Objective—In resistance arteries, there is an emerging view that smooth muscle CaV3.2 channels restrain arterial constriction through a feedback response involving the large-conductance Ca2+-activated K+ channel (BKαca). Here, we used wild-type and CaV3.2 knockout (CaV3.2−/−) mice to definitively test whether CaV3.2 moderates myogenic tone in mesenteric arteries via the CaV3.2-ryanodine receptor-BKαca axis and whether this regulatory mechanism influences blood pressure regulation.

Approach and Results—Using pressurized vessel myography, CaV3.2−/− mesenteric arteries displayed enhanced myogenic constriction to pressure but similar K+-induced vasoconstriction compared with wild-type C57BL/6 arteries. Electrophysiological and myography experiments subsequently confirmed the inability of micromolar Ni2+, a CaV3.2 blocker, to either constrict arteries or suppress T-type currents in CaV3.2−/− smooth muscle cells. The frequency of BKαca-induced spontaneous transient outward K+ currents dropped in wild-type but not in knockout arterial smooth muscle cells upon the pharmacological suppression of CaV3.2 channel. Line scan analysis performed on en face arteries loaded with Fluo-4 revealed the presence of Ca2+ sparks in all arteries, with the subsequent application of Ni2+ only affecting wild-type arteries. Although CaV3.2 channel moderated myogenic constriction of resistance arteries, the blood pressure measurements of CaV3.2−/− and wild-type animals were similar.

Conclusions—Overall, our findings establish a negative feedback mechanism of the myogenic response in which CaV3.2 channel modulates downstream ryanodine receptor-BKαca to hyperpolarize and relax arteries. (Arterioscler Thromb Vasc Biol. 2015;35:1843-1851. DOI: 10.1161/ATVBAHA.115.305736.)

Key Words: arteries ■ calcium-activated potassium channels ■ calcium channels ■ calcium signaling ■ ryanodine receptors ■ T-type calcium channels ■ vascular smooth muscle

The cardiovascular system comprises a muscular pump and a distribution network of arteries, veins, and capillaries. Within this integrated system, resistance arteries control the magnitude and distribution of tissue perfusion and respond to vasoactive stimuli, including mechanical forces, neurotransmitters, and metabolites.1-3 Bayliss first described the inherent ability of resistance arteries to constrict to elevated pressure,4 and studies have shown that the so-called myogenic response is intimately tied to depolarization and the activation of smooth muscle L-type Ca2+ channels. It is often presumed that Ca1,2 is the only Ca2+ channel of functional significance because dihydropyridines, L-type blockers, prominently attenuate myogenic tone.5 This traditional perspective has begun to change with the identification of arterial T-type Ca2+ channels, including Ca3,1 and Ca3,2 subtypes.6,7 Recent findings suggest that the former (ie, Ca3,1) modestly facilitates myogenic constriction at hyperpolarized voltages, whereas the latter (ie, Ca3,2) facilitates a negative feedback response restraining arterial constriction.8,9

Our recent observations have tied the paradoxical ability of rat cerebral arterial CaV3.2 channel to limit myogenic tone to the triggering of ryanodine receptors (RyR) on the sarcoplasmic reticulum. The RyR-mediated generation of Ca2+ sparks subsequently activates the large conductance Ca2+-activated K+ channels (BKαca), eliciting a hyperpolarization to counteract pressure-induced constriction.10 Furthermore, the CaV3,2 conductance in the human cerebral circulation seems to mediate a similar physiological role.10 Although the concept of a voltage-gated Ca2+ channel counterbalancing vasoconstriction is novel and intriguing, it is one delimited by 2 primary concerns. First, current work is heavily reliant on the presumed selectivity of Ni2+ to block Ca3,2 channels.11 Second, there is a lack of corroborative observations, outside the cerebral circulation, in vascular beds known to acutely and sustainably regulate systemic blood pressure.
Here, we used wild-type and CaV3.2 knockout (CaV3.2−/−) mice to definitively test whether CaV3.2 channel moderates myogenic tone in mesenteric arteries via the CaV3.2-RyR-BKCa axis and, more generally, whether this regulatory mechanism influences blood pressure regulation. Experiments ranged from cells to whole animals and encompassed the integrative use of myography, electrophysiology, Ca2+ imaging, and intravascular catheterization. Arteries displayed enhanced myogenic tone when CaV3.2 channels were genetically ablated or pharmacologically suppressed using Ni2+. Subsequent analyses indicated that Ni2+ inhibited BKCa currents and Ca2+ sparks in wild-type but not CaV3.2−/− arteries. Although CaV3.2 channel moderated myogenic constriction, the blood pressure measurements of both animal types were similar. In conclusion, this study establishes a negative feedback response in which CaV3.2 channel modulates downstream activity of the RyR-BKCa complex to hyperpolarize and relax resistance arteries.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Genetic Ablation of CaV3.2 Enhances Arterial Myogenic Tone

Our earlier reports revealed the involvement of CaV3.2 channels in negative feedback control of rat cerebral arterial tone10; thus, channel deletion should enhance myogenic tone. Figure 1A and 1B demonstrates that C57BL/6 and CaV3.2−/− arteries display enhanced myogenic tone. Although CaV3.2 channel moderated myogenic constriction, the blood pressure measurements of both animal types were similar. In conclusion, this study establishes a negative feedback response in which CaV3.2 channel modulates downstream activity of the RyR-BKCa complex to hyperpolarize and relax resistance arteries.

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CaV3.2−/− arteries were myogenically active and constricted as intravascular pressure increased from 20 to 100 mm Hg. Ca2+-free saline (0 mmol/L Ca2++2 mmol/L EGTA) reversed myogenic constriction and evoked passive arterial dilation. Expressing data as a percentage myogenic tone (Figure 1C) revealed the predicted enhancement in CaV3.2−/− arteries (at 60 mm Hg: CaV3.2−/−, 28%±2%; C57BL/6, 19%±3%). Although myogenic tone was significantly different, 60 mmol/L K+-induced vasoconstriction was similar in C57BL/6 (40%±2%) and CaV3.2−/− (43%±2%) pressurized arteries (Figure 1D). Note, the basal diameter of CaV3.2−/− arteries was smaller than that of C57BL/6, a finding consistent with variable myogenic tone. Interpretational caution is, however, warranted because CaV3.2−/− mice display a lighter body mass than wild-type animals.12 Quantitative PCR showed that mRNA expression of CaV1.2, CaV3.1, BKCa-α, or RyR2 was comparable in wild-type and CaV3.2−/− arteries (Figure 1E).

Micromolar Ni2+ Selectively Blocks CaV3.2 Currents
Mesenteric arterial smooth muscle cells from C57BL/6 mice express 3 subtypes of voltage-gated Ca2+ channels (CaV1.2, CaV3.1, and CaV3.2),9 and thus, total inward current is representative of this ensemble of channels. To distinguish between the subcomponents, we used patch clamp electrophysiology in combination with defined pharmacology.10 First, nifedipine (200 nmol/L) was applied to block L-type CaV1.2 channels and reveal a current predominated by T-type conductances (Figure 2A). Subsequent application of 50 μmol/L Ni2+, which is presumed to be a selective CaV3.2 blocker,10–14 reduced the nifedipine-insensitive T-type currents in C57BL/6 but not in CaV3.2−/− smooth muscle cells (Figure 2B and 2C). The absence of an effect of Ni2+ in CaV3.2−/− myocytes is consistent with this pharmacological selectivity. Note that the broad-spectrum T-type blocker (NNC 55–0396, 1 μmol/L) subsequently abolished the residual current15 in both C57BL/6 and CaV3.2−/− myocytes because of the suppression of remaining CaV3.1 current (Figure 2C). Importantly, voltage dependence profiles demonstrated that the T-type current is available for activation at physiological membrane potentials (Figure I in the online-only Data Supplement). As noted previously, CaV3.1 mRNA levels were similar among the 2 groups of animals, whereas CaV1.2 was modestly but insignificantly lower in CaV3.2−/− arteries (Figure 1E).

CaV3.2 Activity Regulates BKCa-Mediated STOCs
Given the enhancement of myogenic constriction in CaV3.2−/− arteries, we next tested whether this T-type channel modifies myogenic reactivity through a negative feedback response that involves downstream BKCa.10,12 We used perforated patch clamp electrophysiology to monitor BKCa-mediated spontaneous transient outward K+ currents (STOCs) in arterial smooth muscle cells from wild-type and knockout animals. In C57BL/6 cells held at the physiological voltage of −40 mV, Ni2+ significantly suppressed STOC frequency. In contrast, Ni2+ had no effect on STOCs when C57BL/6 cells were voltage-clamped at more depolarized potentials (−20 mV), a finding consistent with the voltage profile of CaV3.2 channel (Figure 3A). In CaV3.2−/− cells, Ni2+ had no effect at either −40 or −20 mV (Figure 3B). All STOCs were fully abolished by the

Figure 2. Micromolar Ni2+ suppresses CaV3.2 current. A. Current–voltage (I-V) plots illustrate total inward currents in wild-type C57BL/6 and CaV3.2−/− smooth muscle cells and the effects of CaV1.2 and CaV3.2 blockade using nifedipine (200 nmol/L) and Ni2+ (50 μmol/L), respectively. All recordings used 10 mmol/L Ba2++ as the charge carrier. B and C. Representative traces and summary data illustrate the effect of Ni2+ on nifedipine insensitive T-type currents. Representative traces were evoked using a prepulse (−90 mV) followed by a test pulse (0 mV). Ni2+ significantly suppressed T-type currents in C57BL/6 but not in CaV3.2−/− smooth muscle cells (n=7 each; *P<0.05).
Figure 3. Ca₃.2 channel modulates BKₐ-mediated spontaneous transient outward K⁺ currents (STOCs). A and B, Representative traces of STOCs recorded at −40 and −20 mV in wild-type C57BL/6 (A) or Ca₃.2⁻/⁻ (B) arterial smooth muscle cells. Application of 50 μmol/L Ni²⁺ suppressed STOC frequency only at −40 mV in C57BL/6 cells with no noticeable effect at other conditions. C, Averaged bar graphs illustrate the effect of Ni²⁺ on STOC frequency (Hz) and amplitude (pA) at −40 or −20 mV in C57BL6 or Ca₃.2⁻/⁻ arterial myocytes (n=8–11, *P≤0.05, paired t test).
application of the BKCa inhibitor paxilline (1 μmol/L; Figure II in the online-only Data Supplement). Message expression of the BKCa pore-forming subunit (BKCa-α) was similar in C57BL/6 and CaV3.2−/− arteries (Figure 1E). Further, Ni2+ had no effect on STOC amplitude under different experimental conditions (Figure 3C). Note, basal STOCs tended to fire at lower frequencies in CaV3.2−/− myocytes compared with C57BL/6 counterparts. Statistical analysis was not performed across groups as cells that did not fire sufficient STOCs were eliminated a priori from experimentation.

**CaV3.2 Channel Controls Ca2+ Spark Generation**

Given the ability of CaV3.2 channel to modulate BKCa current (Figure 3) and the reported correlation between RyR-mediated spark generation and BKCa activation,10,16 we next explored the CaV3.2-Ca2+ spark relationship using Ca2+ imaging and line scan analysis of mouse mesenteric arteries (Figure 4). In C57BL/6 arteries loaded with Fluo-4, Ca2+ sparks were observed in 76% of the 454 line scans performed under control conditions. Depolarizing arteries (30 mmol/L K+) increased spark activity (95%), whereas the subsequent addition of Ni2+ significantly reduced firing (52%; Figure 4A and 4B). In comparison, 30 mmol/L K+ increased spark firing in CaV3.2−/− arteries from 58% to 94% (456 line scans) but Ni2+ failed to attenuate sparks (98%; Figure 4A and 4B). In depolarized wild-type C57BL/6 arteries, Ca2+ spark frequency was calculated to be 0.0347 and 0.0153 sparks/μm s in the absence and presence of Ni2+, respectively; spark frequency in knockout tissues was distinctively insensitive to the application of Ni2+ (Figure 4C). The genetic absence of CaV3.2 channels was notably associated with lower percentage firing (CaV3.2−/−, 58%; C57BL/6, 76%) and lower basal Ca2+ spark frequency when compared with wild-type arteries (CaV3.2−/−, 0.0065±0.0014; C57BL/6, 0.0169±0.005 sparks/μm s). The amplitudes and spatiotemporal characteristics of Ca2+ sparks displayed no significant changes before and after the application of Ni2+ on C57BL/6 and CaV3.2−/− arteries (Figure 4D).

**CaV3.2 Activity Restrains Myogenic Constriction by Altering BKCa Feedback**

The application of Ni2+ (50 μmol/L) onto C57BL/6 arteries evoked vasoconstriction at intravascular pressure values between 20 and 60 mmHg, and this vasomotor effect diminished at higher pressures; CaV3.2−/− arteries lacked a similar response (Figure 5A). The percentage of myogenic tone in wild-type C57BL/6 arteries increased after the application of Ni2+ (at 60 mmHg: control 20%±4% versus Ni2+ 25%±1%), but was not altered in CaV3.2−/− arteries (control 30%±5% versus Ni2+ 28%±8%; Figure 5B). Coinciding with vasomotor data, membrane potential measurements showed that Ni2+ only depolarized C57BL/6 but not CaV3.2−/− pressurized arteries (60 mmHg; Figure 5C). In C57BL/6 arteries, the BKCa...
blocker (paxilline, 1 μmol/L) evoked vasoconstriction and enhanced myogenic tone similar to that of Ni²⁺ (Figure 5D), an observation consistent with a common signaling axis between CaV3.2 and BKCa channels. When Ni²⁺ and paxilline were sequentially added to the same wild-type artery, Ni²⁺ evoked vasoconstriction, whereas subsequent paxilline had no additional effect. Similar experiments using CaV3.2−/− arteries demonstrated a lack of vasomotor responses to Ni²⁺ but preserved responsiveness to paxilline (Figure 5E).

**CaV3.2−/− Mice Display Normal Blood Pressure Responses**

Pharmacological and genetic approaches suggested that CaV3.2 channel counterbalances myogenic constriction (Figure 1 and 5) and could as such influence blood pressure regulation. To explore this possibility, we catheterized carotid arteries of C57BL/6 and CaV3.2−/− mice to monitor blood pressure under resting conditions and in response to a vasopressor challenge. As depicted (Figure 6A), basal mean arterial pressure was similar in wild-type and knockout mice (C57BL/6, 100±2 mm Hg; CaV3.2−/−, 103±5 mm Hg), a finding consistent with earlier reports in conscious animals.17,18

Given that CaV3.2 channels seem to be involved in a feedback mechanism, we next assessed whether this conductance can alter mean arterial pressure responsiveness to a vasopressor challenge. The intravenous administration of phenylephrine (α₁-adrenoceptor agonist, 1–16 μg/kg body weight) evoked dose-dependent rises in mean arterial pressure, and these transient responses were similar among the 2 groups (Figure 6B). Analogous to in vivo experiments, phenylephrine (0.01–10 μmol/L)-induced vasoconstriction was similar in C57BL/6 and CaV3.2−/− mesenteric arteries (Figure 6C and 6D).

**Discussion**

This study used wild-type and CaV3.2 knockout mice to examine the purported contribution of CaV3.2 channels to a negative feedback response that counterbalances arterial tone development. Using mesenteric arteries, functional experiments illustrated that the genetic ablation or pharmacological suppression of CaV3.2 channel selectively enhanced myogenic constriction. Subsequent electrophysiological recordings revealed that CaV3.2 channel modulates downstream BKCa−mediated STOCs. Ca²⁺ imaging further demonstrated that...
Ca\(^{2+}\) spark generation is an intermediary step in the Ca\(_{\text{V}3.2}\)-BK\(_{\text{Ca}}\) functional axis. Finally, although Ca\(_{\text{V}3.2}\) moderated myogenic tone, this regulatory mechanism did not influence resting blood pressure or vasopressor-induced responses. In summary, findings from this study establish a model by which Ca\(_{\text{V}3.2}\) channel restrains myogenic constriction by driving a process where Ca\(^{2+}\) influx triggers Ca\(^{2+}\) sparks and downstream activation of BK\(_{\text{Ca}}\) currents (Figure III in the online-only Data Supplement).

Resistance arteries control tissue perfusion and respond to a range of vasoactive stimuli.1–5 The integral ability of resistance arteries to respond to perturbations in arterial pressure, known as the myogenic response,6 is intrinsic to vascular smooth muscle and plays an essential role in maintaining blood flow and capillary pressure to tissues, such as the brain and the heart.19 This response is mechanistically linked to a rise in cytosolic [Ca\(^{2+}\)] driven by a depolarization that activates L-type Ca\(_{\text{V}1.2}\) channels. The traditional perspective that Ca\(_{\text{V}1.2}\) is the only voltage-gated Ca\(^{2+}\) channel of functional significance3 has shifted with the identification of 2 T-type Ca\(^{2+}\) channels,7,8 the first being Ca\(_{\text{V}3.1}\), which modestly facilitates myogenic tone at hyperpolarized voltages.9 In contrast, Ca\(_{\text{V}3.2}\) channels have been reported to drive a paradoxical feedback response that limits arterial constriction.10,12 The feedback response begins with Ca\(^{2+}\) influx through Ca\(_{\text{V}3.2}\) channels triggering ryanodine receptors to initiate Ca\(^{2+}\) sparks. These discrete sarcoplasmic reticulum–driven events in turn activate BK\(_{\text{Ca}}\) channels to hyperpolarize and dilate resistance arteries.10 Although an intriguing concept, data interpretation is singularly dependent on the presumed selectivity of Ni\(^{2+}\) as a pharmacological Ca\(_{\text{V}3.2}\) blocker.11 Current knowledge is also restricted to the cerebral circulation, and experiments have not extended to other vascular beds essential to blood pressure regulation.

One means to better probe the functional properties of arterial Ca\(_{\text{V}3.2}\) channel is to use an animal model in which the channel has been genetically ablated.12 In this regard, we performed pressure myography on arteries from Ca\(_{\text{V}3.2}\) knockout (Ca\(_{\text{V}3.2}^{-/-}\)) mice to study the vascular phenotype. Consistent with the view that Ca\(_{\text{V}3.2}\) channel mediates feedback vasodilation, we observed enhanced myogenic constriction in mesenteric arteries from Ca\(_{\text{V}3.2}^{-/-}\) mice. This finding was somewhat akin to the functional observations in coronary arteries12 and the enhancement of rat cerebral arterial myogenic tone noted in the presence of Ni\(^{2+}\).10 Our present findings overcome concerns raised by past studies that off-target effects of Ni\(^{2+}\) could theoretically have accounted for the vasomotor responses.20–22 In particular, we were unable to elicit Ni\(^{2+}\)-sensitive current in Ca\(_{\text{V}3.2}^{-/-}\) arterial myocytes, and Ni\(^{2+}\) also failed to constrict/depolarize arteries from knockout mice.

The unexpected ability of Ca\(_{\text{V}3.2}\) channel to mediate a negative feedback response has been previously tied to downstream modulation of BK\(_{\text{Ca}}\) channels through intermediary activation of ryanodine receptors.10 In arterial smooth muscle, BK\(_{\text{Ca}}\) is ubiquitously expressed and known to moderate membrane depolarization and arterial constriction.23 To activate arterial BK\(_{\text{Ca}}\), vasoactive stimuli must induce depolarization and elicit a discrete micromolar rise in [Ca\(^{2+}\)] in the subsarcolemma taking the form of a Ca\(^{2+}\) spark.24 Here, we present multiple lines of evidence implicating BK\(_{\text{Ca}}\) as the final downstream effector of Ca\(_{\text{V}3.2}\) channel. First, electrophysiology revealed that BK\(_{\text{Ca}}\)-mediated STOCs were sensitive to Ni\(^{2+}\) in wild-type but not in Ca\(_{\text{V}3.2}^{-/-}\) arterial myocytes. Second, pressure myography illustrated that adding Ni\(^{2+}\) or paxilline (BK\(_{\text{Ca}}\) blocker) to Ca\(_{\text{V}3.2}^{-/-}\) arteries did not reproduce the functional findings observed in wild-type Ca\(_{V3.2}\) arteries.

Ca\(_{\text{V}3.2}\) channel inhibition with Ni\(^{2+}\) or paxilline (Figure 6) was followed by enhanced myogenic constriction, a finding consistent with the Ca\(_{\text{V}3.2}\) knockout studies. In contrast, Ca\(_{\text{V}3.2}\) channel activation with Bay K 86444 (Figure 7) was followed by enhanced arterial dilation. These results are consistent with our electrophysiological findings, which showed that Ca\(_{\text{V}3.2}\) channels were inhibited by Ni\(^{2+}\) in wild-type but not in Ca\(_{\text{V}3.2}^{-/-}\) arteries. These Ca\(_{\text{V}3.2}\) channel blockers also failed to potentiate arterial constriction in Ca\(_{\text{V}3.2}^{-/-}\) arteries. Therefore, Ca\(_{\text{V}3.2}\) channel inhibition with Ni\(^{2+}\) or paxilline is a poor model of Ca\(_{\text{V}3.2}\) channel activation and vice versa.

Our findings also contrast with previous studies that showed enhanced myogenic tone in Ca\(_{\text{V}3.2}^{-/-}\) arteries.20,22 These differences may be due to the presence of Ni\(^{2+}\)-sensitive current in Ca\(_{\text{V}3.2}^{-/-}\) muscular arteries, which we were unable to detect in our Ca\(_{\text{V}3.2}^{-/-}\) arterial myocytes. The presence of Ni\(^{2+}\)-sensitive current in muscular arteries could account for the enhanced myogenic tone observed in previous studies. This suggests that the Ni\(^{2+}\)-sensitive current in muscular arteries could account for the enhanced myogenic tone observed in previous studies.
inhibitor) to the superfusate comparably augmented myogenic tone. Intriguingly, applying paxilline to wild-type arteries pretreated with Ni2+ had no additive effect, consistent with CaV3.2 and BKCa channels being linked through a common sequential pathway.

It has been long established that RyR activation is responsible for the initiation of BKCa-mediated STOCs in arterial smooth muscle,6,24 and this recognized relationship led us to examine the nature of Ca2+ spark generation in mouse mesenteric arteries. Indeed, inhibitors of RyR (eg, ryanodine) has been shown to suppress the generation of STOCs in vascular smooth muscle irrespective of their origin.24 Here and in consistency with CaV3.2 channel driving Ca2+ sparks and subsequently STOC generation, we used Ca2+ imaging and line scan analysis and observed that spark frequency decreased in wild-type arteries when CaV3.2 channels were inhibited with Ni2+. We also found that firing of Ca2+ sparks was unaffected by this divalent cation in CaV3.2−/− arteries and that basal Ca2+ spark frequency was lower than those of wild-type arteries. These findings align well with recent work from the cerebral circulation where a variety of functional, structural, electrophysiological, and computational observations draw a critical relationship between CaV3.2 and RyR and then BKCa (Figure III in the online-only Data Supplement).10 Intriguingly, the preceding work is distinct from neuronal studies where T-type conductances have been suggested to directly activate Ca2+-activated K+ channels independent of RyR.25–28

With CaV3.2 channels playing an intimate role in limiting myogenic constriction, it is logical to argue that the loss of this conductance would alter peripheral resistance and systemic blood pressure regulation. We tested this supposition by catheterizing anesthetized mice and assessing blood pressure at rest and in response to a vasopressor challenge. Resting blood pressure was similar among wild-type and knockout mice, a finding consistent with earlier reports which used different monitoring approaches (eg, catheterization, tail cuff) in conscious animals over longer time spans (days to weeks).17,18 Given the presumed role of CaV3.2 in feedback (rather than active vasodilation), we challenged blood pressure regulation using intravenous phenylephrine. This multidose challenge evoked comparable rises in blood pressure among the 2 animal groups; likewise, mesenteric arteries isolated from wild-type and CaV3.2−/− mice constricted similarly to this α1-adrenergic agonist. These mice in vivo and in vitro results interestingly seem to rule out a direct modulatory role for vascular CaV3.2 channels in agonist-induced vasoconstriction.

It is not clear at present why blood pressure responses fail to change. Perhaps, the loss of smooth muscle feedback in CaV3.2−/− arteries is compensated by enhanced dilatory feedback from the endothelium. Other functional compensation may have occurred in this global knockout independent of vascular smooth muscle25; in this context, establishing a smooth muscle–specific CaV3.2 knockout model would be valuable. It should also be recognized that for the proposed CaV3.2/RyR/BKCa feedback mechanism to effectively drive a blood pressure response, it should be ideally present in a full range of vascular beds. Recent work documenting heterogeneity of BKCa activity,30 because of differences in channel activity and Ca2+ spark generation, challenges this key assumption. Further investigation is warranted to address this intriguing anomaly.

In conclusion, this study showed that arterial CaV3.2 channels retain a unique ability to counterbalance myogenic constriction. This negative feedback response entails a modulatory paradigm in which Ca2+ flux through CaV3.2 channel triggers Ca2+ spark generation and then activates BKCa channels to hyperpolarize and relax arteries (Figure III in the online-only Data Supplement). This novel functional axis, recently described in the human cerebral circulation,31 challenges the traditional view that voltage-gated Ca2+ channels solely facilitate arterial tone development.

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Disclosures

None.

References


### Significance

Vascular smooth muscle cells express T-type Ca2+ channels along with L-type channels. Although the latter have been long implicated in arterial excitation–contraction coupling, studies have only recently begun to assess the role of T-type channels. Using an animal model in which Ca3.2, a T-type channel, was genetically deleted, we tested its role in arterial tone development. We demonstrate that resistance arteries from knockout animals paradoxically display enhanced responsiveness to arterial pressure. This enhancement was mechanistically attributed to the ability of Ca3.2 to modulate downstream K+ channels, which hyperpolarize and relax arteries. This novel data challenge the traditional view that voltage-gated Ca2+ channels are singularly involved in the genesis of arterial constriction.
Genetic Ablation of CaV3.2 Channels Enhances the Arterial Myogenic Response by Modulating the RyR-BK Ca Axis
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SUPPLEMENTAL MATERIALS

Genetic ablation of CaV3.2 channels enhances the arterial myogenic response by modulating the RyR-BKCa axis

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DETAILED METHODS

Animal procedures

Animal procedures were approved by the Animal Care and Use Committee at the University of Calgary and Loma Linda University. Briefly, male or female C57BL/6J (wild-type) and CaV3.2 knockout (CaV3.2−/−) mice (2–4 months old, obtained from Jackson Laboratories) were asphyxiated in a CO2 chamber. The mesentry was carefully removed and placed in cold phosphate-buffered saline solution (pH 7.4) containing (in mM): 138 NaCl, 3 KCl, 10 Na2HPO4, 2 NaH2PO4, 5 glucose, 0.1 CaCl2 and 0.1 MgSO4. Third and fourth order mesenteric arteries were dissected out of surrounding tissues and cut into 2–3 mm segments.

Vessel myography and membrane potential (VM) measurement

Mesenteric arteries were mounted in an arteriograph and superfused with physiological saline solution (PSS; 37°C; pH 7.4; 21% O2, 5% CO2, balance N2) containing (in mM): 119 NaCl, 4.7 KCl, 20 NaHCO3, 1.1 KH2PO4, 1.2 MgSO4, 1.6 CaCl2 and 10 glucose. To limit the endothelial influence, air bubbles were passed through the lumen for 1–2 min. Arteries were equilibrated at 15 mmHg and contractile responsiveness assessed by briefly applying 60 mM KCl. Following equilibration, intravascular pressure was incrementally elevated from 20 to 100 mmHg and external diameter monitored. Maximal diameter was assessed in Ca2+-free PSS (zero Ca2+ + 2 mM EGTA). Percentage myogenic tone was calculated as follows: % Myogenic tone=100*(D0–D)/D0; where D is external diameter under control conditions (Ca2+ PSS) or treated conditions (Ca2+ PSS) or treated conditions, and D0 is external diameter in Ca2+-free PSS. Smooth muscle membrane potential (VM) was ascertained as previously published1 by inserting a glass microelectrode backfilled with 1 M KCl (tip resistance ~120-150 MΩ) into the vessel wall while pressurized at 60 mmHg. Criteria for successful impalement included: 1) a sharp negative VM deflection upon insertion; 2) a stable VM for ≥1 min after entry; and 3) a sharp return to baseline upon electrode removal.

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from mouse C57BL/6 or CaV3.2−/− mesenteric arteries using the RNeasy plus micro kit (Qiagen) following manufacturer’s recommendations. Reverse transcription was performed with 20 ng per sample of total RNA using the Quantitect reverse transcription kit (Qiagen). For the negative control groups, all components except the reverse transcriptase were included in the reaction mixtures. Real-Time PCR using intron-spanning primer sequences was performed using the Kapa SYBR Fast Universal qPCR Kit (Kapa Biosystems). Mouse beta-actin gene was utilized as the reference gene. Control reactions and those containing cDNA from arteries were performed with 1 ng of template per reaction. The running protocol extended to 45 cycles consisting of 95°C for 5 s, 55°C for 10 s and 72°C for 10 s using an Eppendorf Realplex 4 Mastercycler. PCR specificity was checked by dissociation curve analysis, and assay validation was confirmed by testing serial dilutions of pooled template cDNAs suggesting a linear dynamic range of 50-0.05 ng template and yielded percent efficiencies ranging from 85-95%. No template controls yielded no detectable fluorescence. Expression levels of the various genes of interest in arteries from CaV3.2−
relative to C57BL/6 mice were determined using the relative expression software tool (REST) version 2.0.13.2

Quantitative PCR primer sequences and validation parameters.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
<th>Amplicon</th>
<th>Efficiency (%)</th>
<th>Linear Dynamic Range ng template (Cq values)</th>
<th>Cq values ± SEM</th>
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</thead>
<tbody>
<tr>
<td>Cav1.2</td>
<td>ATTGCACGTGAAGGCACGTG GAGTTAGCAGGACCCGTGGAG</td>
<td>90</td>
<td>95</td>
<td>50-0.05 (23.41-33.75)</td>
<td>33.22±0.43</td>
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<td>31.93±0.24</td>
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<tr>
<td>CaV3.1</td>
<td>TCCTGGTCATACCTCACG GAGGCTGGAAGAGATGT</td>
<td>98</td>
<td>85</td>
<td>50-0.005 (24.09-39.29)</td>
<td>38.98±0.21</td>
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<td>36.68±1.05</td>
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<tr>
<td>BKCaα</td>
<td>TCTCGACTTGGGTGCCCTCGGAT GTAGAGGAAGACACGTTGAA</td>
<td>127</td>
<td>92</td>
<td>33-0.0033 (22.48-36.48)</td>
<td>34.56±0.06</td>
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<td>32.30±0.14</td>
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<tr>
<td>RyR2</td>
<td>CTGAGCTGGGCATCAAGGAG AGGCTTCTTGTGAGCCAGCAAG</td>
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<td>88</td>
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<td>35.11±0.50</td>
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<td>33.47±0.02</td>
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<tr>
<td>ACTB</td>
<td>ACTGTCGAGTCCGCTCCA GCAGCCATATCGTCCATCATCAT</td>
<td>100</td>
<td>88</td>
<td>50-0.0005 (20.61-39.35)</td>
<td>31.15±0.43</td>
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<td></td>
<td></td>
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<td>28.71±0.59</td>
</tr>
</tbody>
</table>

Isolation of arterial smooth muscle cells

As previously described3, arterial segments were placed in an isolation medium containing (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl2, 10 glucose and 10 HEPES with 1 mg/ml bovine serum albumin (pH 7.4, 37 °C, 10 min). Vessels were then exposed to a two-step digestion process: 1) 13 min incubation in isolation medium containing 0.5 mg/ml papain and 1.5 mg/ml dithioerythritol; and 2) 10 minutes incubation in isolation medium containing 100 μM Ca2+, 0.7 mg/ml type F collagenase and 0.4 mg/ml type H collagenase. Following incubation, tissues were washed repeatedly with ice-cold isolation medium and triturated with a fire-polished pipette. Liberated cells were stored in ice-cold isolation medium for use within ~6 hr.

Electrophysiological recordings

Conventional patch-clamp electrophysiology was used to monitor whole-cell voltage-gated Ca2+ channel currents in isolated smooth muscle cells.3 Recording electrodes (5-8 MΩ) were pulled from borosilicate glass microcapillary tubes using a micropipette puller, and backfilled with pipette solution (in mM): 135 CsCl, 5 Mg-ATP, 10 HEPES, and 10 EGTA (pH 7.2). Cells were voltage-clamped and equilibrated in bath solution (in mM): 110 NaCl, 1 CsCl, 10 BaCl2, 1.2 MgCl2, 10 glucose, and 10 HEPES (pH 7.4). A 1 M NaCl–agar salt bridge between the reference electrode and the bath solution was used to minimize offset potentials. To record whole-cell Ba2+ currents, isolated cells held at -60 mV were exposed to a pre-pulse (-90 mV, 200 ms) and then test pulses (-50 to 40 mV, 10 mV intervals, 300 ms).
Perforated patch-clamp electrophysiology was used to measure spontaneous transient outward K⁺ currents (STOCs) in freshly isolated smooth muscle cells. The bath solution contained (in mM): 134 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM): 110 K aspartate, 30 KCl, 10 NaCl, 2 MgCl₂, 10 HEPES, and 0.05 EGTA (pH 7.2) with 200 µg/ml amphotericin B. Currents were recorded while cells were held at -40 or -20 mV. STOC analysis was performed using Clampfit 10.3; threshold for detection was set to be ~3 times the BKCa single channel conductance. Whole-cell currents were recorded using an Axopatch 200B patch-clamp amplifier, filtered at 1 kHz, digitized at 5 kHz, and were stored on a computer for offline analysis. Whole-cell capacitance averaged ~13 pF and all experiments were performed at room temperature.

Ca²⁺ spark measurement

Ca²⁺ sparks were recorded in mouse arterial myocytes loaded with the Ca²⁺ sensitive dye Fluo-4 AM and using a Zeiss LSM 710 NLO laser scanning confocal imaging workstation on an inverted microscope platform (Zeiss Axio Observer Z1). Fluo-4 AM was dissolved in DMSO and added from a 1 mM stock to the arterial suspension at a final concentration of 10 µM, along with 0.1% pluronic F127 for 1-1.5 hr at room temperature in the dark in balanced salt solution. Arterial segments were then washed (30 min) to allow dye esterification and then cut into linear strips. Arterial segments were pinned to Sylgard blocks and placed in an open bath imaging chamber mounted on the confocal imaging stage. Arteries were illuminated at 488 nm with a krypton argon laser, and emitted light was collected using a photomultiplier tube. Line scans were imaged at 529 fps with the emission signal recorded at 493-622 nm. The acquisition period for Ca²⁺ spark recordings was 18.9 s and the resultant pixel size ranged from 0.0148 to 0.0911 µm per pixel. To ensure that sparks within the cell were imaged, the pinhole was adjusted to provide an imaging depth of 2.5 µm, this is roughly equivalent to 50% the width of the cell based on morphological examination of live preparations. Analysis was performed to characterize the percentage of scans with Ca²⁺ sparks (% firing), Ca²⁺ spark frequency, amplitude and spatiotemporal characteristics using SparkLAB 4.2.1. Threshold for spark detection was 3.2 times the standard deviation of the background noise above mean background. Prior to analysis, background fluorescence was subtracted from each image assuming homogeneous background levels in each cell.

Intravascular catheterization

Male C57BL/6 or Cav3.2⁻/⁻ mice were anesthetized with intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Anesthesia was extended by additional ketamine (10 mg/kg) as required. Anesthesia adequacy was verified by the abolition of the withdrawal and blink reflexes. A small neck incision was made and the left common carotid artery was ligated and catheterized using an arterial cannula. Similarly, a small incision was made to expose and catheterize the right jugular vein for intravenous drug administration. Changes in mean arterial pressure (ΔMAP) were monitored using a data acquisition system (ACQKnowledge). Mice were sacrificed at the end of the experiment using a high dose of anesthetic.
Statistical analysis
Data are expressed as means±S.E.M., and n indicates the number of cells, arteries or mice. Where appropriate, paired/unpaired t-tests or one way ANOVA were performed to compare the effects of a given condition/treatment on arterial diameter, or whole-cell current. *P values ≤ 0.05 were considered statistically significant. Averaged current-voltage relationships were fit to the following Peak Gaussian function: \[ I(V) = I_{\text{max}} \cdot \exp[-0.5 \{(V - V_{\text{max}})/b\}^2] \]; where \( I_{\text{max}} \) is peak current (I), \( V_{\text{max}} \) is \( V \) at \( I_{\text{max}} \), and \( b \) is the slope of the distribution.

Solutions and Chemicals
Drugs, enzymes and buffer reagents were purchased from Sigma-Aldrich, unless otherwise mentioned.

SUPPLEMENTAL REFERENCES
SUPPLEMENTAL MATERIALS

Genetic ablation of Cav3.2 channels enhances the arterial myogenic response by modulating the RyR-BKCa axis

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Running Title: Cav3.2 counterbalances myogenic constriction

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Supplementary Figure I: Voltage-dependence of activation and steady-state inactivation of T-type current. In C57BL/6 mesenteric arterial smooth muscle cells, voltage dependence of activation and steady-state inactivation (availability) of T-type current was assessed. Barium (10 mM) was employed as the charge carrier and recordings were made in the presence of an L-type blocker (nifedipine, 200 nM). Upper insets depict protocols employed to assess availability (left) and activation (right). The red bar denotes physiological potentials that overlaps with the window current. Note that in physiological Ca²⁺ curves are expected to shift rightward by ~10-20 mV and window currents are expected to remain overlapping with physiological potentials.
Supplementary Figure II: Paxilline abolishes STOCs. Spontaneous transient outward K⁺ currents were fully abolished in wild-type (C57BL/6) or knockout Cav3.2⁻/⁻ smooth muscle cells by paxilline. This BKCa inhibitor was equally effective at holding potentials -40 or -20 mV (n=10 cells each).
Supplementary Figure III: A diagram highlighting the proposed role of Cav3.2 channels in vascular smooth muscle. A stimulus, such as an increase in intravascular pressure, elicits membrane potential (V_M) depolarization of the smooth muscle cells. This electrical stimulus triggers the activation of the voltage-gated Ca^{2+} channel, Cav3.2. Ensuing Ca^{2+} influx through Cav3.2 pores activates RyR on the sarcoplasmic reticulum to release Ca^{2+} sparks. The latter release events then activate BK_{Ca} to generate hyperpolarizing K^{+} currents. This hyperpolarizing stimulus feedbacks upon membrane depolarization (red) and Ca^{2+} influx responsible for smooth muscle contraction.