Genetic ablation of caveolin-1 modifies Ca\(^{2+}\) spark coupling in murine arterial smooth muscle cells

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Caveolin-1 modifies Ca\(^{2+}\) spark coupling in murine arterial smooth muscle cells. Am J Physiol Heart Circ Physiol 290: H2309–H2319, 2006. First published January 20, 2006; doi:10.1152/ajpheart.01226.2005.—L-type, voltage-dependent calcium (Ca\(^{2+}\)) channels, ryanodine-sensitive Ca\(^{2+}\) release (RyR) channels, and large-conductance Ca\(^{2+}\)-activated potassium (K\(_{Ca}\)) channels comprise a functional unit that regulates smooth muscle contractility. Here, we investigated whether genetic ablation of caveolin-1 (cav-1), a caveole protein, alters Ca\(^{2+}\) spark to K\(_{Ca}\) channel coupling and Ca\(^{2+}\) spark regulation by voltage-dependent K\(_{Ca}\) channels in murine cerebral artery smooth muscle cells. Caveolae were abundant in the sarcolemma of control (cav-1\(^{+/+}\)) cells but were not observed in cav-1-deficient (cav-1\(^{-/-}\)) cells. Although voltage-dependent K\(_{Ca}\) current density was similar in cav-1\(^{+/-}\) and cav-1\(^{-/-}\) cells, diltiazem and Cd\(^{2+}\), voltage-dependent Ca\(^{2+}\) channel blockers, reduced transient K\(_{Ca}\) current frequency to near 55% of control in cav-1\(^{-/-}\) cells but did not alter transient K\(_{Ca}\) current frequency in cav-1\(^{+/-}\) cells. Furthermore, although K\(_{Ca}\) channel density was elevated in cav-1\(^{-/-}\) cells, transient K\(_{Ca}\) current amplitude was similar to that in cav-1\(^{+/-}\) cells. Higher Ca\(^{2+}\) spark frequency in cav-1\(^{-/-}\) cells was not due to elevated intracellular Ca\(^{2+}\) concentration, sarcoplasmic reticulum Ca\(^{2+}\) load, or nitric oxide synthase activity. Similarly, Ca\(^{2+}\) spark amplitude and spread, the percentage of Ca\(^{2+}\) sparks that activated a transient K\(_{Ca}\) current, the amplitude relationship between sparks and transient K\(_{Ca}\) currents, and K\(_{Ca}\) channel conductance and apparent Ca\(^{2+}\) sensitivity were similar in cav-1\(^{+/-}\) and cav-1\(^{-/-}\) cells. In summary, cav-1 ablation elevates Ca\(^{2+}\) spark and transient K\(_{Ca}\) current frequency, attenuates the coupling relationship between voltage-dependent K\(_{Ca}\) channels and RyR channels that generate Ca\(^{2+}\) sparks, and elevates K\(_{Ca}\) channel density but does not alter transient K\(_{Ca}\) current activation by Ca\(^{2+}\) sparks. These findings indicate that cav-1 is required for physiological Ca\(^{2+}\) spark and transient K\(_{Ca}\) current regulation in cerebral artery smooth muscle cells.

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calCium (Ca\(^{2+}\)) is a signaling messenger that regulates a wide variety of cellular functions, including secretion, proliferation, contractation (2). To regulate specific Ca\(^{2+}\)-dependent functions, cells can generate a variety of intracellular Ca\(^{2+}\) signals with distinct frequency, amplitude, and spatial-temporal characteristics.

Smooth muscle cells generate several different modes of Ca\(^{2+}\) signaling (15, 20). The global intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) arises because of extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release. An elevation in global [Ca\(^{2+}\)], stimulates contraction, whereas a reduction in global [Ca\(^{2+}\)], results in relaxation. Localized [Ca\(^{2+}\)], transients, termed “Ca\(^{2+}\) sparks,” also occur in smooth muscle cells (20, 27). Ca\(^{2+}\) sparks occur due to the opening of several ryanodine-sensitive Ca\(^{2+}\) release (RyR) channels on the sarcoplasmic reticulum (SR) (15, 27). In smooth muscle cells, a Ca\(^{2+}\) spark does not directly elevate global Ca\(^{2+}\) but activates a number of nearby large-conductance Ca\(^{2+}\)-activated potassium (K\(_{Ca}\)) channels, resulting in a transient K\(_{Ca}\) current. Membrane hyperpolarization induced by asynchronous transient K\(_{Ca}\) currents reduces voltage-dependent Ca\(^{2+}\) channel activity, leading to a decrease in global [Ca\(^{2+}\)], and relaxation. An elevation in [Ca\(^{2+}\)], activates Ca\(^{2+}\) sparks and transient K\(_{Ca}\) currents, forming a negative-feedback loop that limits Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels (15, 27). Thus voltage-dependent Ca\(^{2+}\) channels, RyR channels, and K\(_{Ca}\) channels comprise a functional unit that regulates smooth muscle contractility (17). The differential regulation of contractility by local and global Ca\(^{2+}\) signaling is also effective because of differences in the frequency and amplitude of the Ca\(^{2+}\) signals and the Ca\(^{2+}\) sensitivities of the downstream targets for each signal mode (15). For local Ca\(^{2+}\) signaling mechanisms to operate, downstream targets must also be located in the proximity of the Ca\(^{2+}\) source. In smooth muscle cells, whether membrane proteins maintain spatial organization of voltage-dependent Ca\(^{2+}\) channels, RyR channels, and RyR channels to permit Ca\(^{2+}\) signaling between these proteins is unclear.

Compartmentalization of signaling molecules can occur in small, cholesterol-enriched, flask-shaped membrane invaginations termed “caveolae” (11, 13, 34, 39). Caveolins, of which three isoforms have been cloned (cav 1–3), are structural components required for caveolae formation (11, 13, 34, 39). Although all three caveolins have been identified in vascular smooth muscle cells, caveolin-1 is the primary isoform (9, 18, 28, 34, 39). Supporting a role for caveolae in the regulation of arterial smooth muscle local Ca\(^{2+}\) signaling is evidence that acute cholesterol depletion with dextrin inhibits Ca\(^{2+}\) sparks (23). Similarly, transient K\(_{Ca}\) current frequency is reduced in cerebral artery smooth muscle cells of cav-1-deficient (cav-1\(^{-/-}\)) mice, when compared with wild-type controls (10). In ureter smooth muscle cells, K\(_{Ca}\) channels are found in buoyant membrane fractions, and in cultured myometrial cells K\(_{Ca}\) channels are localized to caveolae (1, 3). Thus cav-1 and caveolae may regulate Ca\(^{2+}\) sparks and K\(_{Ca}\) channels in smooth muscle.

Here, the communication between voltage-dependent Ca\(^{2+}\) channels and RyR channels that generate Ca\(^{2+}\) sparks and sig-
naling between Ca\(^{2+}\) sparks and KC\(_{\text{a}}\) channels was investigated in cerebral artery smooth muscle cells of cav-1\(^{+/+}\) and cav-1\(^{-/-}\) mice. Data indicate that cav-1 ablation abolishes caveolae, elevates Ca\(^{2+}\) spark frequency, and attenuates Ca\(^{2+}\) spark regulation by voltage-dependent Ca\(^{2+}\) channels. In contrast, transient KC\(_{\text{a}}\) current activation by Ca\(^{2+}\) sparks is unaltered in cav-1\(^{-/-}\) cells, even though there is an increase in KC\(_{\text{a}}\) channel density.

**MATERIALS AND METHODS**

Tissue preparation. Animal procedures used were reviewed and approved by the Animal Care and Use Committee policies at the University of Tennessee. Age-matched (4–6 wk) cav-1\(^{-/-}\) mice (Cav1tm1Mls, stock no. 004585; see Ref. 33) or wild-type control (cav-1\(^{+/+}\)) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were killed by peritoneal injection of a pentobarbital sodium overdose (130 mg/kg). The brain was removed and placed into ice-cold (4°C) physiological saline solution (PSS) containing (in mM) 112 NaCl, 4.8 KCl, 26 NaHCO\(_3\), 1.8 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), and 10 glucose and gassed with 74% N\(_2\)-21% O\(_2\)-5% CO\(_2\) (pH 7.4). Posterior cerebral, cerebellar, and middle cerebral arteries (~150 \(\mu\)m in diameter) were removed, cleaned of connective tissue, and maintained in ice-cold Ca\(^{2+}\)-free buffer (solution A) containing (in mM) 55 NaCl, 80 Na glutamate, 5.6 KCl, 2 MgCl\(_2\), 10 HEPES, and 10 glucose (pH 7.3 with NaOH). For single cell isolation, smooth muscle cells were dissociated from cerebral arteries with the use of enzymes, as previously described (14).

Electron microscopy. The brain was fixed in PSS containing 2.5% glutaraldehyde for 1 h. Cerebral arteries were dissected from the fixed brain, cleaned of connective tissue, and postfixed in 1% osmium tetroxide in PSS for 4 h. Arteries were then rinsed briefly in deionized water and en bloc stained with 2% uranyl acetate in 0.85% sodium chloride overnight at 4°C. Arteries were dehydrated in graded solutions of ethanol, from 30% through 100% for 1 h each, infiltrated with 50% Spurr in 100% ethanol overnight at room temperature, 100% Spurr over an 8-h period involving at least three changes of Spurr, and then cured at 60°C for 2 days. Transverse sections (75 nm) were cut with a Reichert Ultracut E microtome and poststained with uranyl acetate and lead citrate. Cells were observed and photographed with a JEOL 2000EX TEM located in the Electron Microscope Facility at the University of Tennessee Health Science Center.

Patch-clamp electrophysiology. Potassium currents were measured by using the conventional whole cell, perforated-patch or inside-out patch-clamp configurations with an Axopatch 200B amplifier and Clampex 8.2 (Axon Instruments, Union City, CA). For transient KC\(_{\text{a}}\) current measurement, the bath solution (solution B) contained (in

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**Fig. 1.** Genetic ablation of caveolin-1 (cav-1) abolishes caveolae in cerebral artery smooth muscle cells. A: electron micrograph illustrates sarcolemma of smooth muscle cell in cerebral artery from cav-1\(^{+/+}\) mouse. Abundant caveolae are present within sarcolemma. B: magnification of area illustrated by black box in A showing caveolae. C: caveolae were absent in sarcolemma of cav-1\(^{-/-}\) cerebral artery smooth muscle cells. D: magnification of area highlighted by black box in C.
mM) 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPEs, and 10 glucose (pH 7.4 with NaOH), and the pipette solution contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPEs, and 0.05 EGTA (pH 7.2 with KOH). Amphotericin B (Sigma) was dissolved in DMSO and diluted into the pipette solution to give a final concentration of 200 μg/ml. For whole cell K⁺ current measurement, the bath solution was solution B and the pipette solution contained (in mM) 135 KCl, 5 EGTA, 1.0 BAPTA, 3.5 MgCl₂, 2.5 Na₂ATP, and 10 HEPES (pH 7.2 with KOH). For inside-out patch-clamp experiments to measure single KCa channel currents, the pipette and bath solution both contained (in mM) 140 KCl, 10 HEPES, 5 EGTA, and 1.6 1- N-(2-hydroxyethyl)ethylenediamine- N,N',N''-triacetic acid (pH 7.2 with KOH). MgCl₂ and CaCl₂ were supplemented to bath solution to provide 1 mM free Mg²⁺ and the required free Ca²⁺ concentration (1–100 μM). Free Ca²⁺ concentration in the pipette solution was 10 μM. Free Ca²⁺ concentrations were measured by using a Ca²⁺-sensitive and reference electrode (Corning). For voltage-dependent Ba²⁺ current measurements, the pipette solution contained (in mM) 125 CsCl, 20 tetraethylammonium chloride (TEACl), 1.0 MgCl₂, 0.5 EGTA, 10 HEPEs, and 10 glucose (pH 7.2 with CsOH), and the bath solution contained (in mM) 60 NaCl, 60 TEACl, 1.0 MgCl₂, 20 BaCl₂, 10 glucose, and 10 HEPEs (pH 7.4 with NaOH). Pipette resistance was measured by applying a 5-mV pulse using the seal test function of pClamp 8.2. Transient KCa currents were measured at a steady membrane potential of −40 mV. Voltage-dependent Ba²⁺ currents were activated from a holding potential of −80 mV by applying 300-ms voltage steps to voltages between −50 and +60 mV in increments of 10 mV. Whole cell K⁺ (Ik) currents were activated from a holding potential of −80 mV by applying 250-ms voltage steps to voltages between −70 and +80 mV in 10-mV increments. In inside-out patches, single KCa channel currents were measured at steady voltages of −40 or +40 mV. Transient KCa currents were...
filtered at 1 kHz and digitized at 5 kHz. Voltage-dependent Ba2+ currents were filtered at 1 kHz and digitized at 4 kHz. Other current measurements were filtered at 2 kHz and digitized at 10 kHz. Transient KCa current analysis was performed off-line using methodology described elsewhere (6). A transient KCa current was defined as the simultaneous opening of three or more KCa channels. Open probability (P<sub>o</sub>) was calculated from the following equation: 

\[ P_o = \frac{\Sigma t_i}{nT} \]

where \( t_i \) is the time at each channel level, \( n \) is the number of channels in the patch, and \( T \) is the total time of analysis. The total number of KCa channels in an inside-out patch was determined at a voltage of +40 mV with 100 \( \mu \)M free Ca2+ in the bath solution. In each patch under each condition, at least 5 min of continuous data were analyzed to calculate transient KCa current frequency and amplitude, and 2–5 min were analyzed to determine single KCa channel open probability.

Confocal Ca2+ imaging. Isolated smooth muscle cells were incubated in solution A containing fluo-4 AM (10 \( \mu \)M) for 20 min at room temperature, followed by a 30-min wash to allow indicator deesterification. Smooth muscle cells were imaged with the use of a Noran Oz laser scanning confocal microscope (Noran Instruments, Middleton, WI) and a ×60 water immersion objective (1.2 numerical aperture) attached to a Nikon TE300 microscope. Fluo-4 was excited by using the 488 nm line of a krypton-argon laser, and emitted light >500 nm was captured. Images (56.3 \( \times \) 52.8 \( \mu \)m) were recorded every 8.3 ms (120 images/s). The laser intensity used did not alter transient KCa current frequency or amplitude. Current and fluorescence measurements were synchronized by using a light-emitting diode placed above the recording chamber that was triggered during acquisition. Each cell was imaged for 15 s. Ca2+ sparks in smooth muscle cells were analyzed with the use of software kindly provided by Dr. M. T. Nelson (University of Vermont). Detection of Ca2+ sparks was performed by dividing an area 1.54 \( \mu \)m (7 pixels) \( \times \) 1.54 \( \mu \)m (7 pixels) (i.e., 2.37 \( \mu \)m²) in each image (F) by a baseline (F<sub>0</sub>) that was determined by averaging 10 images without Ca2+ spark activity. The entire area of each image was analyzed to detect Ca2+ sparks. A Ca2+ spark was defined as a local increase in F/F<sub>0</sub> that was >1.2.

Fura-2 imaging. Cerebral arteries were incubated with the ratiometric fluorescent Ca2+ indicator fura-2 AM (5 \( \mu \)M) and 0.05% pluronic F-127 for 20 min, followed by a 15-min wash. All experiments were performed using solution B (composition described in Patch-clamp electrophysiology). Fura-2 was alternately excited at 340 or 380 nm using a PC-driven hyperswitch (Ionoptix, Milton, MA). Background corrected ratios were collected every 1 s at 510 nm using a photomultiplier tube (Ionoptix). SR Ca2+ load ([Ca2+]<sub>SR</sub>) was estimated by rapidly applying a high concentration of caffeine (10 mM), an RyR channel activator, and measuring the amplitude of [Ca2+]<sub>i</sub> transients (i.e., A[Ca2+]<sub>i</sub>). Intracellular Ca2+ concentrations were calculated by using the following equation (12):
where \( R \) is the 340/380 nm ratio; \( R_{\text{min}} \) and \( R_{\text{max}} \) are the minimum and maximum ratios determined in "Ca\(^{2+}\)"-free and saturating "Ca\(^{2+}\)" solutions, respectively, \( S_{3}/S_{2} \) is the ratio of "Ca\(^{2+}\)"-free to "Ca\(^{2+}\)"-replete emissions at 380 nm excitation, and \( K_{d} \) is the dissociation constant for fura-2 (282 nM) (19). \( R_{\text{min}}, R_{\text{max}}, S_{3}, \) and \( S_{2} \) were determined at the end of experiments and in separate experiments by increasing the "Ca\(^{2+}\)"-free, HEPES-containing patch-clamp bath solution (composition described in Patch-clamp electrophysiology). Cell images were acquired with the use of a Zeiss LSM5 confocal microscope. Cell length and width were calculated using Pascal software (Zeiss).

**Measurement of cellular dimensions.** Isolated smooth muscle cells were allowed to settle on a glass coverslip in a chamber with "Ca\(^{2+}\)"-free, HEPES-containing patch-clamp bath solution (composition described in Patch-clamp electrophysiology). Cell images were acquired with the use of a Zeiss LSM5 confocal microscope. Cell length and width were calculated using Pascal software (Zeiss).

**Statistics.** Values are expressed as means ± SE. Student’s t-test and Student-Newman-Keuls test were used for comparing paired or unpaired data and multiple data sets, respectively. Simultaneous "Ca\(^{2+}\)" spark and transient \( K_{\text{Ca}} \) current amplitude data were fit with a linear regression function, and the slope ± SE of each fit was compared using Student’s t-test and Graphpad Prizm (San Diego, CA). \( P < 0.05 \) was considered significant.

**RESULTS**

Caveolae are absent in cerebral artery smooth muscle cells of cav-1\(^{-/-}\) mice. Electron microscopy revealed that abundant caveolae were present in the sarcolemma of smooth muscle cells of small-diameter (~150 \( \mu \)m) cerebral arteries of cav-1\(^{+/+}\) mice (Fig. 1, A and B). In contrast, caveolae were not observed in cerebral artery smooth muscle cells of cav-1\(^{-/-}\) mice (Fig. 1, C and D). These data indicate that cav-1 is necessary for caveolae formation in smooth muscle cells of small cerebral arteries.

**Transient \( K_{\text{Ca}} \) current frequency is higher in cav-1\(^{-/-}\) than in cav-1\(^{+/+}\) cerebral artery smooth muscle cells.** To investigate the effects of cav-1 ablation on "Ca\(^{2+}\)" spark-to-\( K_{\text{Ca}} \) channel signaling, transient \( K_{\text{Ca}} \) currents were measured in cerebral artery smooth muscle cells of cav-1\(^{-/+}\) and cav-1\(^{-/-}\) mice. Transient \( K_{\text{Ca}} \) currents were measured at ~40 mV, which is the membrane potential of cerebral arteries pressurized to 60 mmHg (19). Mean transient \( K_{\text{Ca}} \) current frequency was 1.11 ± 0.14 Hz in cav-1\(^{+/+}\) cells (\( n = 35 \)) and 2.20 ± 0.30 Hz in cav-1\(^{-/-}\) cells (\( n = 31 \)), or approximately twofold higher (Fig. 2, A and B). In contrast, transient \( K_{\text{Ca}} \) current amplitude was similar in cav-1\(^{+/+}\) and cav-1\(^{-/-}\) cells (Fig. 2, A and C).

![Image](http://ajpheart.physiology.org/DownloadedFromhttp://ajpheart.physiology.org%2Fby10.220333onJuly6,2017)

Fig. 4. Intracellular "Ca\(^{2+}\)" concentration (["Ca\(^{2+}\)]\(_{i}\)) and sarcoplasmic reticulum "Ca\(^{2+}\)" load are similar in cav-1\(^{+/+}\) and cav-1\(^{-/-}\) cerebral arteries. A: ["Ca\(^{2+}\)]\(_{i}\) and caffeine (10 mM)-induced "Ca\(^{2+}\)" transients were measured in cav-1\(^{+/+}\) and cav-1\(^{-/-}\) cerebral arteries using fura-2. B: ["Ca\(^{2+}\)]\(_{i}\) was similar in cav-1\(^{+/+}\) (\( n = 7 \)) and cav-1\(^{-/-}\) (\( n = 9 \)) cerebral arteries. C: caffeine-induced "Ca\(^{2+}\)" transients were similar in cav-1\(^{+/+}\) (\( n = 7 \)) and cav-1\(^{-/-}\) (\( n = 9 \)).
Cell capacitance of cav-1 

+ /− cells (−8.4 pF) was smaller than for cav-1 

+ /− cells (−12.1 pF; Fig. 2D). To investigate whether cell size underlies the difference in cav-1 

+ /− and cav-1 

− /− cell capacitance, cellular dimensions were measured. Cells were observed in a Ca

2

++ -free bath solution to induce maximal relaxation. Cav-1 

+ /− and cav-1 

− /− cell length [in μm: cav-1 

+ /− , 37.1 ± 1.6 (n = 32); cav-1 

− /− , 39.4 ± 2.6 (n = 33)] and width [in μm: cav-1 

+ /− , 9.6 ± 0.3 (n = 32); cav-1 

− /− , 9.4 ± 0.3 (n = 33)] were not different (P > 0.05 for each). These data suggest that cav-1 ablation reduces the cell surface area but does not alter the dimensions of cerebral artery smooth muscle cells.

In arterial smooth muscle cells, transient K

Ca currents occur due to SR Ca

2

++ release (38). We sought to determine mechanisms that activate transient K

Ca currents in cav-1 

− /− cells. In cav-1 

− /− cells, thapsigargin (100 nM), an SR Ca

2

++ ATPase inhibitor, abolished transient K

Ca currents, indicating that these events occur due to SR Ca

2

++ release (Fig. 2E; n = 4).

Ca

2

++ spark frequency is elevated in cav-1 

− /− cells when compared with cav-1 

+ /− cells, but the amplitude relationship between Ca

2

++ sparks and transient K

Ca currents is similar. Elevated transient K

Ca current frequency in cav-1 

− /− cells could occur because of an increase in Ca

2

++ spark frequency or an increase in the percentage of Ca

2

++ sparks that activate a transient K

Ca current (i.e., percent coupling). To investigate these possibilities and to compare Ca

2

++ spark properties in cav-1 

+ /− and cav-1 

− /− cells, simultaneous measurements of Ca

2

++ sparks and evoked transient K

Ca currents were obtained by performing confocal Ca

2

++ imaging in combination with patch-clamp electrophysiology.

At −40 mV, Ca

2

++ spark frequency was ~1.8-fold higher in cav-1 

− /− cells than in cav-1 

+ /− cells (Fig. 3, A and B). In contrast, Ca

2

++ spark amplitude (Fig. 3C) and spatial spread [full width at half-maximal amplitude; cav-1 

+ /− , 1.82 ± 0.13 μm (n = 40); cav-1 

− /− , 1.94 ± 0.11 μm (n = 59)] were similar in cav-1 

+ /− and cav-1 

− /− cells (P > 0.05 for each). The percentage of Ca

2

++ sparks that activated a transient K

Ca current (cav-1 

+ /− , 67 ± 8%; cav-1 

− /− , 71 ± 4%) and the amplitude relationship between Ca

2

++ sparks and transient K

Ca currents were also similar (Fig. 3, A and D). Taken together, these data indicate that genetic ablation of cav-1 

− /− elevates Ca

2

++ spark frequency, leading to an increase in transient K

Ca current frequency.
Cav-1+/- and Cav-1-/- cells. Cytosolic [Ca2+]i and [Ca2+]S regulate Ca2+ sparks in arterial smooth muscle cells (6, 38). However, [Ca2+]i and [Ca2+]S, as determined by caffeine (10 mM)-induced Ca2+ transients, were not different in Cav-1+/- and Cav-1-/- cerebral arteries (Fig. 4, A–C). Thus the higher Ca2+ spark frequency in Cav-1-/- cells is not because of elevated cytosolic or SR Ca2+ concentration.

N^G-nitro-L-arginine does not inhibit transient Ca2+ currents in Cav-1+/- or Cav-1-/- cells. Cav-1 ablation leads to nitric oxide (NO) synthase (NOS) activation and an increase in NO induced Ca2+ sparks (10, 34). NO donors activate Ca2+ oxide (NO) synthase (NOS) activation and an increase in NO induced Ca2+ sparks (10, 34). NO donors activate Ca2+ synthase (NOS) activation and an increase in NO induced Ca2+ sparks (10, 34). NO donors activate Ca2+ synthase (NOS) activation and an increase in NO induced Ca2+ sparks (10, 34).

Voltage-dependent Ba2+ current density is similar in Cav-1+/- and Cav-1-/- cells. In Cav-1-/- cells, voltage-dependent Ca2+ channel density may be reduced or voltage-dependent Ca2+ channels may be insensitive to blockers. To test these hypotheses, voltage-dependent Ca2+ current density relationships were compared in Cav-1+/- and Cav-1-/- cells using Ba2+ as the charge carrier. Voltage-dependent Ba2+ current density was similar, and Cd2+ abolished voltage-dependent Ba2+ currents in both Cav-1+/- and Cav-1-/- cells (Fig. 6, A and B). Thus the absence of Ca2+ spark regulation by voltage-dependent Ca2+ entry in Cav-1-/- cells cannot be explained by alterations in sarcolemma voltage-dependent Ca2+ current density or Ca2+ sensitivity. Ca2+ and K+ current density is higher in Cav-1-/- than in Cav-1+/- cells. In cardiac myocytes, coupling between voltage-gated Ca2+ channels and RyR channels is tight, whereas in smooth muscle cells, this communication is loose (5, 20, 24, 36). Conceivably, Ca2+ spark regulation by voltage-dependent Ca2+ channels may be abolished in Cav-1-/- cells because of

Fig. 6. Voltage-dependent Ca2+ current density is similar in Cav-1+/- and Cav-1-/- cells. A: original traces illustrating voltage-dependent Ba2+ currents (I_{Ba}) elicited by a voltage step from a holding potential of -80 to +20 mV in Cav-1+/- and Cav-1-/- cerebral artery smooth muscle cells. CdCl2 (250 μM) abolished (I_{Ba}) in Cav-1+/- and Cav-1-/- cells. B: current density-voltage relationship of CdCl2 (250 μM)-sensitive currents in Cav-1+/- (n = 9) and Cav-1-/- (n = 9) cells. P > 0.05 for each voltage when compared with cav-1+/-.
an increase in the distance between the sarcolemma and the
SR. Therefore, we sought to study the distance between Ca$^{2+}$
spark sites and a sarcolemmal Ca$^{2+}$ spark target in cav-1$^{+/+}$
and cav-1$^{-/-}$ cells. To do this, $K_{Ca}$ channel properties were
measured, thereby allowing further investigation of Ca$^{2+}$ spark
to $K_{Ca}$ channel coupling.

Transient $K_{Ca}$ current amplitude is dependent on $K_{Ca}$
channel conductance and Ca$^{2+}$ sensitivity (4). In excised inside-out
patches, single $K_{Ca}$ channel slope conductance between -40
and +40 mV [cav-1$^{+/+}$, 268 ± 3 pS ($n = 9$); cav-1$^{-/-}$, 260 ±
3 pS ($n = 7$); $P > 0.05$] and $K_{Ca}$ channel-apparent Ca$^{2+}$ sensitivity
were similar in cav-1$^{+/+}$ and cav-1$^{-/-}$ cells (Fig. 7, A and B). Transient $K_{Ca}$ current amplitude also depends on the
number of $K_{Ca}$ channels activated by a Ca$^{2+}$ spark. On average,
inside-out patches pulled from cav-1$^{-/-}$ cells contained
~1.5-fold more $K_{Ca}$ channels than patches obtained from
cav-1$^{+/+}$ cells (Fig. 7C). This finding was not due to differences
in the size of patch pipettes used for these experiments
[cav-1$^{+/+}$, 28.6 ± 1.2 MΩ ($n = 17$); cav-1$^{-/-}$, 27.1 ± 0.7 MΩ
($n = 22$); $P > 0.05$]. These data suggest that $K_{Ca}$ channel density is higher in cav-1$^{+/+}$ cells than in cav-1$^{-/-}$ cells.

To further examine $K_{Ca}$ channel properties, whole cell $K^+$
current density was measured in cav-1$^{+/+}$ and cav-1$^{-/-}$ cells. $K^+$ currents were measured by using a strongly buffered
Ca$^{2+}$-free pipette solution to abolish Ca$^{2+}$-dependent $K_{Ca}$
channel activation. Whole cell $K^+$ current density was larger in
cav-1$^{+/+}$ cells than in cav-1$^{-/-}$ cells (Fig. 8, A and D). Paxilline (300 nM), a selective $K_{Ca}$ channel blocker (21, 35),
partially inhibited $K^+$ currents in both cav-1$^{+/+}$ and cav-1$^{-/-}$
cells (Fig. 8B). Paxilline-insensitive current density, which arises because of KV channel activation, was larger in cav-1$^{-/-}$
cells than in cav-1$^{+/+}$ cells (Fig. 8, B and E). Similarly,
paxilline-sensitive current density, which occurs through $K_{Ca}$ channel activation, was larger in cav-1$^{-/-}$ cells than in cav-
1$^{+/+}$ cells (Fig. 8, C and F). These data suggest that cav-1
ablation elevates the sarcolemmal current density of both $K_{Ca}$
and KV channels.

**DISCUSSION**

The impact of cav-1 ablation on the regulation of Ca$^{2+}$
sparks by voltage-dependent Ca$^{2+}$ channels and the commun-
ication between Ca$^{2+}$ sparks and $K_{Ca}$ channels were investi-
gated in arterial smooth muscle cells. Caveolae were not
observed in cav-1$^{-/-}$ cerebral artery smooth muscle cells,
consistent with a role for this protein in caveolae formation
(10, 34, 39). Cav-1 ablation attenuated coupling between
voltage-dependent Ca$^{2+}$ channels and RyR channels that gen-
erate Ca$^{2+}$ sparks. Cav-1 ablation also elevated Ca$^{2+}$ spark
frequency, although this was not due to an increase in voltage-
dependent Ca$^{2+}$ current density, cytosolic or SR Ca$^{2+}$ concen-

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**Fig. 7.** Single $K_{Ca}$ channel properties in cav-
1$^{+/+}$ and cav-1$^{-/-}$ cells. A: original records illustrate single $K_{Ca}$ channel openings in 10
μM Ca$^{2+}$ at -40 and +40 mV in inside-out
patches from a cav-1$^{+/+}$ and cav-1$^{-/-}$ cell. B: at -40 and +40 mV, apparent Ca$^{2+}$ sensitivity
of $K_{Ca}$ channels was similar for cav-1$^{+/+}$ ($n =
8–9$ for each [Ca$^{2+}$]) and cav-1$^{-/-}$ ($n = 7$ for
each [Ca$^{2+}$]) cells ($P > 0.05$ for each). $P_{o}$
open probability. C: inside-out patches from
cav-1$^{-/-}$ cells ($n = 22$) contained more $K_{Ca}$
channels than patches from cav-1$^{+/+}$ cells ($n =
17$). C, closed; O, open. *$P < 0.05$. 

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tration, or NOS activity. Although there was an increase in KCa channel density in cav-1\(^{-/-}\)/H11002 cells, transient KCa current amplitude was similar to that in cav-1\(^{-/+}\)/H11001 cells. Taken together, the data suggest that cav-1 abolishment leads to uncoupling between voltage-dependent Ca\(^{2+}\)/H11001 channels and RyR channels that generate Ca\(^{2+}\) sparks but does not alter transient KCa current activation by Ca\(^{2+}\) sparks.

In smooth muscle cells, coupling between voltage-dependent Ca\(^{2+}\)/H11001 channels and RyR channels is "loose" (20). Although structural and molecular mechanisms that establish this coupling process are unclear, loss of voltage-dependent Ca\(^{2+}\)/H11001 channel to RyR channel communication in cav-1\(^{-/-}\)/H11002 cells likely occurs because of spatial separation of these proteins. Supporting this hypothesis is evidence that although voltage-dependent Ca\(^{2+}\)/H11001 current density was similar in cav-1\(^{-/+}\)/H11001 and cav-1\(^{-/-}\)/H11002 cells, regulation of transient KCa currents by voltage-dependent Ca\(^{2+}\)/H11001 channel blockers was abolished in cav-1\(^{-/-}\)/H11002 cells. In addition, although KCa channel density was elevated in cav-1\(^{-/-}\)/H11002 cells, mean transient KCa current amplitude was similar to that in cav-1\(^{-/+}\)/H11001 cells. KCa channel conductance, Ca\(^{2+}\) spark amplitude and spatial spread, the effective coupling of Ca\(^{2+}\) sparks to KCa channels, and KCa channel-apparent Ca\(^{2+}\) sensitivity were similar in cav-1\(^{-/+}\)/H11001 and cav-1\(^{-/-}\)/H11002 cells. Caveolae are structural membrane invaginations that may reduce the sarcolemma to SR distance and physically localize RyR channels nearby voltage-dependent Ca\(^{2+}\)/H11001 channels and KCa channels in cav-1\(^{-/+}\)/H11001 cells. A flattening of the sarcolemma in cav-1\(^{-/-}\)/H11002 cells may explain the proposed increase in the signaling distance between RyR channels and voltage-dependent Ca\(^{2+}\)/H11001 channels and KCa channels. In cav-1\(^{-/-}\)/H11002 cells, a distant spark would impact a smaller membrane area and induce a lower subsarcolemmal [Ca\(^{2+}\)]i elevation. Consistent with our observations, higher KCa channel density would be required for distant Ca\(^{2+}\) sparks to activate transient KCa currents of similar amplitude to those in cav-1\(^{-/+}\)/H11001 cells. Another explanation for the proposed increase in signaling distance is that cav-1 abolishment leads to delocalization of voltage-dependent Ca\(^{2+}\)/H11001 channels and KCa channels from within the vicinity of the spark site. Several lines of evidence support such a proposal. In smooth muscle cells, caveolae compartmentalize voltage-dependent Ca\(^{2+}\)/H11001 channels and KCa channels (3, 8). In arterial smooth muscle cells, KCa channels

![Fig. 8. Whole cell K\(^{+}\), KCa, and KV current density is elevated in cav-1\(^{-/-}\) cells. A: original recordings of whole cell K\(^{+}\) currents activated by depolarizing voltage steps in a cav-1\(^{-/+}\) and cav-1\(^{-/-}\) cell. B: paxilline (300 nM) inhibits K\(^{+}\) currents in the same cells illustrated in A. C: paxilline-sensitive K\(^{+}\) currents. D: mean whole cell K\(^{+}\) current density (pA/pF) in cav-1\(^{-/+}\) (n = 11) and cav-1\(^{-/-}\) (n = 9) cells. E: paxilline-insensitive K\(^{+}\) current density. F: paxilline-sensitive K\(^{+}\) current density. *P < 0.05 when compared with cav-1\(^{-/+}\) cells.](http://ajpheart.physiology.org/Download/fig8.png)
are proposed not to cluster above Ca\(^{2+}\) sparks sites, although in *Bufo marinus* stomach smooth muscle cells, such clustering may occur (30, 42). In guinea pig bladder smooth muscle, peripheral SR and caveolae are in close proximity, and L-type Ca\(^{2+}\) channels located in caveolae strips may be closely opposed to SR RyR channels (25). It is not clear what causes the elevation in KCa channel density in cav-1/−/− cells, but in cultured human myometrial smooth muscle cells, KCa channels associate with caveolins 1 and 2, and cholesterol depletion with cycloheximide leads to an increase in whole cell KCa current (23), consistent with the results here. Furthermore, in bovine endothelial cells, cav-1 physically interacts with and inhibits KCa channels and this effect can be removed by cholesterol depletion, leading to channel activation (37).

Investigating the effects of chronic caveolae deficiency on smooth muscle Ca\(^{2+}\) signaling can provide insights into physiological regulation by these membrane organelles in cav-1/−/− cells and potential changes that occur during pathophysiology and ontogeny (11, 29, 32, 34, 39). In contrast to the observations here, acute cholesterol depletion with dextrin inhibited arterial smooth muscle Ca\(^{2+}\) sparks (23). Acute cholesterol depletion and chronic cav-1 ablation may differentially regulate Ca\(^{2+}\) spark frequency through opposing effects on signaling pathways that regulate these events, and in the case of the genetic model, through developmental changes that occur in the sustained absence of cav-1 (10, 39). However, dextrin treatment also induces a change in membrane fluidity that may inhibit Ca\(^{2+}\) sparks (26). In agreement, both dextrin and cav-1/−/− abolishment coupled between voltage-dependent Ca\(^{2+}\) channels and RyR channels that generate sparks (present data and Ref. 23). Whereas dextrin treatment disrupts both caveolar and noncaveolar lipid rafts, in the absence of cav-1, noncaveolar lipid rafts would remain (see Ref. 40). Thus our data suggest that caveolae-localized voltage-dependent Ca\(^{2+}\) channels regulate Ca\(^{2+}\) sparks in arterial smooth muscle cells. In another previous study, transient KCa current frequency was reduced in arterial smooth muscle cells of cav-1/−/− mice (10). It is unclear why data here are contradictory to those observations, but in the previous study, effects of cav-1 deficiency on Ca\(^{2+}\) sparks were not measured (10).

Through its amino-terminal scaffolding domain, cav-1 inhibits the activity of many binding partners, including NOS, protein kinase C, and adenylyl cyclase (28, 34). Cav-1 is found in the cytosol (22) and, conceivably, may interact with and inhibit RyR channels. Cav-1 is also found in mitochondria, which regulate Ca\(^{2+}\) sparks in cardiac artery smooth muscle cells (7, 41). Cav-1 abolishment may also activate RyR channels indirectly by modulating the activity of signaling pathways that regulate Ca\(^{2+}\) sparks (e.g., see Refs. 15 and 41). Data here indicate that cav-1 ablation does not activate Ca\(^{2+}\) sparks through NOS activation (31, 33). Because cav-1 ablation may activate RyR channels directly and indirectly, future studies will be required to determine the specific underlying mechanisms. The physiological impact of the transient KCa current frequency elevation in cav-1/−/− cells would be a membrane hyperpolarization that reduces voltage-dependent Ca\(^{2+}\) channel activity, leading to a decrease in global [Ca\(^{2+}\)], and vasodilation (15). Cav-1/−/− aortas contract less in response to vasoconstrictors and relax more in response to acetylcholine, a vasodilator, when compared with cav-1+/- arteries (10, 33), supporting upregulation of a vasodilatory pathway.

In summary, data show that genetic ablation of cav-1 leads to loss of caveolae, an increase in Ca\(^{2+}\) spark frequency, abolishment of Ca\(^{2+}\) spark regulation by voltage-dependent Ca\(^{2+}\) channels, and an increase in KCa channel density in cerebral artery smooth muscle cells. In contrast, cav-1 ablation does not attenuate transient KCa current activation by Ca\(^{2+}\) sparks.

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