Title
Kv2.1 channels play opposing roles in regulating membrane potential, Ca2+ channel function, and myogenic tone in arterial smooth muscle.

Permalink
https://escholarship.org/uc/item/07s8q2k5

Journal
Proceedings of the National Academy of Sciences of the United States of America, 117(7)

ISSN
0027-8424

Authors
O'Dwyer, Samantha C
Palacio, Stephanie
Matsumoto, Collin
et al

Publication Date
2020-02-03

DOI
10.1073/pnas.1917879117

Peer reviewed
Kv2.1 channels play opposing roles in regulating membrane potential, Ca\(^{2+}\) channel function, and myogenic tone in arterial smooth muscle

Samantha C. O’Dwyer\(^\text{a}\), Stephanie Palacio\(^\text{b}\), Collin Matsumoto\(^\text{b}\), Laura Guarina\(^\text{a}\), Nicholas R. Klug\(^\text{a,b,1}\), Sendoa Tajada\(^\text{a}\), Barbara Rosati\(^\text{a,b}\), David McKinnon\(^\text{a,b}\), James S. Trimmer\(^\text{a}\), and L. Fernando Santana\(^\text{a,2}\)

\(^\text{a}\)Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, CA 95616; and \(^\text{b}\)Department of Physiology and Biophysics, Renaissance School of Medicine, Stony Brook University, The State University of New York, Stony Brook, NY 11794

Edited by Mark T. Nelson, University of Vermont, Burlington, VT, and approved January 9, 2020 (received for review October 11, 2019)

The accepted role of the protein Kv2.1 in arterial smooth muscle cells is to form K\(^{+}\) channels in the sarcolemma. Opening of Kv2.1 channels causes membrane hyperpolarization, which decreases the activity of L-type Ca\(_{\text{V}1.2}\) channels, lowering intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and causing smooth muscle relaxation. A limitation of this model is that it is based exclusively on data from male arterial myocytes. Here, we used a combination of electrophysiology as well as imaging approaches to investigate the role of Kv2.1 channels in male and female arterial myocytes. We confirmed that Kv2.1 plays a canonical conductive role but found it also has a structural role in arterial myocytes to enhance clustering of Ca\(_{\text{V}1.2}\) channels. Less than 1% of Kv2.1 channels are conductive and induce membrane hyperpolarization. Paradoxically, by enhancing the structural clustering and probability of Ca\(_{\text{V}1.2}\)–Ca\(_{\text{V}1.2}\) interactions within these clusters, Kv2.1 increases Ca\(^{2+}\) influx. These functional impacts of Kv2.1 depend on its level of expression, which varies with sex. In female myocytes, where expression of Kv2.1 protein is higher than in male myocytes, Kv2.1 has conductive and structural roles. Female myocytes have larger Ca\(_{\text{V}1.2}\) clusters, larger [Ca\(^{2+}\)]\(_i\), and larger myogenic tone than male myocytes. In contrast, in male myocytes, Kv2.1 channels regulate membrane potential but not Ca\(_{\text{V}1.2}\) channel clustering. We propose a model in which Kv2.1 function varies with sex: in males, Kv2.1 channels control membrane potential but, in female myocytes, Kv2.1 plays dual electrical and Ca\(_{\text{V}1.2}\) clustering roles. This contributes to sex-specific regulation of excitability, [Ca\(^{2+}\)]\(_i\), and myogenic tone in arterial myocytes.

The smooth muscle cells lining the walls of small resistance arteries and arterioles contract in response to increases in intravascular pressure (1). Due to its important role in regulating blood pressure, the molecular and biophysical mechanisms underlying this myogenic response have been the subject of intense investigation for decades. This work has led to the formulation of a model in which the myogenic response is initiated when membrane stretch activates Na\(^{+}\)-permeable canonical TRPC6, melastatin-type TRPM4, and TRPP1 (PKD2) channels (2–4). The opening of these channels depolarizes arterial smooth muscle cells, thereby activating voltage-gated, dihydropyridine-sensitive L-type Ca\(_{\text{V}1.2}\) Ca\(^{2+}\) channels. Ca\(^{2+}\) influx through Ca\(_{\text{V}1.2}\) channels causes a local elevation in intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) called a “Ca\(_{\text{V}1.2}\) sparklet” (5–7). The simultaneous activation of multiple Ca\(_{\text{V}1.2}\) sparklets produces a global increase in [Ca\(^{2+}\)]\(_i\), that triggers contraction.

A salient property of Ca\(_{\text{V}1.2}\) channels is their intrinsic ability to form clusters via a stochastic self-assembly mechanism (8). Channels within these clusters undergo functional coupling in response to local elevations in [Ca\(^{2+}\)]\(_i\) (9–11). Coupled gating of clustered Ca\(_{\text{V}1.2}\) channels is dynamic and involves cytoplasmic C-tail–C-tail interactions initiated by Ca\(^{2+}\) binding to calmodulin (11, 12). The consequence of Ca\(_{\text{V}1.2}\) channel coupling is that it allows more Ca\(^{2+}\) influx than random openings of independently gating channels. Coupled gating of Ca\(_{\text{V}1.2}\) channels accounts for ~50% of Ca\(^{2+}\) influx in arterial smooth muscle (7). At present, however, the mechanisms controlling clustering of Ca\(_{\text{V}1.2}\) channels are unknown.

Negative feedback regulation of Ca\(^{2+}\) influx via Ca\(_{\text{V}1.2}\) channels occurs through the activation of voltage-dependent Kv2.1 and Kv1.5 K\(^{+}\) channels as well as large-conductance, Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels (13–15). In cerebral arterial myocytes, Kv2.1 associates with “silent” Kv9.3 subunits in heteromeric complexes. This association has important physiological consequences, as it results in hyperpolarizing shifts in the voltage-dependent activation of Kv2.1 currents, making Kv2.1/Kv9.3 channel currents the predominant K\(^{+}\) conductance determining membrane potential over the lower physiological range of intravascular pressures (40 to 80 mmHg) (16). Acute pharmacological blockade or knockdown of Kv2.1 eliminates a substantial component of delayed rectifier current in arterial myocytes, suggesting that Kv2.1 channels are activated by membrane depolarization and conduct K\(^{+}\) in these.
cells (14, 16–19). Accordingly, acute inhibition of Kv2.1 with stromatoxin 1 (ScTx1) caused constriction in rat cerebral artery smooth muscle (14).

Intriguingly, almost all plasma membrane Kv2.1 channels are nonconductive in heterologous cells (20–22). If true for native cells, it would raise an important but difficult question: What is the function of these nonconductive Kv2.1 channels? Recent studies suggest a potential answer to this question. Like CaV1.2 channels, Kv2.1 channels are expressed in clusters in the surface membrane of neurons and mammalian heterologous expression systems (23–29). Importantly, Vierra et al. (30) recently showed that clustered Kv2.1 colocalizes with CaV1.2 channels and that coexpression with Kv2.1 translates into an increase in the cluster size and activity of coexpressed CaV1.2 channels in both HEK-293 cells and hippocampal neurons. In smooth muscle cells, CaV1.2 clustering is critical for functional coupling and the development of myogenic tone (6, 7). It is currently unknown whether Kv2.1 channels have similar roles in smooth muscle to regulate CaV1.2 channel clustering and hence function.

Here, we tested the hypothesis that Kv2.1 channels have dual conducting and structural roles in arterial smooth muscle. Our data challenge the conventional view that Kv2.1 has solely a conductive role in arterial myocytes. Rather, our data support our hypothesis that in arterial myocytes, Kv2.1 plays both conductive and structural roles with opposing functional consequences. Conductive Kv2.1 channels oppose vasoconstriction by inducing membrane hyperpolarization. Paradoxically, by promoting the structural clustering of the CaV1.2 channel, Kv2.1 enhances Ca²⁺ influx and induces vasoconstriction. We found that Kv2.1 protein is expressed to a larger extent in female than in male arterial smooth muscle. Sex-specific disparities in Kv2.1 expression shift the balance between the membrane hyperpolarization and Ca²⁺ entry actions of Kv2.1, leading to differences in the regulation of membrane potential, [Ca²⁺], and myogenic tone between males and female arterial myocytes.

**Results**

**Sexual Dimorphism in the Expression of Kv2.1 Channels in Arterial Smooth Muscle.** We began our study by recording voltage-gated K⁺ (IK) currents elicited in response to 500-ms depolarizations to voltages ranging from −50 to +50 mV in male and female mesenteric artery smooth muscle cells (SI Appendix, Fig. S1). In these cells, IK currents are produced by the opening of Kv and BK channels. To quantify the contribution of Kv (IKv) and BK (IKB) currents to IK, K⁺ currents were recorded before and after the application of the specific BK channel blocker iiberiotoxin (IBTX; 100 nM). Accordingly, we identified IKB as the IBTX-sensitive component of IK (15), and BK channel protein levels were determined by western blotting (30), indicating sex-specific differences in IKv2.1 traces were obtained by subtracting the currents after the application of ScTx1 from control currents (i.e., ICK). (B) Current-voltage relationship of IKv2.1. (C) Images of immunoblots of Kv2.1 and β-actin using WT and Kv2.1−/− mesenteric arteries. (D) The bar plot shows the intensities of the Kv2.1 band in all arteries examined. **P < 0.01, ****P < 0.0001. Error bars indicate mean ± SEM.

**Most Kv2.1 Channels in the Sarcolemma of Male and Female Arterial Myocytes Are Nonconductive.** A limitation of our immunoblot analysis is that it was performed using whole-artery homogenates and hence did not allow us to determine the level of plasma membrane Kv2.1 expression in mesenteric myocytes. To determine the number of plasma membrane Kv2.1 channels, we calculated the time integral of the ON gating charge associated with activation of Kv2.1 channels (Qon,Kv2.1), which is proportional to the number of these voltage-gated channels in the plasma membrane. The ON gating charge is described by the equation Qon,Kv2.1 = Ns,Kv2.1 * qKv2.1, where N is the number of channels and q is the number of elementary charges per channel, and is independent of the open probability (Po) and amplitude of elementary Kv2.1 currents (IKv2.1). Thus, Qon,Kv2.1 can be used to estimate the number of Kv2.1 channels in the sarcolemma of isolated mesenteric myocytes. We recorded Qon under conditions in which K⁺, Ca²⁺, Na⁺, and Cl⁻ ionic conductances were eliminated before and after the application of the tarantula venom peptide guanxotoxin-1E.
Next, we determined the total number of sarcolemma Kv2.1 channels in female and male myocytes. The number of Kv2.1 channels equals the total Kv2.1 charge movement per cell divided by the charge movement per channel. According to Islas and Sigworth (40), a total of 12.5 gating charges (e/q) move per Kv2.1 channel gating cycle. Thus, the total amount of charge movement per channel is 12.5 * 1.6 * 10^{-19} C (i.e., 2 * 10^{-18} C). Therefore, using our maximal Qon,Kv2.1, we calculated that the total number of Kv2.1 channels was 183,000 ± 27,716 in female myocytes versus 75,000 ± 21,628 in male myocytes (P < 0.05), assuming that the charge movement in native myocyte Kv2.1/Kv9.3 channels is similar to that previously determined for homeric Kv2.1 (39).

The number of conducting channels could be calculated using the equation I = N * i * P_o, where N is the number of conducting channels, i is the amplitude of elementary currents, P_o is the open probability, and I is the amplitude of the macroscopic current. For heteromeric Kv2.1/Kv9.3 channels, the iKv2.1 at +60 mV is 1.8 pA (37). To our knowledge, the maximum P_o of Kv2.1/Kv9.3 heteromeric channels has not been reported. However, because differences in the amplitude of macroscopic Kv2.1 and Kv2.1/Kv9.3 currents at voltages where the maximum P_o has been reached (e.g., +60 mV) seem to be exclusively due to differences in the amplitude of elementary currents only (e.g., Kv9.3 expression does not increase Kv2.1 expression) (16, 37), it is reasonable to assume that P_o of Kv2.1 and Kv2.1/Kv9.3 channels are likely similar. Accordingly, we used the maximum P_o value (i.e., 0.7) reported by Islas and Sigworth (40).

Assuming that the maximum P_o and amplitude of elementary current of Kv2.1 channels in male and female WT myocytes are similar, and using our IKv2.1 values, the number of conducting Kv2.1 channels was 194 (N_{Kv2.1,female} = 244 pA/[1.8 pA * 0.7]) and 62 (N_{Kv2.1,male} = 79 pA/[1.8 pA * 0.7]) in female and male myocytes, respectively. The percentage of functional Kv2.1-containing channels is therefore about 0.1% in both male and female myocytes. Thus, as is the case in heterologous expression systems (20–22), the vast majority of Kv2.1 channels in the sarcolemma of arterial myocytes are nonconductive.

Kv2.1 Channels Differentially Regulate the Membrane Potential, [Ca2⁺]_i, and Myogenic Tone of Male and Female Arterial Myocytes. A key question raised by the electrophysiological data above is whether such a small fraction of conducting Kv2.1 channels (i.e., ~0.1%) have a functional impact on pressurized male and female mesenteric artery smooth muscle (Fig. 3). Because the generally accepted role of Kv2.1 channels is to mediate K⁺ flux and hence regulate membrane excitability, we began by recording membrane potential, V_m, from pressurized, denuded WT and Kv2.1⁻/⁻ mesenteric artery segments using sharp electrodes (Fig. 3). At the physiological intravascular pressure of 80 mmHg, the smooth muscle cells of WT female arteries were more depolarized (V_m = -28 ± 2 mV) than those in male arteries (V_m = -40 ± 2 mV) (Fig. 3B). This was unexpected, as female myocytes express a larger number of conducting Kv2.1 channels than male myocytes.

Notably, chronic loss of Kv2.1 expression and function had different effects on the membrane potential of male and female smooth muscle (Fig. 3B). Consistent with their generally accepted role of opposing membrane depolarization, the membrane potential of male Kv2.1⁻/⁻ artery smooth muscle was more depolarized (V_m = -33 ± 2 mV) than that of their WT counterparts (V_m = -40 ± 2 mV). In contrast, female Kv2.1⁻/⁻ arteries were more hyperpolarized (V_m = -33 ± 2 mV) than WT female artery smooth muscle (V_m = -28 ± 2 mV). As such, the differences between the V_m of WT male and female myocytes were eliminated in myocytes lacking Kv2.1 expression.

We also investigated the acute effects of blocking Kv2.1 channels on smooth muscle membrane potential (Fig. 3C). Application of ScTx1 (100 nM) depolarized male and female...
can depolarize smooth muscle and also directly increase [Ca\(^{2+}\)].

Membrane potential data, male Kv2.1 arteries had higher [Ca\(^{2+}\)] than female arteries. Consistent with our showing [Ca\(^{2+}\)] in WT and Kv2.1 female and male arteries. (D) Bar plot showing [Ca\(^{2+}\)] in WT and Kv2.1 female and male arteries at intravascular pressures ranging from 20 to 120 mmHg. (E) Pressure–tone relationships of WT and Kv2.1 female and male arteries. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars indicate mean ± SEM.

arteries by 7 ± 2 and 7 ± 1 mV, respectively. ScTx1 had no effect on Kv2.1−/− arteries.

Next, we measured wall [Ca\(^{2+}\)] (Fig. 3D) and myogenic tone (Fig. 3E) in male and female WT and Kv2.1−/− pressurized arteries. Our data indicate that [Ca\(^{2+}\)] and myogenic tone are higher in female than in male arteries. Consistent with our membrane potential data, male Kv2.1−/− had higher [Ca\(^{2+}\)], and myogenic tone than WT arteries. In sharp contrast, female Kv2.1−/− had lower [Ca\(^{2+}\)] and myogenic tone than female WT arteries. Together, these data suggest that while acute changes in Kv2.1 function alter male and female smooth muscle excitability, chronic loss of these channels has sex-specific effects on smooth muscle function.

Expression of Kv2.1 Increases the Activity and Open Times of Ca\(_{\text{v}}\)1.2 Channels in Female but Not in Male Arterial Myocytes. The membrane potential of arterial myocytes is set by a balance of outward and inward currents. In principle, our observation that female WT myocytes are more depolarized than male WT myocytes could be the result of lower hyperpolarizing currents and/or up-regulation of an inward depolarizing current in female cells. One current we recently showed to be up-regulated by Kv2.1 channel expression in HEK-293 cells and hippocampal neurons is the L-type Ca\(_{\text{v}}\)1.2 channel current (I\(_{\text{Ca}}\)) (30), which can depolarize smooth muscle and also directly increase [Ca\(^{2+}\)].

Thus, we recorded macroscopic ion Ca\(_{\text{v}}\)1.2 currents (I\(_{\text{Ca}}\)) from WT and Kv2.1−/− male and female arterial myocytes (Fig. 4A). I\(_{\text{Ca}}\) were evoked by 300-ms step depolarizations from the holding potential of −80 mV to voltages ranging from −70 to +60 mV. We found that the amplitude of I\(_{\text{Ca}}\) was larger in female than in male WT myocytes. Furthermore, loss of Kv1.2 channels was associated with a decrease in I\(_{\text{Ca}}\) amplitude in cells of both sexes, but the decrease in I\(_{\text{Ca}}\) was larger in female than in male Kv2.1−/− myocytes. The overall impact of eliminating Kv2.1 expression is that the differences between the I\(_{\text{Ca}}\) amplitude of WT male and female myocytes were eliminated in myocytes lacking Kv2.1 expression.

We also determined the voltage dependence of the normalized I\(_{\text{Ca}}\) conductance (G/G\(_{\text{max}}\)) (Fig. 4B). This analysis indicates that the I\(_{\text{Ca}}\) G/G\(_{\text{max}}\) relationship in WT female myocytes (V\(_{1/2}\) = −10.2 ± 0.8 mV) was shifted toward more negative membrane potentials than in male myocytes (V\(_{1/2}\) = −1.8 ± 1.1 mV). The V\(_{1/2}\) of the G/G\(_{\text{max}}\) relationship of female Kv2.1−/− myocytes (−1.9 ± 2.0 mV) was similar to that of WT (P = 0.98) and Kv2.1−/− male myocytes (−5.8 ± 1.1 mV, P = 0.06) (Fig. 4B).

We next investigated the basis of the different I\(_{\text{Ca}}\) amplitudes of WT male and female myocytes. Immunoblot analysis showed that Ca\(_{\text{v}}\)1.2 protein expression was similar in WT and
Kv2.1−/− male and female arteries (Fig. 4C). As done above for Kv2.1, we followed up these experiments by recording CaV1.2 gating currents, which provide a direct measurement of the channels in the sarcolemma of arterial myocytes (Fig. 4D). CaV1.2 gating currents were recorded before and after the CaV1.2 voltage sensor immobilizer nitrendipine (10 μM) was applied (SI Appendix, Fig. S4) (41) under conditions in which ionic current was eliminated. Consistent with our immunoblot data, the maximal (at +20 mV) Qon,Cav1.2 was similar in WT and Kv2.1−/− male and female myocytes (P = 0.67). Finally, we found that the voltage dependence of the normalized Qon,Cav1.2 was fit with a sigmoidal function and that V1/2 and k values were similar in WT and Kv2.1−/− male and female myocytes (P = 0.9). We used Qon,Cav1.2 data to calculate the number of Cav1.2 channels in the membrane as was done for Kv2.1 channels above. According to Noceti et al. (42), a total of 9.1 q, gating charges move per Cav1.2 channel gating cycle. Because Qon,Cav1.2 was similar in male and female myocytes, we combined the data into a single group. This analysis suggested that the number of Cav1.2 channels in mesenteric myocytes was 192,612 ± 19,168 channels.

Next, we recorded elementary CaV1.2 currents (iCa) (Fig. 5). Cav1.2 channel openings were evoked by 2-s step depolarizations to potentials ranging from −30 to +20 mV. Currents were recorded in cell-attached patches, with 110 mM Ba2+ in the pipette solution used as the charge carrier. Fig. 5A shows representative records of iCa from female and male WT and Kv2.1−/− myocytes at −30, −10, and +10 mV with the expected amplitude for this voltage and ionic conditions. The voltage dependence of unitary Cav1.2 current and (at +20 mV) is shown in Fig. 5B. These data were fit with a linear function that revealed that the conductance of iCa was similar in WT (female 15 ± 1 pS; male 14 ± 1 pS) and Kv2.1−/− (female 14 ± 1 pS; male 16 ± 1 pS) myocytes.

We determined the P0 of single Cav1.2 channels in WT and Kv2.1−/−/− female and male myocytes (Fig. 5C). Note that the P0 at the voltage where the peak of iCa was recorded (i.e., +10 mV; Fig. 4A) was largest in WT female myocytes but similar among WT male and Kv2.1−/− male and female cells. A consistent observation during analysis was that WT female iCa openings appeared to be longer than those in WT male and Kv2.1−/− male and female myocytes. Thus, we analyzed the open times of single Cav1.2 channel openings in these cells at −10 mV (SI Appendix, Fig. S5). Although the Cav1.2 channel open-time histogram for all cells could be fit with the sum of 2 exponential log-transformed functions with a short and long time constant of 0.7 and 20 ms, respectively, the proportion of short and long openings varied among individual myocytes. The fraction of long CaV1.2 openings was nearly 12-fold larger in WT female cells (12%) than in WT male (1%) and Kv2.1−/− myocytes from mice of either sex (1%).

**Kv2.1 Channel Expression Increases CaV1.2 Channel Cluster Size in Female Myocytes to a Larger Extent than in Male Myocytes.** We have previously shown that physical coupling of clustered CaV1.2 channels shifts the voltage dependence of iCa toward more negative potentials and increases the P0, and open times of CaV1.2 channels (9, 11, 12). Thus, the electrophysiological differences between male and female WT and Kv2.1−/− myocytes are similar to those observed with varying degrees of CaV1.2 clustering and functional Cav1.2 coupling (9, 11, 12, 43). Three previously published lines of evidence support the hypothesis that Kv2.1 regulates the spatial organization and functional state of CaV1.2 channels. First, Kv2.1 channels exist in large clusters in diverse brain neurons (25–36). A recent study indicated that Kv2.1 colocalizes with CaV1.2 channels coexpressed in heterologous HEK-293 cells (29). Finally, Vierra et al. (30) have recently shown that Kv2.1 coexpression leads to increased clustering and activity of coexpressed CaV1.2 channels in both hippocampal neurons and HEK-293 cells. Thus, we tested the hypothesis that Kv2.1 expression increases CaV1.2 clustering in arterial myocytes using ground-state depletion (GSD) super-resolution imaging (Fig. 6).

**SI Appendix, Fig. S6** shows representative total internal reflection fluorescence (TIRF) images of immunolabeled CaV1.2 clusters in male and female WT and Kv2.1−/− arterial myocytes. We found that CaV1.2-associated fluorescence intensity was similar in WT and Kv2.1−/− female and male myocytes, consistent with the immunoblot and gating current data (Fig. 4 C and D), leading to the conclusion that overall CaV1.2 expression does not vary among these samples. Fig. 6 illustrates representative superresolution maps of immunolabeled CaV1.2 clusters in male and female WT and Kv2.1−/− myocytes. Note that CaV1.2 clusters were significantly larger in female WT (4,131 ± 209.4 nm2) than in male WT (3,492 ± 192 nm2) myocytes. When we compared the cluster size of Cav1.2 channels in WT vs. Kv2.1−/− myocytes, there was a significant difference in females (WT, 4,131 ± 209 nm2; Kv2.1−/−, 3,395 ± 178 nm2) but not in males (WT, 3,492 ± 192 nm2; Kv2.1−/−, 3,870 ± 228 nm2); note that the sex-specific differences in cluster size observed in WT myocytes were eliminated in the Kv2.1−/− cells. These data are consistent with the view that, as in hippocampal neurons and HEK-293 cells (30), Kv2.1 channels promote Cav1.2 channel clustering in arterial myocytes, and that this effect is more prominent in female myocytes that express higher levels of Kv2.1.

**Kv2.1 Channels Colocalize with CaV1.2 Channels and Promote CaV1.2–CaV1.2 Channel Interactions.** We used a proximity ligation assay (PLA) to determine if Kv2.1 and Cav1.2 are within 50 nm of each other in arterial myocytes (Fig. 7A). We detected multiple puncta, indicative of CaV1.2–Kv2.1 channel proximity in male and female WT but not Kv2.1−/− myocytes. These observations suggest that Kv2.1 channels colocalize with CaV1.2 channels in mesenteric arterial myocytes.

Having determined that Kv2.1 and Cav1.2 channels are in close association in arterial myocytes, we investigated whether Kv2.1 promotes Cav1.2 channel–channel interactions. To test this hypothesis, we applied a bimolecular fluorescence complementation approach using CaV1.2 channels fused with either the
CaV1.2-Venus increased by 5% upon stimulation, while CaV1.2-
7
G/Gmax in cells coexpressing CaV1.2 and Kv2.1 but not in cells
membrane depolarization shifted the voltage dependence of
their C tails for the duration of the experiment. A fluorescent
Venus, the channels will likely remain coupled at
a conditioning protocol involving five 500-ms pulses from
larger in cells coexpressing Kv2.1 and CaV1.2-VN/VC versus
Venus cotransfected with Kv2.1 increased by 27% (Fig. 7
VN and CaV1.2-VC channels with or without Kv2.1. Because
fluorescent areas (Fig. 7
2 ways: noninteracting Venus proteins dimerized to create new,
adjacent CaV1.2-VN and CaV1.2-VC physically interact to form
Venus reconstitution is virtually irreversible, once the C tails of
ever, when brought into close proximity by interaction of the
isolation, VN155(I152L) and VC155 are nonfluorescent. How-
these experiments so as not to confound any ICa recordings. The
form of Kv2.1, Kv2.1P404W (46), was used for transfection in
female myocytes. The bar plot shows the mean ± SEM CaV1.2 cluster area per
cell. *P < 0.05.

N or C terminus of the split-Venus fluorescent protein system to
yield CaV1.2-VN155(I152L) and CaV1.2-VC155, respectively. In
isolation, VN155(I152L) and VC155 are nonfluorescent. How-
ever, when brought into close proximity by interaction of the
separately tagged CaV1.2 subunits, they can reconstitute a full,
fluorescent Venus protein. Thus, split-Venus fluorescence can be
used to report spontaneous interactions between adjacent
CaV1.2 channels (12, 44, 45). Accordingly, we compared basal
split-Venus fluorescence in HEK-293 cells expressing CaV1.2-
VN and CaV1.2-VC channels with or without Kv2.1. Because
Venus reconstitution is virtually irreversible, once the C tails of
adjacent CaV1.2-VN and CaV1.2-VC physically interact to form a
fluorescent Venus, the channels will likely remain coupled at
their C tails for the duration of the experiment.

For these experiments, Venus fluorescence and ICa were
recorded from the same cells before and after the application of
a conditioning protocol involving five 500-ms pulses from −80 to
+20 mV. CaV1.2 clusters increased in Venus fluorescence in 2 ways: noninteracting Venus proteins dimerized to create new,
fluorescent areas (Fig. 7B, ROI 1), or preexisting clusters display
additional channel—channel interactions post stimulation (Fig.
7B, ROI 2 and 3). Indeed, fluorescence in cells transfected with
CaV1.2-Venus increased by 5% upon stimulation, while CaV1.2-
Venus cotransfected with Kv2.1 increased by 27% (Fig. 7C).

Basal (i.e., before preconditioning pulse) ICa was nearly 3-fold
larger in cells coexpressing Kv2.1 and CaV1.2-VN/VC versus
CaV1.2-VN/VC alone, respectively (Fig. 7D). The nonconducting
form of Kv2.1, Kv2.1P404W (46), was used for transfection in these
experiments so as not to confound any ICa recordings. The conditioning protocol increased both ICa and Venus fluorescence to a larger extent in cells coexpressing Kv2.1 and CaV1.2 than in
cells expressing CaV1.2 alone (Fig. 7D). Furthermore, note that
membrane depolarization shifted the voltage dependence of
G/Gmax in cells coexpressing CaV1.2 and Kv2.1 but not in cells
transfected with CaV1.2 only (Fig. 7E). These data suggest that
coopexression of nonconducting Kv2.1P404W increases the
probability of CaV1.2–CaV1.2 channel interactions, both at rest and
during membrane depolarization. Together, these data support
a model that Kv2.1 can enhance CaV1.2 activity by increasing
CaV1.2 clustering and interaction.

Discussion
Kv2.1 protein is expressed in female and male arterial myocytes,
where its assumed functional role has been as a voltage-gated ion
channel that, upon opening, hyperpolarizes the membrane poten-
tial of these cells to impact myocyte [Ca2+], and myogenic
tone (16). Here, we propose a model in which Kv2.1 channels
have a more complex function to exert opposing actions on
vascular smooth muscle. In its canonical role, the opening of
conducting Kv2.1 hyperpolarizes arterial myocytes, which
decreases the P o of CaV1.2 channels. This lowers [Ca2+], inducing
relaxation. Our data indicate that Kv2.1 protein has an addi-
tional nonconducting structural role in arterial myocytes: to en-

tance CaV1.2 clustering and activity, thereby increasing [Ca2+],
and inducing contraction. It is paradoxical that Kv2.1 could
control both relaxation and contraction in arterial smooth mus-
cle. Notably, we find that the relative contribution of the elec-
trical and structural roles of Kv2.1 to the control of membrane potential and CaV1.2 activity, respectively, varies with sex. In
male myocytes, the dominant role for Kv2.1 channels is as an ion
channel regulating membrane potential. In female myocytes,
however, Kv2.1 channels have dual electrical and structural roles
in both controlling membrane potential and enhancing CaV1.2
function, respectively.

Our data suggest that Kv2.1 enhances ICa by increasing the P o
of CaV1.2 channels, as the number of CaV1.2 channels and the amplitude of their unitary currents remain unchanged, regardless of differences in Kv2.1 expression in WT and Kv2.1−/−
myocytes. Kv2.1 also shifted the voltage dependence of activation
of CaV1.2 channels toward more hyperpolarized potentials and
increased their open times, suggesting that Kv2.1 facilitates
CaV1.2 gating and stabilizes its open conformation. In combi-
nation with recently published work (11, 12), the data presented
here suggest a potential mechanism by which Kv2.1 acts to in-
crease the P o of CaV1.2 channels. We found by PLA that Kv2.1
and CaV1.2 are in close proximity (i.e., 40 to 60 nm) to one
another in arterial myocytes. The increase in CaV1.2, P o and open
times along with the shift of their voltage dependence of acti-
vation are similar to those observed during physical coupling of
clustered CaV1.2 channels (11, 12). Our data support this model
in that female myocytes, which have higher Kv2.1 expression,

Our recent paper describes a potential mechanism by which
Kv2.1 could increase CaV1.2 channel clustering in arterial
smooth muscle (8). In these cells, CaV1.2 cluster formation is
determined by a stochastic self-assembly process in which cluster
size is determined by 3 distinct biological probabilities: cluster
nucleation (P n), removal (P R), and growth (P G). P n is the first
step in cluster formation and is the probability that an ion
channel-containing vesicle will be randomly inserted at any site
in the membrane. Changes in P n lead to variations in cluster
density. Changes in density as well as cluster size are determined
by P R, the probability of clusters being endocytosed or degraded
from the membrane. Lastly, P G is the probability of a channel
being inserted immediately adjacent to preexisting channels. The
growth probability of a cluster is P G multiplied by the number of
available neighbors. Thus, an increase in P G increases cluster
area. In Figs. 2 and 6, we show that the level of Kv2.1 expression
in female myocytes increased CaV1.2 channel cluster area,
without significant changes in the number of channels in the

Fig. 6. Kv2.1 channel expression increases CaV1.2 channel cluster size in
female myocytes to a larger extent than in male myocytes. Superresolution
GSD images of regions of interest from representative Kv2.1−/− male and
female myocytes. The bar plot shows the mean ± SEM CaV1.2 cluster area per
cell. *P < 0.05.
membrane (i.e., similar $P_g$) or total Cav1.2 protein expression. This suggests that Kv2.1 could be acting to increase the $P_g$ of Cav1.2 channels.

Importantly, loss of Kv2.1 expression decreased but did not eliminate Cav1.2 channel clusters in arterial myocytes of both sexes. This suggests that Kv2.1 expression is not required for the formation of Cav1.2 clusters. Indeed, Cav1.2 cluster size in male WT and Kv2.1−/− myocytes is similar, indicating that the relationship between Kv2.1 expression, Cav1.2 cluster size, and Ca2+ influx is not linear. Thus, a critical number of Kv2.1 channels or ratio of Kv2.1 to Cav1.2 expression levels could be a key determinant for how Cav2.1 alters Cav1.2 organization and function. We note that this nonlinearity could also suggest that, in addition to Kv2.1 expression levels, other factors might contribute to the observed sex-specific differences in Cav1.2 function. Future studies should investigate whether the ~1:1 Kv2.1:Cav1.2 channel ratio observed in female myocytes is the lower limit or optimal point for the modulation of Cav1.2 cluster size and channel–channel interactions, and any additional mechanisms that may act in concert with Kv2.1 expression level to impact Cav1.2 function.

An intriguing finding in this study is that, as in heterologous expression systems (20–22), the vast majority of Kv2.1 channels in male and female arterial myocytes are nonconductive. One potential hypothesis is that conductive and nonconductive Kv2.1 channels have separate functional roles, with conductive channels regulating membrane potential and nonconductive channels...
acting to increase the $P_f$ and $P_o$ of clustered Cav1.2 channels. Several observations are consistent with this hypothesis. First, our data suggest that Kv2.1 and Cav1.2 colocalize. Second, we show that at sites where Kv2.1 and Cav1.2 channels cocluster, Cav1.2–Cav1.2 interactions take place. Third, it has been proposed that the bulk of clustered Kv2.1 channels may be in a nonconductive state (21). These observations suggest the hypothesis that nonconductive Kv2.1 channels are involved in the clustering and activity of Cav1.2 channels.

While the evidence for nonconductive Kv2.1 channels playing a role in Cav1.2 clustering is strong, our data do not allow us to determine if the sole function of clustered Kv2.1 is to bring Cav1.2 channels together. However, our recent paper (30) as well as other work from the Trimmer (46, 47) and Tamkun (48) laboratories support that clustered Kv2.1 channels are involved in the formation of endoplasmic reticulum–plasma membrane junctions in HEK-293 cells and hippocampal neurons. In hippocampal neurons, Kv2.1-expressing endoplasmic reticulum–plasma membrane junctions contain functionalryanodine receptors that produce $Ca^{2+}$ sparks at both rest and during membrane depolarization (30). Whether this same phenomenon happens in arterial smooth muscle cells is currently unknown. Future experiments should investigate whether Kv2.1 promotes the formation of sarcoplasmic reticulum–plasma junctions in arterial myocytes as it does in HEK-293 cells and hippocampal neurons. Further studies should consider if, in arterial myocytes, these Kv2.1-containing junctions form in combination with junctophilin-2 (49), and whether due to their differing levels of Kv2.1 expression these structures are more prominent in female than in male myocytes.

An additional question raised by this study is the nature of the mechanisms underlying the differential expression of Kv2.1 channels in male and female arterial myocytes. To our knowledge, there are no published studies addressing whether the expression of Kv2.1 or Kv9.3 in smooth muscle is regulated by sex hormones. Interestingly, Vierra et al. (30) did not detect sex-specific differences in Kv2.1 expression or Cav1.2 clustering and function in hippocampal neurons. However, others have suggested that estrogen down-regulates Kv1.5 but has no effect on Kv2.1 expression in female mouse ventricular myocytes (50). Interestingly, we find that Kv1.5 expression is similar in male and female arterial myocytes. Thus, the control of expression of these channel sequences in arterial smooth muscle cells is currently unknown. Future experiments should investigate whether Kv2.1 promotes the formation of sarcolemmal junctions in arterial myocytes as it does in HEK-293 cells and hippocampal neurons. Further studies should consider if, in arterial myocytes, these Kv2.1-containing junctions form in combination with junctophilin-2 (49), and whether due to their differing levels of Kv2.1 expression these structures are more prominent in female than in male myocytes.

Materials and Methods
A detailed version of this study’s materials and methods can be found in SI Appendix. Briefly, male and female WT C57BL/6J and Kv2.1+− mice were used in this study. Experiments were conducted within Institutional Animal Care and Use Committee guidelines. Electrophysiological recording techniques were performed using Axopatch amplifiers. Protein levels were determined using immunoblot approaches. $Ca^{2+}$ imaging was performed using an Andor Discovery spinning disk system. Immunofluorescence TIRF, superresolution, and confocal images were acquired using a Leica GSD or Olympus FV3000 microscope.

Data Availability Statement. All data are available in the manuscript and SI Appendix.

ACKNOWLEDGMENTS. We thank Delaney Rudolph-Gandy for technical assistance. Thanks also to Manuel F. Navedo for help with the analysis of data. 

1. W. M. Bayliss, On the local reactions of the arterial wall to changes of internal pressure. J. Physiol. 28, 220–231 (1902).
2. A. L. Gonzales et al., A PLC1-dependent, force-sensitive signaling network in the myogenic constriction of cerebral arteries. Sci. Signal. 7, ra49 (2014).
3. M. A. Spassova, T. Hewavitharana, W. Xu, J. Soboloff, D. L. Gill, A common mechanism underlies stretch activation and receptor activation of TRPC6 channels. Proc. Natl. Acad. Sci. U.S.A. 103, 16586–16591 (2006).
4. S. Bulley et al., Arterial smooth muscle cell PKD2 (TRPP3) channels regulate systemic blood pressure. elife 7, e46286 (2018).
5. M. F. Navedo, L. F. Santana, Cav1.2 sparklets in heart and vascular smooth muscle. J. Mol. Cell. Cardiol. 58, 67–76 (2013).
6. M. F. Navedo, G. C. Amberg, V. S. Votava, L. F. Santana, Constitutively active L-type $Ca^{2+}$ channels. Proc. Natl. Acad. Sci. U.S.A. 102, 11112–11117 (2005).
7. G. C. Amberg, M. F. Navedo, M. Nieves-Cintrón, J. D. Molkentin, L. F. Santana, Calcium sparklets regulate local and global calcium in murine arterial smooth muscle. J. Physiol. 579, 187–201 (2007).
8. D. Sato et al., A stochastic model of ion channel cluster formation in the plasma membrane. J. Gen. Physiol. 181, 1116–1134 (2019).
9. M. F. Navedo et al., Increased coupled gating of L-type $Ca^{2+}$ channels during hyper-tension and Timothy syndrome. Circ. Res. 106, 748–756 (2010).
10. E. P. Cheng et al., Restoration of normal L-type $Ca^{2+}$ channel function during Timothy syndrome by ablation of an anchoring protein. Circ. Res. 109, 255–261 (2011).
11. R. E. Dixon, C. Yuan, E. P. Cheng, M. F. Navedo, L. F. Santana, Cav1.2 signaling amplification by oligomerization of L-type Cav1.2 channels. Proc. Natl. Acad. Sci. U.S.A. 109, 1749–1754 (2012).
12. R. E. Dixon et al., Graded Cav1.2 and Cav1.3 channel-dependent coupling of voltage-gated Cav1.2 channels. eLife 4, e05608 (2015).
13. M. T. Nelson et al., Relaxation of arterial smooth muscle by calcium sparks. Science 270, 633–637 (1995).
14. G. C. Amberg, L. F. Santana, Kv2 channels oppose myogenic constriction of rat cerebral arteries. Am. J. Physiol. Cell Physiol. 291, C348–C356 (2006).
15. F. Plane et al., Heteromultimeric Kv1 channels contribute to myogenic control of arterial diameter. Circ. Res. 96, 216–224 (2005).
16. X. Z. Zhong et al., Stromatoxin-sensitive, heteromultimeric Kv2.2/Kv9.3 channels contribute to myogenic control of cerebral arterial diameter. J. Physiol. 588, 4519–4537 (2010).
17. S. L. Archer et al., Molecular identification of the role of voltage-gated K+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J. Clin. Invest. 101, 2319–2330 (1998).
18. Y. Lu, S. T. Hanna, G. Tang, R. Wang, Contributions of Kv1.2, Kv1.5 and Kv2.1 subunits to the native delayed rectifier K+ current in rat mesenteric artery smooth muscle cells. Life Sci. 71, 1465–1473 (2002).
19. G. C. Amberg, C. F. Rosowe, M. F. Navedo, L. F. Santana, NFATc3 regulates Kv2.1 expression in arterial smooth muscle. J. Biol. Chem. 279, 47326–47334 (2004).
20. K. Benndorf, R. Koopmann, C. Lorra, O. Pongs, Gating and conductance properties of a human delayed rectifier K+ channel expressed in frog oocytes. J. Physiol. 477, 1–14 (1994).
21. K. M. O’Connell, R. Loftus, M. M. Tamkun, Localization-dependent activity of the Kv2.1 delayed-rectifier K+ channel. Proc. Natl. Acad. Sci. U.S.A. 107, 12351–12356 (2010).
22. P. D. Fox, R. J. Loftus, M. M. Tamkun, Regulation of Kv2.1 K(+)-conductance by cell surface channel density. J. Neurosci. 33, 1259–1270 (2013).
23. J. S. Trimmer, Immunological identification and characterization of a delayed rectifier K+ channel polypeptide in rat brain. Proc. Natl. Acad. Sci. U.S.A. 88, 10764–10768 (1991).
24. D. E. Antonucci, S. T. Lim, S. Vassanelli, J. S. Trimmer, Dynamic localization and clustering of dendritic Kv2.1 voltage-dependent potassium channels in developing hippocampal neurons. Neuroscience 108, 69–81 (2001).
25. S. T. Lim, D. E. Antonucci, R. H. Scannevin, J. S. Trimmer, A novel targeting signal for the proximal clustering of the Kv2.1 K+ channel in hippocampal neurons. Neuron 25, 385–397 (2000).
26. D. P. Mohapatra, J. S. Trimmer, The Kv2.1 C terminus can autonomously transfer Kv2.1-like phosphorylation-dependent localization, voltage-dependent gating, and muscarinic modulation to diverse Kv channels. J. Neurosci. 26, 685–695 (2006).
27. M. Cobb, D. C. Austin, J. T. Sack, J. S. Trimmer, Cell cycle-dependent changes in localization and phosphorylation of the plasma membrane Kv2.1 K+ channel impact endoplasmic reticulum membrane contact sites in COS-1 cells. J. Biol. Chem. 290, 29189–29201 (2015).
28. N. C. Vierra, M. Kirmiz, D. van der List, L. F. Santana, J. S. Trimmer, Kv2.1 mediates spatial and functional coupling of L-type calcium channels and ryanodine receptors in mammalian neurons. eLife 8, e49953 (2019).
29. J. Gayet-Primo, D. B. Yaeger, R. A. Khanjari, T. Puthussery, Heteromeric Kv2.2/K8.2 channels mediate delayed rectifier potassium currents in primate photoreceptors. J. Neurosci. 38, 3414–3427 (2018).
30. D. C. Tilley et al., The tarantula toxin GxTx detains K+ channel gating charges in their resting conformation. J. Gen. Physiol. 151, 292–315 (2019).
31. M. Taglialatela, E. Stefani, Gating currents of the cloned delayed-rectifier K+ channel DRK1. Proc. Natl. Acad. Sci. U.S.A. 90, 4758–4762 (1993).
32. A. Scholle et al., Effects of Kv1.2 intracellular regions on activation of Kv2.1 channels. Biophys. J. 87, 873–882 (2004).
33. A. Jara-Oseguera et al., Uncoupling charge movement from channel opening in voltage-gated potassium channels by ruthenium complexes. J. Biol. Chem. 286, 16414–16425 (2011).
34. E. Bocksteins, A. J. Labro, D. J. Sanders, D. P. Mohapatra, The electrically silent Kv6.4 subunit confers hyperpolarized gating charge movement in Kv2.1/Kv6.4 heterotetrameric channels. Proc. Natl. Acad. Sci. U.S.A. 7, e37143 (2012).
35. A. J. Patel, M. Ladunska, E. Honore, Kv2.1/Kv9.3, a novel ATP-dependent delayed-rectifier K+ channel in oxygen-sensitive pulmonary artery myocytes. EMBO J. 16, 6615–6625 (1997).
36. R. H. Cox, S. Fromme, Functional expression profile of voltage-gated K(+)-channel subunits in rat small mesenteric arteries. Cell Biochem. Biophys. 74, 263–276 (2016).
37. C. M. Armstrong, F. Bezanilla, Currents related to movement of the gating particles of the sodium channels. Nature 242, 459–461 (1973).
38. R. W. Hadley, W. J. Lederer, Properties of L-type calcium channel gating current in isolated guinea pig ventricular myocytes. J. Gen. Physiol. 98, 265–285 (1991).
39. F. Noceti et al., Effective gating charges per channel in voltage-dependent K+ and Ca2+ channels. J. Gen. Physiol. 108, 143–155 (1996).
40. D. W. Ito et al., ß-adrenergically mediated dynamic augmentation of sarcolemmal Ca2+ clustering and co-operativity in ventricular myocytes. J. Physiol. 597, 2139–2162 (2019).
41. Y. Kodama, C. D. Hu, An improved bimolecular fluorescence complementation assay with a high signal-to-noise ratio. Biotechniques 49, 793–805 (2010).
42. C. M. Moreno et al., Ca2+ entry into neurons is facilitated by cooperative gating of clustered CaV1.3 channels. eLife 5, e15744 (2016).
43. M. Kirmiz et al., Remodeling neuronal ER-PM junctions is a conserved nonconducting function of Kv2 plasma membrane ion channels. Mol. Biol. Cell 29, 2410–2432 (2018).
44. M. Kirmiz, N. C. Vierra, S. Palacio, J. S. Trimmer, Identification of VAPA and VAPB as Kv2 channel-interacting proteins defining endoplasmic reticulum-plasma membrane junctions in mammalian brain neurons. J. Neurosci. 38, 7562–7584 (2018).
45. B. Johnson et al., Kv2 potassium channels form endoplasmic reticulum/plasma membrane junctions via interaction with VAPA and VAPB. Proc. Natl. Acad. Sci. U.S.A. 115, E7331–E7340 (2018).
46. H. A. T. Pritchard et al., Nanoscale coupling of junctophilin-2 and ryanodine receptors regulates vascular smooth muscle cell contractility. Proc. Natl. Acad. Sci. U.S.A. 116, 21874–21881 (2019).
47. T. Saito et al., Estrogen contributes to gender differences in mouse ventricular re-polarization. Circ. Res. 105, 343–352 (2009).