RESEARCH ARTICLE | Cell-to-Cell Communication and Signaling Pathways

Na-K-ATPase regulates intercellular communication in the vascular wall via cSrc kinase-dependent connexin43 phosphorylation

Lise Hangaard,¹* Elena V. Bouzinova,¹* Christian Staehr,¹ Vibeke S. Dam,¹ Sukhan Kim,¹ Zijian Xie,³ Christian Aalkjaer,¹,² and Vladimir V. Matchkov¹
¹Department of Biomedicine, Aarhus University, Aarhus, Denmark; ²Department of Biomedicine, University of Copenhagen, Copenhagen, Denmark; and ³Marshall Institute for Interdisciplinary Research, Marshall University, Huntington, West Virginia

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Hangaard L, Bouzinova EV, Staehr C, Dam VS, Kim S, Xie Z, Aalkjaer C, Matchkov VV. Na-K-ATPase regulates intercellular communication in the vascular wall via cSrc kinase-dependent connexin43 phosphorylation. Am J Physiol Cell Physiol 312: C385–C397, 2017. First published January 25, 2017; doi:10.1152/ajpcell.00347.2016.—Communication between vascular smooth muscle cells (VSMCs) is dependent on gap junctions and is regulated by the Na-K-ATPase. The Na-K-ATPase is therefore important for synchronized VSMC oscillatory activity, i.e., vasomotion. The signaling between the Na-K-ATPase and gap junctions is unknown. We tested here the hypothesis that this signaling involves cSrc kinase. Intercellular communication was assessed by membrane capacitance measurements of electrically coupled VSMCs. Vasomotion in isometric arteries in vitro. Phosphorylation of cSrc kinase and connexin43 (Cx43) were semiquantified by Western blotting. Micromole concentration of ouabain reduced the amplitude of norepinephrine-induced vasomotion and desynchronized Ca²⁺ transients in VSMC in the arterial wall. Ouabain also increased input resistance in the arterial wall. These effects of ouabain were antagonized by inhibition of tyrosine phosphorylation with genistein, PP2, and by an inhibitor of the Na-K-ATPase-dependent cSrc activation, pNaKtide. Moreover, inhibition of cSrc phosphorylation increased vasomotion amplitude and decreased the resistance between cells in the vascular wall. Ouabain inhibited the electrical coupling between A7r5 cells, but pNaKtide restored the electrical coupling. Ouabain increased cSrc autophosphorylation of tyrosine 418 (Y418) required for full catalytic activity whereas pNaKtide antagonized it. This cSrc activation was associated with Cx43 phosphorylation of tyrosine 265 (Y265). Our findings demonstrate that Na-K-ATPase regulates intercellular communication in the vascular wall via cSrc-dependent Cx43 tyrosine phosphorylation.

vasoconstriction; gap junctions; Na-K-ATPase; cSrc kinase signaling; vasomotion

NA-K-ATPASE is a ubiquitous membrane protein responsible for establishing and maintaining cellular ionic homeostasis, which is critical for numerous cellular functions and processes. There is growing evidence for an additional function of the Na-K-ATPase as a signal transducer independent of ion translocation (1, 65) in different tissues including blood vessels (62, 70). A number of Na-K-ATPase-dependent signaling pathways have been characterized and shown to be initiated by binding of ouabain and other cardiotonic steroids to the Na-K-ATPase. Changes in ion transport are essential for some signaling (5) whereas other signaling is independent of ion translocation (1, 31). Thus the Na-K-ATPase acts as a receptor for ouabain and activates different signaling pathways, including activation of cSrc kinase and tyrosine phosphorylation of the epidermal growth factor receptors and other proteins (31). cSrc kinase, also designated pp60Src, Src p60, and proto-oncogene tyrosine protein kinase Src, is expressed in a broad range of cells including cells in the vascular wall and requires autophosphorylation of tyrosine 418 (Y418) for full catalytic activity (49). Although there is some concern whether this pathway depends on enzymatic activity of the Na-K-ATPase (16, 61, 68), the Na-K-ATPase-dependent cSrc kinase activation has been shown in several different cell types and tissues (32, 59, 64) including vascular tissues (62, 70). Moreover, a direct interaction between the Na-K-ATPase and cSrc kinase was recently shown in vascular endothelial cells (2). However, the functional consequences of acute ouabain-induced cSrc activation in the vasculature were never studied in detail.

We have previously shown that micromolar concentrations of ouabain uncouple intercellular communication in the vascular wall affecting several important vascular functions, e.g., endothelium-dependent relaxation, arterial contraction, and rhythmic oscillations of the wall tension, i.e., vasomotion (38, 40). The physiological significance of vasomotion is not entirely clear but it has been suggested to be important for tissue clearance and O₂ delivery, and is changed under several severe pathological conditions, e.g., hypertension and diabetes (see reviews: 37, 71).

Vasomotion is a useful readout for intercellular coupling in the vascular wall (41, 42). Although endothelium is essential for generation of vasomotion in rat mesenteric small arteries (46, 48), vasomotion is the product of synchronization of intracellular Ca²⁺ oscillations in vascular smooth muscle cells (VSMCs) (45, 71, 72). Synchronization between cells in the vascular wall is enabled by tight electrical coupling via intercellular channels, i.e., gap junctions, which are formed by connexin proteins (22). At least three types of connexins (Cx37, 40 and 43) have been identified in the vasculature (12) where they form highly complex, dynamically regulated gap junction channels (29). In our previous studies we have shown that ouabain reduces electrical conductance through gap junc-

* L. Hangaard and E. V. Bouzinova contributed equally to this work.

Address for reprint requests and other correspondence: V. V. Matchkov, Dept. of Biomedicine, Aarhus Univ., Ole Worms Alle bygn.4, 1163, Aarhus C 8000, Denmark (e-mail: vvm@biomed.au.dk).

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Na-K-ATPase-dependent cSrc regulation of gap junctions. Drugs were applied a minimum of 15 min before measurements/interventions. Ouabain (Sigma-Aldrich, Denmark) stock solution was prepared at the day of the experiment in a concentration of 10−2 M in water. Stock solutions of genistein and PP2 were prepared in DMSO (10−2 M), divided into aliquots, and stored at −20°C.

The sequence of pNaKtide is GRKKRRQRRRPPQSATWLALSIAGLCNRAVFQ (27, 30, 32, 51, 64). This 33-amino-acid peptide is composed of 20 amino acids from the N-domain of the α1 isoform Na-K-ATPase and a TAT leader peptide sequence of 13 amino acids that allows cell membrane penetration. This N-domain of the α1 isoform Na-K-ATPase differs from the α2 isoform by 3 amino acids, which probably explains its 10 times less efficiency for Src kinase inhibition (64). The pNaKtide is water soluble, and prior studies have shown that it resides in the plasma membrane and is effective in blocking the Na-K-ATPase-dependent Src activation in both cell culture and in live animals (27, 30, 32, 51, 64). Stock solution of pNaKtide (HD Biosciences, Shanghai, China; purity >95%) was prepared in water (10−2 M), divided into aliquots, and stored at −20°C. In the experiment, pNaKtide was applied at least 15 min before measurements/interventions.

Simultaneous measurements of isometric force, membrane potential, and input resistance. Measurements of membrane potential and input resistance in VSMCs in the intact vascular wall were performed in arteries mounted in an isometric myograph (Danish Myo Technology) using glass KCl-filled microelectrodes with resistance in the range 40–100 MΩ as previously described (41, 42). An Ag-AgCl electrode in the organ bath was used as a reference electrode. Input resistance was evaluated semiquantitatively by injecting 1-nA current pulses (25 ms) into the VSMC and measuring the subsequent potential change; electrode resistance was routinely compensated by balancing the Wheatstone bridge of the amplifier (Intro-710, WPI) before impalements.

Simultaneous measurements of isometric force and intracellular Ca2+. An inverted confocal laser scanning microscope (LSM 5 Pascal, Zeiss, Germany) was used for intracellular Ca2+ imaging. Arteries mounted in specially designed confocal myograph (Danish Myo Technology) were loaded with Calcium Green-1/AM (3 μM) and Fura Red/AM (6 μM) for 1.5 h at 37°C. Dyes were dissolved in DMSO with 0.1% cremophor and 0.02% Pluronic F-127 (final DMSO concentration 0.5%). Images were acquired with a water immersion objective (×60, NA 1.2, Nikon Instech, Japan) as described previously (38).

The emissions from Fura Red and Calcium Green-1 (excited at 488 nm) were collected above 560 nm and in the range from 505 to 530 nm, respectively. Elevated intracellular free Ca2+ results in increased fluorescence intensity of Calcium Green-1 and decreased fluorescence intensity of Fura Red. The combined use of these two Ca2+ indicators allows ratiometric analysis of intracellular Ca2+ signaling. The emission signals were analyzed using the Zeiss LSM Image Examiner program (Zeiss, Germany).

Membrane capacitance measurements to access cellular coupling. Rat aortic VSMCs (A7r5) were cultured in DMEM medium (In Vitro, Denmark) supplemented with 10% fetal calf serum, 1% t-glutamine and 0.1% KPS (kanamycin 2 g, penicillin 1 million IU, streptomycin 1 g in 20 ml PBS). Confluent cells were detached from the culture dishes by nonenzymatic cell dissociation solution (Sigma-Aldrich, Denmark) and pipetted into new culture dishes (Falcon, Becton Dickson, Denmark) with DMEM medium supplemented with 10% fetal calf serum, 1% t-glutamine and 0.1% KPS. After 4 h the medium was replaced with extracellular solution and paired cells used for capacitance measurements.

Cellular coupling was measured by recording the membrane capacitance (11, 13, 38, 40, 41). Since the membrane capacitance area of biological membranes is nearly constant, a decrease in capacitance would reflect a reduction in membrane area. Capacitance measurements were performed using the Membrane Test tool of AJP-Cell Physiol • doi:10.1152/ajpcell.00347.2016 • www.ajpcell.org

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Calculations) was unaffected by neither ouabain nor genistein nor their combination. **P**

Frequency of vasomotion (only oscillating arteries were taken into account) and potentiated by genistein or by a combination of genistein and ouabain. Arteries from 8 rats. Amplitude of rhythmic contractions under control conditions (8 of 11 arteries oscillated) was suppressed by ouabain (only 1 of 7 arteries oscillated), genistein (second NE stimulation; 4 arteries/rats) and a combination of ouabain and genistein (third NE stimulation; 11 arteries from 8 rats).

Western blotting. A7r5 cells were fixed directly in cultured bottle in ice-cold 10 mM dithiothreitol (DTT) in acetone with 10% trichloroacetic acid and kept on dry ice. Mesenteric arteries were dissected as described above and mounted in wire myograph. After 30 min equilibration arteries were challenged pharmacologically for 15 min and fixed immediately in ice-cold 10 mM dithiothreitol (DTT) in acetone with 10% trichloroacetic acid. Arterial segments or cells were lysed in SL mixture of lysis buffer (10 mM Tris·HCl, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, and 2% Triton X-100, pH 7.4; 1 tablet protease inhibitor per 10 ml and 10 mM of phosphatase inhibitors) and 2X trisglycine sodium dodecyl sulfate (SDS) sample buffer (Invitrogen, Denmark) adjusted with 1 M dithiothreitol (DTT) was added. The homogenates were heated for 10 min at 95°C, ultrasonicated for 45 s and centrifuged at 10,000 g for 10 min.

Ten microliters of the supernatant were loaded to gels for identification of phosphorylated proteins, and 2 μl (for cells) and 5 μl (for arteries) were loaded to gels for identification of total (i.e., phosphorylated and nonphosphorylated) protein. Note that this procedure does not permit detection of the protein concentration in lysate before gel loading. Proteins were separated on 4–15% trisglycine gels and electrotransferred onto nitrocellulose membranes. Membranes for identification of phosphorylated proteins were blocked in 3% bovine serum albumin (BSA) in Tris-buffered solution (TBS: 10 mM Tris·HCl, 100 mM NaCl, pH = 7.6) with 0.5% vol/vol Tween 20 (TBST). Membranes for identification of total protein where blocked in 0.3% iBlock in TBS for 2 h.

Membranes were cut at ~50 kDa and 35 kDa, with the upper part for cSrc detection (expected band ~60 kDa), middle part for connexin43 detection (expected band ~43 kDa), and lower part for detection of thioredoxin 2 (expected band ~12 kDa) as a housekeeping protein.

The membranes were incubated with primary antibodies (pY418 cSrc 1:200, Invitrogen (cat. no. 44660G), diluted in 3% BSA in TBS; total cSrc 1:500, Santa Cruz Biotechnology (cat. no. sc-8056), diluted in 0.3% iBlock in TBST; Y265 Cx43 1:500, Invitrogen (cat. no. 71-0700), diluted in 0.3% iBlock in TBST, thioredoxin 2 1:10,000, Abcam (cat. no. ab185544), diluted in 0.3% iBlock in TBST) overnight at 4°C. After washing was completed, the membranes were incubated with horseradish peroxidase-(HRP-) conjugated secondary antibody (1:2000; Dako) for 1.5 h in TBST. Excess antibody was removed by extensive washing, and bound antibodies were detected by an enhanced chemiluminiscence kit (ECL, Amer sham UK).

Intensities of specific Western blot bands were measured using ImageJ software (NIH) and individually normalized to the corresponding band intensity of thioredoxin 2 from the same load. The

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**Fig. 1.** Vasomotion is induced by 10⁻⁵ M norepinephrine (NE) and inhibited by 10⁻⁵ M ouabain. Addition of 5·10⁻⁵ M genistein recovered vasomotion. A and B show representative traces from two experimental protocols where vasomotion was induced by NE and, then, rhythmic activity was tested in the presence of either ouabain (A) or genistein (B). Then, vasomotion in the presence of both ouabain and genistein was explored. C–E show averaged data for the experiments shown in A and B. No significant difference in contraction to NE (C) was seen under control conditions (11 arteries from 8 rats), in the presence of ouabain (second NE stimulation; 7 arteries/rats), genistein (second NE stimulation; 4 arteries/rats) and a combination of ouabain and genistein (third NE stimulation; 11 arteries from 8 rats). Amplitude of rhythmic contractions under control conditions (8 of 11 arteries oscillated) was suppressed by ouabain (only 1 of 7 arteries oscillated), and potentiated by genistein or by a combination of genistein and ouabain (D). Frequency of vasomotion (only oscillating arteries were taken into calculations) was unaffected, by neither ouabain nor genistein nor their combination. ***P < 0.001.
normalized control (i.e., in absence of drugs) was set to 100% to compare the effects of drug interventions. Protein phosphorylations were semiquantified as a ratio between normalized phosphoprotein and total protein. The average ratio in the control experiments (i.e., in the absence of drugs) was set to 100% to compare the effects of drug interventions.

**Data analyses.** Microsoft Excel and GraphPad Prism software (version 5.02 for Windows) were utilized for graphing and statistical analysis. Data are summarized as means ± SE of the sample group. Significant differences between means were determined by either unpaired or paired one-way ANOVA followed by Bonferroni posttest or by Student’s t-test. A probability (P) level of <0.05 was considered significant.

**RESULTS**

Rat mesenteric small arteries activated with NE (10−5 M) demonstrated rhythmic contractions (Fig. 1, A and B), vasomotion, in 8 of 11 arteries (Fig. 1D). The frequency (Fig. 1E) and the amplitude (Fig. 1D) of these oscillations were similar to previously reported (7, 9, 41, 42, 46). Ouabain (10−5 M) significantly reduced vasomotion amplitude (Fig. 1, A and D), as reported previously (19), consistent with an effect on intercellular coupling in the arterial wall (38). An unspecific tyrosine kinase inhibitor, genistein (5·10−5 M) prevented this effect of ouabain (Fig. 1A). Genistein alone increased the

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**Fig. 2.** Vasomotion is modulated by ouabain and cSrc kinase. A–C show an effect of vehicle (DMSO) and 10−5 M ouabain on arterial contraction to 10−5 M NE (A), and amplitude (B) and frequency (C) of vasomotion (13 arteries/rats). An effect of 10−6 M PP2 on NE-induced arterial contraction and vasomotion is shown on D–F (6 arteries/rats). G–I show the effect of 10−6 M PP2 on NE-induced arterial contraction and vasomotion (6 arteries/rats). *P < 0.05, **P < 0.01, ***P <0.001, respectively.
amplitude of vasomotion in comparison with control conditions (Fig. 1, B and D) but was without effect on vasomotion frequency (Fig. 1E) and arterial tone induced by 10^{-5} M NE (Fig. 1C).

Vasomotion prevalence, its amplitude and frequency were unaffected by the vehicle (DMSO), but 10^{-5} M ouabain still reduced vasomotion amplitude in the presence of vehicle (Fig. 2, B and C). Another tyrosine kinase inhibitor, PP2 (10^{-6} M), did not affect the contractile response to NA (10^{-5} M) (Fig. 2D). However, PP2 significantly increased the amplitude of vasomotion (Fig. 2E) without modifying the frequency (Fig. 2F). Ouabain still inhibited vasomotion amplitude in the presence of 10^{-6} M PP2 (Fig. 2, E and F) similar to the control experiments (Fig. 2D). To test whether higher PP2 concentration could antagonize the effect of ouabain, we increased the concentration of PP2 to 10^{-5} M. This concentration partially antagonized the inhibitory effect of ouabain on vasomotion amplitude; 2 of 6 arteries oscillated in the presence of both PP2 and ouabain (Fig. 2H). Amplitude (Fig. 2H) and frequency (Fig. 2I) of vasomotion in the presence of both 10^{-5} M PP2 and 10^{-5} M ouabain were similar to those under control conditions. However, 10^{-5} M PP2 significantly suppressed arterial contraction (Fig. 2G).

Incubation with 2·10^{-6} M pNaKtide, a peptide designed to target the Na-K-ATPase-interacting pool of cSrc kinase (30), significantly potentiated the amplitude of vasomotion while no effect on the frequency was seen (Fig. 3, A, D, and E). Importantly, pNaKtide increased the prevalence of vasomotion by inducing vasomotion in the arteries which did not oscillate under control conditions (2 of 8 arteries without vasomotion under control conditions vs. all 8 oscillating arteries in the presence of pNaKtide; Fig. 3D). Arterial contraction to 10^{-5} M

![Fig. 3. Vasomotion is modulated by pNaKtide. A and B show representative traces for vasomotion under control conditions in arteries constricted with 10^{-5} M NE (left traces), after incubation with 2·10^{-6} M pNaKtide or 10^{-5} M ouabain, and both chemicals simultaneously. C–E show average results from the experiments in A (8 arteries/rats). F–I show average results from the experiments in B (8 arteries/rats). C and F demonstrate NE-induced contractions. D and G show the amplitude of vasomotion; and E and I show vasomotion frequency (only oscillating arteries were taken into calculations). *P < 0.05, **P < 0.01.

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NE was slightly suppressed by pNaKtide although some variability of this suppressing effect was observed. (Fig. 3, C and F). Vasomotion amplitude and frequency were similar under control conditions and in the presence of a combination of pNaKtide and ouabain (Fig. 3, D and E). When pNaKtide was applied to arteries where vasomotion was suppressed by ouabain, vasomotion amplitude recovered to the level seen under control conditions (Fig. 3, G and I).

The level of tone is important for the ability of arteries to oscillate (28). We found, however, that arteries were oscillating at different levels of vascular tone under control conditions and in the presence of a combination of ouabain and pNaKtide, whereas oscillations at the same levels of tone were absent in the presence of ouabain (Fig. 4). This was also found with genistein (11 arteries/8 rats) and PP2 (6 arteries/6 rats) (data not shown).

Suppression of vasomotion could be a consequence of either inhibition of intracellular Ca^{2+} transients in VSMCs or their synchronization (46). Previous studies demonstrated that ouabain inhibits intercellular communication between VSMCs and, consequently, desynchronized Ca^{2+} transients (38, 40). Consistent with this, we found that vasomotion is associated with synchronized Ca^{2+} oscillations in the VSMC (Fig. 5A) and that ouabain desynchronized the Ca^{2+} transients leading to suppression of rhythmic contractions of the arterial wall (Fig. 5B). Importantly, incubation with pNaKtide recovered synchronization of the intracellular Ca^{2+} oscillations and vasomotion (Fig. 5C).

Improvement of intercellular communication by pNaKtide was further supported by measurements of input resistance in the vascular wall, which is a function of membrane conductance and intercellular communication (41, 42). Incubation with pNaKtide significantly reduced input resistance in the arterial wall in the presence of NE (Fig. 6, A and B). This suggests an improvement of intercellular coupling by pNaKtide. Incubation with pNaKtide slightly but significantly depolarized the membrane potential of arterial smooth muscles stimulated by NE (Fig. 6C), and this was associated with reduced contraction (Fig. 6D). Ouabain (10^{-5} M) increased input resistance in the arterial wall under resting conditions and in the presence of NE (Fig. 6E). Ouabain slightly depolarized resting membrane potential [from −56.3 ± 1.3 to −54.3 ± 1.2 mV (n = 6); Fig. 6F] but was without significant effect on membrane potential and force response to 10^{-5} M NE (Fig. 6, F and G).

Our results suggest that ouabain-induced suppression of intercellular coupling can be reversed by pNaKtide, and we tested this suggestion using electrically coupled cultured smooth muscle cells. Electrical coupling between cell pairs was estimated using membrane capacitance measurements (11, 13, 41). A pair of cells has a membrane capacitance which is approximately twice that of the capacitance of a solitary cell (Fig. 7A). Application of 10^{-5} M ouabain to a cell pair reduced their capacitance to the level of solitary cells, whereas no significant effect of ouabain was seen on solitary cells (Fig. 7, A and C). This is consistent with previous findings (38). Because of the nonlinear relation between intercellular resistance and measured capacitance it is difficult to quantify intermittent reductions in gap junction conductance. However, the reduction of capacitance to the level of a solitary cell suggests a complete electrical uncoupling of cell pair by 10^{-5} M ouabain (38). Preincubation with 2·10^{-6} M pNaKtide prevented the uncoupling effect of ouabain on cell pairs (Fig. 7B).
Moreover, incubation with pNaKtide significantly increased membrane capacitance of paired cells by 4.2 ± 0.5% (P < 0.05, n = 5; Fig. 7C). This suggests that pNaKtide further decreased electrical resistance between cell pairs. Since pNaKtide has no effect on capacitance of solitary cells, this increase in capacitance of paired cells does not seem to be due to cell swelling or changes in membrane conductance.

The relative amount of phosphorylated cSrc was measured in cultured smooth muscle cells (Fig. 8) and in rat mesenteric small arteries (Fig. 9) under control conditions, after incubation with 10⁻⁵ M ouabain, with 2·10⁻⁶ M pNaKtide, and in the presence of both drugs (Figs. 8A and 9A). Neither ouabain nor pNaKtide affected the amount of total cSrc protein in smooth muscle cells (Figs. 8B and 9B). Ouabain significantly potentiated cSrc phosphorylation at Y418 suggesting its activation (35), and this effect was antagonized by pNaKtide (Figs. 8C and 9C). No significant effect of pNaKtide alone was seen. When the lysates were probed with connexin43 (Cx43) antibodies for total Cx43 content (Figs. 8D and 9D) and for Cx43 tyrosine phosphorylated at Y265, a significant increase in Cx43 phosphorylation was seen in the presence of ouabain, and this effect was also antagonized by pNaKtide (Figs. 8E and 9E).
In another study (23), where A7r5 cells were incubated with ouabain for 15 min, similar to the current study, ouabain had a concentration-dependent effect on cSrc activation with the maximal response above 1 mM. The concentration of ouabain we have used in the current study, 10 μM, induced ~25% activation (23). However, we (38, 40) and others (20) have reported that 10 μM completely uncouples VSMCs. Importantly, in rodents the α2 and α3 isoforms of Na-K-ATPase are sensitive to ouabain, whereas the α1 isoform is relatively insensitive to ouabain concentrations below 30 μM (58). This might suggest that the important cSrc activation in the vascular wall is derived from the α2 isoform [the α3 isoform is not expressed in VSMCs (39)].

The Na-K-ATPase has been implicated in regulation of many cellular signaling pathways (6) including cSrc kinase activation (16, 33, 61, 62) which results in subsequent tyrosine phosphorylation of multiple effectors (31). Although there is some controversy regarding the interaction between the Na-K-ATPase and cSrc, activation of cSrc by phosphorylation at Y418 is well-established in response to ouabain. The way by which this signaling is activated is under debate. Several studies suggest that cSrc kinase interacts with the Na-K-ATPase and that cSrc kinase is specifically activated by ouabain (4, 23, 27, 30, 33, 56, 67). Immunoprecipitation experiments have failed, however, to identify interaction between the Na-K-ATPase and cSrc (10), although interaction of the Na-K-ATPase with Src-associated phosphoprotein is reported (2). This direct interaction model is different from the conclusion based on experiments, which suggest that ouabain-induced cSrc autophosphorylation may be the result of increased ATP concentration consequent to Na-K-ATPase inhibition (16, 61, 68). Even though Na-K-pump activity may not affect the global ATP concentration in VSMCs (21), local changes in ATP/ADP ratio can be suggested (18). Studies in skeletal muscle showed that submicromolar and micromolar concentrations of ouabain do not affect the global intracellular ATP/ADP ratio, while causing phosphorylation of cSrc kinase and activation (26). Moreover, in contrast to ouabain, another Na-K-ATPase inhibitor, digoxin failed to induce cSrc phosphorylation in VSMCs arguing against an essential role of ATPase pumping activity in this signaling (70). The reason for this difference between these Na-K-ATPase inhibitors is unknown (53).

We found that in VSMCs micromolar concentrations of ouabain phosphorylates cSrc kinase at Y418 site within a few minutes of incubation consistent with findings in other cell types (33, 34, 56, 64, 69), including cultured smooth muscle cells (23). We have then addressed the functional consequences of this phosphorylation for intercellular communication and tested possibilities for its pharmacological modulation.

Tyrosine kinase inhibitors antagonize inhibition of intercellular coupling by ouabain. The potential importance of cSrc activity for intercellular communication in the arterial wall was tested with two tyrosine kinase inhibitors, genistein and PP2. We found that tyrosine kinase inhibition prevented the inhibitory effect of ouabain on prevalence and amplitude of vaso-motion. A water-soluble, membrane-permeable peptide conjugate, pNaKtide, was previously designed to target specifically the Na-K-ATPase-interacting pool of cSrc kinase (30). The specificity of pNaKtide has been supported by several func-

DISCUSSION

Several important novel findings addressing the mechanism of Na-K-ATPase-dependent modulation of intercellular communication have been made in this study. We have found that an inhibition of cSrc kinase improves intercellular coupling and antagonizes the inhibition of intercellular coupling by ouabain. More specifically, we have found that pNaKtide, a peptide designed to target the Na-K-ATPase-interacting pool of cSrc kinase (30), reduces intercellular resistance in the vascular wall and improves electrical coupling between smooth muscle cells. We have shown that the inhibition of intercellular coupling by ouabain is associated with cSrc autophosphorylation and tyrosine phosphorylation of Cx43, and that pNaKtide inhibits these phosphorylations. Thus our results suggest that the Na-K-ATPase modulates intercellular communication in the vascular wall and vasmotion via cSrc-dependent phosphorylation of gap junctions.

Inhibition of the Na-K-ATPase by ouabain initiates cSrc phosphorylation. In our study, we have shown that inhibition of the Na-K-ATPase by ouabain increases cSrc phosphorylation at Y418, which suggests full catalytic activation of cSrc (49). This is consistent with studies showing increased cSrc phosphorylation in VSMCs by chronic ouabain treatment (70). In contrast to the previous results, the effects observed here occurred within minutes, suggesting acute regulatory signaling.

Fig. 7. Ouabain uncouples electrically coupled A7r5 cells while pNaKtide improves electrical coupling and prevents the inhibition of intercellular coupling by ouabain. Membrane capacitance is proportional to the number of cells

A shows representative traces of cell capacitance measurements for a solitary cell and for two electrically coupled cells. Ouabain (10^{-8} M) uncoupled electrically coupled cells and reduced membrane capacitance to the level of a solitary cell. No significant effect of ouabain was detected on a solitary cell. B shows representative trace of membrane capacitance of paired cells which were pretreated with 2·10^{-8} M pNaKtide before ouabain (ouab) application. C shows averaged results expressed as percentage of initial membrane capacity measured before drug application. Coupled (5 cell pairs/culture dishes) and solitary (3 cells/culture dishes) cells were treated with ouabain under control conditions or after incubation with pNaKtide. *P < 0.05.
tional studies, and pNaKtide has been shown to antagonize ouabain-induced responses (14, 27, 30, 32, 51, 60, 64). Consistent with previous reports we have shown that pNaKtide antagonizes ouabain-induced phosphorylation of cSrc kinase. Importantly, pNaKtide also antagonized tyrosine phosphorylation of Cx43 and the functional effects of ouabain, i.e., improved intercellular coupling and “normalized” rhythmic contractions in the vascular wall. These biochemical and functional results strongly suggest that the Na-K-ATPase modulates intercellular coupling in the vascular wall via cSrc kinase activity controlling phosphorylation of Cx43.

Although both genistein and PP2 have many different effects on cell functions, the similar action of three nonrelated chemicals strongly suggests that tyrosine phosphorylation is important for inhibition of intercellular coupling by ouabain. Phosphorylation by cSrc is known to affect membrane conductances including the voltage-gated Ca$^{2+}$ channels (63) and the Ca$^{2+}$-activated K$^+$ channels (3, 66) as it is also evident from intermediate suppression of the contraction by inhibitors of tyrosine phosphorylation. This can affect the interpretation of the input resistance measurements. Thus NE-induced depolarization and increase in input resistance were previously reported (55) and attributed to changes in membrane conductance. Accordingly, ~10 mV hyperpolarization and decrease in input resistance by pNaKtide can be a result of increased K$^+$ conductance. It has previously been shown that cSrc phosphorylation suppressed the Ca$^{2+}$-activated K$^+$ channels (3), although another study reported an opposite effect (66). However, the current results from patch-clamp study argue against significant changes in membrane con-
ductance and suggest mediation of the effect via intercellular coupling.

**Na-K-ATPase-dependent cSrc signaling and its role for intercellular communication.** Vasomotion in mesenteric small arteries (46) is a result of interplay between an intracellular Ca\(^{2+}\)/H\(^{+}\)oscillator (37) and intercellular synchronization enabled by electrical coupling via gap junctions (40–42). The intracellular oscillator is represented by waves of [Ca\(^{2+}\)/H\(^{+}\)]i released from the sarcoplasmic reticulum (24, 25). These Ca\(^{2+}\)/H\(^{+}\)waves are not synchronized between neighboring cells but can be synchronized into global [Ca\(^{2+}\)/H\(^{+}\)]i oscillations because of ion current (46) running through the gap junctions and synchronizing Ca\(^{2+}\) release in neighboring cells. This leads to oscillations of membrane potential and consequently vasomotion (see reviews, see 37, 71).

Consistent with our previous reports (38, 40) we have shown that micromolar concentrations of ouabain inhibit vasomotion due to uncoupling of intracellular Ca\(^{2+}\) oscillators as it is evident from the lack of synchronized VSMC Ca\(^{2+}\) transients in the presence of ouabain (41). This interpretation was further supported by direct demonstration of ouabain-induced inhibition of intercellular coupling of the electrical communication between two smooth muscle cells and an increase of input resistance in the vascular wall.

We have previously reported that spatially restricted submembrane Ca\(^{2+}\) events following ouabain treatment are necessary for inhibition of intercellular coupling (38). We were unable to identify whether these Ca\(^{2+}\) events have a direct action on gap junction conductance since inhibition of intercellular coupling by ouabain can be inhibited by both fast (i.e.,
BAPTA) and slow (i.e., EGTA) Ca^{2+} buffers (15). Ouabain can induce submembrane Ca^{2+} events via modulation of Na\(^+\) homeostasis and the Na/Ca exchanger activity (5), and this might be important for the effect of cSrc. Another possibility is that ouabain-induced cSrc signaling modulates IP\(_3\) receptors either directly or via activation of PLC\(_{\gamma}\) as reported previously and induces localized Ca\(^{2+}\) release (31, 69). The detailed understanding of interaction between cSrc and Ca\(^{2+}\) signaling in modulation of gap junctions needs further investigation.

In this study we showed that the inhibition of intercellular coupling by ouabain was associated with cSrc kinase phosphorylation and that pNaKtide antagonized both effects. Moreover, pNaKtide alone slightly improved electrical coupling between smooth muscle cells, reduced intercellular resistance in the vascular wall, and increased the amplitude of vasomotion, strongly suggesting an improvement of VSMC synchronization. Electrical coupling can be improved either by increased electrical conductance through existing gap junctions or by incorporation of new hemichannels into the cell membrane.

Of the connexin isoforms expressed in the vascular wall (41), Cx43 is known to be expressed also in A7r5 cells (43) and is one of the most strongly regulated gap junction proteins (29). This makes Cx43 a suitable candidate for modulation of gap junctional conductance by ouabain-dependent signaling. However, the involvement of other connexins expressed in the smooth muscle cells, e.g., connexin37 and connexin40, cannot be excluded. Cx43 is shown to be regulated by a broad range of intracellular signaling pathways, including the Src kinase protein family (29). In fact, a direct interaction between Cx43 and cSrc was shown previously (17). Cx43 is known to be phosphorylated by cSrc directly on Y247 and Y265, and indirectly via mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase, and protein kinase C on several other sites (52). The net effect of these phosphorylations is reduction of the open probability and altered selectivity of gap junctions and suppression of intercellular communication. Moreover, gap junction phosphorylation triggers recruitment of the disassembly apparatus and reduction in intercellular communication (52). The mechanistic importance of different phosphorylation sites is not known yet, but multiple cSrc-initiated phosphorylations seem to act synergistically to reduce intercellular communication (52). We have detected in the current study increased phosphorylation of Cx43 on Y265 and suggest that this is a result of cSrc activation. We also suggest that this Cx43 phosphorylation is one of the reasons for reduced intercellular coupling in the presence of ouabain. Accordingly, prevention of this phosphorylation by tyrosine kinase inhibitors increased intercellular coupling, providing further support for this hypothesis. Consistent with the inhibitory effect of ouabain on intercellular coupling Martin et al. (36) reported for several cells types, including cultured VSMCs, that ouabain reduces coupling and expression and trafficking of Cx43. However, in a study on epithelial cells ouabain stimulates translocation of Cx43 from the cytosol into the plasma membrane in a cSrc-dependent manner and improves intercellular coupling (47). The reason for this discrepancy is unknown.

Thus we propose that the Na-K-ATPase modulates, at least in part, intercellular communication in the vascular wall through the cSrc kinase signaling pathway and tyrosine-phosphorylation of Cx43. Micromolar ouabain activates cSrc kinase and reduces VSMC synchronization in the vascular wall while inhibition of cSrc antagonizes this effect and improves intercellular coupling.

**Perspectives.** Vasomotion in small arteries has been reported in many vascular beds from different species and is caused by synchronized contractions of VSMCs in the vascular wall (37). Although the physiological significance of vasomotion is under debate, it has been suggested that rhythmic oscillations in blood flow improves tissue perfusion and oxygenation levels (71). The prevalence of vasomotion is changed significantly during pathology and depending on experimental conditions (71). For example, vasomotion is more prevalent during ischemia and may improve oxygenation of the ischemic area (50). This study demonstrated that synchronization of VSMCs and vasomotion can be modulated by the Na-K-ATPase-associated cSrc kinase signaling. We suggest that specific inhibition of signaling via the Na-K-ATPase-cSrc complex might be used to promote vasomotion and thus potentially improve tissue oxygenation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

L.H., E.V.B., C.S., V.S.D., S.K., and V.V.M. performed experiments; L.H., E.V.B., and V.V.M. analyzed data; L.H., E.V.B., C.S., V.S.D., S.K., Z.X., C.A., and V.V.M. edited and revised manuscript; E.V.B., Z.X., C.A., and V.V.M. conceived and designed research; E.V.B. and V.V.M. prepared figures; C.A. and V.V.M. approved final version of manuscript; C.A. and V.V.M. interpreted results of experiments; V.V.M. drafted manuscript.

**REFERENCES**


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