Cav3.2 Channels and the Induction of Negative Feedback in Cerebral Arteries

Osama F. Harraz1,2,*, Rasha R. Abd El-Rahman1,*, Kamran Bigdely-Shamloo1,3, Sean M. Wilson4, Suzanne E. Brett1, Monica Romero4, Albert L. Gonzales5, Scott Earley6, Edward J. Vigmond3,7, Anders Nygren3, Bijoy K. Menon8, Rania E. Mufti1, Tim Watson8, Yves Starreveld8, Tobias Furstenhaupt9, Philip R. Muellerleile10, David Kurjiaka10, Barry D. Kyle1, Andrew P. Braun1 and Donald G. Welsh1

1Dept. of Physiology and Pharmacology, Hotchkiss Brain and Libin Cardiovascular Institutes, University of Calgary, Calgary, AB, Canada; 2Dept. of Pharmacology and Toxicology, Alexandria University, Alexandria, Egypt; 3Dept. of Electrical and Computer Engineering, University of Calgary, Calgary, AB, Canada; 4Division of Pharmacology, Loma Linda University, Loma Linda, CA, USA; 5Dept. of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA; 6Dept. of Pharmacology, University of Nevada, Reno, NV, USA; 7LIRYC Institute and Lab IMB, University of Bordeaux, Bordeaux, France; 8Dept. of Clinical Neurosciences, University of Calgary, Calgary, AB, Canada; 9Microscopy Imaging Facility, University of Calgary, Calgary, AB, Canada; 10Dept. of Biomedical Sciences, Grand Valley State University, Allendale, MI, USA

* O.F.H. and R.R.A. are both first authors and contributed equally to the manuscript

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Corresponding Author:
Donald G. Welsh, PhD.
Department of Physiology & Pharmacology
GAA-14, Health Research Innovation Center
3280 Hospital Dr. N.W.
Calgary, Alberta, Canada, T2N-4N1
Phone: (403)-210-3819
Email: dwelsh@ucalgary

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ABSTRACT

Rationale: T-type (CaV3.1/CaV3.2) Ca^{2+} channels are expressed in rat cerebral arterial smooth muscle. While present, their functional significance remains uncertain with findings pointing to a variety of roles.

Objective: This study tested whether CaV3.2 channels mediate a negative feedback response by triggering Ca^{2+} sparks, discrete events that initiate arterial hyperpolarization by activating BKCa channels.

Methods and Results: Micromolar Ni^{2+}, an agent that selectively blocks CaV3.2 but not CaV1.2/CaV3.1, was first shown to depolarize/constrict pressurized rat cerebral arteries; no effect was observed in CaV3.2-- arteries. Structural analysis using 3D-tomography, immunolabeling, and a proximity ligation assay next revealed the existence of microdomains in cerebral arterial smooth muscle comprised of sarcoplasmic reticulum and caveolae. Within these discrete structures, CaV3.2 and RyR resided in close apposition to one another. Computational modeling revealed that Ca^{2+} influx through CaV3.2 could repetitively activate RyR, inducing discrete Ca^{2+}-induced Ca^{2+} release events in a voltage dependent manner. In keeping with theoretical observations, rapid Ca^{2+} imaging and perforated patch clamp electrophysiology demonstrated that Ni^{2+} suppressed Ca^{2+} sparks and consequently spontaneous transient outward K+ currents, BKCa mediated events. Additional functional work on pressurized arteries noted that paxilline, a BKCa inhibitor, elicited arterial constriction equivalent, and not additive, to Ni^{2+}. Key experiments on human cerebral arteries indicate that CaV3.2 is present and drives a comparable response to moderate constriction.

Conclusions: These findings indicate for the first time that CaV3.2 channels localize to discrete microdomains and drive RyR-mediated Ca^{2+} sparks enabling BKCa activation, hyperpolarization and attenuation of cerebral arterial constriction.

Key Words: Ca^{2+} channels, Ca^{2+} sparks, Potassium channels, BKCa, Cerebral circulation, Vascular smooth muscle, T-type channel, Myogenic tone.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>BKCa</td>
<td>Large Conductance Ca^{2+} activated K+ Channel</td>
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<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
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<tr>
<td>STOC</td>
<td>Spontaneous Transient Outward K+ Current</td>
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<td>TRPV4</td>
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INTRODUCTION

Cerebral arteries form an integrated network that controls the magnitude and distribution of tissue blood flow. Tone within these structures is regulated by multiple stimuli including blood flow, neuronal activity, tissue metabolism and intraluminal pressure. These vasoactive stimuli alter myosin light chain phosphorylation through a dynamic process controlled by myosin light chain kinase and phosphatase. While the precise signaling mechanisms vary among stimuli, a global rise in cytosolic [Ca^{2+}] is generally thought to be a key mediating step. This global rise is in turn intimately tied to changes in the smooth muscle membrane potential (V_M) and the subsequent activation of voltage-gated Ca^{2+} channels.

Voltage-gated Ca^{2+} channels are heteromultimeric complexes comprised of a pore-forming α subunit and auxiliary subunits that influence gating and protein trafficking to the plasma membrane. In cerebral arterial smooth muscle, the L-type CaV1.2 channel is the dominant Ca^{2+} entry pathway by which vasoactive stimuli set cytosolic [Ca^{2+}], and consequently tone development. Recent studies have noted that, in addition to CaV1.2, T-type channels (i.e. CaV3.1 and CaV3.2) are also expressed in rat and mouse cerebral arteries. It has been argued that the T-type conductance, like that of CaV1.2, plays a direct role in elevating cytosolic [Ca^{2+}], albeit at hyperpolarized potentials due to a leftward shift in their voltage dependence. Earlier reports have, however, suggested that the relationship between T-type channels and arterial tone is more complex with Ca^{2+} influx via CaV3.2 potentially acting in a discrete fashion to influence a defined target. Speculation of the downstream effector does vary, ranging from nitric oxide synthase in the endothelium to Ca^{2+}-activated channels in the smooth muscle.

The large conductance Ca^{2+} activated K^+ channel (BK_{Ca}) is expressed in cerebral arterial smooth muscle and its principal role is to feedback upon and limit excessive constriction. Vasoconstrictor stimuli enhance BK_{Ca} activity through arterial depolarization and augmentation of Ca^{2+} spark generation. Ca^{2+} sparks are discrete sarcoplasmic reticulum (SR) driven events that arise in response to the transient opening of ryanodine receptors (RyR). While the functional significance of Ca^{2+} sparks is recognized, the mechanistic events that initiate repetitive SR release remains ambiguous, with current theories suggesting a role for CaV1.2 or transient receptor potential (TRPV4) in triggering the cytosolic or luminal gate of RyR. Decidedly absent from this discussion has been a potential role for a T-type conductance.

The present study tested whether CaV3.2 channel triggers Ca^{2+} spark generation, BK_{Ca} channel activation, and ultimately negative feedback control of cerebral arterial tone. Our examination progressed from cellular to tissue level and involved the integrative use of pressurized vessel myography, electrophysiology, confocal and electron microscopy, and computational modeling. In rat cerebral arteries, we specifically show that Cav3.2 and RyR co-localize within a microdomain and that steady-state depolarization activates Cav3.2 to trigger Ca^{2+} sparks. We subsequently show that Cav3.2-evoked Ca^{2+} sparks activate BK_{Ca} channels, contributing to hyperpolarization that attenuates myogenic constriction. We further demonstrate that this negative feedback mechanism is not limited to rat arteries but extends to the human cerebral circulation. Overall, this study is the first to illustrate that localized Ca^{2+} influx through T-type Ca^{2+} channels in vascular smooth muscle plays an important but indirect role in setting arterial tone by targeting key conductances involved in V_M regulation.
METHODS

Female Sprague Dawley rats were euthanized by CO2 asphyxiation as approved by the Animal Care and Use Committee at the University of Calgary. Rat brains were removed, placed in cold phosphate-buffered saline and middle and posterior cerebral arteries were isolated. Human cerebral arteries were extracted from resected brain tissue according to the University of Calgary Institutional Review Board. Structural analysis was performed employing confocal, electron and epifluorescence approaches. Vasomotor/VM responses were subsequently assessed with the aid of pressure myography. Conventional, perforated and on-cell patch clamp electrophysiology was used to record whole-cell and single channel voltage-gated Ca\(^{2+}\) and BK\(_{Ca}\) currents. Data are presented as means ± SEM; paired or unpaired t-tests were performed where appropriate and a P<0.05 was considered statistically significant. An expanded version of the Materials and Methods can be found in the Supplementary Material.

RESULTS

CaV3.2 inhibition: Vasomotor and electrical responses in rat cerebral arteries

Our examination began by confirming the ability of 50 µM Ni\(^{2+}\) or 200 nM nifedipine to respectively block CaV3.2 and CaV1.2 currents in tSA-201 cells. Figure 1A illustrates that Ni\(^{2+}\) effectively abolished inward Ba\(^{2+}\) current through CaV3.2 channels without impacting charge movement through CaV1.2 or CaV3.1 (Online Figure I). In comparison, nifedipine selectively blocked CaV1.2 channels (Online Figure I). Moving to rat cerebral arterial smooth muscle cells, we next monitored the nifedipine-insensitive Ba\(^{2+}\) current, a conductance that is dominated by T-type channels and is stable over time (Online Figure IIA-IIIB). Nickel partially attenuated this native inward current, a finding consistent with CaV3.2 channel expression (Figure 1B). On-cell recordings further denoted T-type activity in rat cerebral arterial smooth muscle. While CaV1.2 channels were blocked, single channel activity was observed at hyperpolarized voltages (-50 to -20 mV), and slope conductance was 8.5 pS. The subsequent application of Ni\(^{2+}\) to endothelial-denuded arteries enhanced myogenic tone at 20-80 mmHg (Figures 1C-1D). Control experiments confirmed that arterial responses to pressure were repeatable over time (Online Figure IIC). Coincident with arterial constriction was a Ni\(^{2+}\)-induced depolarization of 5±0.9 mV in pressurized arteries (Figures 1E-1F). The latter observation inferred that CaV3.2-mediated Ca\(^{2+}\) influx could elicit hyperpolarization and dilation through a smooth muscle signaling mechanism. In theory, this hyperpolarization could be triggered through localized Ca\(^{2+}\) entry initiating RyR-mediated Ca\(^{2+}\) sparks, SR events that activate BK\(_{Ca}\) channels. Nickel’s effects on the nifedipine-insensitive Ba\(^{2+}\) current and arterial tone were reversible (not shown).

Microdomains and the co-localization of CaV3.2 and RyR

CaV3.2 and RyR reside in the plasma and SR membranes, respectively. For these proteins to functionally interact there must be regions where the two membranes come into close apposition. With this in mind, 3-D electron tomography assayed for microdomains; image analysis and model reconstruction revealed the presence of microstructures comprised of caveolae and SR (Figures 2A-2C). These discrete regions were ~500-600 nm in length and were circumferentially discontinuous. Immunogold labeling subsequently confirmed that RyR localized to regions underneath caveolae whereas CaV3.2 was confined to the plasma membrane in-or-close to caveolae (Figures 2D-2F).
To strengthen the emerging relationship between Ca V3.2 and RyR, the preceding structural work was supplemented with an immunohistochemical analysis of fixed cerebral arteries using antibodies against actin, CaV3.2 and RyR. Findings in Figure 3A first illustrate that actin labeling runs lengthwise in cerebral arterial smooth muscle cells, fading every 7-10 µm as actin leaves the viewing plane. CaV3.2 staining was circumferential and often observed in regions devoid of smooth muscle actin. A similar circumferential pattern was observed for RyR2, a finding indicative although not definitive for co-localization with CaV3.2 (Figure 3B). Unlike CaV3.2 and RyR, CaV1.2 labeling was ribbon-like and ran lengthwise in smooth muscle (Figure 3C). A proximity ligation assay was subsequently performed and consistent with CaV3.2 and RyR2 residing within 40 nm of one another, punctate red fluorescent product was observed in myocytes treated with both primary and secondary antibodies (Figure 4A). Reaction product was absent in control experiments where one or both primary antibodies were removed (Figures 4B-4D).

**CaV3.2, RyR-mediated Ca^{2+} release and the induction of BK_{Ca} activity**

To ascertain at a conceptual level whether Ca^{2+} flux through CaV3.2 channels could activate RyR to initiate Ca^{2+} sparks, a computational model was designed. The microdomain model (Figure 5A) was developed based upon the preceding structural data, measurements of CaV channel activity and mathematical representations of other Ca^{2+} transporters/binding proteins. Findings illustrate that a depolarizing stimulus (from -60 to -40 mV) elicits Ca^{2+} spark-like events in the subspace between the plasma membrane and the SR (Figure 5B). These repetitive events fire at a frequency of ~0.11 Hz and are fully abolished with RyR blockade (Figure 5C). In keeping with a role for CaV3.2, the elimination of this conductance attenuated these spark-like events (~59% inhibition, Figure 5D). A broader voltage dependent analysis also revealed that the frequency of Ca^{2+} spark-like events rose with depolarization (Figure 5E).

Moving forward to experimentally explore the CaV3.2/RyR relationship, Ca^{2+} imaging and line scan analysis were used to monitor Ca^{2+} sparks in rat cerebral arteries (Figure 6A). In opened tissues, Ca^{2+} sparks were observed in 57% of 291 line scans with a mean frequency of 0.0148 sparks/µm/sec. Subsequent application of Ni^{2+} reduced event frequency by 53% (Figure 6A-6C) and had a significant effect on the spatial/temporal characteristics of Ca^{2+} sparks (Online Figure III). Given these positive observations, we next used perforated patch clamp electrophysiology to monitor spontaneous transient outward K^+ currents (STOC), BK_{Ca}-mediated events in response to Ca^{2+} spark generation. Findings in Figure 6D show that STOC were robustly observed in cerebral arterial myocytes and their frequency increased as the holding membrane potential was stepped from -40 to -20 mV. The subsequent application of 50 µM Ni^{2+} reduced STOC frequency at -40 but not -20 mV, a finding consistent with the voltage dependence of CaV3.2 channels. The reduction in STOC frequency occurred without effect on amplitude (Figure 6D). In comparison, STOCs were abolished by 1 µM paxilline, a BK_{Ca} inhibitor (Figure 6E). Control experiments (Online Figure IV) confirmed that peak inward/outward current in myocytes, slowly ramped from -60 to +20 mV, was unaffected by Ni^{2+}. They also confirmed that 200 nM nifedipine does not reduce STOC frequency at -40 mV (n=4: control, 70±15 events/min; nifedipine, 62±12 events/min). Overall, these results support the view Ca^{2+} influx via CaV3.2 channels drives BK_{Ca} activity via a mechanism involving RyR and the induction of Ca^{2+} sparks.

Further functional experiments were sought to emphasize the relationship between CaV3.2, BK_{Ca} activity, and the attenuation of arterial constriction. First, Figures 7A-7B reveal that BK_{Ca} blockade (paxilline, 1 µM) enhanced myogenic tone at intravascular pressures <80 mmHg, akin to Ni^{2+} (Figures 1C-
1D). Second, when Ni\(^{2+}\) and paxilline were sequentially added, the first agent induced constriction while the second had little or no additional effect (Figures 7C-7F). Control experiments confirmed that Ni\(^{2+}\)-induced constriction at 60 mmHg was absent in mesenteric arteries isolated from Ca\(_{V3.2}^{-/-}\) mice (Figure 7G-7H). Overall, these results are consistent with Ca\(_{V3.2}\) and BK\(_{Ca}\) channels working cooperatively within a common signaling pathway.

**Ca\(_{V3.2}\) in human cerebral arteries**

A final set of experiments was conducted on human cerebral arteries to ascertain the translational impact of the preceding findings. Cerebral arteries were isolated from brain tissues resected from patients undergoing temporal lobectomy (Figure 8A). PCR analysis on isolated smooth muscle cells, pre-screened for endothelial contamination, illustrated that Ca\(_{V3.2}\) mRNA was expressed (Figure 8B). Whole-cell patch clamp electrophysiology subsequently confirmed the presence of a nifedipine-insensitive current that was partially sensitive 50 \(\mu\)M Ni\(^{2+}\) (Figure 8C). Analogous to rat, Ni\(^{2+}\) application to human cerebral arteries elicited constriction and enhanced myogenic tone at pressure values \(\leq 60\) mmHg (Figure 8D-8E). These findings confirm that Ca\(_{V3.2}\) is not only expressed in human myocytes but its paradoxical role in tone development is likely akin to the rat.

**DISCUSSION**

This study delineated Ca\(_{V3.2}\) channels in cerebral arterial smooth muscle and determined whether this T-type conductance triggers Ca\(^{2+}\) sparks and consequently BK\(_{Ca}\) channels to elicit feedback control of arterial tone. Experiments progressed from cells to tissues and incorporated electrophysiology, cellular imaging and computational modeling. Patch clamp electrophysiology confirmed the presence of a Ca\(_{V3.2}\) current in cerebral arterial smooth muscle cells, a conductance selectivity blocked by micromolar Ni\(^{2+}\). In pressurized arteries, Ca\(_{V3.2}\) blockade induced unexpected depolarization and constriction, a result indicative of Ca\(_{V3.2}\) involvement in a dilatory process. A combination of structural and protein localization techniques revealed that Ca\(_{V3.2}\) channels localize to microdomains in close apposition to RyR. Computational modeling then conceptually revealed that Ca\(_{V3.2}\) could gate RyR, elicit Ca\(^{2+}\) sparks and activate BK\(_{Ca}\) channels. Consistent with these predictions, Ni\(^{2+}\) inhibited Ca\(^{2+}\) spark production and STOC generation at physiological voltages. Further functional analysis reinforced this linkage by extending experiments to humans. In summary, this study is the first to demonstrate that Ca\(_{V3.2}\) drives a local Ca\(^{2+}\)-induced Ca\(^{2+}\) release event that restrains cerebral arterial constriction by triggering Ca\(^{2+}\) sparks and BK\(_{Ca}\) channel activation.

**Background**

The depolarization of cerebral arterial smooth muscle augments extracellular Ca\(^{2+}\) influx through the activation of voltage-gated Ca\(^{2+}\) channels. This response elevates global [Ca\(^{2+}\)]\(_i\), enhances myosin light chain phosphorylation and augments arterial tone development\(^6,9\). Ca\(^{2+}\) channels are categorized according to the pore-forming \(\alpha_1\)-subunit\(^12\) and in cerebral arterial smooth muscle the L-type Ca\(_{1.2}\) is considered the primary conductance governing Ca\(^{2+}\) entry\(^6\). While Ca\(_{1.2}\) is a dominant conductance, recent studies have begun to acknowledge the expression of low-voltage activated Ca\(^{2+}\) channels in cerebral arteries\(^14,15,28\). T-type channels are the sole members of this subfamily and as their name suggests, their activation/inactivation profiles are leftward shifted compared to the high-voltage activated L-type Ca\(^{2+}\)
Channels12,28. CaV3.1 and CaV3.2 are expressed in arterial smooth muscle and recent work suggests that Ca2+ entry through one or both T-type channel(s) could elevate global [Ca2+]i, albeit at more hyperpolarized potentials, to modestly facilitate myogenic tone14-17,29. While Ca2+ entry through T-type channels could drive bulk [Ca2+]i changes, it could also elicit localized increases to gate conductances tied to Vm regulation18,19. To date, evidence of discrete Ca2+ signaling is limited in vascular tissue although studies have alluded to this possibility given the unexpected impairment of arterial dilation following CaV3.2 blockade19,30.

CaV3.2 Channels in Cerebral Arteries

Studying vascular T-type channels is challenging as pharmacological tools display minimal subtype selectivity. The one exception is Ni2+ which, at low micromolar concentrations, selectively blocks CaV3.2 over CaV3.1 or CaV1.2, the primary Ca2+ channels in vascular smooth muscle28,31. We confirmed Ni2+ selectivity by transfecting the preceding CaV.x.x subunits into tsA-201 cells and monitoring the inward Ba2+ current (Figure 1; Online Figure I). Moving into cerebral arterial myocytes and focusing on the nifedipine-insensitive current dominated by T-type activity28,29, Ni2+ attenuated but did not abolished this conductance, consistent with expression of both CaV3.2 and CaV3.1. On-cell electrophysiology further confirmed successful single channel recordings with a slope conductance consistent with T-type channels32. While only a handful of vascular studies have exploited differential Ni2+ sensitivity to isolate CaV3.2 currents19,28,33, this approach is commonly used in cardiac/neuronal tissues to isolate this conductance and to ascertain its cellular function34,35. In this context, we show for the first time that selective CaV3.2 blockade augmented myogenic tone, findings paradoxical to typical vasodilatory effects of Ca2+ channel blockers6,14,15. The enhancement of tone resulted from the ability of Ni2+ to depolarize arterial Vm. These observations along with earlier reports19 indicate that CaV3.2 might elicit localized rise in cytosolic [Ca2+]i that gates a K+ conductance that limits arterial constriction.

In the cerebral circulation, BKCa channels moderates vasoconstriction to agonists and elevated intravascular pressure. The channel is comprised of four pore forming α1 subunits and four β1 subunits to confer Ca2+ sensitivity24,36. To activate BKCa, [Ca2+]i must discretely rise to micromolar levels and this is achieved through Ca2+ spark generation, SR events dependent on RyR gating21,22,24. The opening of RyR is an integrated process, partially reliant on extracellular Ca2+ entry triggering the RyR cytosolic Ca2+ sensor. The identity of this entry channel is uncertain although past studies have alluded to candidates including TRPV425 and L-type channels26,27,37. While both are plausible candidates, their intrinsic properties are somewhat inconsistent with a triggering role. TRPV4 displays voltage independent properties yet Ca2+ spark generation is graded in a voltage dependent manner. L-type channels exhibit Ca2+-dependent inactivation and if positioned in a diffusion-restriction microdomain, high [Ca2+]i would elicit strong inactivation, impinging upon its ability to activate the RyR cytosolic gate. As CaV3.2 channels are voltage-gated, free of Ca2+ dependent inactivation, and display a voltage window that overlaps with physiological membrane potentials12,28, this conductance appears best suited for microdomain localization and functioning as a trigger of Ca2+-induced Ca2+ release.

Microdomains and Ca2+ Channel Localization

For Ca2+ influx via CaV3.2 to trigger RyR and initiate Ca2+ sparks, the SR and plasma membranes must form a discrete signaling domain and then the proteins of interest must localize in close apposition to one another. In this context, we began our examination of a potential CaV3.2-RyR relationship using
electron tomography, a structural technique that permits intercellular structures to be viewed in high 3-D resolution. With this approach, microdomains comprised of caveolae and SR were readily identified (Figure 2). These discrete signaling regions were observed periodically along smooth muscle cells and were discontinuous longitudinally and circumferentially. Immunogold labeling subsequently placed Cav3.2 intra- or near caveolae and RyR in regions beneath these invaginated structures. In light of these findings, a broader immunohistochemical analysis was performed where further evidence of Cav3.2-RyR colocalization was observed. First, using whole mounted cerebral arteries, this study found that both Ca2+ pores were expressed in regions devoid of smooth muscle actin (Figure 3). Their circumferential labelling pattern was intriguing and strikingly distinct from Cav1.2. Second, a proximity ligation assay yielded a fluorescent product in isolated cells, consistent with Cav3.2 and RyR2 co-localizing within 40 nm of one another (Figure 4). Immunolabelling controls were negative and antibody specificity was characterized, a priori, by western blot analysis14.

**Cav3.2 channels, Ca2+ sparks, STOCs, and arterial tone development**

In order to forward the stated hypothesis, it is important to consider Ca2+ flux in context with channel localization and the spatial compartments. Accordingly, our next step was to build a computational model to ascertain whether Ca2+ flux through Cav3.2 could, on a theoretical level, trigger the opening of RyR (Figure 5). Simulations revealed that at physiological VM, Ca2+ spark-like events could be repetitively generated. Event frequency was coupled to voltage, an observation that aligns with experimental literature23,38,39. RyR blockade abolished these events while Cav3.2 inhibition reduced frequency by 59%. In keeping with theory, analysis of opened arteries confirmed that Cav3.2 blockade decreased the frequency of Ca2+ spark (Figure 6); it also had a significant effect on their spatial/temporal characteristics (Online Figure III). The inability of Ni2+ to completely block discrete events indicates a complexity to RyR gating that extends beyond our focused analysis of Cav3.2. Future studies will need to consider whether other Ca2+ transporters are present in microdomains and able to trigger the cytosolic Ca2+ gate of RyR. Ca2+ transport proteins external to the microdomain could also foster Ca2+ sparks generation by altering SR refilling or the rate of Ca2+ diffusion from the subspace26,27.

The predicted consequence of Ni2+ blockade and reduced Ca2+ spark generation should be decreased BKCa activity. We assessed the latter using perforated patch clamp electrophysiology to monitor STOC in arterial myocytes. As denoted in Figure 6D-E, Ni2+ application reduced STOC frequency at physiological voltages (-40 mV) and had an insignificant effect at depolarized potentials where Cav3.2 channels reside in the inactive state. In comparison, paxilline abolished all STOC activity at both voltages. The voltage dependent effect of Ni2+ is intriguing and one which suggests that the ability of Cav3.2 to drive a feedback response might be confined to a specific VM range. Functional observations in Figure 1 align with this perspective in that the Ni2+ effect on myogenic was greatest at intravascular pressures (40-60 mmHg) where arterial VM will overlap with the peak window current of Cav3.2. Given that paxilline augmented myogenic tone in an analogous manner to Ni2+, we can further suggest that Cav3.2 channels are likely a dominant trigger of BKCa in intact cerebral arteries (Figure 7). This view is further supported by our observations that placing one blocker upon another had no additive effect on arterial tone.

In interpreting the preceding findings, it is important to consider the possible off-target effects of Ni2+. Past studies have noted that under certain conditions this divalent can affect voltage-gated K+ and depolarizing TRP currents40-43. Two lines of evidence indicate that such off-target effects are minimal in this study. First, electrophysiology revealed that Ni2+ had no effect on peak inward/outward current in
smooth muscle cells ramped from -60 to 20 mV (Online Figure IV). Second, Ni$^{2+}$ failed to alter tone in paxilline-pretreated cerebral arteries or in vessels isolated from Cav3.2 knockout mice (Figure 7).

**Translation to Humans**

Our work in rat cerebral arteries highlights a structural and functional association between Cav3.2, RyR and BK$_{Ca}$, whereby voltage-dependent Ca$^{2+}$ influx drives Ca$^{2+}$ sparks generation and consequently arterial hyperpolarization. While these foundational observations are unique and provocative, questions remained as to whether they translate to human tissue. In this context, we harvested human cerebral arteries from resection surgeries and repeated key experiments. We show that Cav3.2 mRNA is indeed present in human cerebral arterial myocytes. Further, Cav3.2 is functionally expressed as patch clamp electrophysiology delineated a Ni$^{2+}$-sensitive T-type current. Finally, consistent with Cav3.2 driving arterial hyperpolarization, we found that Ni$^{2+}$ constricted pressurized human cerebral arteries with an effect peaking at 60 mmHg where arterial $V_m$ likely resides at -45 mV. These findings are the first to note T-type Ca$^{2+}$ channel expression in human cerebral circulation and that it has a unique physiological role.

**Summary**

Vascular Ca$^{2+}$ channels have been targets of investigative interest with Cav1.2 receiving particular attention given that dihydropyridines induce profound arterial dilation. With the recent isolation of T-type Ca$^{2+}$ channels$^{14,28,33}$, interest has begun to shift towards defining their physiological function$^{17,18,33}$. Vascular studies using blockers that don’t discriminate among the T-type subunits have argued that they contribute modestly to global [Ca$^{2+}$], albeit at hyperpolarized potentials$^{14,17}$. The present study challenges this stereotypic view by arguing that Ca$^{2+}$ influx through Cav3.2 acts in a localized manner to alter Ca$^{2+}$ sensitive conductances involved in $V_m$ regulation. While this study focused specifically on Cav3.2, RyR and BK$_{Ca}$, it is intriguing to speculate that Cav3.1 might also regulate a Ca$^{2+}$-activated target such as TRPM4 or TMEM16A, a Ca$^{2+}$ activated Cl$^{-}$ conductance$^{44,45}$. Both conductances have been identified in arterial smooth muscle and linked to pressure-induced depolarization$^{44,46}$.

In summary, this study delineated Cav3.2 channels, explored their cellular expression and examined their relationship to tone development in the cerebral circulation. Our examination employed theoretical and experimental approaches from computational modeling to structural analysis, electrophysiology, and pressure myography. Cav3.2 channels were readily identified, shown to co-localize in microdomains with RyR to initiate Ca$^{2+}$ sparks. These discrete events activate BK$_{Ca}$ channels to facilitate arterial hyperpolarization and drive a feedback response that moderates constrictor events including those initiated by intravascular pressure. As Cav3.2 channels are present in other vascular beds$^{17,19,20,30}$, their feedback mechanism likely extends beyond the cerebral circulation. These findings also provide a straightforward explanation how Cav3.2 deletion paradoxically impacts arterial relaxation$^{19,30}$. This atypical Cav3.2 vasomotor response entails further attention given the emerging potential use of therapeutic T-type blockers for hypertension, cerebral vasospasm, or pain$^{47-49}$.

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DISCLOSURES
None

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FIGURE LEGENDS

**Figure 1. Effects of Ni2+ on CaV3.2 currents, myogenic tone and membrane potential.** (A) Representative traces and summary data of inward currents in CaV3.2-transfected tSA-201 cells in the absence and presence of Ni2+ (CaV3.2 blocker, 50 μM). A voltage step from -90 to -10 mV was used to evoke inward Ba2+ current (n=5, *P<0.05, paired t-test). (B) T-type current in rat cerebral arterial smooth muscle before and after Ni2+ (50 μM). Experiments were performed in the presence of nifedipine (200 nM) to block L-type Ca2+ channels. A voltage step from -90 to 0 mV was used to elicit inward current (n=8, *P<0.05, paired t-test). (C,D) Single channel recordings and summary current-voltage relationship (n=5, slope conductance=8.5 pS) of T-type Ca2+ currents in cerebral arterial myocytes. On-cell recording were performed at -50 to -10 mV in the presence nifedipine (200 nM), 60 mM Ca2+ and 50 mM TEA-Cl to block K+ channels. (E,F) Rat middle or posterior cerebral arteries were pressurized from 20 to 100 mmHg while diameter was monitored under control conditions, in the presence of Ni2+ (50 μM), and in Ca2+-free medium. Representative traces (E) and summary data (F) display augmented arterial tone in response to Ni2+ (n=7, *P<0.05, paired t-test). (G,H) Arterial VM in pressurized cerebral arteries (60 mmHg) in the absence and presence of Ni2+ (50 μM). Illustrative traces (G) and summary data (H) reveal the depolarizing effect of Ni2+ (n=6, *P<0.05, paired t-test).

**Figure 2. Electron microscopic imaging of rat cerebral arterial smooth muscle cells.** (A) Tissue sections (300 nm thick) were used to generate a contiguous stack of 2-D photomicrographs (~3.5 nm resolution); subcellular structures were subsequently traced on each section. (B,C) 3-D models of discrete membranous regions where caveolae and SR are in close apposition to one another. (D,E) Transmission electron microscopy and immunogold labeling of RyR (D) or CaV3.2 channels (E) in rat cerebral arteries. RyR labeling (arrowheads) can be observed in membranes localized beneath the plasma membrane. CaV3.2 labeling (arrowheads) was confined to the plasma membrane in association with caveolae. Boxed areas were magnified in the lower micrographs. (F) Control experiments showed no electron dense particles. Each photomicrograph is representative of three independent preparations.

**Figure 3. CaV3.2 displays localization patterns similar to RyR2 in rat cerebral arteries.** (A) Cerebral arteries were labeled with antibodies against smooth muscle actin (green) and CaV3.2 (red). Labeling of CaV3.2 ran perpendicular (arrowheads) to the longitudinal axis of smooth muscle cells (arrow) in regions devoid of smooth muscle actin. Bottom panels display smooth muscle actin and CaV3.2, separately. (B) Immunohistochemical staining of RyR (red) localized to areas where actin (green) was absent. Magnified panels show RyR was perpendicular to the longitudinal axis of smooth muscle cells. (C) CaV1.2 labeling (arrowheads) was parallel to the longitudinal axis of smooth muscle cells (arrow). The boxed area (middle) was magnified in the right panel. Photomicrographs are representative of 3 independent experiments.

**Figure 4. Proximity ligation assay of CaV3.2 and RyR2 in rat cerebral arterial smooth muscle cells.** (A) A gallery representation reveals the presence of red fluorescent product consistent with CaV3.2 and RyR co-localization within 40 nm of one another. Nuclei were labeled with Hoechst 33342 (blue). (B) Assay was performed with no primary antibodies. (C,D) Assay controls were developed with one primary antibody. Scale bars are 10 μm, optical section depth in each image is 0.3–0.5 μm. Photomicrographs are representative of ~10-20 smooth muscle cells and the assay was tested 2-3 times for each panel.
Figure 5. Computational modeling of the role of CaV3.2 in smooth muscle Ca2+ dynamics. (A) A computational model was developed using structural and electrophysiological data. The model consists of an 8.5 μm slice of an arterial smooth muscle cell. The microdomain is 600 nm in length and 15 nm from the SR. Membrane proteins have been distributed and the level of expression was set by optimization procedures. Key proteins include CaV1.2, CaV3.1, CaV3.2, RyR, Na+/Ca2+ exchanger (NCX), SERCA/PMCA pumps, calmodulin and calsequestrin. Concentration of Ca2+ was calculated in the subspace region ([Ca2+]ss). (B) Simulations display repetitive Ca2+ spark-like events in response to depolarization from -60 to -40 mV. (C,D) Spark-like events were fully abolished by RyR inhibition and attenuated by CaV3.2 blockade. (E) Frequency of Ca2+ sparks increased with depolarization.

Figure 6. CaV3.2 suppression attenuates the generation of Ca2+ sparks and spontaneous transient outward K+ currents (STOC). (A) Line scan imaging performed on posterior and middle rat cerebral arteries to ascertain Ca2+ spark generation under control conditions and in the presence of Ni2+ (50 μM). Arrowheads denote the presence of Ca2+ sparks. (B,C) Summary data highlight Ca2+ sparks frequency (sparks/μm/sec) and the number of line scans in which Ca2+ sparks were detected (n=6 arteries, 291 line scans in total, *P<0.05, paired t-test). (D) Representative traces and summary data of STOC measurements under control conditions and in the presence of Ni2+ (50 μM, n=8, *P<0.05, paired t-test). Holding membrane potentials were set at -40 or -20 mV. (E) STOC were monitored before and after the application of paxilline (1 μM, n=8, *P<0.05, paired t-test).

Figure 7. Effects of Ni2+ and paxilline on myogenic tone in rat cerebral arteries. (A) Middle and posterior cerebral arteries were gradually pressurized from 20 to 100 mmHg while diameter was monitored. The experiment was performed under control conditions and in the presence of paxilline (1 μM) or in Ca2+-free media. (B) Summary data of the experiment in panel A (n=6, *P<0.05, paired t-test). (C,D) Traces and summary data illustrate the effects of sequential exposure to Ni2+ (50 μM) followed by paxilline (1 μM, n=7, *P<0.05, paired t-test). (E,F) The order of Ni2+ and paxilline in panel (C) was reversed (n=6, *P<0.05, paired t-test). (G,H) Traces and summary data illustrate the effects of Ni2+ (50 μM) on pressurized mesenteric arteries (60 mmHg) from wildtype (n=4) and CaV3.2 KO (n=6) mice (*P<0.05, unpaired t-test).

Figure 8. Expression and function of CaV3.2 in human cerebral arteries. (A) Brain tissues were excised from patients undergoing lobectomy. Whole cerebral arteries and cerebral arterial smooth muscle cells were subsequently isolated for experimental assessments. (B) PCR analysis of whole brain and isolated smooth muscle cells highlights the presence of CaV3.2. Data is representative of cells obtained from two human subjects. (C) A voltage step from -90 to 0 mV was used to monitor inward Ba2+ current (n=6 cells from 4 subjects) in human cerebral arterial smooth muscle cells in the absence or presence of Ni2+ (50 μM, *P<0.05, paired t-test). Currents were monitored in the presence of nifedipine (200 nM) to block L-type Ca2+ channels. (D,E) Human cerebral arteries were pressurized from 20 to 100 mmHg while diameter was sequentially monitored under control conditions and in the presence of Ni2+ (50 μM) or in Ca2+ free media (n=4 arteries from 3 subjects, *P<0.05, paired t-test).
Novelty and Significance

What Is Known?

- T-type (CaV3.1/CaV3.2) Ca^{2+} channels are expressed in cerebral arterial smooth muscle.
- T-type Ca^{2+} channels are thought to mediate arterial tone development although the mechanisms remain uncertain.

What New Information Does This Article Contribute?

- CaV3.2 channels mediate a paradoxical dilation in pressurized cerebral arteries.
- CaV3.2 channels are located in microdomains in association with the sarcoplasmic reticulum and ryanodine receptors.
- Ca^{2+} influx through CaV3.2 channels triggers ryanodine receptors, generates transient Ca^{2+} sparks and activates large conductance Ca^{2+}-activated K^{+} (BKCa) channels to elicit hyperpolarization.

T-type (CaV3.1/CaV3.2) Ca^{2+} channels are present in rat cerebral arterial smooth muscle but their functional significance is uncertain. We tested whether CaV3.2 channels might mediate dilation rather than constriction by triggering Ca^{2+} sparks, discrete events that initiate arterial hyperpolarization by activating BKCa channels. Micromolar Ni^{2+}, a CaV3.2 blocker, constricted pressurized rat cerebral arteries. Structural analysis revealed microdomains comprised of sarcoplasmic reticulum and caveolae, with CaV3.2 and ryanodine receptors residing next to each another. Modeling showed that Ca^{2+} influx through CaV3.2 could activate ryanodine receptors and consistent with theory, Ca^{2+} imaging and electrophysiology demonstrated that Ni^{2+} suppressed Ca^{2+} sparks and downstream BKCa activity. CaV3.2 channels are also present in human cerebral arteries and drive a comparable response. In summary, we show for the first time that Ca^{2+} influx through CaV3.2 channels discretely activates Ca^{2+} sparks and BKCa channels, to elicit arterial hyperpolarization and dilation. This feedback mechanism will prevent cerebral arteries from overly constricting to strong stimuli such as intravascular pressure. This new knowledge challenges the stereotypical view that Ca^{2+} channels are singularly involved in mediating arterial constriction.
Figure 2
Figure 3
Figure 4

**Anti-Ca\textsubscript{v}3.2/Anti-RyR**

- A
- B

**Secondary Alone**

- B
- C

**Anti-RyR Alone**

- C
- D

**Anti-Ca\textsubscript{v}3.2 Alone**

- D
Figure 7

(A) Intravascular Pressure (mmHg)

(B) Diameter (µm)

(C) Intravascular Pressure (mmHg)

(D) Diameter (µm)

(E) Intravascular Pressure (mmHg)

(F) Diameter (µm)

(G) WT

(H) Ni²⁺ Response (µm)

Ca,3.2⁻
Figure 8
CaV3.2 Channels and the Induction of Negative Feedback In Cerebral Arteries

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CaV3.2 Channels and the Induction of Negative Feedback in Cerebral Arteries

Osama F. Harraz1,2,*, Rasha R. Abd El-Rahman1,*, Kamran Bigdely-Shamloo1,3, Sean M. Wilson4, Suzanne E. Brett1, Monica Romero4, Albert L. Gonzales5, Scott Earley6, Edward J. Vigmond3,7, Anders Nygren3, Bijoy K. Menon8, Rania E. Mufti1, Tim Watson8, Yves Starreveld8, Tobias Furstenhaupt9, Philip R. Muellerleile10, David Kurjiaka10, Barry D. Kyle1, Andrew P. Braun1 and Donald G. Welsh1,#

* OFH and RRA are co-first authors and contributed equally to the manuscript

1 Dept. of Physiology and Pharmacology, Hotchkiss Brain and Libin Cardiovascular Institutes, University of Calgary, Calgary, AB, Canada
2 Dept. of Pharmacology and Toxicology, Alexandria University, Alexandria, Egypt
3 Dept. of Electrical and Computer Engineering, University of Calgary, Calgary, AB, Canada
4 Division of Pharmacology, Loma Linda University, Loma Linda, CA, USA
5 Dept. of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA
6 Dept. of Pharmacology, University of Nevada, Reno, NV, USA
7 LIRYC Institute and Lab IMB, University of Bordeaux, Bordeaux, France
8 Dept. of Clinical Neurosciences, University of Calgary, Calgary, AB, Canada
9 Microscopy Imaging Facility, University of Calgary, Calgary, AB, Canada
10 Dept. of Biomedical Sciences, Grand Valley State University, Allendale, MI, USA

Running Title: Vascular CaV3.2 and Negative Feedback

# Corresponding Author: Donald G. Welsh, PhD (dwelsh@ucalgary.ca)
SUPPLEMENTAL MATERIALS AND METHODS

Animal Procedures

Animal procedures were approved by the Animal Care and Use Committee at the University of Calgary. Briefly, female Sprague–Dawley rats (10–12 weeks of age) were killed via CO₂ asphyxiation. The brain was carefully removed and placed in cold phosphate-buffered saline (PBS, pH 7.4) solution containing (in mM): 138 NaCl, 3 KCl, 10 Na₂HPO₄, 2 NaH₂PO₄, 5 glucose, 0.1 CaCl₂ and 0.1 MgSO₄. Middle and posterior cerebral arteries were carefully dissected out of surrounding tissue and cut into 2–3 mm segments. Human brain samples, from which small cerebral arteries (150 to 250 μm-diameter), were obtained with institutional review board approval and written informed consent according to the Declaration of Helsinki.

Isolation of Cerebral Arterial Smooth Muscle Cells

Smooth muscle cells from middle and posterior cerebral arteries were enzymatically isolated as previously described. Briefly, arterial segments were placed in an isolation medium (37 °C, 10 min) 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). Vessels were then exposed to a two-step digestion process that involved: 1) 12-15 min incubation in isolation medium (37 °C) containing 0.5 mg/ml papain and 1.5 mg/ml dithioerythritol; and 2) a 15 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 the same day within ~5 hr.

Electrophysiological Recordings

Conventional patch-clamp electrophysiology was used to monitor whole-cell voltage-gated Ca²⁺ channel currents in isolated smooth muscle cells as reported earlier. Recording electrodes (5-8 MΩ) were pulled from borosilicate glass microcapillary tubes (Sutter Instruments, Novato, CA) using a micropipette puller (Narishige PP-830, Tokyo, Japan), 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 NaCl-agar bridge was used between the reference electrode and the bath solution. To record whole-cell currents, isolated cells held at -60 mV were exposed to a pre-pulse (-90 mV, 200 ms) and then voltage steps ranging from -50 to +40 mV (10 mV interval, 300 ms).

Perforated patch-clamp electrophysiology was used to measure whole-cell K⁺ currents in 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. Membrane currents were recorded while the cells were: 1) held at a steady membrane potential of -40 and -20 mV; or 2) slowly ramped from -60 to +20 mV (4mV/s). Whole-cell currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA), filtered at 1 kHz, digitized at 5 kHz, and were stored on a computer for offline analysis with Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA). Whole-cell capacitance averaged 14-18 pF and was measured with the cancellation circuitry in the voltage-clamp amplifier. A 1 M NaCl-agar salt bridge between the reference electrode and the bath
solution was used to minimize offset potentials (<2 mV). All electrophysiological experiments were performed at room temperature (~22°C). STOC analysis was performed with customized software provided by B. Hald (University of Copenhagen); threshold for detection was set at 3 times the BKCa single channel conductance.

In a small complement of experiments, single channel currents were monitored using the on-cell configuration. The bath solution contained: 145 mM KCl, 1 mM CaCl2, 10 mM HEPES, and 200 nM nifedipine (pH 7.4). The pipette solution contained: 60 mM CaCl2, 50 mM TEA-Cl, 10 mM HEPES, and 200 nM nifedipine (pH 7.2). Single channel currents were filtered at 2 kHz, digitized at 5 kHz, and stored on a computer for offline analysis with Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA). Cellular patches were voltage-clamped at a holding V_M of -60 mV. To elicit single channel currents, a pre-pulse (-90 mV, 250 ms) was followed by test pulses (300 ms, -60 to -10 mV; 10 mV intervals). Recordings were performed at 22 °C. Slope conductance was calculated as described previously (Fox AP, Nowycky MC, Tsien RW (1987) Single-channel recordings of three types of calcium channels in chick sensory neurons. J Physiol 394:173-200).

Vessel Myography and Membrane Potential Measurement

Arterial segments were mounted in a customized arteriograph and superfused with warm (37 °C) physiological salt solution (PSS; pH 7.4; 21% O_2, 5% CO_2, balance N_2) containing (in mM): 119 NaCl, 4.7 KCl, 20 NaHCO_3, 1.1 KH_2PO_4, 1.2 MgSO_4, 1.6 CaCl_2 and 10 glucose. To limit the endothelial influence on myogenic tone development, air bubbles were passed through the vessel lumen (1–2 min); successful removal of the endothelium was confirmed by the loss of bradykinin-induced dilation. Arteries were equilibrated at 15 mmHg and the contractile responsiveness was assessed by brief application of 60 mM KCl. Following equilibration, intravascular pressure was incrementally elevated from 20 to 100 mmHg and arterial external diameter was monitored under control conditions and in the presence of paxilline (1 μM, BKCa inhibitor) and/or Ni^{2+} (50 μM, CaV3.2 blocker). Maximal arterial diameter was subsequently assessed in Ca^{2+}-free PSS (zero externally added Ca^{2+} + 2 mM EGTA). Smooth muscle membrane potential (V_M) was assessed by inserting a glass microelectrode backfilled with 1 M KCl (tip resistance = 120–150 MQ) into the vessel wall. Measurements of V_M were first made at 60 mmHg and then in the presence of Ni^{2+} (50 μM). The criteria for successful cell impalement included: 1) a sharp negative V_M deflection upon insertion; 2) a stable recording for at least 1 min following entry; and 3) a sharp return to baseline upon electrode removal.

Electron Tomography

Whole animals were fixed similarly to immunohistochemical analysis. Brains were then removed and post-fixed in 4% paraformaldehyde (22 °C, 1 h) and cerebral arteries were isolated. Arteries were immersed in 1.6% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 at 4 °C overnight. After washing three times with the same previous buffer, the samples were post-fixed in 1% osmium tetroxide buffered with cacodylate for 1 h at 22 °C, dehydrated through a graded series of acetone concentration (30–100%) and embedded in Epon 812 mixture resin. Thick sections (300-400 nm) were cut on a Reichert-Jung Ultracut E microtome using a diamond knife and collected on single hole grids with Formvar supporting film. The sections were first stained with 20% aqueous uranyl acetate/Reynolds’s lead citrate and then placed on one side of a transmission electron microscopy slot grid (1x2 mm slot) covered with a continuous formvar film (~40 nm) to dry (10 min). Colloidal gold particles (10 nm
diameter) were then placed on both sides of the grid to serve as fiducial markers, and a thin carbon coating was applied for mechanical stabilization and to reduce electric charging. Once prepared, sections were viewed on a Tecnai F20 transmission electron microscope (200 keV), regions of interest were defined, and images were captured on a 1,024 × 1,024 charge-coupled device camera (GIF 794, Gatan, Pleasanton, CA). To perform dual-axis transmission electron-microscopic tomography, Serial EM software was employed to capture one image per degree of sample rotation (136 degrees in total). Tomographic reconstruction was performed by weighted back-projection with the IMOD software package; this yielded a contiguous stack of two-dimensional photomicrographs with ~4 nm resolution. The same software was used to trace subcellular structures on each section of the contiguous stack. We then compiled the traces to produce a 3-D rendition of the microdomain structure.

**Immunogold Labeling**

Whole animals were fixed similarly to immunohistochemical analysis. Brains were then removed and post-fixed in 4% paraformaldehyde (22 °C, 1 h) and cerebral arteries were isolated. In accordance with the Aurion immunogold reagent kit, fixed arteries were exposed to 0.1% sodium borohydride in PBS (15 min), 0.05% Triton X-100 (30 min), blocking solution 1 h at 4 °C and then PBS (2x10 min). Primary antibodies (1:100 dilution) were subsequently added to the buffer and incubated for 48 h at 4 °C. Tissues were subsequently washed with buffer (6x10 min), exposed to incubation medium containing ultra-small gold particles (0.8 nm labeled goat anti rabbit, 1:100 dilution, 48 h at 4 °C) and then washed with incubation buffer (6x10 min) and PBS (2x10 min). Prepared arteries were fixed again in PBS containing 2.5% glutaraldehyde (2 h) and then washed in PBS (2x10 min). Arteries were sequentially processed as follows: 1) enhancement conditioning solution (ECS, 4x10 min); 2) Silver enhancement solution (2 h); 3) 0.03 M sodium thiosulphate in ECS (10 min); 4) ECS wash (4x10 min); and 5) PBS wash (2x10 min). Tissues were then post-fixed for 1 h in a 1% osmium tetroxide-PBS solution, dehydrated in ethanol, and embedded in Epon resin. Ultrathin sections of about ~70 nm were cut, lightly stained with 2% aqueous uranyl acetate and Reynold’s lead citrate (15 min), and viewed/photographed using a Hitachi H7650 transmission electron microscope (80 keV) and an AMT 16000 digital camera.

**Immunohistochemistry**

Rats were anaesthetized with sodium pentobarbital and perfused intra-cardially with 250 ml of PBS (pH 7.4), followed by 100 ml of 4% paraformaldehyde (pH 7.4) in PBS at room temperature. Brains were then removed and post-fixed in 4% paraformaldehyde for 1 h (22 °C). Posterior and middle cerebral arteries were subsequently excised and placed in a conical vial with standard working PBS solution containing 3% donkey, horse or goat serum, 0.1% Tween, and 1% dimethylsulphoxide (DMSO). Primary antibodies against smooth muscle actin (1:25 dilution), CaV1.2 (1:25 dilution), CaV3.1 (1:100 dilution), CaV3.2 (1:100 dilution), and RyR (1:100 dilution) were added to the working solution and incubated for 48 h (4 °C). Next, tissues were washed 3 times with PBS (15 min each, 22 °C) and incubated with working PBS solutions containing secondary antibodies (4 hr, 22 °C). Following washes with PBS, sections were mounted on gel-coated slides and covered with anti-fade medium, covered slips were sealed with nail polish and stored (-20 °C). Controls were obtained by omitting the 1ry antibodies or pre-adsorbing excess purified peptide. All reactions involved the use of fluorophore-conjugated secondary antibodies (1:1000 dilution): Alexa Fluor 488-donkey anti-mouse IgG, and Alexa
Fluor 555-goat anti-rabbit IgG. Immunolabelling was assessed using an Olympus FV300 BX50 confocal microscope equipped with Cy3 (555 nm, red) and FITC (488 nm, green) filter sets.

**Proximity Ligation Assay (PLA)**

The Duolink in situ PLA detection kit was employed using freshly isolated smooth muscle cells. Briefly, cells were fixed in PBS containing 4% paraformaldehyde (15 min), permeabilized in PBS containing 0.1% Tween (15 min), and quenched in PBS containing 100 mM glycine (5 min). Cells were then washed with PBS, blocked in Duolink blocking solution (30 min, 37 °C), and incubated overnight (4 °C) with primary antibodies (rabbit anti-CaV3.2 and mouse RyR2) in Duolink antibody diluent solution. Control experiments employed no primary antibody or only one primary antibody. Cells were labelled with Duolink PLA PLUS and MINUS probes for 2 h (37 °C). The secondary antibodies of PLA PLUS and MINUS probes are attached to synthetic oligonucleotides that hybridize when in close proximity (<40 nm). The hybridized oligonucleotides are then ligated prior to rolling circle amplification. The amplification products extending from the oligonucleotide arm of the PLA probes were detected using red fluorescent fluorophore-tagged, complementary oligonucleotide sequences and a Zeiss Apotome epifluorescence microscope.

**Computational Modeling**

We have constructed a mathematical model that incorporates crucial ultrastructure and calcium handling features of the vascular smooth muscle cell in cerebral arteries. The smooth muscle cell is described as a cylinder, 76.5 μm in length and 5 μm in diameter, as shown in Fig. 5. For simulation purposes, a cell was subdivided into segments with each 8.5 μm in length. Based on microscopic data, in each segment there is a circumferential band, which is interrupted in approximately two locations, thus forming two discrete sections circumferentially. In the model, each segment is therefore further sub-divided into two semi-cylindrical “slices”. Simulations presented in this paper are based on the behavior of one slice. The frequency of Ca2+ spark-like events have been scaled to represent the whole cell, assuming that individual slices act independently. The model includes only mechanisms responsible for dynamic Ca2+ handling in the smooth muscle cell. This includes the sodium-calcium exchanger (NCX), plasma membrane Ca2+ ATPase (PMCA), sarco/endoplasmic reticulum Ca2+ ATPase (SERCA), ryanodine receptor (RyR), calmodulin, calsequestrin, and the Ca2+ channels CaV1.2, CaV3.1 and CaV3.2. In its present form, the model is limited to Ca2+ dynamics evoked by voltage-clamp stimuli. As illustrated in Fig. 5, the model explicitly accounts for an interaction between CaV3.2 and RyR in a restricted microdomain (subspace), representing the ~15 nm space between caveolae in the plasma membrane and the sarcoplasmic reticulum. Mathematically, the model comprises 12 ordinary differential equations, which were solved using the “ode15s” ODE solver in MATLAB (The MathWorks, Natick, MA, USA). See Supplemental appendix for a complete description of the model’s formulation.

**Ca2+ Spark Measurements**

Ca2+ sparks were measured in rat myocytes in situ using the en-face preparation technique38 with the Ca2+ sensitive dye Fluo-4 AM 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 h 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. The arterial segments were pinned to Sylgard blocks and placed in an open bath imaging chamber mounted on the confocal imaging stage. Cells were illuminated at 488 nm with a krypton argon laser and the emitted light was collected using a photomultiplier tube. Line scans were imaged at 529 fps with the emission signal recorded at 493-622. The acquisition period for Ca\(^{2+}\) spark recordings was 18.9 s. The resultant pixel size ranged from 0.068 to 0.11 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 depth is roughly equivalent to the width of 50% of the cell based on morphological examination of live preparations, and was equivalent to our previous studies (data not shown). We performed analysis to characterize the percentage of cells with Ca\(^{2+}\) sparks and the frequency of firing. Sparks per 100 μm per second and the percentage of cells firing were computed from these observed sparks. Spatial and temporal characteristics of the Ca\(^{2+}\) spark events were calculated using the SparkMaster plug-in for ImageJ. These characteristics included: fractional fluorescence intensity; full duration at half maximum; full width at half maximum; and time to peak. The threshold for spark detection was 3.2 times the standard deviation of the background noise above the mean background level. Prior to analysis the background fluorescence was subtracted from each image assuming homogeneous background levels in each cell.

**PCR Analysis**

Smooth muscle cells (~200) isolated from cerebral arteries were placed in RNase- and DNase-free collection tubes. Total RNA was isolated from human cerebral artery smooth muscle cells using the RNeasy mini kit (Qiagen) whereas total RNA from human brain was purchased from Clontech. The RNA was reverse-transcribed using the Quantitect reverse transcription kit (Qiagen) according to manufacturer's instructions. A total of 1 ng cDNA was used to PCR amplify a 98 bp amplicon corresponding to Ca\(_{\mathrm{V}}\)3.2 using the primer sequences Ca\(_{\mathrm{V}}\)3.2-F (TGATTACCAGCATGCTCACG) and Ca\(_{\mathrm{V}}\)3.2-R (GGTCTTCTTCTGCCTCGGTC) with Q5 DNA polymerase (New England Biolabs) according to manufacturer's instructions. The PCR reactions were electrophoresed on a 1% agarose-TAE gel. Lane 1 is the PCR marker, lane 2 is the Ca\(_{\mathrm{V}}\)3.2 amplicon from brain and lane 3 is the Ca\(_{\mathrm{V}}\)3.2 amplicon from isolated smooth muscle cells.

**Statistical Analysis**

Data are expressed as means ± S.E., and n indicates the number of vessels or cells. No more than two different experiments were performed on vessels from a given animal. 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 (see figure legends for specific details). *P values ≤ 0.05 were considered statistically significant.

**Solutions and Chemicals**

All buffers, chemicals and reagents originated from Sigma-Aldrich unless otherwise stated. Donkey and goat serum was purchased from Jackson Immuno-Research. Primary antibodies against smooth muscle actin were obtained from Abcam Inc. whereas those directed against Ca\(_{\mathrm{V}}\)1.2, Ca\(_{\mathrm{V}}\)3.1, Ca\(_{\mathrm{V}}\)3.2, and RyR were purchased from Alomone. Secondary antibodies, which included Alexa Fluor 488-goat anti-mouse IgG and Alexa Fluor 555-goat anti-
rabbit IgG, were obtained from Invitrogen life Technologies. Aurion immunogold reagent and the PLA detection kit were purchased from Electron Microscopy Sciences and Olink, respectively.
Online Figure I. Effects of Ni\textsuperscript{2+} and nifedipine on Ca\textsubscript{V}1.2, Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.2 currents. Summary data of inward currents in tSA-201 cells transfected with rCa\textsubscript{V}1.2, rCa\textsubscript{V}3.1 or rCa\textsubscript{V}3.2-cDNA. Experiments were performed in the absence and presence of Ni\textsuperscript{2+} (50 µM) or Nifedipine (200 nM). A voltage step from -90 to 0 mV was used to evoke inward Ba\textsuperscript{2+} current (n=5 each, * \(P<0.05\), paired t-test). Ni\textsuperscript{2+} and nifedipine selectively block Ca\textsubscript{V}1.2 and Ca\textsubscript{V}3.2, respectively.
Online Figure II. Time control experiments. (A,B) Representative traces and summary data of T-type current in rat cerebral arterial smooth muscle cells over time (n=8). Experiments were performed in the presence of nifedipine (200 nM) to block L-type Ca$^{2+}$ channels. A voltage step from -90 to 0 mV was used to elicit inward current. (C) Rat middle or posterior cerebral arteries were pressurized from 20 to 100 mmHg twice under control conditions; passive responses were ascertained in Ca$^{2+}$-free media (n=7). Electrical and vasomotor measurements were stable and repeatable over time.
Online Figure III. Effects of Ni$^{2+}$ on the kinetic characteristics of Ca$^{2+}$ sparks. Line scan imaging performed on posterior and middle rat cerebral arteries to ascertain Ca$^{2+}$ spark generation under control conditions and in the presence of Ni$^{2+}$ (50 μM). Summary data characterizes the influence of Ni$^{2+}$ on spatial (A & B) and temporal (C & D) characteristics of Ca$^{2+}$ sparks (n=6 arteries, 291 line scans in total, *$P<0.05$, paired t-test). Ni$^{2+}$ elicited a limited but significant effect on the spatial and temporal characteristics of Ca$^{2+}$ sparks.
Online Figure IV. Ni$^{2+}$ control experiments. (A,B) Representative traces and summary data of inward and outward current in rat cerebral arterial smooth muscle cells under control conditions and in the presence of Ni$^{2+}$ (50 μM, n=10). Cells were slowly ramped from -60 to +20 mV (4 mV/s). Ni$^{2+}$ had no effect on peak inward/outward current.
Supplemental Model Summary

1 Model and Method

Recent work has revealed a microdomain in cerebral arterial smooth muscle comprised of caveolae and sarcoplasmic reticulum. Immunolabeling techniques indicate that T-type Ca\(^{2+}\) channels and ryanodine receptors localize to this microdomain while L-type Ca\(^{2+}\) channels do not [3]. Given these observations, Ca\(_{v3.2}\) channels were hypothesized to regulate a CICR response. To address this hypothesis, a mathematical model of a microdomain of rat cerebral vascular smooth muscle Ca\(^{2+}\) handling was developed. This model was based on parameters arising from tomographic, immunolabeling and electrical measurements of our lab and previous studies. It includes only the components responsible for Ca\(^{2+}\) dynamics in the SMC. In the following sections, the details of the model are explained.

2 Slice

A typical vascular smooth muscle cell from rat cerebral artery has a fusiform shape. In our model, we consider it as a cylinder with a length of about 80 \(\mu\)m and a diameter of 5 \(\mu\)m (Figure 1). A slice is considered to be the volume of the cell that is devoid of actin and includes the peripheral SR, its adjacent caveolae and the space between them which is denoted the subspace. Based on microscopic data [3], in each \(\approx 8.5\) \(\mu\)m length of the cell, there is a circumferential band which includes 2 discrete slices. Therefore, each slice is a half cylinder of length of 8.5 \(\mu\)m as illustrated in Figure 1.

The model includes only the components responsible for Ca\(^{2+}\) dynamics in the SMC. This comprises the Sodium-Calcium Exchanger (NCX), Plasma membrane Ca\(^{2+}\) ATPase (PMCA), Sarcoplasmic reticulum, Sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), Ryanodine receptor, Calmodulin, Calsequestrin, ans the calcium channels Ca\(_{v1.2}\), Ca\(_{v3.1}\) and Ca\(_{v3.2}\). The model is based on a set of 11 ordinary differential equations (Equations 1, 3, 6, 8, 13, 18, 24, 27, 32, 33 and 34).

3 Myoplasmic Calcium

The subspace (the dark blue area in Figure 1 immediately under the cell membrane) has a depth of 10 to 15 nm [3]. Two channels inject their currents into the subspace: T-type Ca\(^{2+}\) (\(I_{Ca_{v3.2}}\)), and RyR (\(J_{rel}\)). The Ca\(^{2+}\) concentration within the subspace, denoted \([Ca^{2+}]_{SS}\), diffuses into the cytosol (\(J_{xfer}\)). The buffer calmodulin (CMDN) was also assumed present in the subspace:

\[
\frac{d[Ca^{2+}]_{SS}}{dt} = B_{SS} \left( J_{rel} \frac{V_{ISR}}{V_{SS}} - J_{xfer} \frac{V_{myo}}{V_{SS}} + I_{Ca_{v3.2}} \frac{A_{cap}C_{m}}{2V_{SS}F} \right) \tag{1}
\]

\[
B_{SS} = \left\{ 1 + \frac{[CMDN]_{tot}K_{m}^{CMDN}}{K_{m}^{CMDN} + [Ca^{2+}]_{SS}^2} \right\}^{-1} \tag{2}
\]

Calcium in the cytosol, \([Ca^{2+}]_{i}\), is further influenced by leak from the sarcoplasmic reticulum (\(J_{leak}\)), uptake by the SERCA pump (\(J_{up}\)), extrusion by the PMCA pump (\(I_{PMCA}\)), L-type Ca\(^{2+}\) channels (\(I_{Ca,L}\)), and a background Ca\(^{2+}\) channel (\(I_{Ca,b}\)). Again, calmodulin was present:

\[
\frac{d[Ca^{2+}]_{i}}{dt} = B_{i} \left( J_{leak} + J_{xfer} - J_{up} - (I_{Ca,b} + I_{PMCA} + I_{Ca,L} + I_{Ca_{v3.1}} - 2I_{NCX}) \frac{A_{cap}C_{m}}{2V_{myo}F} \right) \tag{3}
\]

\[
B_{i} = \left\{ 1 + \frac{[CMDN]_{tot}P_{m}^{CMDN}}{K_{m}^{CMDN} + [Ca^{2+}]_{i}^2} \right\}^{-1} \tag{4}
\]

\[
J_{xfer} = \frac{[Ca^{2+}]_{SS} - [Ca^{2+}]_{i}}{\tau_{xfer}} \tag{5}
\]
Modeling Figure 1: **Top:** One smooth muscle cell (A) is divided into cylindrical segments (B) which are composed of two slices(C). **Bottom:** Schematic representation of a slice. The slice includes Sodium-Calcium Exchanger (NCX), Plasma membrane Ca$^{2+}$ ATPase (PMCA), the Sarcoplasmic Reticulum which has two parts: Uptake (lower pink) and Release (upper pink), Sarco/Endoplasmic Reticulum Ca$^{2+}$ ATPase (SERCA), Ryanodine Receptor (RyR), Buffers: Calmodulin, Calsequestrin, and Ca$^{2+}$ Channels: Ca$_{v}$.1.2, Ca$_{v}$.3.1 and Ca$_{v}$.3.2
### Myoplasmic Calcium parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>V&lt;sub&gt;myo&lt;/sub&gt;</td>
<td>Volume of the cytoplasm</td>
<td>6.5450e-08</td>
<td>µL</td>
</tr>
<tr>
<td>V&lt;sub&gt;JSR&lt;/sub&gt;</td>
<td>Junctional SR volume</td>
<td>3.2725e-10</td>
<td>µL</td>
</tr>
<tr>
<td>V&lt;sub&gt;NSR&lt;/sub&gt;</td>
<td>Network SR volume</td>
<td>6.54509e-10</td>
<td>µL</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>Subspace volume</td>
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<td>µL</td>
</tr>
<tr>
<td>C&lt;sub&gt;m&lt;/sub&gt;</td>
<td>membrane capacitance</td>
<td>1.0</td>
<td>µF/cm²</td>
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<td>A&lt;sub&gt;cap&lt;/sub&gt;</td>
<td>Capacitive membrane area</td>
<td>5.2360e-07</td>
<td>cm²</td>
</tr>
<tr>
<td>F</td>
<td>Faraday’s constant</td>
<td>96.5</td>
<td>Col/mol</td>
</tr>
<tr>
<td>[CMDN]&lt;sub&gt;total&lt;/sub&gt;</td>
<td>Total myoplasmic calmodulin concentration</td>
<td>50</td>
<td>µM</td>
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<tr>
<td>K&lt;sub&gt;CMDN&lt;/sub&gt;</td>
<td>half-saturation constant for calmodulin</td>
<td>0.238</td>
<td>µM</td>
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<tr>
<td>τ&lt;sub&gt;xfer&lt;/sub&gt;</td>
<td>Time constant for transfer from subspace to cytoplasm</td>
<td>6.96</td>
<td>ms</td>
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### SR Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
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</thead>
<tbody>
<tr>
<td>ν&lt;sub&gt;leak&lt;/sub&gt;</td>
<td>maximum Ca&lt;sup&gt;2+&lt;/sup&gt; leak rate from the NSR to cytosol</td>
<td>2.5463e-05</td>
<td>ms&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ν&lt;sub&gt;up&lt;/sub&gt;</td>
<td>maximum uptake rate of SERCA pump</td>
<td>0.5352</td>
<td>µM/ms</td>
</tr>
</tbody>
</table>

## 4 Sarcoplasmic Reticulum

The model of sarcoplasmic reticulum consists of two parts: uptake (also called the network sarcoplasmic reticulum, NSR) and release (also called the junctional sarcoplasmic reticulum, JSR). The uptake part contains the SERCA pump. Moreover, Ca<sup>2+</sup> passive release, also called Ca<sup>2+</sup> leak, is modeled to take place in the uptake portion. The release compartment contains the Ca<sup>2+</sup> buffer calsequestrin (CSQN) and RyR channels. The Ca<sup>2+</sup> flux between these two subcompartments is designated by J<sub>tr</sub>.

Ca<sup>2+</sup> uptake into the SR (J<sub>up</sub>) was described by the Hill equation (Eq. 11). The value of SR Ca<sub>2+</sub>-ATPase maximum pump rate and half-saturation constant have been adjusted. The mathematical relations describing the sarcoplasmic reticulum are derived from [2]:

\[
\frac{d[Ca^{2+}]_{JSR}}{dt} = B_{JSR}(J_{tr} - J_{rel})
\]

\[
B_{JSR} = \left(1 + \frac{[CSQN]_{tot}K_{m}^{CSQN}}{K_{m}^{CSQN} + [Ca^{2+}]_{JSR}}\right)^{-1}
\]

\[
\frac{d[Ca^{2+}]_{NSR}}{dt} = (J_{up} - J_{leak})\frac{V_{myo}}{V_{NSR}} - J_{tr}\frac{V_{JSR}}{V_{NSR}}
\]

\[
J_{leak} = \nu_{leak}([Ca^{2+}]_{NSR} - [Ca^{2+}]_{i})
\]

\[
J_{tr} = \frac{[Ca^{2+}]_{NSR} - [Ca^{2+}]_{JSR}}{\tau_{tr}}
\]

\[
J_{up} = \nu_{up}\frac{[Ca^{2+}]_{i}^2}{K_{m,up}^2 + [Ca^{2+}]_{i}^2}
\]
5 T-type Ca\(^{2+}\) Channels

Two isoforms of the T-type Ca\(^{2+}\) channel exist in vascular smooth muscle cell: Ca\(_{v}\)3.1 and Ca\(_{v}\)3.2 [3]. Based on Immunolabeling data [3], the former has a diffusive distribution throughout the membrane. In contrast, Ca\(_{v}\)3.2 is mainly expressed in the caveolae. They are found mostly in clusters directly opposed to ryanodine receptors on the sarcoplasmic reticulum [3]. We hypothesized that Ca\(^{2+}\) which enters the cell as I\(_{Cav3.2}\) results in a localized increase in \([Ca^{2+}]_{ss}\). We put the Ca\(_{v}\)3.1 splice variant along the cell and Ca\(_{v}\)3.2 were localized only in the caveolae. We have adopted the T-type Ca\(^{2+}\) channel mathematical model from Purkinje fibers in rabbit heart [1] for both isoforms.

The equations describing the channel are given below with \(b\) and \(g\) being gating variables. For channel \(X\), \(x_\infty\) is the steady state value, \(\tau_X\) is the time constant, \(\alpha_X\) is the transition rate from non-permissive to permissive state, and \(\beta_X\) is rate of the opposite transition.

\[
I_{Ca_{v,3.X}} = g_{Ca_{v,3.X}} b g (V - E_{CaT})
\]

\[
\frac{db}{dt} = \frac{b_\infty - b}{\tau_b}
\]

\[
b_\infty = \frac{1}{1 + e^{(V+28)/6.1}}
\]

\[
\tau_b = \frac{1}{\alpha_b + \beta_b}
\]

\[
\alpha_b = 1.068e^{(V+16.3)/30}
\]

\[
\beta_b = 1.068e^{-(V+16.3)/30}
\]

\[
\frac{dg}{dt} = \frac{g_\infty - g}{\tau_g}
\]

\[
g_\infty = \frac{1}{1 + e^{(V+60)/6.6}}
\]

\[
\tau_g = \frac{1}{\alpha_g + \beta_g}
\]

\[
\alpha_g = 0.015e^{-(V+71.7)/83.33}
\]

\[
\beta_g = 0.015e^{-(V+71.7)/15.38}
\]

6 L-type Ca\(^{2+}\) Channel

L-type Ca\(^{2+}\) channels (Ca\(_{v}1.2\)) are spread along the long axis of the smooth muscle cell [3] and have a distinct distribution in comparison with Ca\(_{v}3.2\) channels which run perpendicular to the long axis of SMC.
Modeling Figure 2: T-type Ca\(^{2+}\) channel (both Ca\(_{v}\)3.1 and Ca\(_{v}\)3.2) activation and inactivation curves

Thus, we have placed L-type Ca\(^{2+}\) channel throughout the slice but out of the caveolae as there is no spatial correlation between them and the caveolae.

Innate smooth muscle L-type Ca\(_{v}\)1.2 calcium channels have been shown to contain a fraction of Ca\(^{2+}\) currents with a window current that is close to resting potential\[8\]. The voltage-dependence of the activation curve of smooth muscle L-type Ca\(^{2+}\) channels shows a hyperpolarized shift of -15 mV in comparison with those of cardiac cells [7]. If the voltage-dependence of L-type Ca\(^{2+}\) channels of smooth muscle cells were similar to that of cardiac cells, only a small amount of contraction would be generated by smooth muscle cells [7]. Therefore, we modified an L-type model of rabbit cardiac cells [1] by shifting the activation to the left by 15 mV (Figure 3) and scaling the conductance, \(g_{CaL}\), according to the experimental data obtained from rat cerebral arteries in our lab [3]. Equations for the channel are given below with \(d\) and \(f\) being the gating variables:

\[
I_{CaL} = g_{CaL}(1 - f_{Ca}) d f (V - E_{CaL}) \tag{23}
\]

\[
\frac{dd}{dt} = \frac{d_\infty - d}{\tau_d} \tag{24}
\]

\[
d_\infty = \frac{1}{1 + e^{-(v+11)/6.74}} \tag{25}
\]

\[
\tau_d = \frac{0.59 + 0.8e^{0.52(v+28)}}{1 + e^{0.132(v+28)}} \tag{26}
\]

\[
\frac{df}{dt} = \frac{f_\infty - f}{\tau_f} \tag{27}
\]

\[
f_\infty = \frac{1}{1 + e^{(v+40)/10}} \tag{28}
\]
\[ \tau_f = 0.005(v + 12.5)^2 + 4 \]  

(29)

<table>
<thead>
<tr>
<th>L-type Channel parameters</th>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_{CaL} )</td>
<td>maximum conductance</td>
<td>0.0641</td>
<td>mS/cm²</td>
<td></td>
</tr>
<tr>
<td>( E_{CaL} )</td>
<td>Reversal potential</td>
<td>63</td>
<td>mV</td>
<td></td>
</tr>
</tbody>
</table>

Modeling Figure 3: L-type Ca\textsuperscript{2+} Channel Activation and Inactivation Curves. \( f \) and \( d \) are the inactivation and activation parameters. The inset zooms in on the product of \( d \) and \( f \).

There is another important difference between smooth muscle L-type Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channels and those of the cardiac tissue. Data obtained in our lab show that the smooth muscle splice does not exhibit Ca\textsuperscript{2+}-dependent inactivation in steady-state current (Figure 4). It is known that Ba\textsuperscript{2+} does not cause Ca\textsuperscript{2+}-dependent inactivation because it does not affect the channel chemically to inactivate it. Thus, if the Ba\textsuperscript{2+} and Ca\textsuperscript{2+} traces are the same for the channel, it means the channel does not go through Ca\textsuperscript{2+}-dependent inactivation. Figure 4 shows the percentage of the channels which are in the inactivation state for Ba\textsuperscript{2+} and Ca\textsuperscript{2+} at different times. At 1500 ms, there is no difference between the Ba\textsuperscript{2+} and Ca\textsuperscript{2+} traces, indicating that the steady-state Ca\textsuperscript{2+}-dependent inactivation does not exist for smooth muscle L-type Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channels.

7 Background Calcium Current

The background calcium current was modelled as a simple ohmic leak:

\[ I_{Ca,b} = g_{Ca,b}(V - E_{CaL}) \]  

(30)

<table>
<thead>
<tr>
<th>Background Ca\textsuperscript{2+} Channel parameters</th>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( g_{CaB} )</td>
<td>Maximum conductance</td>
<td>3.670e-05</td>
<td>( \mu S/cm² )</td>
</tr>
</tbody>
</table>

8 Ryanodine Receptor Channel
Modeling Figure 4: Some smooth muscle splice variants show minimal Ca\(^{2+}\)-dependent steady state inactivation

The model of Keizer, J et al.\cite{5} with some modifications in their rates has been adopted. The modifications were determined using parallel multi-objective genetic algorithm \cite{4} in order to reproduce experimental data. More specifically, the object functions were set to produce repetitive CICR responses with an appropriate period, \([Ca^{2+}]_{ss}\) amplitude, average \([Ca^{2+}]_i\) amplitude and basal \([Ca^{2+}]_i\) level.

This RyR model consists of two close and two open states. See Figure 5. State C\(_1\) and C\(_2\) are closed states while O\(_1\) and O\(_2\) are open states. These states are considered to be states of the entire RyR rather than states of the four subunits. A channel is in one of the states, \(X\), with probability \(P_X\). Transition from C\(_1\) to O\(_1\) and from O\(_1\) to O\(_2\) were considered to be Ca\(^{2+}\) dependent. The second open state, O\(_2\) has the important role of increasing the open probability during the plateau as \([Ca^{2+}]_{ss}\) is elevated. The plateau open probability is shown in Figure 7.

Figure 6 shows RyR open probability when \([Ca^{2+}]_{ss}\) is stepped from 0.1 \(\mu\)M to 5 \(\mu\)M. The initial increase to peak happens on the millisecond time scale, whereas the decline to the plateau level occurs over several hundreds of milliseconds. The extent of opening immediately after Ca\(^{2+}\) stimulation (the peak open probability) and that after inactivation (the plateau) both increase with increasing \([Ca^{2+}]_{ss}\). Figure 7 illustrates the peak and the plateau (steady-state value) open probabilities at different \([Ca^{2+}]_{ss}\) levels. At lower concentrations, the open probability rises quickly and then decreases to the plateau slowly. However, at higher \([Ca^{2+}]_{ss}\), after reaching the peak, RyR stays open and inactivation is negligible.

\[J_{rel} = \nu(P_{O_1} + P_{O_2})([Ca^{2+}]_{JSR} - [Ca^{2+}]_{SS})\]  \hspace{1cm} (31)
\[\frac{dP_{C_1}}{dt} = k_a^- P_{O_1} - k_a^+[Ca^{2+}]_{SS} P_{C_1}\]  \hspace{1cm} (32)
\[\frac{dP_{O_2}}{dt} = k_b^+[Ca^{2+}]_{SS} P_{O_1} - k_b^- P_{O_2}\]  \hspace{1cm} (33)
\[\frac{dP_{C_2}}{dt} = k_c^+ P_{O_1} - k_c^- P_{C_2}\]  \hspace{1cm} (34)
\[P_{O_1} = 1 - (P_{C_1} + P_{C_2} + P_{O_2})\]  \hspace{1cm} (35)

<table>
<thead>
<tr>
<th>RyR Channel parameters</th>
<th>Definition</th>
<th>Value</th>
<th>Unit</th>
</tr>
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<tbody>
<tr>
<td>(\nu)</td>
<td>Maximum permeability</td>
<td>4.5</td>
<td>(ms^{-1})</td>
</tr>
</tbody>
</table>
Modeling Figure 5: Schematic diagram of transition among the four states of RyR channel. C₁ and C₂ are closed states whereas O₁ and O₂ are open states. Transition from C₁ to O₁ and also transition from O₁ to O₂ are Ca²⁺ dependent.

<table>
<thead>
<tr>
<th>k⁺</th>
<th>Reaction</th>
<th>Rate</th>
<th>Unit</th>
</tr>
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<tbody>
<tr>
<td>kᵃ</td>
<td>PₐC₁ - PₐO₁ rate</td>
<td>0.02050</td>
<td>µM⁻ⁿ ms⁻¹</td>
</tr>
<tr>
<td>kᵇ</td>
<td>PₐO₁ - PₐO₂ rate</td>
<td>0.05205</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>kᶜ</td>
<td>PₐO₁ - PₐO₂ rate</td>
<td>0.0094</td>
<td>µM⁻ⁿ ms⁻¹</td>
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<tr>
<td>kᵇ</td>
<td>PₐO₁ - PₐO1 rate</td>
<td>0.1920</td>
<td>ms⁻¹</td>
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<tr>
<td>kᶜ</td>
<td>PₐO₁ - PₐO₂ rate</td>
<td>0.0778</td>
<td>ms⁻¹</td>
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<tr>
<td>kᶜ⁻</td>
<td>PₐO₂ - PₐO₁ rate</td>
<td>0.0035</td>
<td>ms⁻¹</td>
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<tr>
<td>m</td>
<td>PₐO₁ - PₐO₂ cooperativity</td>
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<td>dimensionless</td>
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<td>n</td>
<td>PₐC₁ - PₐO₂ cooperativity</td>
<td>4</td>
<td>dimensionless</td>
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</table>

This RyR model consists of two close and two open states. State C₁ and C₂ are closed states. States O₁ and O₂ are open states. These states are considered to be states of the entire RyR rather than states of the four subunits. As it is illustrated in Figure 5, transition from C₁ to O₁ and from O₁ to O₂ are considered to be Ca²⁺ dependent. The second open state, O₂ has an important role of keeping the plateau open probability increasing as [Ca²⁺]ₚₚ is elevated [5]. The plateau open probability is shown in Figure 7.

Figure 6 shows the RyR open probability when [Ca²⁺]ₚₚ is elevated from 0.1 µM to 5 µM. The initial increase to peak happens on the millisecond time scale, whereas the decline to the plateau occurs within several hundreds of milliseconds. The extent of opening immediately after Ca²⁺ stimulation (the peak open probability) and that after inactivation (the plateau) both increase with increasing [Ca²⁺]ₚₚ. Figure 7 illustrate the peak and the plateau (steady-state value) of open probability at different [Ca²⁺]ₚₚ level. At lower concentrations, the open probability rises quickly and then decreases to the plateau slowly. However as the figures indicates, at higher [Ca²⁺]ₚₚ, after reaching the peak, RyR stays open and inactivation is negligible. This shows that RyR inactivation is Ca²⁺-dependent in the model.

9 Ca²⁺ Extrusion Mechanisms

The model of NCX and PMCA were adopted from Bondarenko et al. [2] and the exchanger scaling factor (kₐCa) was adjusted (by using GA) so that the basal level of [Ca²⁺]ᵢ agreed with physiological values.

\[
I_{NCX} = k_{NaCa} \frac{1}{k_{m,Na} + [Na^+]_o^3} \frac{1}{k_{m,Ca} + [Ca^{2+}]_o} \frac{1}{1 + \kappa_{sat}(\eta - 1)VF/RT} \times \left\{ e^{\eta VF/RT [Na^+]_o^3 [Ca^{2+}]_o} - e^{(\eta - 1)VF/RT [Na^+]_o^3 [Ca^{2+}]_o} \right\} \\
I_{PCMA} = I_{PCMA}^{max} \frac{[Ca^{2+}]_l^2}{K_{m,PCa} + [Ca^{2+}]_l^2} \times \left(36\right)
\]

\[
I_{PCMA} = I_{PCMA}^{max} \frac{[Ca^{2+}]_l^2}{K_{m,PCa} + [Ca^{2+}]_l^2} \times \left(37\right)
\]
Modeling Figure 6: A simulation of the rapid rise to peak open probability of a single RyR following an increase of $[\text{Ca}^{2+}]_{\text{os}}$ from 0.1 to 5 µM.

### NCX parameters

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
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<td>$k_{NaCa}$</td>
<td>scaling factor</td>
<td>$1.464e+02$</td>
<td>$\mu A/cm^2$</td>
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<tr>
<td>$K_{mNa}$</td>
<td>Na$^+$ half-saturation constant</td>
<td>87500</td>
<td>$\mu M$</td>
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<tr>
<td>$K_{mCa}$</td>
<td>Ca$^{2+}$ half-saturation constant</td>
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<td>$\mu M$</td>
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<td>$K_{sat}$</td>
<td>saturation factor for large negative potentials</td>
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<td>dimensionless</td>
</tr>
<tr>
<td>$\eta$</td>
<td>voltage dependence</td>
<td>0.35</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$T$</td>
<td>temperature</td>
<td>293.15</td>
<td>K</td>
</tr>
<tr>
<td>$R$</td>
<td>Ideal gas constant</td>
<td>8.314</td>
<td>$JK^{-1}mol^{-1}$</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_{o}$</td>
<td>Extracellular Ca$^{2+}$ concentration</td>
<td>1800</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$[\text{Na}]_{o}$</td>
<td>Extracellular Na concentration</td>
<td>$1.400e+04$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$[\text{Na}]_{i}$</td>
<td>Intracellular Na concentration</td>
<td>$1.4237e+04$</td>
<td>$\mu M$</td>
</tr>
</tbody>
</table>

### PMCA parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{\text{max}_{PMCA}}$</td>
<td>max rate</td>
<td>1.2</td>
<td>$\mu A/cm^2$</td>
</tr>
<tr>
<td>$K_{m,\text{pCa}}$</td>
<td>Ca$^{2+}$ half-saturation constant</td>
<td>0.500</td>
<td>$\mu M$</td>
</tr>
</tbody>
</table>

10 Initial Conditions
Modeling Figure 7: The peak and the plateau open probabilities increases with increasing $[\text{Ca}^{2+}]_s$ indicating RyR inactivation is a $\text{Ca}^{2+}$-dependent process.
### Initial Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t )</td>
<td>Time</td>
<td>0.0</td>
<td>ms</td>
</tr>
<tr>
<td>( V )</td>
<td>Membrane Potential</td>
<td>-60</td>
<td>mV</td>
</tr>
<tr>
<td>([Ca^{2+}]_i)</td>
<td>Cytosolic (Ca^{2+}) concentration</td>
<td>0.1209</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>([Ca^{2+}]_{SS})</td>
<td>Subspace (Ca^{2+}) concentration</td>
<td>0.1505</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>([Ca^{2+}]_{JSR})</td>
<td>JSR (Ca^{2+}) concentration</td>
<td>0.300</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>([Ca^{2+}]_{NSR})</td>
<td>NSR (Ca^{2+}) concentration</td>
<td>0.300</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>( P_{C1} )</td>
<td>Fraction of channels in ( P_{C1} )</td>
<td>0.4298</td>
<td>dimensionless</td>
</tr>
<tr>
<td>( P_{C2} )</td>
<td>Fraction of channels in ( P_{C2} )</td>
<td>0.4298</td>
<td>dimensionless</td>
</tr>
<tr>
<td>( P_{O1} )</td>
<td>Fraction of channels in ( P_{O1} )</td>
<td>0.1404</td>
<td>dimensionless</td>
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<tr>
<td>( P_{O2} )</td>
<td>Fraction of channels in ( P_{O2} )</td>
<td>1.0030e-05</td>
<td>dimensionless</td>
</tr>
</tbody>
</table>

### 11 Numerical Methods

The model was implemented in MATLAB™ and 12 differential equations were solved with the ode15s routine for stiff systems [6]. We used multi-objective parallel genetic algorithm to adjust various parameters in the model in order to obtain experimentally-consistent results. To implement the optimization, the Parallel Computing toolbox and Global Optimization toolbox of MATLAB™ software package were used.

We chose this multi-objective optimization as there were several (and conflicting) objective functions. These objective functions rewards sets of parameters that cause the behaviors of model to be to be consistent with the experimental data including the following items:

- Frequency of oscillation ~1 Hz
- Steady-state \([Ca^{2+}]_i\) of 100 nM
- Average \([Ca^{2+}]_i\) in the range 200-250 nM at -40 mV
- No oscillations at ~-60 mV
- Sustained oscillations at a reduced frequency after \(Ca_{v.1.2}\) blockade

The optimization was used to modify the RyR transition rates \((k_{a}, k_{b}, k_{c})\), leakage current from SR into the cytosol \((\nu_{leak})\), the NCX maximum rate \((k_{NaCa})\) and the diffusion rate from the subspace into the cytosol \((\tau_{xfer})\). Changes in those parameters were mostly within 20% of the original values. The most appropriate solution was selected among the Pareto optima.

As the each run of simulation took 10s, for speeding up the GA convergence, we used a parallel multi-objective GA. This optimization algorithm was run on an Intel(R) 4-Core(TM) i7-2600K CPU @ 3.40GHz.

### References


