 1.20, $NaHCO_3$ 25, $CaCl_2$ 1.60, EDTA 0.03, glucose 5.50 and gassed with 5% CO_2 in air and pH adjusted to 7.4. The high-potassium PSS (K-PSS) had the same composition as PSS but NaCl was replaced with KCl. Stock solutions of noradrenaline (10^{-2} M) and methoxamine (10^{-2} M) were prepared in MilliQ-water. Stock solution of IbTX (10^{-4} M) was prepared in isotonic (0.9 %) NaCl solution. Stock solution of U46619 was prepared in 96% ethanol. The aliquots of these stock solutions were stored at -20°C. Ouabain stock solution (10^{-2} M) was prepared in MilliQ-water and its aliqouts were stored for a maximum of 7 days at -20°, with each aliquot only thawed once.

Intracellular Ca^{2+} changes were assessed with FURA-2/AM dye. FURA-2/AM was dissolved in a loading mix at a concentration of $2.5 \cdot 10^{-6}$ M immediately prior to the experiment. The loading mix contained dimethyl sulfoxide (DMSO) with 0.1% weight/volume of Cremophor EL and 0.02% weight/volume of Pluronic F127. The loading mix with FURA-2/AM was added to the myograph bath PSS in a proportion of 1:1,000 for two consecutive incubation periods of 30 min each.

FURA-2/AM was acquired from TermoFisher Scientific (Roskilde, Denmark), U46619 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), iberiotoxin was purchased from Latoxan (Portes-lès-Valence, France), other chemicals were acquired from Sigma-Aldrich (Søborg, Denmark).

2.3. Preparation of experimental arterial segments and isometric myography

Immediately after euthanasia, the intestines, including the mesentery, were excised from the rat and placed in ice-cold PSS. Third-order mesenteric arteries were carefully dissected free from surrounding tissue under a stereomicroscope. Arterial segments approximately 2 mm in length were mounted in a four-chamber wire myograph system (620M, Danish Myograph Technology A/S, Denmark) for isometric tension recordings. The myograph chambers were filled with PSS maintained at 37°C and continuously aerated with 5% CO_2 in air. Arterial segments were mounted as ring preparations on two 40 µm stainless steel wires, as previously described [33].

The arterial segments were normalized based on their passive length-tension relationship to an internal circumference corresponding to 90% (IC_{90}) of that achieved at a transmural pressure of 100 mmHg (IC_{100}), which provides optimal conditions for maximal active force development [33]. Each experiment began with a standardization protocol to verify arterial viability. This involved exposure to 10^{-5} of the agonists that would later be used for the concentration–response experiments (methoxamine, U46619, or noradrenaline). After washout, the arteries were stimulated with KPSS for 3 minutes. Following another washout period, stimulation with 10^{-5} M agonist was repeated.

An initial cumulative concentration-response relationship was constructed in the absence of inhibitors by cumulative increases in agonist concentration. U46619 was applied at the following concentrations (M): 10^{-8}, $3\cdot10^{-8}$, 10^{-7}, $3\cdot10^{-7}$, 10^{-6}, $3\cdot10^{-6}$, 10^{-5} and $3\cdot10^{-5}$. Noradrenaline was administered at 10^{-7}, $3\cdot10^{-7}$, 10^{-6}, $3\cdot10^{-6}$ and 10^{-5} M. Methoxamine was used at $3\cdot10^{-7}$, 10^{-6}, $3\cdot10^{-6}$, 10^{-5} and $3\cdot10^{-5}$ M. After washout, the four myograph chambers containing arterial segments from the same rat were randomly assigned to one of four experimental conditions: incubation with 10^{-7} M IbTX for 15 minutes; 10^{-5} M ouabain for 5 minutes; a combination of IbTX (15 minutes) with ouabain added during the final 5 minutes; or vehicle (PSS) as a time control. The concentration-response protocol was then repeated under each of these conditions.

Force development (mN) was recorded using a PowerLab/200 data acquisition system and analyzed with LabChart version 8 software (ADInstruments, Dunedin, New Zealand). Baseline tension was defined as the wall tension measured prior to the initial concentration–response experiment. Active wall tension (mN/mm) was calculated as active force divided by twice the segment length and was used for comparison of arterial contractility [33].

2.4. Simultaneous measurement of intracellular Ca^{2+} concentration and isometric force

Mesenteric arterial segments were isolated, mounted, and normalized as described above. For fluorescence measurements, arteries were mounted in a wire myograph equipped with a glass-bottom

chamber (Danish Myograph Technology A/S, Denmark) positioned on the stage of an inverted fluorescence microscope. Intracellular Ca^{2+} in the outer layers of vascular smooth muscle cells were measured using FURA-2/AM [34]. FURA-2/AM was added to the 5 mL myograph chamber at a final concentration of $2.5 \cdot 10^{-6}$ M for 30 minutes. This loading procedure was performed twice with an intervening washout period.

Following washout of FURA-2/AM and completion of the standardization protocol (as described above), intracellular Ca^{2+} measurements were performed. Details of the equipment used (Photon Technology) have been given elsewhere [34]. The dye was alternately excited at 340 and 380 nm using a 75 W xenon light source, and emitted fluorescence was collected at 515 nm. Background fluorescence, obtained prior to FURA-2/AM loading, was determined and subtracted from all measurements. Fluorescence signals were acquired using Felix32 software (version 1.2, Photon Technology, USA). Intracellular Ca^{2+} was expressed as the ratio of fluorescence intensity at 340 nm to that at 380 nm (F340/F380), where an increase in the ratio reflects an elevation in intracellular Ca^{2+} concentration.

For simultaneous assessment of intracellular Ca^{2+} and isometric force, each experiment included three cumulative concentration-response relationships separated by washout periods: (1) an initial concentration–response relationship in the absence of inhibitors; (2) a second concentration–response relationship following incubation with either 10^{-7} M IbTX for 15 minutes or 10^{-5} M ouabain for 5 minutes (randomly assigned); and (3) a final concentration–response relationship after combined incubation with both IbTX and ouabain.

2.5. Quantification of protein phosphorylation by western blot

Isolated arterial segments were mounted on a single wire myograph system (Danish Myograph Technology A/S, Denmark) containing PSS maintained at 37°C and continuously aerated with 5% CO_2 in air. The vessels were equilibrated for at least 30 minutes before incubation with either 10^{-7} M M iberiotoxin (IbTX; 15 minutes), 10^{-5} M ouabain (5 minutes), a combination of both, or vehicle (MilliQ water). Four myograph chambers containing arterial segments from the same rat were included in each experiment. Following incubation, arteries were stimulated by direct addition of either $3 \cdot 10^{-6}$ M U46619 or 10^{-5} M methoxamine to the chamber. After 15 minutes of stimulation, arterial segments were fixed in ice-cold acetone containing 10 mM DL-dithiothreitol (DTT) and 10% trichloroacetic acid and stored at -80°C for 24 hours. Subsequently, the tissues were washed in ice-cold acetone with 10 mM DTT and stored again at -80°C until further processing.

For protein extraction, arterial segments were lysed in buffer containing (in mM): Tris-HCl 10, sucrose 250, EDTA 1, EGTA 1, DTT 1, and 2% Triton X-100 (pH 7.4), supplemented with protease inhibitor (1 tablet/10 mL), 10^{-5} Halt phosphatase inhibitor cocktail, and 2× Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Denmark). Homogenates were heated to 95°C for 10 minutes, ultrasonicated for 45 seconds, and centrifuged at 10,000×g. As this protocol does not permit reliable determination of protein concentration, equal gel loading was performed under the assumption of comparable protein content across samples.

Proteins were separated on 4–20% precast polyacrylamide stain-free gels (Criterion TGX Stain-Free Precast Gel; Bio-Rad). Total protein loading was visualized directly on the stain-free gels using UV illumination in an imaging system (c600; Azure Biosystems, Dublin, CA) to verify equal loading. Proteins were then electro-transferred onto nitrocellulose membranes. Membranes were blocked for 2 hours either in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS: 10 mM Tris-HCl, 100 mM NaCl, pH 7.6) with 0.5% Tween 20 (TBST) for detection of phosphorylated proteins, or in 0.3% iBlock in TBS for detection of total cSrc and MYPT. The upper portion of the membrane (>100 kDa) was used for MYPT detection (expected band ~130 kDa), and the lower portion for cSrc detection (expected band ~65 kDa). The following primary antibodies were used [3, 32]: phosphorylated cSrc (pY418; 1:200 in 3% BSA in TBS; Invitrogen, no. 44660G), total cSrc (1:500 in 0.3% iBlock in TBST; Santa Cruz Biotechnology, no. sc-8056), phosphorylated MYPT (pT850; 1:500 in 3% BSA in TBS; Millipore, no. 04-773), and total MYPT (1:6000 in 0.3% iBlock in TBST; Santa Cruz Biotechnology, no. sc-25618). Membranes were incubated overnight at +4°C washed and subsequently incubated for 1.5 hours with horseradish peroxidase–conjugated secondary antibodies (1:2000; Dako, Denmark) in TBST. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham, UK). Protein phosphorylation was semi-quantified as the ratio of phosphorylated cSrc or MYPT band intensity to the corresponding total (bulk) cSrc or MYPT protein signal, respectively.

2.6. Data analysis

Statistical analyses and graphing were performed using GraphPad Prism software (version 8.4.3 for Windows). Data are presented as mean ± SEM or as median with confidence intervals (CI), as specified; n is the number of vessels tested. Normal data distribution was supported with Q-Q plots. As only one vessel from each rat was used per interventional group, and all interventional groups included the vessel segments from the same rat. n represents number of rats, i.e. biological replicates.

Concentration-response relationships were fitted using a four-parameter nonlinear regression model. From these fits, the EC_{50} (the concentration producing 50% of the maximal response), Hill slope, and maximal response (E_{max}) were derived. Comparisons between fitted individual curves were performed using the extra sum-of-squares *F* test with the derived parameters, i.e., EC_{50}, Hill slope and E_{max}. The relationship between intracellular Ca^{2+} concentration and wall tension was analyzed using simple linear regression. Regression equations were compared with respect to both slopes and intercepts. Other parameters were analyzed using one- or two-way analysis of variance (ANOVA) for normally distributed data, or the Friedman test for nonparametric data, as appropriate. A probability value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Similar initial contractile responses in the experimental groups

In this study, 4 experimental groups of rat mesenteric small arteries dissected from the same animals but exposed to different pharmacological interventions were compared. These include arteries pre-treated with 100 nM IbTX, arteries exposed to 10 μM ouabain, arteries in the presence of both IbTX and ouabain, and control arteries incubated with vehicle only, i.e., PSS. To ensure similar initial functional properties of arterial segments allocated to these different experimental groups, we first examined their responses to the contractile agonists in the absence of IbTX and ouabain. No significant difference in the initial contractile responses to the thromboxane A_2 analogue U46619; the non-selective adrenoceptor agonist, noradrenaline; and the α_1 adrenoceptor agonist, methoxamine was detected between the arteries allocated to the 4 experimental groups (Suppl. Fig. 1A-C). Thus, these arteries were functionally similar under control conditions.

3.2. A combination of ouabain and IbTX potentiates basal vascular tone

After the initial stimulation with contractile agonists and washout, these arteries were incubated with IbTX (15 min), and/or ouabain (5 min) and/or vehicle prior to repeating the agonist-induced concentration-response assessment. When IbTX (100 nM) was applied, no statistically significant effect was detected on basal tone (Fig. 1A). The pre-incubation with ouabain (10 μM) also did not result in a detectable change in basal tone. However, the combination of both ouabain and IbTX elevated basal vascular tone (Fig. 1A).

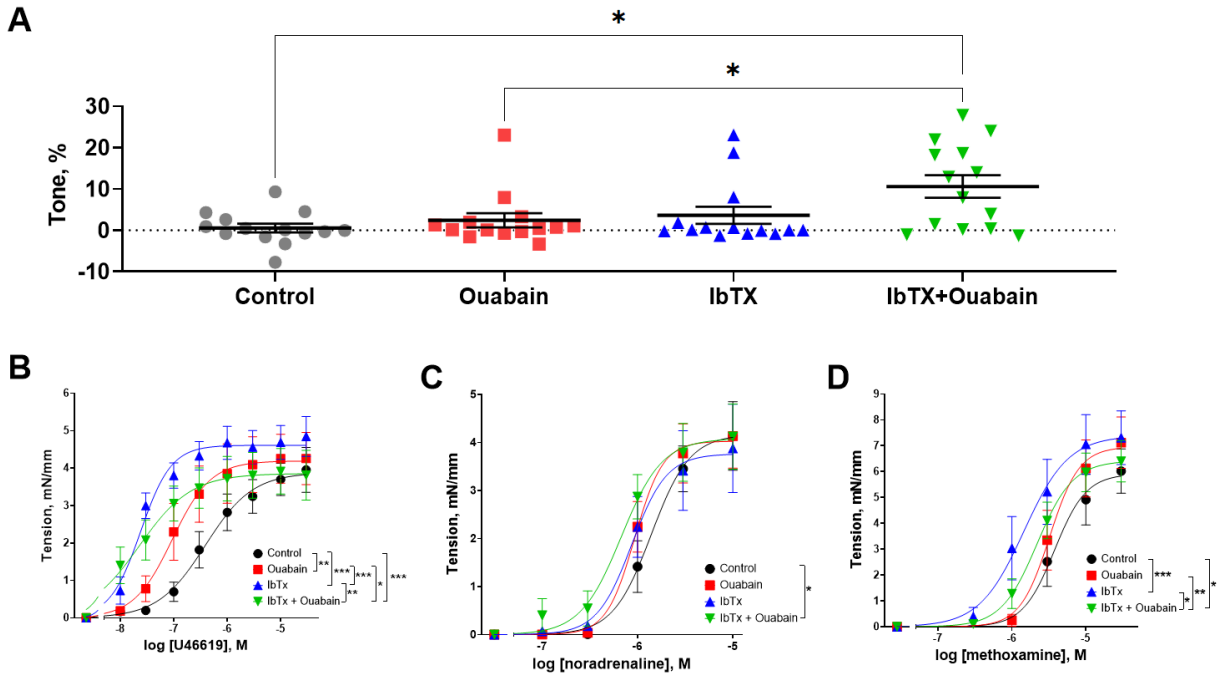


Figure 1. Pre-incubation of arteries with ouabain (10 µM) or IbTX (100 nM) did not affect basal tone, but their combination increased vascular tone (A; n = 14). The data were compared using one-way ANOVA followed with Šidák correction for multiple comparisons and * indicates $P < 0.05$. The differential effects of ouabain and IbTX on agonist-induced contraction with U46619 (B; n = 7), noradrenaline (C; n = 6) and methoxamine (D; n = 7). The concentration-response curves were compared with extra sum-of-squares F test. *, ** and *** show P <0.05, <0.01 and <0.001, as indicated.

3.3. Micromolar ouabain potentiated U46619-induced contraction of rat mesenteric artery, while no statistically significant effect was seen on the noradrenaline- and methoxamine-induced contractions

Ten µM ouabain potentiated U46619-induced contraction (Fig. 1B). This potentiation was due to an increased agonist sensitivity, while the maximal contractile response was not affected (Table. 1). In contrast, no statistically significant effect of pre-incubation with ouabain was detected on noradrenaline-induced (Fig. 1C) or methoxamine-induced (Fig. 1D) contractions (Tables 2 and 3).

3.4. A combination of ouabain and IbTX potentiated contractions in response to U46619, noradrenaline and methoxamine.

Pre-incubation with IbTX shifted the concentration-response relationships for U46619 (Fig. 1B) and methoxamine (Fig. 1D) to the left suggesting an increased sensitivity of the arteries to agonists as indicated by increased -LogEC_{50} values (Tables 1 and 3). In contrast, IbTX pre-incubation was without detectable effect on the noradrenaline-induced concentration-response relationship (Fig. 1C).

However, when a combination of IbTX and ouabain was applied, the responses to all three agonists, U46619, noradrenaline and methoxamine, were more sensitive to stimulation than those under control conditions (Fig. 1 and Tables 1-2). The sensitivity to methoxamine in the presence of both IbTX and ouabain was, however, not different from the control ($P = 0.083$) (Fig. 1 and Table 3).

3.5. Ouabain but not IbTX sensitizes smooth muscle cells for intracellular Ca^{2+} during U46619 stimulation

Previous studies suggested that the potentiating action of micromolar ouabain on noradrenaline- [3] and methoxamine-induced [31] contractions is mediated, at least in part, because of sensitization of the contractile machinery to intracellular Ca^{2+}. Accordingly, we assessed intracellular Ca^{2+} changes in U46619-induced contractions of rat mesenteric arteries in the presence of ouabain or IbTX or a combination of these drugs (Fig. 2). Arteries loaded with the Ca^{2+}-sensitive dye, FURA-2, were first constricted with U46619 under control conditions and then, in the presence of either ouabain (Fig. 2A) or IbTX (Fig. 2D). No statistically significant difference was observed between the concentration-response curves obtained under control conditions prior to exposure to either ouabain (Fig. 2A) or IbTX (Fig. 2D). Incubation with ouabain did not affect resting intracellular Ca^{2+} ($P = 0.063$), nor basal wall tension ($P = 0.466$). In contrast, IbTX elevated resting intracellular Ca^{2+} ($P = 0.009$) but did not affect basal wall tension ($P = 0.265$). This suggests that the sensitivity of the contractile machinery to Ca^{2+} is relatively low in the absence of agonist stimulation. Ouabain increased the sensitivity for U46619-induced contraction ($-\text{LogEC}_{50}$ was shifted from 7.16 [95% CI: 7.50 – 6.15] to 7.64 [95% CI: 7.86 – 7.36], $P = 0.027$, $n = 5$; Fig. 1A). Intracellular Ca^{2+} changes during the stimulation with U46619 were different in the presence of ouabain in comparison with control conditions ($P = 0.006$, $n = 5$; Fig. 2B). When wall tension was plotted against the intracellular Ca^{2+} concentrations in the presence of different U46619 concentrations, the linear slope was steeper for the relationship in the presence of ouabain than under control conditions (41.5 [95% CI: 36.9 – 46.0] vs. 30.8 [95% CI: 26.0 – 35.6], $P = 0.003$; Fig. 2C). This suggests that in the presence of ouabain, smooth muscle cells stimulated with U46619 are more sensitive to intracellular Ca^{2+} changes than under control conditions.

The effect of pre-incubation with IbTX on U46619-induced arterial contraction ($P = 0.07$, $n = 6$; Fig. 2D) was associated with shifting the U46619-intracellular Ca^{2+} relationship upwards ($P = 0.006$; Fig. 2E). The relationship between intracellular Ca^{2+} and wall tension suggests that IbTX-potentiated U46619-induced contraction was due to intracellular Ca^{2+} elevation with no significant changes in

the Ca^{2+} sensitivity in comparison with the control conditions (21.1 [95% CI: 13.4 – 28.7] vs. 22.2 [95% CI: 16.7 – 27.6], $P = 0.752$; Fig. 2F).

Incubation with both ouabain and IbTX did not affect the intracellular Ca^{2+} responses to U46619 in comparison with the effect of ouabain alone ($P = 0.842$, $n = 5$; Fig. 2B). Incubation with both ouabain and IbTX further shifted the U46619 concentration - intracellular Ca^{2+} relationship upwards in comparison with the effect of IbTX alone ($P = 0.042$, $n = 6$; Fig. 2E), although elevation of the basal intracellular Ca^{2+} ($P = 0.085$) did not achieved statistical significance and no difference in -$LogEC_{50}$ was seen ($P = 0.841$). When both ouabain and IbTX were present in the bath, the steepness of intracellular Ca^{2+} - wall tension relationship was increased in comparison with the effect of IbTX alone (43.8 [95% CI: 33.3 – 54.4] vs. 21.1 [95% CI: 13.4 – 28.7], $P = 0.004$; Fig. 2F) but unchanged in comparison with ouabain alone (40.3 [95% CI: 35.3 – 45.3] vs. 41.5 [95% CI: 36.9 – 46.0], $P = 0.637$; Fig. 2C). This again suggests that IbTX potentiated the U46619-induced contraction due to elevation of intracellular Ca^{2+} while the effect of ouabain is mediated via Ca^{2+} sensitization (Fig. 2C and F).

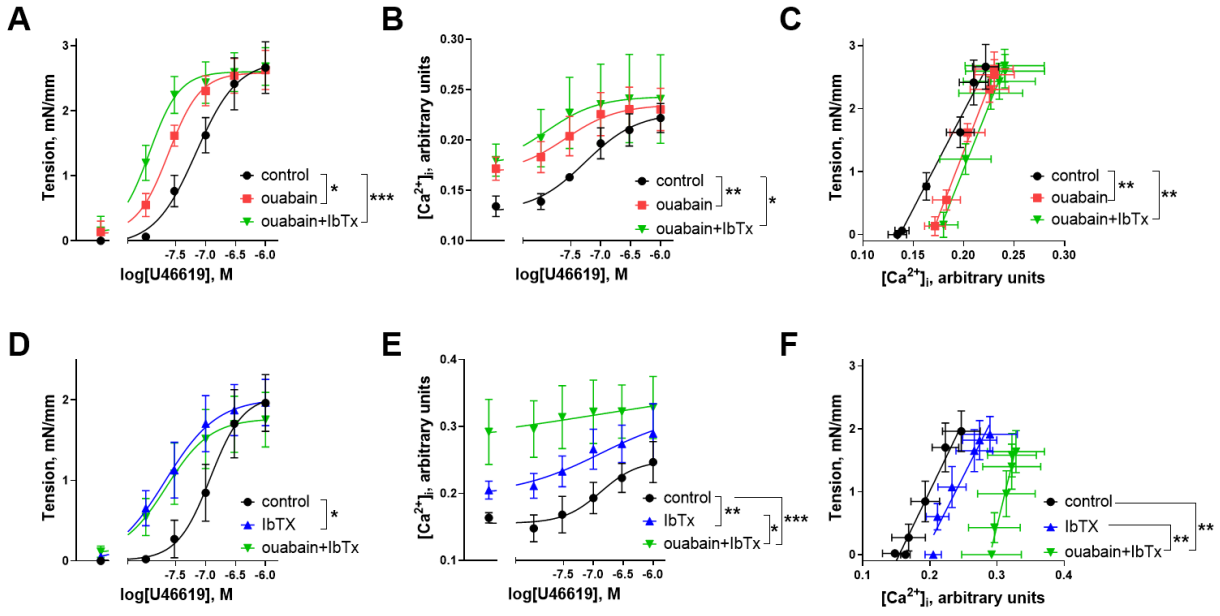


Figure 2. Simultaneous measurements of wall tension (**A** and **D**) and intracellular Ca^{2+} (**B** and **E**) revealed different mechanisms for potentiation of U46619-induced contraction by ouabain (**A-C**; 10 µM) and IbTX (**D-F**; 100 nM). Ouabain potentiated contraction (**A**) and elevated intracellular Ca^{2+} responses (**B**) induced by U46619. This was associated with increased steepness of the intracellular Ca^{2+}-tension relationship in the presence of ouabain in comparison with the control condition, suggesting an increased sensitivity of the contractile machinery to Ca^{2+} (**C**). Addition of IbTX in the presence of ouabain did not affect the contraction (**A**), and intracellular Ca^{2+} (**B**), neither was it associated with changes in the steepness of the Ca^{2+} - tension relation (**C**). IbTX also potentiated the contractile response to U46619 (**D**) and elevated intracellular Ca^{2+} (**E**)

induced by U46619 but did not change the steepness of the Ca^{2+} - tension relationship (**F**). Addition of ouabain to IbTX did not further affect the contraction (**D**), but elevated intracellular Ca^{2+} (**E**) and increased the steepness of the Ca^{2+} - tension relationship (**F**). The relationships were compared using *F*-test considering non-lineal regression's maximum, LogEC50 and Hill slope for **A** and **D**; non-lineal regression's baseline, maximum, LogEC50 and Hill slope for **B** and **E**, and lineal regression's slope and intercept for **C** and **F**. *, ** and ***, $P < 0.05$, < 0.01 and < 0.001, as indicated in the figure legends.

3.6. Ouabain but not IbTX sensitizes the contractile machinery via cSrc-dependent MYPT phosphorylation

Contraction induced by U46619 was previously proposed to be due to sensitization of the contractile machinery of vascular smooth muscle cells to intracellular Ca^{2+} through an increase in myosin light chain (MLC) phosphorylation [35]. Incubation with micromolar ouabain has also been shown to sensitize smooth muscle cells to intracellular Ca^{2+}, a mechanism mediated via cSrc-dependent phosphorylation of myosin phosphatase target subunit (MYPT) [3, 32]. We tested whether cSrc and MYPT phosphorylation contribute to ouabain- and IbTX-dependent potentiation of U46619- and methoxamine-induced contractions (Fig. 3). Ouabain potentiated cSrc phosphorylation upon U46619 stimulation in rat mesenteric arteries in comparison with control arteries stimulated with U46619 alone (Fig. 3A and C). This was associated with phosphorylation of MYPT (Fig. 3B and D). Similarly, when arteries were stimulated with methoxamine, ouabain also potentiated the phosphorylation of both cSrc and MYPT (Fig. 3E and F).

In contrast, IbTX alone did not affect the phosphorylation state of either cSrc or MYPT when arteries were preconstricted with U46619 (Fig. 3C and D) or methoxamine (3E and F). A combination of ouabain and IbTX led to elevation in phosphorylation of both cSrc and MYPT in arteries stimulated with methoxamine (3E and F). Stimulation with U46619 also increased MYPT phosphorylation in the presence of both ouabain and IbTX, though there was no statistical difference in cSrc phosphorylation (Fig. 3C and D). Thus, ouabain increase cSrc and MYPT phosphorylation to a statistically similar level as that observed in the presence of both ouabain and IbTX upon U46619 or methoxamine stimulation. No statistical difference in the phosphorylation states after U46619 or methoxamine was observed in arteries treated with IbTX alone compared with controls (Fig. 3C-F).

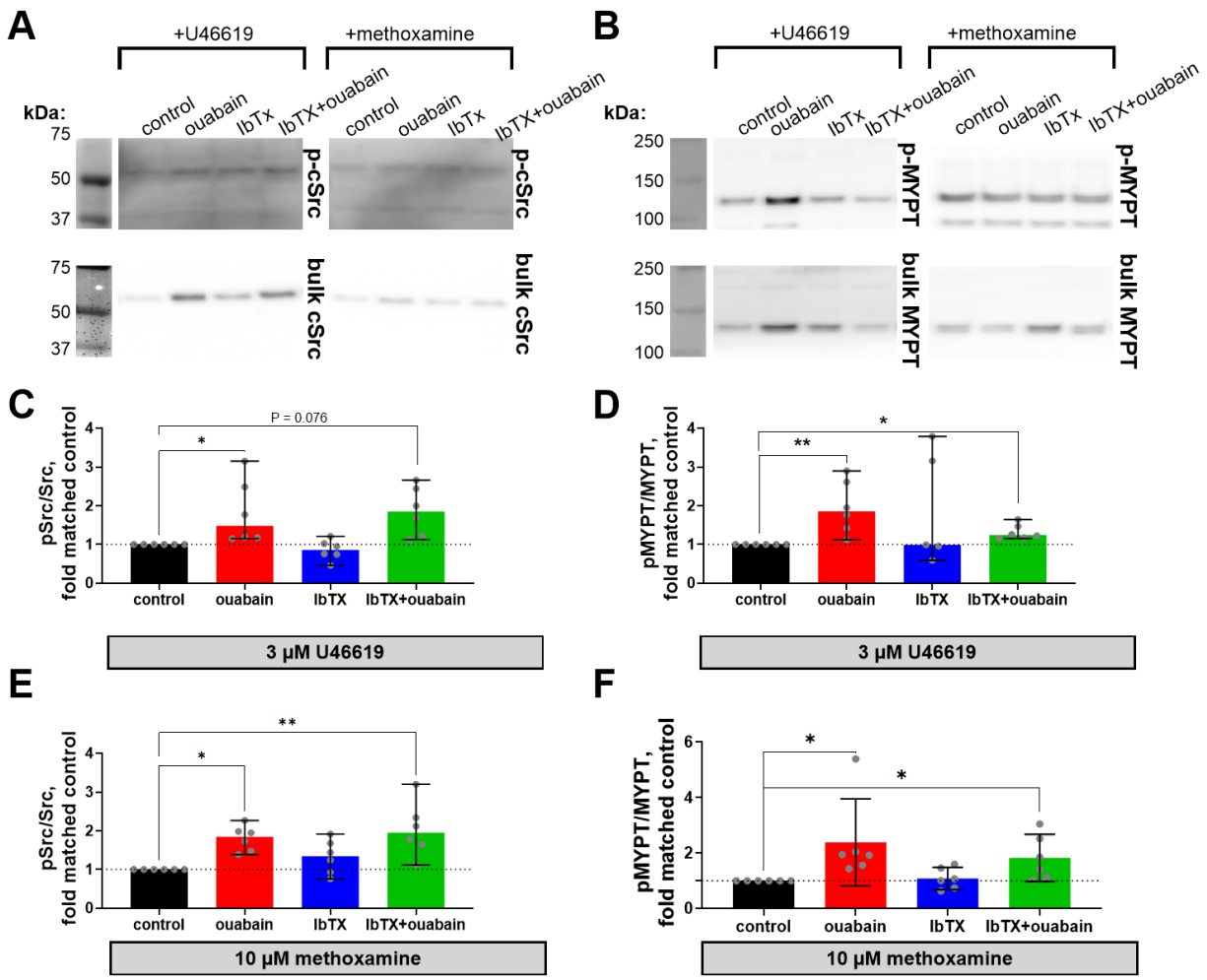


Figure 3. Ouabain but not IbTX potentiates the phosphorylation of both cSrc kinase and myosin phosphatase target subunit (MYPT) in arteries pre-constricted with either U46619 (3 µM) or methoxamine (10 µM). Representative Western blots of arteries exposed to either 10 µM ouabain, 100 nM IbTX, or their combination, as indicated (**A** and **B**). The lysates of arterial segments preconstricted with either U46619 or methoxamine were tested with either phospho-specific cSrc (pSrc) or phospho-specific MYPT (pMYPT) antibodies or with antibodies recognizing bulk cSrc and MYPT in the lysates (**A** and **B**, as indicated). Different mesenteric artery segments from the same rat were exposed to three different interventions or were studied under control conditions, i.e., without ouabain and IbTX. The corresponding band densities were normalized to the band density for the control arterial segments from the same rats, given as 100%. (**C**) The averaged results for cSrc phosphorylation relative to the bulk cSrc expression (pSrc/Src), normalized for the control conditions in the arteries stimulated with U46619. (**D**) The averaged results for MYPT phosphorylation relative to the bulk MYPT expression (pMYPT/MYPT), normalized for the control conditions in the arteries stimulated with U46619. (**E**) The averaged results for pSrc/Src in methoxamine-stimulated arteries. (**F**) The averaged results for pMYPT/MYPT in methoxamine-stimulated arteries. n = 6 (except **D**, where one of the bands in the presence of IbTX was not detectable and was excluded). Because of the normalization procedure used, the

results were compared with Friedman test followed with Dunn's correction for multiple comparison and presented as median with confidence intervals. * and **, $P < 0.05$ and 0.01.

4. Discussion

This study investigated the potentiating effect of ouabain on vascular contraction by examining the roles of changes in intracellular Ca^{2+} and Ca^{2+} sensitization of the contractile machinery in response to different agonists (U46619, methoxamine, and noradrenaline), as well as the contribution of BK_{Ca}-channel-dependent negative feedback signaling [31]. We found that micromolar ouabain, at concentrations known to selectively inhibit the Na/K-ATPase α_2 subunit [36], potentiated contractions induced by U46619. In contrast, the same concentration of ouabain did not enhance contractions elicited by methoxamine or noradrenaline, indicating that the potentiation is agonist dependent. Inhibition of BK_{Ca} channels increased arterial sensitivity to U46619 and methoxamine but had no statistically significant effect on noradrenaline-induced contraction. Notably, the pro-contractile effect of ouabain was augmented in the presence of IbTX, resulting in enhanced sensitivity to all three agonists. Previous studies have shown that potentiation of noradrenaline- [3] and methoxamine-induced [31] contractions by micromolar ouabain is mediated, at least in part, by increased Ca^{2+} sensitization of the contractile apparatus. To clarify the mechanism underlying potentiation of U46619-induced contraction, we examined the relationship between intracellular Ca^{2+} and wall tension following incubation with ouabain and IbTX. We found that ouabain increased Ca^{2+} sensitivity during U46619 stimulation, whereas IbTX primarily elevated intracellular Ca^{2+} levels. The ouabain-induced increase in Ca^{2+} sensitivity in response to agonist stimulation was associated with enhanced phosphorylation of cSrc and MYPT. No significant differences in ouabain-induced phosphorylation levels were detected between U46619- and methoxamine-induced contractions. In contrast, IbTX alone did not alter cSrc or MYPT phosphorylation during pre-constriction with either U46619 or methoxamine. Importantly, although the combination of IbTX and ouabain increased phosphorylation of cSrc and MYPT, this effect did not exceed that observed with ouabain alone, underscoring the distinct and mechanistically separate potentiating actions of ouabain and BK_{Ca} channel inhibition.

The potentiation of vascular contraction by ouabain exhibits agonist-dependent characteristics, a finding consistent with previous publications and explaining some discrepancies between these reports. Micromolar ouabain has been shown to potentiate agonist-induced contractions, e.g., U46619-induced contraction in murine cerebral arteries [32] and noradrenaline-induced contraction in rat mesenteric arteries [3, 14]. In accordance with previous reports [3, 22, 37], this study demonstrates that ouabain increased the smooth muscle cell sensitivity to Ca^{2+} during U46619 stimulation. In contrast, IbTX produced an increase in baseline intracellular Ca^{2+}, but there was no

significant shift in the Ca^{2+}-wall tension relationship. This indicates that the pro-contractile effect during inhibition of the BK_{Ca} channel is due to the elevation of intracellular Ca^{2+}. Furthermore, a comparison of the simultaneous inhibition of the Na/K-ATPase and BK_{Ca} channels, to their selective inhibition suggested that IbTX potentiated the U46619-induced contraction due to elevation of intracellular Ca^{2+} while the effect of ouabain is mainly mediated by Ca^{2+} sensitization.

In accordance with the previous study [31], ouabain was without effect on methoxamine-induced contraction of endothelium-denuded rat mesenteric arteries, unless the BK_{Ca}-channels were inhibited. These findings align with the current data, where micromolar ouabain potentiated the pro-contractile effect of U46619, and a combination of ouabain and IbTX also potentiated methoxamine- and noradrenaline-induced contractions [3, 22, 31]. This could be, at least, in part, due to an interaction between the Na/K-ATPase and the BK_{Ca} channels. The BK_{Ca}-channels in the vascular smooth muscle cells are involved in negative feedback response to increased intracellular Ca^{2+} [11, 23]. A potential interplay between the pro-contractile α-adrenoceptor and vasorelaxant β-adrenoceptor pathways in the arterial wall [38, 39] could also explain the lack of ouabain-induced potentiation of agonist-evoked contraction when ouabain was applied alone. However, contractions induced by the α-adrenoceptor-selective agonist methoxamine and the non-selective adrenoceptor agonist noradrenaline were similarly insensitive to ouabain alone, making this explanation less likely.

Both the thromboxane A_2 receptors [11, 40] and the α_1 adrenergic receptors [41] initiate intracellular signaling pathways interfering with the BK_{Ca} channel activity. Thromboxane A_2 receptors have been reported to physically interact with BK_{Ca} channels, thereby reducing their open probability and promoting smooth muscle cell depolarization [40], although protein kinase C (PKC) dependent signaling has also been implicated in this response [42]. Consistent with this, PKC-dependent phosphorylation has been shown to inhibit BK_{Ca} activity [43, 44]. Similarly, noradrenaline inhibits BK_{Ca} channels primarily via PKC signaling, while simultaneously increasing intracellular Ca^{2+} levels, which can counterbalance or even override this inhibitory effect [44]. Notably, in neurons, α_1 adrenoceptor-dependent PKC activation has been shown to involve downstream Src-kinase activation [45]. This effect has been proposed to be mediated through PKC-dependent activation of a protein tyrosine phosphatase, which in turn activates Src kinase [46].

Additional kinase pathways may also contribute to thromboxane A_2 response, including the Na/K-ATPase-dependent Src signaling. However, in the present study, ouabain further enhanced Src kinase activation and MYPT1 phosphorylation in U46619-stimulated arteries. These findings suggest that

the pathways operate independently; one through ouabain-induced Ca^{2+} sensitization and the other through IbTX-mediated BK_{Ca} channel inhibition. Consequently, their effects may be additive. The present study did not directly assess the effects of methoxamine or noradrenaline on Src signaling in the vascular wall, while previous studies in rat mesenteric arteries have demonstrated that noradrenaline alone increases Src activation and MYPT1 phosphorylation [3]. Ouabain was able to further potentiate Src/MYPT1 signaling in noradrenaline-stimulated rat and mouse mesenteric arteries [3, 37] as well as in methoxamine-stimulated arteries (present study). However, this potentiation was comparable to the effect of noradrenaline alone [3]. Moreover, despite noradrenaline-induced Src/MYPT1 phosphorylation in mouse mesenteric arteries [37], no corresponding enhancement of arterial contraction was observed. This suggests that ouabain-induced Src activation in mesenteric arteries may not be sufficient, on its own, to substantially augment contractile responses, or that mesenteric artery smooth muscle cells lack the downstream signaling mechanisms that remain to be studied.

Taken together, these findings suggest that α_1 adrenoceptor stimulation elevates intracellular Ca^{2+}, which enhances BK_{Ca} channel activity, while concurrently activating PKC to inhibit BK_{Ca} channels and sensitize the contractile apparatus to Ca^{2+} via Src activation. This dynamic balance may limit the effect of ouabain on noradrenaline-induced contraction alone but contribute to the pro-contractile effect observed when ouabain is combined with IbTX. Furthermore, the modulatory influence of BK_{Ca} channels on noradrenaline-induced contraction depends on the availability of functional α and β adrenoceptors, i.e., receptor reserve [47], which may further explain the variability observed across vascular beds and species. Moreover, the functional interaction between the BK_{Ca} channels and the Na/K-ATPase has been previously reported [31]. The inhibition of BK_{Ca} channels was shown to potentiate methoxamine-induced contraction in rat mesenteric arteries and this potentiation is further enhanced by simultaneous inhibition of the α_2 isoform of the Na/K-ATPase [31]. This functional interaction is supported by close proximity of the Na/K-ATPase α_2 isoform and the BK_{Ca} channels in rat mesenteric artery smooth muscle cells, as demonstrated by the proximity ligation assay [31]. Previously, other reports suggested similar functional interactions between the Na/K-ATPase and the BK_{Ca} channels involved in transmembrane potassium ion circulation [21]. Accordingly, the present findings support this functional microdomain between these two membrane transporters.

Previous studies suggest that both the Na/K-ATPase and BK_{Ca} channels regulate vascular contraction through an interaction with the NCX [1, 12, 31]. The physical co-localization of the Na/K-ATPase and the NCX has also been demonstrated, suggesting the involvement of NCX in this signaling

microdomain [48-50]. In addition, BK_{Ca} channels have been shown to co-localize with the Na/K-ATPase [31], while Src kinase physically interacts with the Na/K-ATPase [51]. Together, these findings support the existence of a functional membrane microdomain in which the Na/K-ATPase serves as a scaffold for signaling proteins [19, 52-54]. Inhibition of the Na/K-ATPase impairs Na^+ extrusion, thereby reducing the transmembrane Na^+ gradient and promoting reversal of NCX polarity [12, 18, 19]. As a consequence, NCX facilitates Ca^{2+} influx, leading to an increase in intracellular Ca^{2+}. Consistent with this mechanism, the present study demonstrated that inhibition of the Na^+/K^+-ATPase with ouabain leads to elevation of resting intracellular Ca^{2+} level. Inhibition of BK_{Ca} channels with IbTX also increased resting intracellular Ca^{2+}, likely as a result of membrane depolarization. However, simultaneous inhibition of both the Na/K-ATPase and BK_{Ca} channels produced a further elevation in intracellular Ca^{2+} only when ouabain was added to IbTX-treated arteries. In contrast, administration of IbTX to arteries already treated with ouabain had no substantial additional effect on intracellular Ca^{2+}. This asymmetry may reflect the dual voltage- and Ca^{2+}-dependent nature of BK_{Ca} channel activation [44]. Under resting conditions, only a limited number of BK_{Ca} channels are active. Therefore, IbTX induces only modest depolarization. Subsequent addition of ouabain can then further elevate intracellular Ca^{2+}, potentially via activation of voltage-gated Ca^{2+} channels [37, 45] or modulation of NCX activity [55]. Conversely, when intracellular Ca^{2+} is already elevated by ouabain, the depolarizing impact of BK_{Ca} channel inhibition may be attenuated, limiting any further increase in intracellular Ca^{2+}.

In conclusion, this study addressed the discrepancy in the effects of ouabain on rat mesenteric small artery responses to different pro-contractile agonists, with particular focus on the roles of intracellular Ca^{2+}, Ca^{2+} sensitization, and BK_{Ca}-channel-mediated negative feedback. We found that ouabain alone did not considerably modify contractions induced by adrenoceptor stimulation, whereas IbTX potentiated these responses. In contrast, thromboxane A_2 receptor-mediated contractions were enhanced by both IbTX and ouabain. The effects of IbTX and ouabain were additive. The pro-contractile action of IbTX was primarily mediated through enhanced voltage-dependent Ca^{2+} influx following BK_{Ca} channel inhibition, whereas ouabain increased Ca^{2+} sensitivity of contractile machinery via activation of the Src-kinase pathway.

5. Acknowledgements

Authors thank Jane Holbæk Rønn and Viola Mose Larsen for excellent technical assistance.

6. Funding sources

The research was financially supported by the Lundbeck Foundation grants R344-2020-952 and R412-2022-449, the Novo Nordisk Foundation grants NNF18OC0052021 and NNF19OC0056371, the Independent Research Fund Denmark grant 3101-00103B and the Riisfort Foundation.

7. Author contributions

Daniel Løgstrup Nielsen: data curation, formal analysis, visualization, writing – original draft, writing – review and editing; Josef Ali Khalid: data curation, formal analysis, writing – review and editing; Anna Giné Martínez: data curation, formal analysis, writing – review and editing; Elizaveta V. Melnikova: data curation, formal analysis, supervision, writing – review and editing; Benjamin Hørning Risager: data curation, formal analysis, writing – review and editing; Rudolf Schubert: visualization, writing – original draft, supervision, writing – review and editing; Christian Staehr: supervision, writing – original draft, writing – review and editing; Vladimir V. Matchkov: conceptualization, data curation, formal analysis, funding acquisition, visualization, project administration, supervision, writing – original draft, writing – review and editing

8. Conflicts of Interest

The authors declare no conflicts of interest.

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Intervention	-Log EC_{50} [95% CI]	*P* value						Max. contraction, mN/mm [95% CI]	*P* value						n
Control	6.41 [6.70; 5.93]		0.015					3.88 [3.31; 4.96]		0.594					7
Ouabain	7.03 [7.34; 6.60] #	0.015		0.003				4.20 [3.56; 5.12]	0.594		0.296				7
IbTX	7.62 [7.79; 7.42] ***, $$		0.003		0.912	0.001		4.61 [4.25; 5.03]		0.296		0.046	0.144		7
IbTX and Ouabain	7.64 [8.07; 7.18] +, †††			0.912		0.001	0.038	3.86 [3.33; 4.63]			0.046		0.956	0.461	7

Table 1. Parameters of the concentration-response relationships for the thromboxane A_2 analogue, U46619 under control conditions, in the presence of ouabain (10 μM), IbTX (100 nM) or their combination. The data are presented as the averaged values of arterial sensitivity for U46619 (expressed as -LogEC_{50}) and maximal contraction with their 95% confidence intervals (95% CI). The parameters were compared with the *F*-test. #, P<0.05 control vs. ouabain; ***, P<0.001 control vs. IbTX; $$, P<0.01 ouabain vs. IbTX; +, P<0.05 ouabain vs. IbTX+ouabain; †††, P<0.001 control vs. IbTX+ouabain. See also Figure 1B.

Intervention	-Log EC_{50} [95% CI]	*P* value						Max. contraction, mN/mm [95% CI]	*P* value					n
Control	5.86 [6.02; 5.62]		0.127					4.17 [3.44; 5.33]		0.786				6
Ouabain	6.03 [6.17; 5.85]	0.127		0.796				4.03 [3.47; 4.85]	0.786		0.629			6
IbTX	6.06 [6.30; 5.64]		0.796		0.439	0.189		3.77 [3.01; 5.46]		0.629		0.578	0.519	6
IbTX and Ouabain	6.17 [6.36; 5.92] †			0.439		0.029	0.215	4.08 [3.46; 5.09]			0.578	0.519	0.873	0.909	6

Table 2. Parameters of the concentration-response relationships for noradrenaline under control conditions, in the presence of ouabain (10 μM), IbTX (100 nM) or their combination. The data are presented as the averaged values of arterial sensitivity for noradrenaline (expressed as -LogEC_{50}) and maximal contraction with their 95% confidence intervals (95% CI). The parameters were compared with the *F*-test. †, P<0.05 control vs. IbTX+ouabain. See also Figure 1C.

Intervention	-Log EC_{50} [95% CI]	*P* value						Max. contraction, mN/mm [95% CI]	*P* value					N
Control	5.43 [5.61; 5.07]		0.690					5.91 [4.83; 8.19]		0.328				7
Ouabain	5.49 [5.65; 5.19]	0.690		0.056				6.96 [5.86; 9.11]	0.328		0.683			7
IbTX	5.84 [6.09; 5.45] *		0.056		0.261	0.032		7.39 [6.12; 9.70]		0.683		0.274	0.247	7
IbTX and Ouabain	5.66 [5.81; 5.47]			0.261		0.083	0.178	6.42 [5.64; 7.55]			0.274		0.548	0.489	7

Table 3. Parameters of the concentration-response relationships for methoxamine under control conditions, in the presence of ouabain (10 μM), IbTX (100 nM) or their combination. The data are presented as the averaged values of arterial sensitivity for methoxamine (expressed as -LogEC_{50}) and maximal contraction with their 95% confidence intervals (95% CI). The parameters are compared with the *F*-test. *, P<0.05 control vs. IbTX. See also Figure 1D.

Interplay between Na^+/K^+-ATPase-dependent Src-mediated Ca^{2+} sensitization and BK_{Ca} channel negative feedback regulates rat mesenteric artery contraction.

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Daniel Løgstrup Nielsen: data curation, formal analysis, visualization, writing – original draft, writing – review and editing; **Josef Ali Khalid**: data curation, formal analysis, writing – review and editing; **Anna Giné Martínez**: data curation, formal analysis, writing – review and editing; **Elizaveta V. Melnikova**: data curation, formal analysis, supervision, writing – review and editing; **Benjamin Hørning Risager**: data curation, formal analysis, writing – review and editing; **Rudolf Schubert**: visualization, writing – original draft, supervision, writing – review and editing; **Christian Staehr**: supervision, writing – original draft, writing – review and editing; **Vladimir V. Matchkov**: data curation, formal analysis, visualization, project administration, supervision, funding acquisition, writing – original draft, writing – review and editing

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Declaration of interests

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.