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RESEARCH ARTICLE

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# Vascular smooth muscle BK channels limit ouabain-induced vasocontraction: Dual role of the Na/K-ATPase as a hub for Src-kinase and the Na/Ca-exchanger

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## Abstract

Large-conductance, calcium-activated potassium channels (BK channels) and the Na/K-ATPase are expressed universally in vascular smooth muscle. The Na/K-ATPase may act via changes in the intracellular $Ca^{2+}$ concentration mediated by the Na/Ca exchanger (NCX) and via Src kinase. Both pathways are known to regulate BK channels. Whether BK channels functionally interact in vascular smooth muscle cells with the Na/K-ATPase remains to be elucidated. Thus, this study addressed the hypothesis that BK channels limit ouabain-induced vasocontraction. Rat mesenteric arteries were studied using isometric myography, FURA-2 fluorimetry and proximity ligation assay. The BK channel blocker iberiotoxin potentiated methoxamine-induced contractions. The cardiotonic steroid, ouabain ($10^{-5}$ M), induced a contractile effect of IBTX at basal tension prior to methoxamine administration and enhanced the pro-contractile effect of IBTX on methoxamine-induced contractions. These facilitating effects of ouabain were prevented by the inhibition of either NCX or Src kinase. Furthermore, inhibition of NCX or Src kinase reduced the BK channel-mediated negative feedback regulation of arterial contraction. The effects of NCX and Src kinase inhibition were independent of each other. Co-localization of the Na/K-ATPase and the BK channel was evident. Our data suggest that BK channels limit ouabain-induced vasocontraction by a dual mechanism involving the NCX and Src kinase signaling. The data propose that the NCX and the Src kinase pathways, mediating the ouabain-induced activation of the BK channel, act in an independent manner.

**KEYWORDS**
artery, BK channel, Na/K-ATPase, smooth muscle, vasocontraction

**Abbreviations:** AUC, area under the curve; BK channel, large-conductance, calcium-activated potassium channels; IBTX, iberiotoxin; Mx, methoxamine; NCX, Na/Ca exchanger; PBS, phosphate-buffered saline; PLA, proximity ligation assay.

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# 1 | INTRODUCTION

Large-conductance, calcium-activated potassium channels (BK channels) are expressed universally in vascular smooth muscle where they contribute to negative feedback regulation on vasocontraction and mediate vasodilation.[1] This function is altered in several diseases, e.g. hypertension and diabetes (for a comprehensive overview on BK channel function and dysfunction see e.g. [1]). The activity of BK channels depends on a number of intracellular signaling molecules (for reviews see [1,2]), including Src-kinase.[3,4]

The current mediated by the BK channels is enabled by the $K^+$ transmembrane gradient created by the Na/K-ATPase, an ubiquitous membrane transporter expressed in all living cells.[5] The Na/K-ATPase is a P-type ATPase moving 3 sodium ions out of the cell and 2 potassium ions into the cell, i.e., this transport is electrogenic.[6] The Na/K-ATPase consists of α-, β- and FXYD subunits.[7] The α-subunit is responsible for ion translocation and ATP hydrolysis and contains binding sites for cardiotonic steroids, e.g., ouabain and digoxin, which are its specific inhibitors (for review see [8,9]). The β-subunit is required for stability and trafficking, and FXYD is responsible for modulation of enzymatic activity.[7]

The $Na^+$ transmembrane gradient created by the Na/K-ATPase serves as a driving force for other transporters, in particular the sodium/calcium exchanger (NCX).[10] The functional interaction between the Na/K-ATPase and the NCX contributes to the regulation of the intracellular $Ca^{2+}$ concentration, which mediates the effects of cardiotonic steroids. For example, inhibition of the Na/K-ATPase diminishes the $Na^+$ gradient and, thus, suppresses $Ca^{2+}$ extrusion by the NCX leading to intracellular accumulation of $Ca^{2+}$ ions and potentiation of vasoconstriction.[9,11,12] As the reversal potential of the NCX is slightly more positive than the resting membrane potential, further accumulation of intracellular $Na^+$ and membrane depolarization due to Na/K-ATPase inhibition may change the direction of ion transport from the $Ca^{2+}$ extrusion mode (i.e., forward mode) to the $Ca^{2+}$ influx mode (i.e., reverse mode) and, thus, further potentiate muscle contraction.[9]

In addition to its ion translocation function, the Na/K-ATPase has been shown to act as a signal transduction protein.[13,14] Along with the involvement in several other signal transduction pathways, the Na/K-ATPase keeps Src kinase in its inactive state but it can be released and autophosphorylated upon binding of cardiotonic steroids to the Na/K-ATPase[15,16] (for review see also [17,18]). In vascular smooth muscle, this cardiotonic steroid-induced Src kinase activation has been suggested to be involved in the regulation of, for example, smooth muscle proliferation,[19] intercellular coupling,[20] and vasocontraction.[21–24]

Thus, the Na/K-ATPase may exert a dual influence on cellular function via changes in the intracellular $Ca^{2+}$ concentration and via modulation of Src kinase activity.[25] Importantly, these two pathways are known to regulate BK channel activity.[3,4,26,27] Whether BK channels interact functionally with the Na/K-ATPase and are regulated by the Na/K-ATPase in vascular smooth muscle cells remains to be elucidated. This study addressed the hypothesis that BK channels limit ouabain-induced vasocontraction. We suggested that BK channels are activated in vascular smooth muscle cells by an interaction of cardiotonic steroids with the Na/K-ATPase and aimed to test whether this activation is mediated via Src kinase signaling or intracellular $Ca^{2+}$ changes due to modified activity of the NCX.

# 2 | MATERIALS AND METHODS

## 2.1 | Animals

Albino WISTAR rats (Janvier Labs, Le Genest-Saint-Isle, France) were used for the experiments in this study. The animals were housed under standardized conditions at constant 22°C room temperature and a regulated 12-h light–dark cycle. Rats always had free access to standard pellet chow and drinking water. For experimental purposes, only male rats were used to exclude any influence of the female hormonal cycle. The age of the rats on the experimental day ranged from 9 to 14 weeks. The experiments complied with the Animal Welfare Act and were reported to the Governmental authorities in Karlsruhe (Germany) (I-17/17) and to the Animal Experiments Inspectorate of the Danish Ministry of Environment and Food, where appropriate.

## 2.2 | Vessel preparation

Rats were killed under carbon dioxide anesthesia by decapitation using a guillotine. The intestine including the mesentery was removed from the abdominal cavity and transferred to a 4°C cold physiological salt solution with the following composition (in mM): 145 NaCl, 4.5 KCl, 1.2 $NaH_2PO_4$, 1 $MgSO_4$, 0.1 $CaCl_2$, 0.025 EDTA and 5 HEPES. Third order branches of mesenteric arteries were isolated from surrounding connective tissue and parallel veins.

## 2.3 | Isometric wire myography

A Multi-Wire Myograph (Model 610M & 620M, Danish Myo Technology A/S, Denmark) was used for data

acquisition. Each vessel segment was mounted on two 40 μm thin wires, which were passed through the lumen of arterial segments. One wire was fixed rigidly and the other one was able to detect vessel wall tension via a force transducer reporting to Labchart (ADInstruments) for data acquisition and storage. To eliminate a possible influence of the endothelium, the latter was gently removed by rotating a rat whisker in the vessel lumen with 20 clockwise and counterclockwise rotations each. The four chambers of the myograph were filled with physiological saline solution containing (in mM): 120 NaCl, 26 $NaHCO_3$, 5.5 glucose, 4.5 KCl, 1.6 $CaCl_2$, 1.2 $NaH_2PO_4$, 1.0 $MgSO_4$, 0.025 EDTA and 5 HEPES. This physiological saline solution was continuously bubbled with carbogen (95% $O_2$ and 5% $CO_2$) and heated to 37°C to maintain pH at 7.4. Each arterial segment was stretched to its optimal lumen diameter (90% of the inner diameter it would have at a transmural pressure of 100 mmHg), corresponding to optimal conditions for maximum active force development.[28,29]

Each series of experiments started with the same, well-defined starting protocol to check the viability of the vessel segments and to standardize the responsiveness of different vessel segments with each other. The α1-receptor agonist methoxamine (Mx) at a high concentration ($10^{-5}$) M served as the initial stimulus. A lack of relaxation in response to the following administration of acetylcholine at $10^{-5}$ M was used to ensure that the endothelium was successfully removed. After washout, a modified saline solution containing 120 mM KCl (equimolar replacement with NaCl) was used to provoke a contraction mediated by potassium-induced depolarization, and then the vessel was again washed out to the control saline. Mx at $10^{-5}$ M was given a second time to establish the reference values for standardization of the subsequent experiments. The baseline was defined as complete relaxation of the vessel (0% wall tension) and the value of wall tension after five minutes of the second Mx incubation was defined as maximum contraction (100% wall tension).

## 2.4 | Calcium fluorimetry

Vessels were isolated, mounted and exposed to viability tests as described above. Background fluorescence from the mounted vessel just prior to FURA-2 loading was determined. Thereafter, vessels were loaded with 5 μM FURA-2 AM (dissolved in DMSO and diluted in physiological saline solution) at 37°C for 90 min. During the measurements, the dye was excited with light at 340 and 380 nm using a monochromator and a xenon arc lamp (Horiba Europe GmbH, Germany). Emitted light from the specimen was filtered at 520 nm with a long pass filter, detected by a photomultiplier and intensity data were stored at a computer. Background fluorescence was subtracted from the fluorescence signals at the respective excitation wavelengths, and the ratio of corrected fluorescence evoked at the two excitation wavelengths was calculated (F340/F380). The F340/F380 responses were normalized to the response at the second Mx administration (see also [29]).

## 2.5 | Experimental protocol

All experiments in this study had a standardized protocol. After equilibration for 10 min, the first concentration–response relationship for Mx was determined. The concentration of Mx in the four chambers was increased in a cumulative manner between $10^{-7}$ M and $2\times10^{-5}$ M. In the experiments testing the role of BK channels in ouabain-induced vasocontraction, the concentration steps were $10^{-7}$ M, $2\times10^{-7}$ M, and $5\times10^{-7}$ M. This concentration range was selected based on pilot experiments (data not shown) demonstrating that Mx-induced contractions in this range were accompanied by increases in the intracellular $Ca^{2+}$ concentration, a major activator of the BK channel addressed by our hypothesis. In contrast, at higher Mx concentrations, the augmentation of contraction was not accompanied with a further increase in intracellular $Ca^{2+}$ concentration. The initial average concentration–response relationships to methoxamine had to be similar between the experimental groups, so that any difference in the subsequent concentration–response relationships to Mx could only be attributed to the interventions. In most of the experimental protocols, upon completion of the first concentration–response relationship and washout, two vessels were pretreated with the BK channel inhibitor, iberiotoxin ($10^{-7}$ M, IBTX) and two other vessels were exposed to its vehicle (aqua dest). After 10 min incubation, a second concentration–response relationship to Mx was obtained, and two vessels exposed to IBTX and two control groups had again to be similar in their pairs in the average contraction responses (see also [29]). After washout, one of the IBTX-treated vessels and one vessel of the control group were treated with $10^{-5}$ M ouabain, making two more experimental groups, i.e., the ouabain- and IBTX-treated group, the ouabain-treated group, the IBTX-treated group and the control group. Of note, ouabain was applied 3 to 5 min before the start of the Mx concentration–response relationship, i.e., this study focused on the short-term effects of ouabain as opposed to long-term effects occurring after more than 30 min.[30,31] Then, the third Mx concentration–response relationship was constructed for all four experimental groups (for an example see Supplemental Figure S1).

In isolated cells, the effects of ouabain on BK currents, presumably mediated by a change in intracellular sodium concentration, were reported after <1 min.[32] Moreover, our preliminary experiments (not shown) demonstrated that longer pre-incubation times for ouabain (20–25 min) had a vasorelaxing effect, which is probably due to a reduction in calcium sensitivity of the contractile machinery in smooth muscle cells. This desensitization to intracellular calcium by prolonged Na,K-ATPase inhibition is related to depletion of intracellular $K^+$.[33] Therefore, to assess the pro-contractile effect of ouabain, we used a pre-incubation time of 3–5 min, which is long enough to induce changes in intracellular sodium but allows to avoid the vasorelaxing effect of ouabain.

The aim of the experiments was to examine the influence of IBTX and ouabain on Mx-induced vascular contractions as well as the interaction of these two substances on this contraction. To study the contribution of the Na/K-ATPase to BK channel activity during contraction of mesenteric arteries, the specific BK channel inhibitor, iberiotoxin (IBTX)[1,34–37] and the Na/K-ATPase inhibitor, ouabain[9,38] were used. Data analysis was performed in two steps. First, the effect of an intervention was determined based on the Mx concentration–response relationships. For example, it was found that in the absence of ouabain, IBTX enhanced Mx-induced contraction compared to non-treated, control vessels and that IBTX in the presence of ouabain enhanced the Mx-induced contraction compared to vessels treated with ouabain alone (Supplemental Figure S1A). Second, the effects of IBTX in the absence and in the presence of ouabain were compared. For this purpose, the areas under the concentration–response relationships were determined and the difference between the areas under control conditions and in the presence of IBTX (Supplemental Figure S1A,C) as well as in the presence of ouabain alone and in the presence of IBTX together with ouabain (Supplemental Figure S1B,C) was compared. A similar analysis was done for the effect of ouabain, for more details see the result section. More considerations on experimental design principles can be found in a recent review.[29]

## 2.6 | Proximity ligation assay

The proximity ligation assay (PLA) technique was used to determine the co-localization of the α2 isoform Na,K-ATPase with the BK channel in freshly isolated rat mesenteric artery myocytes. The Duolink in situ PLA detection kit 563 (Sigma-Aldrich, Denmark) was used in accordance with the manufacturer's instructions. Cell isolation from third-order rat mesenteric arteries was conducted as described previously.[39] Briefly, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized in PBS containing 0.1% Triton X-100 for 5 min. Cells were incubated for 1 h at 37°C in Duolink blocking solution to avoid any unspecific binding. The primary antibodies used against the α1 and α2 isoform Na/K-ATPase (1:100; Millipore, United States [Catalog no. 07-674]) and the BK channel (1:100; Neuromab, United States [Catalog no. 75-022]) were diluted in Duolink blocking solution and incubated overnight at 4°C. Combinations of secondary anti-rabbit or anti-mouse antibodies of PLA PLUS and MINUS probes were used followed by hybridization, ligation, and amplification steps. For negative control, cells were stained with only one of two primary antibodies, but the rest of the protocol was unchanged. Red punctae representing proteins located within 40 nm of each other were visualized and quantified using a Zeiss LSM710 upright laser scanning confocal microscope.

## 2.7 | Drugs and chemicals

Digoxin, Iberiotoxin, KB-R7943, SEA0400, ouabain, PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazol o[3,4-d]pyrimidin-4-amine) and XE991 were obtained from Tocris (Wiesbaden, Germany). Acetylcholine chloride and methoxamine hydrochloride were obtained from Sigma-Aldrich (Karlsruhe, Germany). PP3 (1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine) was obtained from Calbiochem, Merck (Darmstadt, Germany). FURA-2 AM was obtained from Fisher Scientific (Schwerte, Germany). All chemicals and agents were stored according to the manufacturer's recommendation. If necessary, a stock solution was prepared in the appropriate solvent and stored at −24°C. On the day of the experiment, the required aliquots were thawed.

## 2.8 | Statistical analysis

Statistical analysis was performed using GraphPadPrism 9.1. All values are given as mean and SEM if the data were normally distributed or median and the interquartile range if the data distribution was not normal. The normality of the data distribution was tested by the Shapiro–Wilk test. Changes in basal tension were analyzed using 1-way ANOVA or the Kruskal–Wallis test, with multiple testing by controlling for the false discovery rate. Concentration–response relationships to methoxamine were compared using repeated measures 2-way ANOVA, with multiple testing by controlling for the false discovery rate. The effect of a certain substance on methoxamine-induced contractions

was evaluated as the changes in the Area Under the Curve (Δ(AUC)) of the methoxamine-induced concentration–response relationships obtained in the presence and in the absence of this substance (see also Supplemental Figure S1). Statistical analyses of changes in the basal tension (Δ(basal tension)) or changes in the AUC (Δ(AUC)) were performed using the Welch's test or the Mann–Whitney test, as appropriate. Differences were accepted as statistically significant if the *p*-value was less than .05. *N* is the number of animals (rats) tested, thus represents biological replicates.

## 3 | RESULTS

### 3.1 | Cardiotonic steroids increase the pro-contractile effect of BK channel inhibition

Vascular responses to $10^{-7}$ M IBTX and $10^{-5}$ M ouabain were first studied at basal tension. We did not detect any effect of IBTX and ouabain alone on basal tone (Figure 1A). Importantly, $10^{-5}$ M ouabain selectively inhibits the

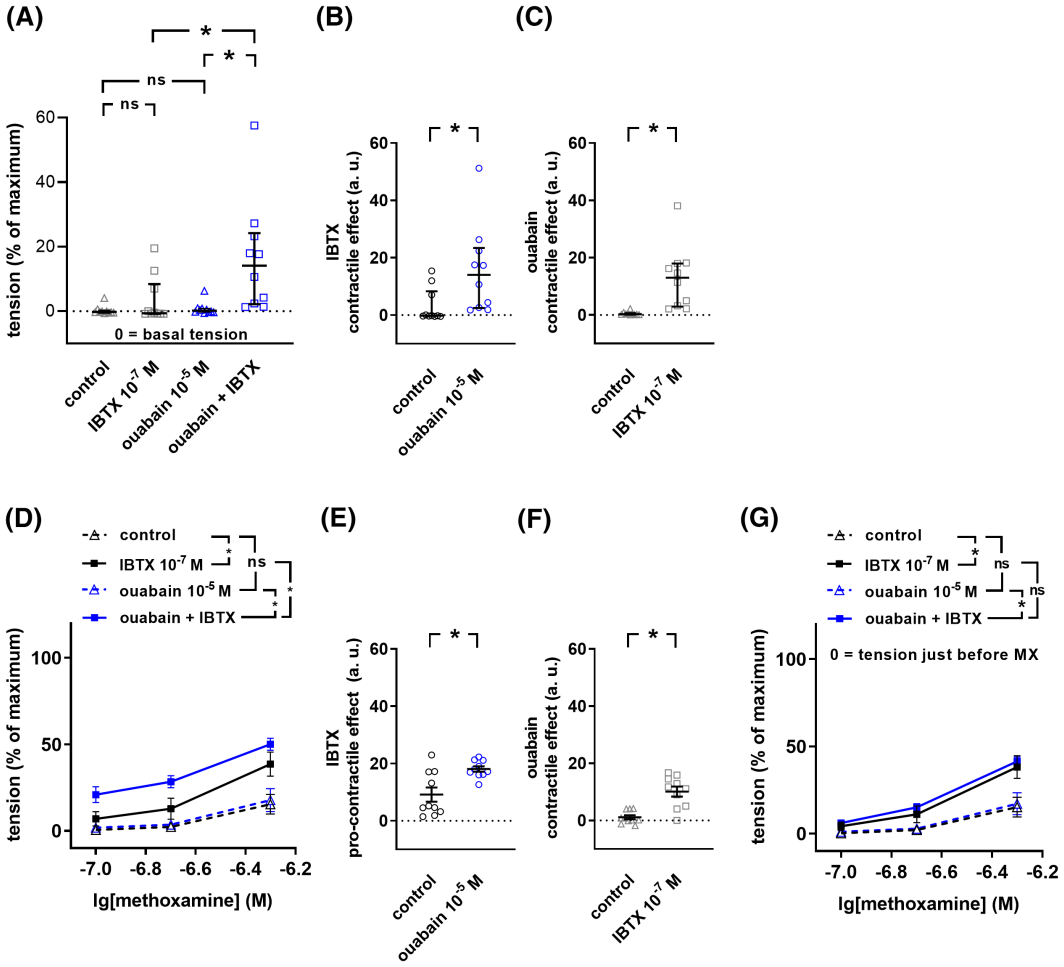


**FIGURE 1** Effects of $10^{-5}$ M ouabain and $10^{-7}$ M IBTX on basal tension and Mx-induced contractions of mesenteric arteries. (A) Basal tension under control conditions, in the presence of the BK channel inhibitor, IBTX ($10^{-7}$ M), in the presence of the Na/K-ATPase inhibitor, ouabain ($10^{-5}$ M) and in the presence of both inhibitors (ouabain + IBTX). Kruskal–Wallis test, controlling for false discovery rate, $n = 10$, $^*p < .05$. (B) Effect of IBTX on basal tension in the absence (control) and presence of $10^{-5}$ M ouabain. Mann–Whitney test, $n = 10$, $^*p < .05$. (C) Effect of ouabain on basal tension was only seen in the presence of $10^{-7}$ M IBTX, but not under control conditions (in the absence of IBTX). Mann–Whitney test, $n = 10$, $^*p < .05$. (D) Mx-induced contractions under control conditions, in the presence of the BK channel inhibitor IBTX ($10^{-7}$ M), in the presence of the Na/K-ATPase inhibitor ouabain ($10^{-5}$ M) and in the presence of both inhibitors (ouabain + IBTX). Repeated-measures ANOVA, controlling for false discovery rate, $n = 10$, $^*p < .05$. (E) Pro-contractile effect of IBTX in the absence (control) and presence of $10^{-5}$ M ouabain. Welsh's test, $n = 10$, $^*p < .05$. (F) Contractile effect of ouabain was only seen in the presence of $10^{-7}$ M IBTX, but not under control conditions (in the absence of IBTX). Welsh's test, $n = 10$, $^*p < .05$. (G) Mx-induced contractions after subtraction of basal tension under control conditions, in the presence of the BK channel inhibitor IBTX ($10^{-7}$ M), in the presence of the Na/K-ATPase inhibitor ouabain ($10^{-5}$ M) and in the presence of both inhibitors (ouabain + IBTX). Repeated-measures ANOVA, controlling for false discovery rate, $n = 10$, $^*p < .05$.

Na,K-ATPase α2 isoform without affecting the α1 isoform. However, in the presence of ouabain, a contractile effect of IBTX appeared (Figure 1A,B). In addition, a contractile effect of ouabain became detectable in the presence of IBTX (Figure 1A,C).

When vasocontraction was evoked by the selective α1-adrenoceptor agonist methoxamine (Mx), preincubation with IBTX ($10^{-7}$ M) enhanced this contraction (Figure 1D). This effect was denoted as the pro-contractile effect of IBTX and represents the activity of the BK channel opposing Mx-induced vasocontraction (in the absence of IBTX), i.e., the BK channel-mediated negative feedback regulation of vasocontraction.[1] We did not detect any relevant effect of $10^{-5}$ M ouabain alone on Mx-induced contractions (Figure 1D). In the presence of $10^{-5}$ M ouabain, the pro-contractile effect of IBTX was further enhanced (Figure 1D,E). In addition, a contractile effect of ouabain became detectable in the presence of IBTX (Figure 1D,F).

To understand whether the effects of IBTX and ouabain on Mx-induced contractions involve an interaction with α-adrenergic mechanisms, Mx-induced contractions were also analyzed after subtracting changes in basal tension from the total tension changes. Under these conditions, IBTX ($10^{-7}$ M) enhanced Mx-induced contractions (Figure 1G), providing additional support for the BK channel-mediated negative feedback regulation of vasocontraction.[1] However, we did no longer detect any relevant effect of $10^{-5}$ M ouabain, either alone or in the presence of IBTX, on Mx-induced contractions (Figure 1G).

Of note, the blocker of Kv7 channels, XE991,[40–42] at a concentration of $3 \times 10^{-6}$ M, increased basal tension and potentiated the Mx-induced contractions, but we did not detect any relevant effect of ouabain on both basal tension or on Mx-induced contractions, neither in the absence nor in the presence of XE991 (Supplemental Figure S2). The observation that the effect of ouabain on basal tension appeared in the presence of IBTX but not in the presence of XE991 suggests a specific functional interaction only between the BK channels and the Na/K-ATPase.

We tested ouabain-specificity of the observed effects using another cardiotonic steroid, digoxin.[38,43] Similar to ouabain, we did not detect any relevant effect of $10^{-5}$ M digoxin alone (Supplemental Figure S3A,D), but it enhanced the pro-contractile effect of IBTX (Supplemental Figure S3A,B,D,E) and a contractile effect of digoxin became detectable in the presence of IBTX (Supplemental Figure S3A,C,D,F). After isolating the Mx-induced changes, IBTX ($10^{-7}$ M) enhanced Mx-induced contractions (Supplemental Figure S3G), but we did no longer detect any relevant effect of $10^{-5}$ M digoxin alone or in the presence of IBTX (Supplemental Figure S3G).

Notably, these effects of ouabain were obtained on endothelium-denuded vessels (see methods section, isometric wire myography). In addition, we did not detect any change in the response of ouabain after functional elimination of sensory nerve endings by pretreatment of arterial segments with $10^{-6}$ M capsaicin and addition of the CGRP-receptor blocker BIBN4096BS at $2 \times 10^{-8}$ M[29] (Supplemental Figure S4).

## 3.2 | Inhibition of the Na/Ca exchanger (NCX) prevents the ouabain-induced increase of the pro-contractile effect of BK channel inhibition

In accordance with our findings in Figure 1, when BK channels were inhibited, administration of ouabain induced contraction (Figure 2). This ouabain-induced increase in basal arterial tension in the presence of IBTX was accompanied by an elevation of the intracellular $Ca^{2+}$ concentration (Figure 2).

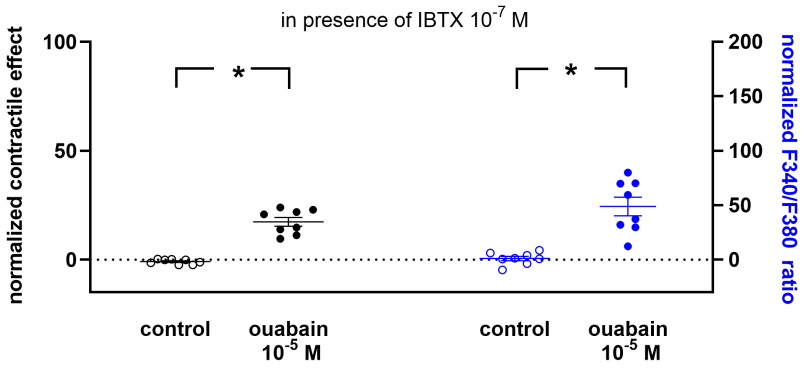


**FIGURE 2** Ouabain ($10^{-5}$ M) elevates vascular wall tension and intracellular $Ca^{2+}$ in mesenteric arteries in the presence of IBTX. Changes in vascular wall tension (left side) and intracellular $Ca^{2+}$ (right side) in response to ouabain ($10^{-5}$ M) or vehicle (control) in the presence of the BK channel inhibitor IBTX ($10^{-7}$ M). The data are expressed relative to values in the presence of IBTX only before vehicle (control) or ouabain administration. Welsh's test, $n = 8$, $^*p < .05$.

In the presence of IBTX, we did not detect any relevant effect of the NCX inhibitor, SEA0400 ($10^{-6}$ M)[44–46] on basal tension (Figure 3A) or Mx-induced contractions (Figure 3C). The contractile effect of ouabain on basal tension in the presence of IBTX was considerably reduced by SEA0400 (Figure 3A,B). The contractile effect of ouabain on Mx-induced contractions in the presence of IBTX was completely abolished by SEA0400 (Figure 3C,D). After subtracting the changes in basal tension from the total tension changes, there was no longer any relevant effect of $10^{-5}$ M ouabain, either in the presence of IBTX alone or in the presence of IBTX and SEA0400, on Mx-induced contractions (Figure 3E). Similar effects were observed with another blocker of the NCX, KB-R7943 ($3 \times 10^{-6}$ M)[47] (Supplemental Figure S5).

Our results suggest that the contractile effect of cardiotonic steroids is mediated, at least in part, by the NCX.[9] Moreover, the NCX may be involved in the BK channel-mediated negative feedback regulation of vasocontraction. SEA0400 ($10^{-6}$ M) reduced Mx-induced contractions both in the absence and

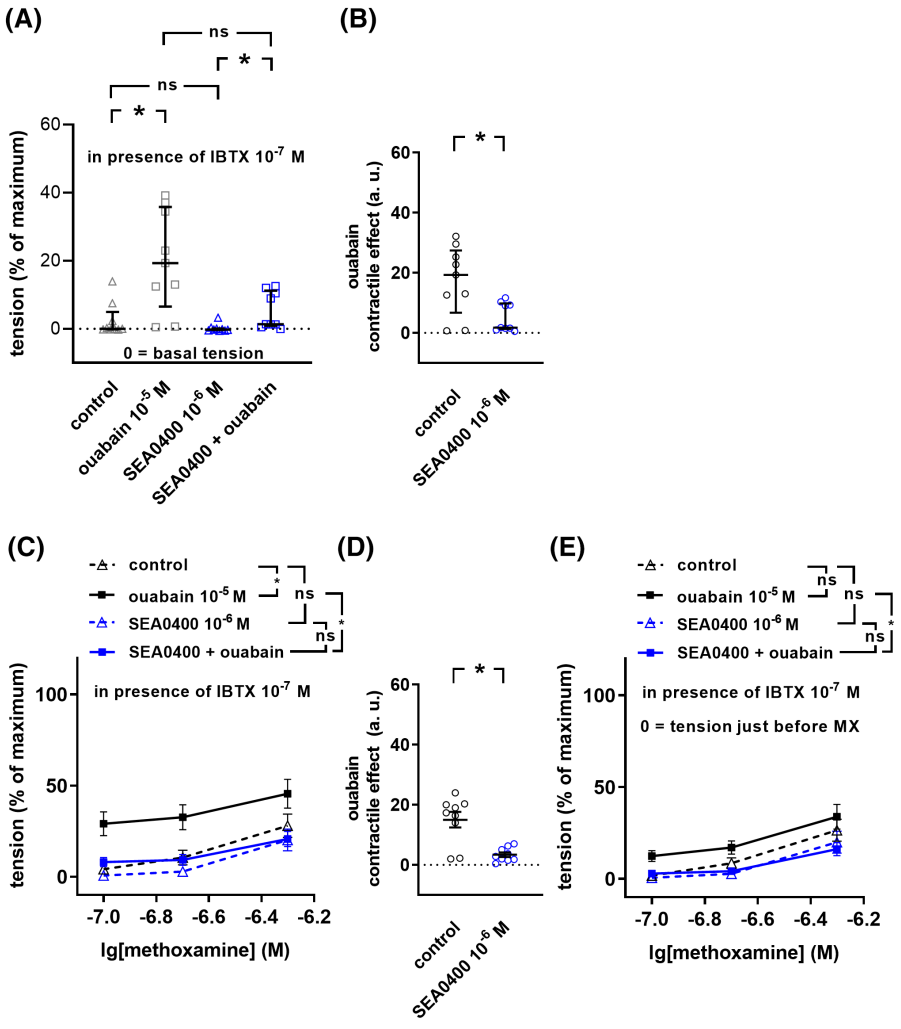


FIGURE 3 SEA0400 ($10^{-6}$ M) suppressed the contractile effect of $10^{-5}$ M ouabain on basal tension and Mx-induced contractions of mesenteric arteries in the presence of IBTX. (A) Basal tension in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain), in the presence of the NCX-inhibitor SEA0400 ($10^{-6}$ M) and IBTX (SEA0400), and in the presence of all three inhibitors (SEA0400 + ouabain). Kruskal–Wallis test, controlling for false discovery rate, $n = 9$, $^*p < .05$. (B) Contractile effect of ouabain on basal tension in the presence of $10^{-7}$ M IBTX only (control) and in the presence of both, $10^{-6}$ M SEA0400 and IBTX (SEA0400). Mann Whitney test, $n = 9$, $^*p < .05$. (C) Mx-induced contractions in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain), in the presence of the NCX-inhibitor SEA0400 ($10^{-6}$ M) and IBTX (SEA0400), and in the presence of all three inhibitors (SEA0400 + ouabain). Repeated-measures ANOVA, controlling for false discovery rate, $n = 9$, $^*p < .05$. (D) Contractile effect of ouabain in the presence of $10^{-7}$ M IBTX only (control) and in the presence of both, $10^{-6}$ M SEA0400 and IBTX (SEA0400). Welsh's test, $n = 9$, $^*p < .05$. (E) Mx-induced contractions after subtraction of basal tension in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain) in the presence of the NCX-inhibitor SEA0400 ($10^{-6}$ M) and IBTX (SEA0400), and in the presence of all three inhibitors (SEA0400 + ouabain). Repeated-measures ANOVA, controlling for false discovery rate, $n = 9$, $^*p < .05$.

presence of IBTX (Figure 4A). The pro-contractile effect of IBTX was reduced in the presence of SEA0400 (Figure 4A,B). Vice versa, the anti-contractile effect of SEA0400 was increased in the presence of IBTX (Figure 4C).

### 3.3 | Src-kinase contributes to the ouabain-induced increase of the pro-contractile effect of BK channel inhibition

To study the role of Src kinase in the ouabain-induced increase of the pro-contractile effect of IBTX, the Src kinase blocker PP2 as well as its inactive analogue PP3 were used.[4,21,48–50]

We did not detect any relevant effect of PP2 and PP3 at $10^{-5}$ M on Mx-induced contractions in the absence of IBTX (Supplemental Figure S6A). In contrast, in the presence of IBTX, $10^{-5}$ M PP2 as well as $10^{-5}$ M PP3 reduced Mx-induced contractions suggesting some unspecific effect (Supplemental Figure S6B). At $3 \times 10^{-6}$ M, PP2 reduced Mx-induced contractions in the presence of IBTX, while no relevant effect of PP3 was detected (Supplemental Figure S6C). However, when the concentration was further decreased to $10^{-6}$ M, no relevant effect of both PP2 and PP3 on the Mx-induced contractions in the presence of IBTX was observed (Supplemental Figure S6D). Thus, to study the role of Src-kinase in the ouabain-induced increase of the pro-contractile effect of IBTX, PP2 at $3 \times 10^{-6}$ M was selected.

In the presence of IBTX, we did not detect any relevant effect of PP2 on basal tension (Figure 5A). However, the contractile effect of ouabain on basal tension in the presence of IBTX was completely abolished by PP2 (Figure 5A,B). PP2 reduced Mx-induced contractions in the presence of IBTX (Figure 5C). The contractile effect of ouabain on Mx-induced contractions in the presence of IBTX was completely abolished by PP2 (Figure 5C,D). After subtracting the changes in basal tension from the total tension changes, there was no longer a relevant effect of $10^{-5}$ M ouabain, either in the presence of IBTX alone or in the presence of IBTX and PP2, on Mx-induced contractions (Figure 5E).

Our results suggest that the contractile effect of cardiotonic steroids is, at least in part, also mediated by Src kinase. Moreover, Src kinase may be involved in the BK channel-mediated negative feedback regulation of vasocontraction. Accordingly, we did not detect any relevant effect of PP2 on Mx-induced contraction in the absence of IBTX (Figure 6A), but the pro-contractile effect of IBTX was reduced in the presence of PP2 (Figure 6A,B). Vice versa, an anti-contractile effect of PP2 appeared only in the presence of IBTX (Figure 6C).

Our data suggest that cardiotonic steroids interfere with the BK channel-mediated negative feedback regulation of vasocontraction by a dual mechanism involving NCX and Src kinase. To explore a possible interaction of NCX and Src kinase mediated signaling, $10^{-6}$ M SEA0400 and $3 \times 10^{-6}$ M PP2 were used. In the presence of IBTX, both SEA0400 and PP2 reduced Mx-induced contractions in the presence of IBTX (Figure 7A). In the presence of PP2, SEA0400 further suppressed the contraction, but we did not detect any relevant difference in the effect of SEA0400 in the presence of PP2 and in its absence (Figure 7B). Accordingly, PP2 had an additive effect in the

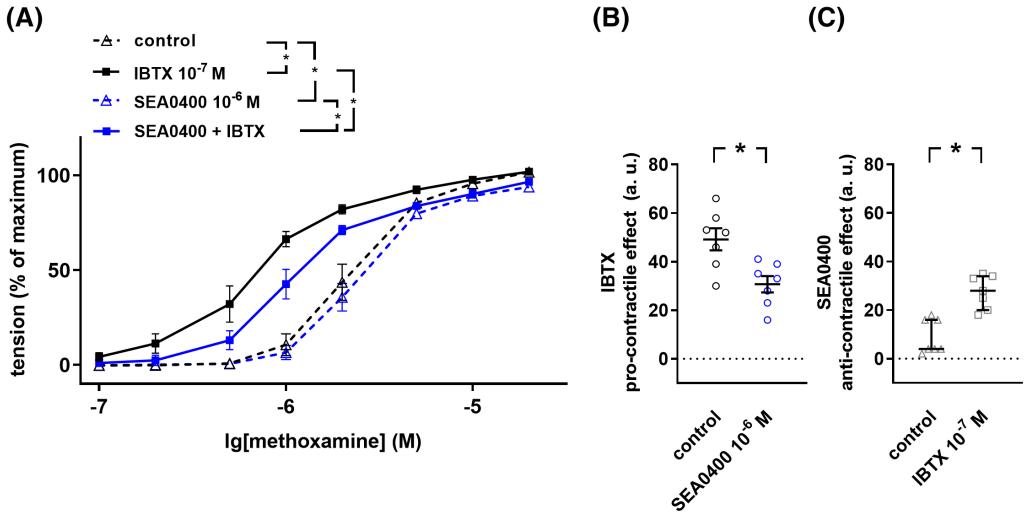


**FIGURE 4** SEA0400 ($10^{-6}$ M) reduced the pro-contractile effect of IBTX in mesenteric arteries. (A) Mx-induced contractions under control conditions, in the presence of IBTX ($10^{-7}$ M), in the presence of the NCX-inhibitor SEA0400 ($10^{-6}$ M), and in the presence of both inhibitors (SEA0400 + IBTX). Repeated-measures ANOVA, controlling for false discovery rate, $n = 7$, $^*p < .05$. (B) Pro-contractile effect of IBTX in the absence (control) and in the presence of $10^{-6}$ M SEA0400. Welsh's test, $n = 7$, $^*p < .05$. (C) Anti-contractile effect of SEA0400 in the absence (control) and in the presence of $10^{-7}$ M IBTX. Mann Whitney test, $n = 7$, $^*p < .05$.

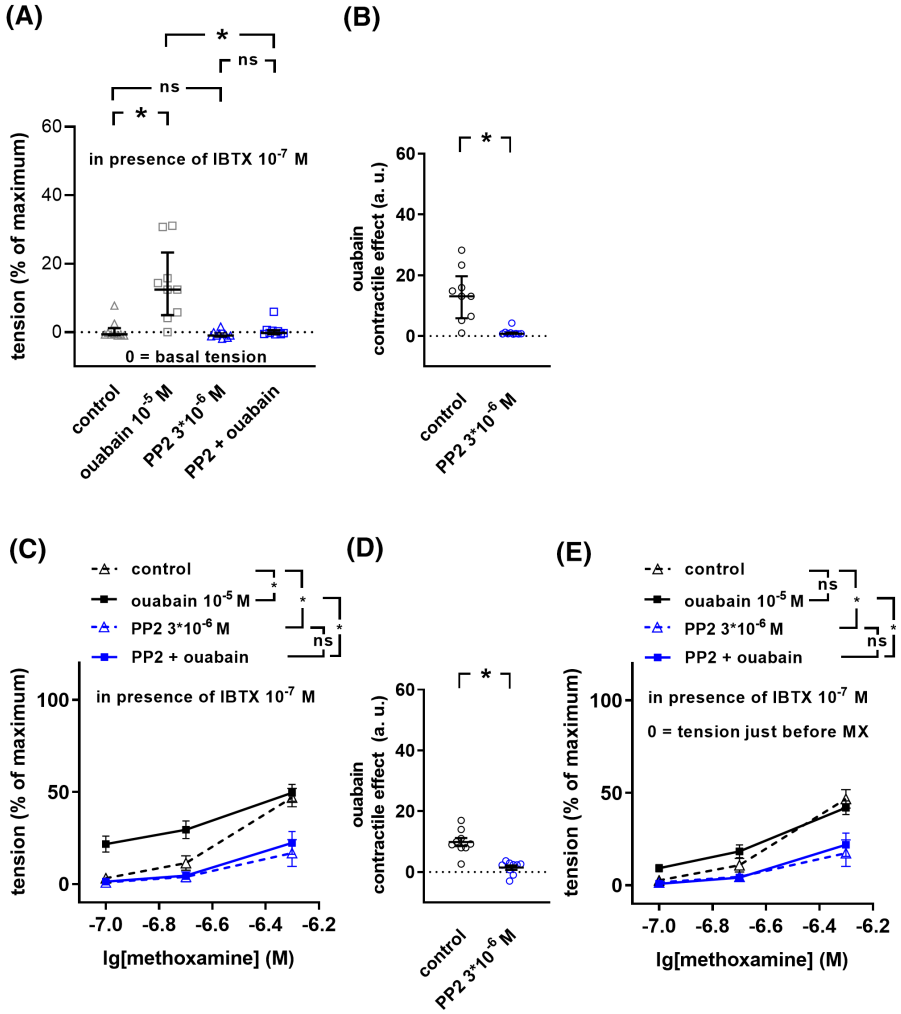


**FIGURE 5** Src kinase inhibition with $3\times10^{-6}$ M PP2 suppressed the contractile effect of $10^{-5}$ M ouabain on basal tension and Mx-induced contractions of mesenteric arteries in the presence of IBTX. (A) Basal tension in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain), in the presence of the Src-kinase-inhibitor PP2 ($3\times10^{-6}$ M) and IBTX (PP2), and in the presence of all three inhibitors (PP2 + ouabain). Kruskal–Wallis, controlling for false discovery rate, $n = 9$, $^*p < .05$. (B) Contractile effect of ouabain on basal tension in the presence of $10^{-7}$ M IBTX only (control) and in the presence of both, $3\times10^{-6}$ M PP2 and IBTX (PP2). Mann Whitney test, $n = 9$, $^*p < .05$. (C) Mx-induced contractions in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain), in the presence of the Src-kinase-inhibitor PP2 ($3\times10^{-6}$ M) and IBTX (PP2), and in the presence of all three inhibitors (PP2 + ouabain). Repeated-measures ANOVA controlling for false discovery rate, $n = 9$, $^*p < .05$. (D) Contractile effect of ouabain in the presence of $10^{-7}$ M IBTX only (control) and in the presence of both, $3\times10^{-6}$ M PP2 and IBTX (PP2). Welsh's test, $n = 9$, $^*p < .05$. (E) Mx-induced contractions after subtraction of basal tension in the presence of IBTX ($10^{-7}$ M, control), in the presence of ouabain ($10^{-5}$ M) and IBTX (ouabain) in the presence of the Src-kinase-inhibitor PP2 ($3\times10^{-6}$ M) and IBTX (PP2), and in the presence of all three inhibitors (PP2 + ouabain). Repeated-measures ANOVA controlling for false discovery rate, $n = 9$, $^*p < .05$.

presence of SEA0400, but we did not detect any relevant difference in the effect of PP2 in the presence of SEA0400 and in its absence (Figure 7C).

## 3.4 | The Na/K ATPase and the BK channel co-localize

Our findings suggest an interaction of the BK channel and the Na/K-ATPase, and we have previously shown that the Na/K-ATPase and Src kinase interact with each other in vascular smooth muscle cells.[24] Indeed, proximity ligation assays on freshly isolated smooth muscle cells from mesenteric arteries demonstrated a co-localization of the α2 isoform Na/K-ATPase and the BK channel (Figure 8) as well as the α1 isoform Na/K-ATPase and the BK channel (Supplemental Figure S7).

# 4 | DISCUSSION

In this study, we explored the interaction between the Na/K-ATPase and the BK channel in vascular smooth muscle cells. We found that the contractile effect of

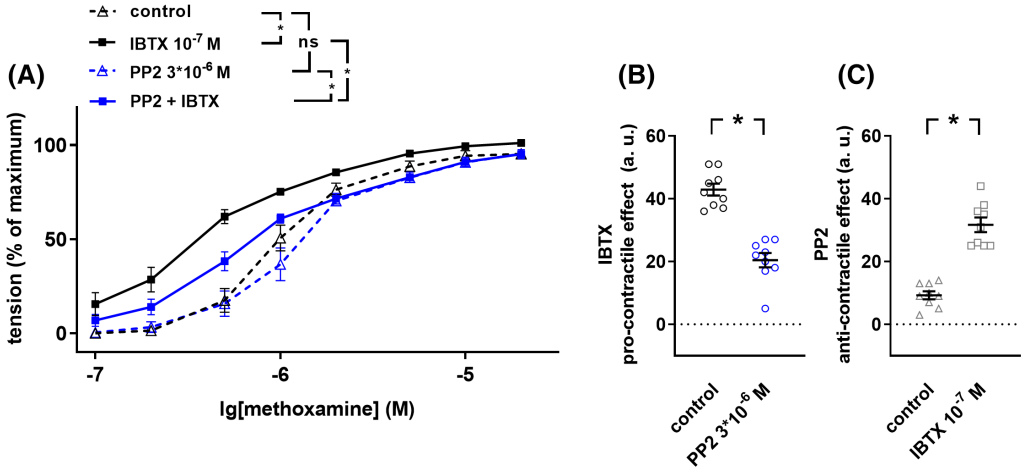


**FIGURE 6** PP2 ($3\times10^{-6}$ M) reduced the pro-contractile effect of IBTX in mesenteric arteries. (A) Mx-induced contractions under control conditions, in the presence of IBTX ($10^{-7}$ M), in the presence of the Src kinase-inhibitor PP2 ($3\times10^{-6}$ M), and in the presence of both inhibitors (PP2 + IBTX). Repeated-measures ANOVA, controlling for false discovery rate, $n = 9$, $^*p < .05$. (B) Pro-contractile effect of IBTX in the absence (control) and in the presence of $3\times10^{-6}$ M PP2. Welsh's test, $n = 9$, $^*p < .05$. (C) Anti-contractile effect of PP2 was only seen in the presence of $10^{-7}$ M IBTX, but not under control conditions (in the absence of IBTX). Welsh's test, $n = 9$, $^*p < .05$.

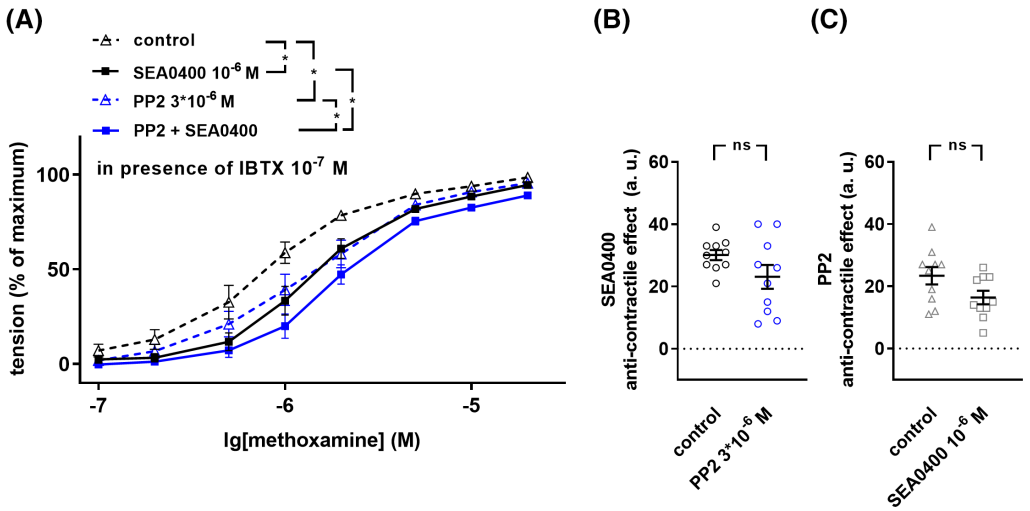


**FIGURE 7** Both, $10^{-6}$ M SEA0400 and $3\times10^{-6}$ M PP2 reduced Mx-induced contractions of mesenteric arteries in the presence of IBTX. (A) Mx-induced contractions in the presence of IBTX ($10^{-7}$ M, control), in the presence of SEA0400 ($10^{-6}$ M) and IBTX (SEA0400), in the presence of PP2 ($3\times10^{-6}$ M) and IBTX (PP2), and in the presence of all three inhibitors (PP2 + SEA0400). Repeated-measures ANOVA, controlling for false discovery rate, $n = 10$, $^*p < .05$. (B) Anti-contractile effect of SEA0400 in the presence of IBTX only (control) and in the presence of $3\times10^{-6}$ M PP2 + IBTX. Welsh's test, $n = 10$, $^*p < .05$. (C) Anti-contractile effect of PP2 in the presence of IBTX only (control) and in the presence of $10^{-6}$ M SEA0400 + IBTX. Welsh's test, $n = 10$, $^*p < .05$.

the BK channel inhibitor IBTX at basal tension and on methoxamine-induced contractions was increased in the presence of ouabain at a concentration that inhibits only the α2 isoform Na/K-ATPase without an effect on the α1 isoform.[51] Another cardiotonic steroid, digoxin, showed a similar contractile effect to ouabain in the presence of IBTX. This action of cardiotonic steroids was specific for the BK channels, as the pro-contractile effect of XE991, a blocker of Kv7 channels, was not affected by ouabain. Moreover, the effect of ouabain on the pro-contractile action of IBTX was abolished after blocking the NCX with two structurally unrelated inhibitors. Inhibition of the NCX also abolished the pro-contractile effect of IBTX alone. Furthermore, the effect of ouabain on the pro-contractile action of IBTX was abolished by inhibition of Src kinase. Importantly, the effects of NCX and Src kinase inhibition were independent from each other. The functional interaction between the Na/K-ATPase and the

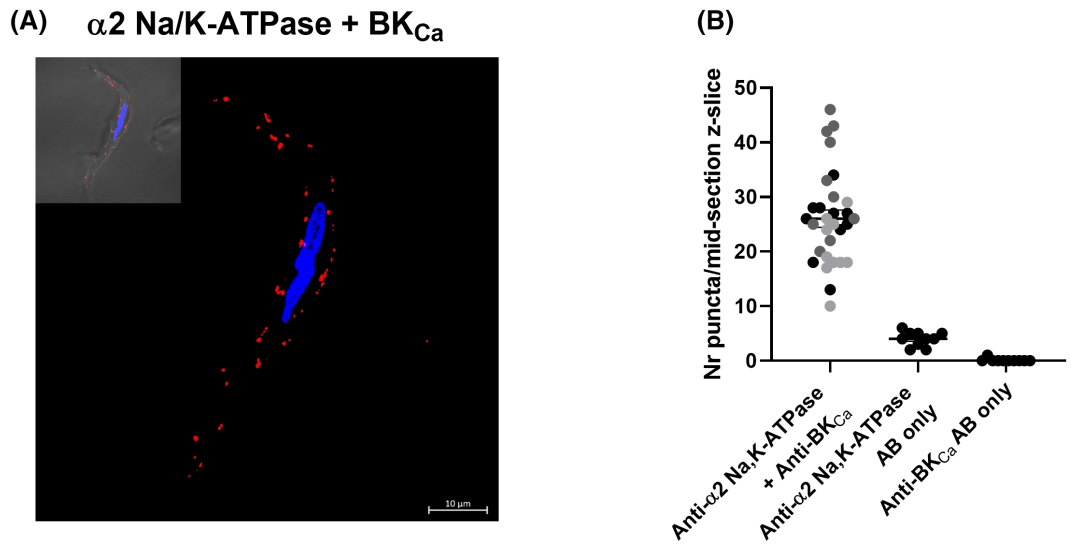


**FIGURE 8** Co-localization of the α2 isoform Na/K-ATPase and the BK channel in freshly isolated mesenteric artery smooth muscle cells. (A) Representative image of proximity ligation assay in rat mesenteric artery myocyte under control conditions. Red punctae indicate a close proximity (<40 nm) between the α2 Na/K-ATPase and the BK channel. Nuclei are stained with DAPI and shown in blue. Scale bar, 10 µm. (B) Quantification of the red punctae in cells (10–30 cells; $n = 3$ rats).

BK channel was structurally supported by the demonstration that these two transporters are in a close proximity (<40 nm) at the membrane.

## 4.1 | The Na/K-ATPase functionally interacts with the BK channel

In the present study, we observed that $10^{-5}$ M ouabain[21,38,52] increased arterial wall tension in the presence of Mx-induced contractions of endothelium-denuded rat mesenteric arteries, and that this effect was observed only when BK channels were inhibited. Importantly, in rodent tissue the α1 isoform Na/K-ATPase is relatively insensitive to ouabain,[51] with $10^{-5}$ M ouabain is only inhibiting the α2 isoform. Previous studies have reported the potentiation of agonist-induced contraction by micromolar concentrations of ouabain in the rodent vasculature[31,53,54] in an endothelium-independent manner.[21,22] Ouabain potentiated the vasocontractile effect of a thromboxane analogue in murine mesenteric and cerebral arteries.[23] Moreover, ouabain was shown to increase myogenic tone in the absence of agonist stimulation in isobaric preparations of mouse mesenteric arteries,[46,55,56] although this was not the case for rat mesenteric arteries under isometric conditions.[54] Importantly, heterozygote mouse knockout for the α1 subunit Na/K-ATPase did not show an altered effect regarding potentiation of myogenic tone by ouabain, whereas heterozygotes for α2 subunit knockout had a decreased sensitivity to ouabain.[56] This further suggests the exclusive importance of the ouabain-sensitive α2 isoform Na,K-ATPase for control of vascular tone.[9]

These previous reports are not entirely in line with our current results, where the contractile effect of ouabain was only observed when BK channels were inhibited. The reason for this discrepancy is uncertain but it seems to be related to the type of contractile agonist applied. We used Mx instead of noradrenaline, thromboxane and other contractile agonists used in the previous studies mentioned above. The functional state of BK channels may also be the reason for this discrepancy. Thus, the functional availability of BK channels in intact vessel preparations depends on a subtle balance of environmental, i.e. experimental factors that may lead to functionally silent BK channels.[1,57] Unfortunately, the previously published reports do not contain information about the functional state of the BK channels. This information is important as BK channel activity depends on multiple intracellular signaling pathways (e.g. [1–4,35,58–63]). Furthermore, most of the previous studies were done with endothelium-intact arterial segments where agonist-induced stimulation of endothelial NO synthesis could mediate BK channel activation via the cGMP/protein kinase G pathway.[64] This issue requires further investigation in future studies.

BK channels in vascular smooth muscle cells are active during agonist-induced membrane depolarization, where intracellular $Ca^{2+}$ is elevated.[1] Thus, the BK current provides negative feedback to agonist-induced membrane depolarization and vasoconstriction.[65] Accordingly, we have observed a considerable potentiation of agonist-induced contraction in the presence of IBTX. Importantly, the procontractile action of IBTX was further enhanced by inhibition of the α2 isoform Na/K-ATPase. This effect is a novel observation of the present study, suggesting a functional

interaction of the Na/K-ATPase with the BK channel. Of note, this was specific for the BK channel as inhibition of another $K^+$ channel, the Kv7 channel[40–42] potentiated Mx-induced contractions but ouabain did not have any additive effect in the presence of XE991. This functional interaction between the Na/K-ATPase and the BK channels has been further supported structurally using the proximity ligation assay. Of note, co-localization of the Na/K-ATPase with the BK channel has been proposed, albeit using different methods, e.g. in neurons and a melanoma cell line.[32,66]

## 4.2 | The Na/Ca exchanger (NCX) mediates the contractile action of the Na/K-ATPase–BK channel functional unit

Our study suggests a functional unit of the Na/K-ATPase and the BK channel that mediates vascular tone at least in part via modulation of NCX activity.[17,52,67] The inhibition of the Na/K-ATPase by cardiotonic steroids reduces the $Na^+$ gradient over the cell membrane and this affects NCX activity, first suppressing its forward mode transport (extrusion of $Ca^{2+}$) and then leading to its reverse activity moving $Ca^{2+}$ into the cell.[9] Altogether, this elevates intracellular $Ca^{2+}$[25,68] that may activate BK channels. Besides, the inhibition of BK channels may depolarize the membrane to an extent facilitating the reverse mode of the NCX and thereby potentiating contraction. This effect will be prominent if intracellular $Na^+$ is elevated, for example, because of Na/K-ATPase inhibition. Accordingly, we observed that in the presence of IBTX, ouabain induced an increase in the intracellular $Ca^{2+}$ concentration. Importantly, previous studies reported that in the absence of IBTX, 1–10 μM ouabain did not affect intracellular $Ca^{2+}$ in the wall of different vascular beds from rodents.[21–23,54]

Consistent with these suggestions, we observed that the contractile effect of ouabain in the presence of IBTX was completely abolished in the presence of KB-R7943 and SEA0400 suggesting a role of NCX in the contractile action of ouabain. Accordingly, the ouabain-induced increase in myogenic tone in mesenteric small arteries was reduced in smooth muscle specific NCX1 knockout mice[46] and in smooth muscle specific, conditional NCX1 knockout mice.[69] Altogether, the data suggest that the vasocontractile effect of ouabain is mediated by a functional microdomain consisting of the NCX, the Na/K-ATPase and the BK channels.[9,70,71]

In addition, KB-R7943 and SEA0400 reduced the pro-contractile effect of IBTX, suggesting a functional interaction between the NCX and the BK channel. Of note, NCX has been shown to increase the sub-plasmalemmal $Ca^{2+}$ concentration that may activate the BK channel.[72] Nevertheless, further studies are required to firmly establish the role of NCX in modulation of BK channel activity.

Alternatively, it is also possible to speculate that inhibition of the NCX reduces intracellular $Ca^{2+}$ and this suppresses the BK channel-induced hyperpolarization, which counteracts the relaxing action of SEA0400. This negative feedback of the BK channels is absent in the presence of IBTX, enabling stronger vasodilating effects of SEA0400. That is, the functional contribution of reverse mode NCX activity is increased in the presence of ouabain after BK channel inhibition due to further membrane depolarization and reduction of the $Na^+$ gradient, making it possible for both KB-R7943 and SEA0400 to inhibit this contractile action of ouabain in a NCX specific manner.

Of note, this study used two structurally unrelated inhibitors that block the NCX preferably in its reverse mode.[44,73] Off-target effects cannot be excluded.[45] This may include other membrane transport, first of all, $Ca^{2+}$ transport that may interfere with arterial contractility.[44] Thus, KB-R7943 at concentrations above 3 μM has been reported to interfere with store-operated $Ca^{2+}$ entrance.[74] At 1 μM, SEA0400 has been proven to be specific for NCX inhibition using smooth muscle-specific conventional NCX1 knockout mice.[46] Although SEA0400[47] at concentrations up to 3 μM was not reported to have considerable off-target effects on ion transport,[44] it has been shown to inhibit voltage-gated $Ca^{2+}$ channels with a half-maximal concentration approximately 10 μM.[75] Accordingly, we have used the concentrations suggested to be specific for inhibition of the NCX and producing considerable NCX blockade.[44–47,75]

## 4.3 | Src kinase mediates the contractile action of the Na/K-ATPase–BK channel functional unit

The Na/K-ATPase has been shown previously to possess an additional cellular function as a signal transducer mediating its effects via Src-kinase.[13,14,76] To explore a possible role of Src-kinase in the NCX-Na/K-ATPase-BK channel signaling, the Src kinase blocker PP2 was used.[4,21,48–50,77] PP2 has previously been shown to inhibit the ouabain-induced contraction of rat mesenteric arteries.[21] Other studies also reported that micromolar PP2 inhibits vascular constriction in different vascular beds and in response to various contractile agonists.[4,21,50,78–80] Accordingly, we observed that 3 μM PP2 reduced Mx-induced contractions, whereas its inactive analog, PP3, was ineffective, suggesting the involvement of Src tyrosine phosphorylation in this contractile signaling pathway.[77]

We also observed that the contractile effect of ouabain in the presence of IBTX was completely abolished by 3 μM

PP2. This finding is consistent with a previous report showing that inhibition of the Na/K-ATPase-dependent activation of Src-kinase prevents the ouabain-induced potentiation of noradrenaline-induced contraction in rat mesenteric arteries.[21,22,81] These findings are also in accordance with previous suggestions[9,21,24,82] that ouabain interaction with the Na,K-ATPase activates intracellular Src kinase signaling. Moreover, a close proximity localization of the Na/K-ATPase and Src kinase, observed in freshly isolated rat mesenteric artery cells,[24] is consistent with these functional data.

Importantly, the inhibitory action of PP2 and SEA0400 on the ouabain-induced potentiation of contraction was independent from each other. This suggests that NCX activity and Src kinase signaling belong to two independent signaling pathways acting on the Na/K-ATPase-BK channel functional domain.

In addition, 3 μM PP2 reduced the pro-contractile effect of IBTX, suggesting a functional interaction between Src kinase and the BK channels. Interestingly, Src kinase can activate BK channels in an heterologous expression system[3,26] and in smooth muscle cells from rat skeletal muscle,[27] whereas an inhibition of BK channels by Src kinase has been reported for human arteries.[4] Our data suggest that Src kinase activates the BK channels, as PP2 suppresses partially the pro-contractile effect of IBTX on Mx-induced contraction but did not affect the contraction in the absence of IBTX.

## 4.4 | Limitations of the present study

It may be considered that an ouabain-induced decrease in intracellular potassium may reduce the chemical gradient for potassium efflux and, thus, the BK current. Moreover, it has been shown in isolated cells that ouabain reduces BK currents via elevation of intracellular sodium.[32] This potential inhibitory actions of ouabain on BK currents contrast with our results suggesting a cardiotonic steroid-induced activation of the BK channels. Importantly, our study was performed in intact arteries and, thus, the integrated effect of ouabain on BK currents should be considered, whereby our study specifically focused on the mechanisms mediating BK channel activation.

An interaction of BK channels with the Na/K-ATPase could also take place in endothelial cells, although the precise role of BK channels in native endothelial cells is less clear and seems to be limited to cultured cells and blood vessels under pathophysiological conditions.[83] This question was however out of scope of the current study, and therefore, the experiments were performed on endothelium-denuded arterial segments to exclude endothelial BK channels as a confounding factor for the interpretation of our results.

Many vessels, in particular mesenteric arteries, are densely innervated. In this study, contractions were induced by methoxamine, a selective α1-adrenoceptor agonist that is not taken up by sympathetic nerves.[84] Cardiotonic steroids, e.g., ouabain, can depolarize nerve endings leading to noradrenaline release and arterial contraction.[85] This noradrenaline release could further potentiate the contractile effect of methoxamine. This will not change the experimental conditions, although it makes the mechanism of ouabain action more complex and therefore represents a limitation of this study. Mesenteric arteries are also innervated by sensory nerve endings that may possess Na/K-ATPases. However, functional elimination of sensory nerve endings[29] did not alter the effect of ouabain. This was demonstrated in our study, in which inhibition of sensory nerve signaling had no effect on the effect of ouabain and IBTX on arterial tone and Mx-induced contraction. The involvement of sensory nerve endings in the observed effects is therefore rather unlikely.

Finally, it should be considered that the BK channel, the Na/K-ATPase and the NCX can contribute to the contractile effect of methoxamine. However, this study focused on the interaction between BK channels, Na/K-ATPase and NCX. This was done by isolating their respective functional effects, focusing on the action of their inhibitors. Accordingly, the mechanisms of how α-adrenergic receptor activation affects BK channels, Na/K-ATPase and NCX were not the subject of this study.

Altogether, our data suggest that BK channels limit cardiotonic steroid-induced vasocontraction by a dual mechanism involving the NCX and Src kinase pathways. These data show that after blocking the NCX, the effect of Src kinase inhibition was unaltered. Conversely, the effect of NCX inhibition was not affected by blocking Src kinase. These data suggest that the NCX and Src kinase pathways mediate the cardiotonic steroid-induced activation of the BK channel in an independent manner. Of note, a similar dual mechanism was reported, whereby the Na/K-ATPase affects gap junction conductivity, on the one hand via NCX activity and alterations in the intracellular $Ca^{2+}$ concentration[86] and on the other hand via Src kinase-dependent phosphorylation.[20]

## AUTHOR CONTRIBUTIONS

Tobias Orth, Thomas A. Jepps, Vladimir V. Matchkov, and Rudolf Schubert conceived and designed the research; Tobias Orth, Anastasia Pyanova, Simon Lux, Peter Kaiser, Isabel Reinheimer, Daniel Løgstrup Nielsen, Josef Ali Khalid, and Salomé Rognant performed the research and acquired the data; Tobias Orth, Anastasia Pyanova, Simon

Lux, Peter Kaiser, Isabel Reinheimer, Daniel Løgstrup Nielsen, Josef Ali Khalid, Salomé Rognant, Thomas A. Jepps, Vladimir V. Matchkov, and Rudolf Schubert analyzed and interpreted the data; All authors were involved in drafting and revising the manuscript.

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## DISCLOSURES

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Materials and Methods, Results, and/or Supplemental Material of this article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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