Caveolin-1 Facilitates the Direct Coupling between Large Conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) and Cav1.2 Ca\(^{2+}\) Channels and Their Clustering to Regulate Membrane Excitability in Vascular Myocytes*\(^{\dagger} \)\(^{\ddagger} \)\(^{\S} \)

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**Background:** The contribution of caveolae to physiological interaction between two major ion channels, large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) and Ca\(^{2+}\) (Cav1.2) channels, is unknown in vascular myocytes.

**Results:** The loss of caveolin by caveolin-1 deficiency reduced BK\(_{Ca}\)-Cav1.2 coupling, Cav1.2 clustering, and membrane excitability regulation.

**Conclusion:** Caveolin-1 provides platform for BK\(_{Ca}\)-Cav1.2 molecular complex.

**Significance:** Caveolin-1-BK\(_{Ca}\)-Cav1.2 in caveola forms a novel Ca\(^{2+}\) signal domain for arterial tonus regulation.

L-type voltage-dependent Ca\(^{2+}\) channels (LVDCC) and large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (BK\(_{Ca}\)) are the major factors defining membrane excitability in vascular smooth muscle cells (VSMCs). The Ca\(^{2+}\) release from sarcoplasmic reticulum through ryanodine receptor significantly contributes to BK\(_{Ca}\) activation in VSMCs. In this study direct coupling between LVDCC (Cav1.2) and BK\(_{Ca}\) and the role of caveolin-1 on their interaction in mouse mesenteric artery SMCs were examined. The direct activation of BK\(_{Ca}\) by Ca\(^{2+}\) influx through coupling LVDCC was demonstrated by patch clamp recordings in freshly isolated VSMCs. Using total internal reflection fluorescence microscopy, it was found that a large part of yellow fluorescent protein-tagged BK\(_{Ca}\) co-localized with the cyan fluorescent protein-tagged Cav1.2 expressed in the plasma membrane of primary cultured mouse VSMCs and that the two molecules often exhibited FRET. It is notable that each BK\(_{Ca}\) subunit of a tetramer in BK\(_{Ca}\) can directly interact with Cav1.2 and promotes Cav1.2 cluster in the molecular complex. Furthermore, caveolin-1 deficiency in knockout (KO) mice significantly reduced not only the direct coupling between BK\(_{Ca}\) and Cav1.2 but also the functional coupling between BK\(_{Ca}\) and ryanodine receptor in VSMCs. The measurement of single cell shortening by 40 mM K\(^{+}\) revealed enhanced contractility in VSMCs from KO mice than wild type. Taken together, caveolin-1 facilitates the accumulation/clustering of BK\(_{Ca}\)-LVDCC complex in caveolae, which effectively regulates spatiotemporal Ca\(^{2+}\) dynamics including the negative feedback, to control the arterial excitability and contractility.

The calcium ion (Ca\(^{2+}\)) is a major second messenger responsible for variety of physiological responses, including neurotransmitter/hormone release, muscular contraction, cell proliferation, apoptosis, etc. It has been demonstrated that voltage-dependent Ca\(^{2+}\) channel (VDCC)\(^{2} \) and Ca\(^{2+}\) effectors often accumulate spatially and form Ca\(^{2+}\) microdomains (1, 2) to increase the local Ca\(^{2+}\) level and activate specific signal transduction events.

In smooth muscle cells (SMCs), it has been established that the L-type VDCC (LVDCC), ryanodine receptor (RyR), and large conductance Ca\(^{2+}\)-activated K\(^{+}\) channel (BK\(_{Ca}\), K\(_{Ca}\)1.1) constitute the Ca\(^{2+}\) microdomains. Two types of local Ca\(^{2+}\) events, Ca\(^{2+}\) hotspots (3) arising from depolarization-evoked Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) (4) and spontaneous Ca\(^{2+}\) release (Ca\(^{2+}\) sparks) (5), play a crucial role in the regulation of SMC contraction and relaxation. These two Ca\(^{2+}\) events occur in the same distinct local areas in SMC so as to couple with BK\(_{Ca}\) activity and, respectively, contribute to the action potential repolarization phase and spontaneous transient outward current (STOC) (6). Thus, BK\(_{Ca}\) is thought to get activated mainly by Ca\(^{2+}\) release from the sarcoplasmic reticulum through RyRs, which loosely couple with LVDCC on the plasma membrane in excitation-contraction coupling (7). In this study the relationship between LVDCC and BK\(_{Ca}\) via RyR activation is termed as “loose coupling.”

Caveolae are composed of an omega-shaped structure on plasma membrane. Caveolin-1 is an essential factor for the properly formed caveola structure and accumulates many

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**Footnotes:**

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\(^{\dagger}\) This article contains supplemental Movies 1–4.

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\(^{\S}\) The abbreviations used are: VDCC, voltage-dependent Ca\(^{2+}\) channel; VSMC, vascular smooth muscle cell; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BK\(_{Ca}\), large conductance Ca\(^{2+}\)-activated K\(^{+}\) channel; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; LVDCC, L-type voltage-dependent Ca\(^{2+}\) channel; Pax, paxilline; RyR, ryanodine receptor; SMC, smooth muscle cell; STOC, spontaneous transient outward current; TIRF, total internal reflection fluorescence; 40 KCl, 40 mM KCI HEPES-buffed solution; pF, picofarads.
types of signaling molecules (8, 9). The BK<sub>Ca</sub> protein contains a binding motif to caveolin-1 and is often co-localized in caveolae (10, 11). Several reports indicate that caveolin-1 is involved in Ca<sup>2+</sup> hotspot and spark generation (12, 13). Caveolin-1 knock-out (KO) mice congenitally lack caveolae and exhibit many types of cardiovascular abnormalities (9, 14, 15).

The interaction of BK<sub>Ca</sub> with several functional molecules in addition to caveolin-1 has been identified (16). In some neurons it has been reported that BK<sub>Ca</sub> is directly activated by Ca<sup>2+</sup> influx through the tightly coupled VDCC (17–20). On the other hand, in tsA-201 cells, which express LVDCC and BK<sub>Ca</sub>, the Ca<sup>2+</sup> entry through single LVDCCs rarely evokes BK<sub>Ca</sub> opening (21). These authors suggested that the BK<sub>Ca</sub> selectively interacts with N-type VDCC rather than L-type VDCC. To our knowledge, the direct molecular and/or functional interaction between BK<sub>Ca</sub> and VDCC has not yet been reported in muscles, including skeletal, cardiac, and smooth muscles, even though BK<sub>Ca</sub> co-localizes with Cav1.1 in the distrophin molecular complex (22) in skeletal muscle and can interact with caveolin-3 (23). In this study this direct coupling between LVDCC and BK<sub>Ca</sub> on the same plasma membrane is known as “tight coupling.” The exact molecular mechanisms underlying direct physical interaction between BK<sub>Ca</sub> and Cav1.2 have not yet been clarified. Subtypes of VDCCs acting as molecular partners for BK<sub>Ca</sub> depend upon the local tissues. The LVDCC encoded by Cav1.2 (α1C) is highly expressed and serves as a predominant Ca<sup>2+</sup> entry pathway in SMCs (24). Because tissue-specific splice variants with differential functions have been found in both BK<sub>Ca</sub> (25) and Cav1.2 (26), it remains to be totally resolved whether BK<sub>Ca</sub> physically couples with Cav1.2 and the coupling is functionally significant in SMCs.

In the previous study we first demonstrated that BK<sub>Ca</sub> forms tetramer and directly interacts with caveolin-1 in living aortic SMCs using total internal reflection fluorescence (TIRF) microscopy. In the TIRF system, an evanescent wave excites fluorescent molecules within a 200-nm depth of a glass bottom. This enables visualization of the fluorescent-labeled molecules localized on the plasma membrane in living cells (27). The present study was undertaken to examine the possible BK<sub>Ca</sub>-Cav1.2 complex and the potential roles of caveolin-1 for this complex formation in vascular SMCs (VSMCs) by use of TIRF microscopy imaging methods. To demonstrate functional coupling between LVDCC and BK<sub>Ca</sub> whole cell patch clamp recording using two different Ca<sup>2+</sup> chelators, EGTA and 1,2-bis(α-amino-phenoxo)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was performed (18, 28). Results suggest that the BK<sub>Ca</sub>-Cav1.2 complex is mostly co-localized in the caveolae of VSMCs. Furthermore, data from KO mice denote the importance of caveolae as a platform of the ion channel complex formation that effectively regulates proper excitability and contractility of VSMCs.

**EXPERIMENTAL PROCEDURES**

**Animals, Cell Isolation, and Culture**—Caveolin-1 knock-out (KO) mice on the C57BL/6 background were obtained from The Jackson Laboratory (stock number 004585) (Bar Harbor, ME). Wild-type (WT) control mice (C57BL/6) were purchased (Japan SLC, Hamamatsu, Japan). All experiments were approved by the Ethics Committee of Nagoya City University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society. The superior mesenteric arteries were removed from male mice (8–14 weeks) and cleansed of connective tissue in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free Krebs solution containing 112 mM NaCl, 4.7 mM KCl, 1.2 mM KH<sub>2</sub.PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 14 mM glucose. From the third to the fifth branch were removed and incubated in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free Krebs solution containing 0.4% collagenase (Amano Enzyme, Nagoya, Japan) and 0.1% papain (Sigma) for 45 min at 37 °C. After incubation, these tissues were washed in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free Krebs solution and dispersed mechanically. For electrophysiological experiments, a few drops of the cell suspension were placed in a recording chamber. For culture, single cells were suspended in DMEM supplemented with 10% heat-inactivated FBS, 20 units/ml penicillin, and 20 μg/ml streptomycin (Sigma). After settling on glass-bottom dishes, cells were transiently transfected with fluorescent-labeled cDNA and cultured for 24–48 h.

**Electrophysiological Recording**—Electrophysiological studies were performed using a whole cell voltage clamp technique with a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), an analog-digital converter (DIGIDATA 1320A; Molecular Devices, Sunnyvale, CA), and a pCLAMP software (Version 8.2; Molecular Devices) in vascular cells as described previously (29). To examine whether the functional coupling between BK<sub>Ca</sub> and Cav1.2 was tight or loose, we used two types of pipette solution containing 1) 140 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM HEPES, 2 mM Na<sub>2</sub>ATP, and 5 mM EGTA or 2) 140 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM HEPES, 2 mM Na<sub>2</sub>ATP, and 10 mM BAPTA (pH 7.2 with KOH). Here 10 mM BAPTA was used to inhibit both tight and loose coupling, and 5 mM EGTA was used to inhibit loose coupling. We defined the EGTA-resistant, BAPTA-sensitive current component as a BK<sub>Ca</sub> current directly activated by Ca<sup>2+</sup> influx through tightly coupled Cav1.2 channels. For simultaneous recording of STOC and Ca<sup>2+</sup> sparks, the pipette solution contained 140 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM HEPES, 2 mM Na<sub>2</sub>ATP, and 0.1 mM fluo-4 (Invitrogen) (pH 7.2 with KOH). The extracellular solution (normal HEPES-buffered solution) had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 4 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH. For VDCC current measurements, the pipette solution contained 120 mM CsCl, 20 mM tetraethylammonium chloride (Tokyo Chemical Industry, Tokyo, Japan), 1 mM MgCl<sub>2</sub>, 10 mM HEPES, 2 mM Na<sub>2</sub>ATP, and 20 mM BAPTA (pH 7.2 with CsOH). The bath solution contained 92 mM NaCl, 5.9 mM KCl, 30 mM BaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH. Whole cell BK<sub>Ca</sub> currents and VDCC currents were activated from a holding potential of −60 mV by applying 150-ms voltage steps to voltages between −50 and +40 mV in increments of 10 mV. To detect tight-coupling between BK<sub>Ca</sub> and Cav1.2, depolarizing stimuli from a holding potential of −60 to 0 or +10 mV for 150 ms were applied. STOCs were measured at a steady membrane potential of −40 mV.

**Plasmid Constructs and Transfection**—The full-length of cDNA encoding the rat KCNMA1 (BK<sub>α</sub> subunit, GenBank™ Accession numbers U93052 and AY330293.2) was subcloned.
into pEYFP-N1 (BKα-YFP), pEYFP-C1 (YFP-BKα), pECFP-N1 (BKα-CFP), and pAcGFP-N1 (BKα-GFP) (Clontech Laboratories, Mountain View, CA). Human CACNA1C (NM_000719), SCN5A (NM_198056.2), and CAV1 (NM_001753) were also subcloned into pECFP-N1 (Cav1.2-CFP), pEYFP-N1 (Cav1.2-YFP), pAcGFP-N1 (Cav1.2-GFP), and pECFP-C1 (CFP-caveolin1 and CFP-Nav1.5). All constructs were confirmed by DNA sequencing. Functions of each channel were examined by whole cell patch clamp recording. Primary-cultured myocytes and HEK293 cells were transiently transfected with fluorescent-labeled cDNA (each 2 μg for co-expression) using Lipofectamine 2000 (Invitrogen). Experiments were performed 24–48 h after transfection. Under these conditions, BKCa current density measured in myocytes expressing yellow fluorescent proteins (YFP) alone. This meant that in our system artifacts by overexpression of fluorescent protein-labeled ion channels were minimized due to their relatively low expression levels (30).

Single-molecule Imaging—Single-molecule imaging was performed using a TIRF imaging system with an objective lens (CFI Plan Apo TIRF 60×/1.45 or CFI Apo TIRF 100×/1.49, oil immersion; Nikon, Tokyo, Japan) as described previously (30). Data were collected with an EM-CCD camera and analyzed by AQUACOSMOS software (Hamamatsu Photonics, Hamamatsu, Japan). Cyan fluorescent proteins (CFP)-fused proteins were excited with a 405-nm blue diode, and YFP- and green fluorescent protein (GFP)-fused proteins were excited with a 488-nm argon laser (Coherent, Santa Clara, CA), respectively, and reflected off dichroic mirrors (427–441/490–510 nm; Omega Optical, Brattleboro, VT). CFP/YFP emissions were collected through dichroic mirrors and dual band-pass filters (454–479/523–567 nm; Omega Optical). A resolution of images was 71 per pixel (x-y) and less than 200 nm (z). TIRF images were collected at 465-ms exposure times and scanned every 1651 ms. Normal HEPES-buffered solution was used during recording. All experiments were carried out at room temperature (25 °C).

Fluorescence Resonance Energy Transfer (FRET) Analysis—The efficiency of FRET (F_{FRET}) was evaluated based on the acceptor photobleaching method, in which the emission of the donor fluorophore is compared before and after the photobleaching of the acceptor (31). The fluorescence of YFP was photobleached using a mercury lamp (100 W, C-SHG1; Nikon) and a G-2A filter cube (Ex510–560/DM575/BA590; Nikon) for 2.5 min. FRET efficiency (F_{FRET}) was calculated as the percentage increase in CFP emission after YFP photobleaching, as described previously (30).

Single-molecule GFP Bleaching—We counted subunits of BKCa and Cav1.2 in membranes of HEK293 cells and arterial myocytes by observing bleaching steps of GFP fused to BKα and VDCCa1C in a single particle, as described previously (30, 32, 33). HEK293 cells or primary-cultured myocytes were transiently transfected with 2 μg of cDNA encoding BKα-GFP or Cav1.2-GFP using Lipofectamine 2000. At 24 h after transfection, cells were fixed for 10 min with 4% paraformaldehyde in PBS, rinsed, and placed in PBS solution before the experiment. GFP was excited with a 488-nm laser and imaged with a B-2A filter cube (DM505/BA520; Nikon) and an objective (100×/1.49). Images (256 × 256 pixels; 1 pixel = 107 nm) were acquired at 10 Hz for 100–120 s. Fluorescence intensity in a region of interest (3 × 3 pixels) was calculated by subtracting the background in 16 pixels around the region of interest. The number of bleaching steps was determined by eye from the fluorescence signal trace. The steps within each single trace were similar in amplitude but varied between different traces. This is a consequence of the Gaussian profile of the laser and complex topology of the plasma membrane, which results in different local illumination intensities of the evanescent field. We used the following criteria for discarding signals: (a) a signal exhibits an elliptical shape, (b) a signal is very close to other signals (<4 pixels), (c) a signal that fluorescence intensity fluctuates too much to be accurately determined bleaching steps, (d) a signal does not show complete bleaching.

Fluorescent Labeling of Freshly Isolated Myocytes—BKCa and LDVCC in freshly isolated mesenteric arterial myocytes were labeled with polyclonal anti-BKα antibody (APC-107, Alomone Laboratories, Jerusalem, Israel) and DM-BODIPY (–)-dihydropyridine (Invitrogen). Cells were immunostained as described previously (34). Dissociated cells settled on glass-bottom dishes (Matsunami Glass Industry, Osaka, Japan) were labeled with 1:100 diluted antibodies for 12 h at 4 °C after fixation and permeabilization. Then cells were washed and incubated with Alexa 405-conjugated anti-rabbit IgG goat antiserum (Invitrogen) for 1 h at room temperature. After washing, cells were loaded with 100 nM DM-BODIPY(–)-dihydropyridine for 5 min and subsequently washed in PBS. Fluorescently labeled cells were observed using a TIRF imaging system and AQUACOSMOS software (mentioned above). Alexa405 and BODIPY were excited with the blue diode and the argon laser, respectively. The emissions of Alexa405 and BODIPY were collected using CFP-HQ (DM450/BA460–510; Nikon) and YFP-HQ (DM510/BA520–560; Nikon) filter cubes, respectively.

Measurement of the Fluo-4 Signal—For simultaneous measurements of Ca2+ sparks and STOCs in VSMCs, the Ca2+ images were obtained using a TIRF imaging system and AQUACOSMOS software (mentioned above) under the whole-cell clamp mode. A myocyte was loaded with 100 μM fluo-4 by diffusion from the recording pipette. An argon laser (488 nm) and a B-2A filter were used for excitation light and emission collection, respectively. Images were collected every 14 ms for 12 s. Resolution of images was 142 or 214 nm/pixel. Fluorescence intensity (F) in the region of interest was measured as an average from the pixels included in the area of a 2.14 × 2.14 μm square. The data show as ΔF/F0 (%), where F0 is the basal F value obtained as the average intensity of the regions of interest acquired during the measurements, and ΔF is the difference between F and F0.

To measure the averaged Ca2+ concentration from whole cell area, myocytes were loaded with 10 μM fluo-4 AM (Invitrogen), and fluorescent images were acquired using AQUACOSMOS software. The minimum fluorescence intensity (F_{min}) and maximum fluorescence intensity (F_{max}) were obtained by applying Ca2+-free HEPES-buffered solution (CaCl2 was removed from and 5 mM EGTA was added to normal HEPES-buffered solution) and 10 μM ionomycin in normal HEPES-buffered solution containing 2 mM Ca2+, respectively. To induce a contraction in a myocyte, 40 mM KCl...
HEPES-buffered solution (40 KCl), which contained 102.9 mM NaCl, 40 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 14 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH), was applied. Ca²⁺ elevation from base line was normalized to the maximum Ca³⁺ change (i.e. the difference between $F_{\text{max}}$ and $F_{\text{min}}$). Images were collected every 2 s. A resolution of images was 267 nm/pixel.

Single Cell Shortening Measurements—Transmitted light images were acquired using the above-mentioned microscope and an objective lens (20×, dry, Nikon). 40 KCl and/or 1 μM paxilline (Pax) (Tocris Bioscience, Bristol, UK) were applied to myocytes. Cellular contraction was estimated as the decrease in cell area from the control condition. Images were collected every 1 s for 20 min. A resolution of images was 533 nm/pixel.

Statistics—Pooled data are shown as the mean ± S.E. Statistical significance between two groups was determined by Student’s t test. Statistical significance among groups was evaluated by Tukey’s test. Significant difference is expressed in the figures as $p < 0.05$ (*) or $p < 0.01$ (**).

RESULTS

BKCa Activation by Ca²⁺ Influx through Tightly Coupled LVDCC in VSMCs—At first, membrane currents elicited by depolarization were measured in mesenteric artery SMCs from WT or KO mice using low Ca²⁺ buffering pipette solution (50 μM EGTA). A large part of the outward current upon depolarization was blocked by the addition of 1 μM Pax (Fig. 1A) or 50 μM Ca²⁺, indicating that BKCa activation as one of the major outward currents is largely due to Ca²⁺ influx through VDCC. In highly excitable SMCs such as those of bladder and vas deferens, the Ca²⁺ source for BKCa activation upon depolarization may also include Ca²⁺ from the sarcoplasmic reticulum via Ca²⁺-induced Ca²⁺ release (CICR) (4, 35). This is also the case in mesenteric SMCs, and the outward currents upon depolarization showed irregular shapes and occurred concomitantly with local subcellular Ca²⁺ transients (Fig. 1B). To prevent the component of BKCa current activation due to CICR during depolarization, the following experiments were performed under much stronger Ca²⁺ buffering conditions (36).

Accordingly, we used two different Ca²⁺ chelators, EGTA and BAPTA, to examine the possible tight-coupling between BKCa and Cav1.2 in VSMCs (18). These Ca²⁺ buffers have similar binding affinities for Ca²⁺, but the binding rate constant of BAPTA is ~150 times faster than EGTA (18, 28). Thus, BAPTA is much more effective in preventing the diffusion of free Ca²⁺ away from the entrance site in LVDCC on the plasma membrane. Based on these distinct characteristics, tight-coupling is effectively interfered by 10 mM BAPTA but not by 5 mM EGTA, whereas loose coupling is equally sensitive to 10 mM BAPTA and 5 mM EGTA (18).

In the presence of 5 mM EGTA in the pipette solution, a fast-activated and inactivated outward current remained in SMCs of the WT but not those of KO (Fig. 2A). When the pipette solution contained 10 mM BAPTA, no such transient outward current was observed in either the WT or KO myocytes (Fig. 2A). Thus, the fast inactivation recorded by the pipette solution containing 5 mM EGTA was presumably due to Ca²⁺ removal by the slow Ca²⁺ chelation by EGTA. The slope of the rising phase of the outward current was compared and is shown in Fig. 2B (in WT: 0.80 ± 0.27 (pA/pF)/ms with EGTA ($n = 5$) and 0.08 ± 0.02 (pA/pF)/ms with BAPTA ($n = 5$); in KO: 0.16 ± 0.04 (pA/pF)/ms with EGTA ($n = 6$) and 0.10 ± 0.02 (pA/pF)/ms with BAPTA ($n = 8$); $p < 0.01$ versus WT with EGTA). The time to the peak of the EGTA-resistant currents was 35.8 ± 11.5 ms ($n = 5$) in WT and 55.2 ± 10.8 ms ($n = 6$) in KO ($p > 0.05$). The peak amplitude of the EGTA-resistant outward currents was 243.3 ± 38.4 pA ($n = 5$) in the WT and 117.7 ± 19.4 pA ($n = 6$) in the KO myocytes ($p < 0.05$). Thus, the EGTA-resistant transient outward current was presumably activated by Ca²⁺ influx via tight-coupled Cav1.2. This EGTA-resistant current was significantly larger and activated faster in the WT than KO myocytes.

Fig. 2C illustrates the I-V relationships of BKCa currents detected as a component sensitive to 1 μM Pax in WT (Fig. 2Ca)
and KO (Fig. 2Cb). In WT, the BK$_{Ca}$ current density resistant to 5 mM EGTA in the pipette solution was higher than that of the 10 mM BAPTA-resistant component at potentials positive to 0 mV. The summarized data at +10 mV in WT show that the EGTA-resistant and BAPTA-resistant BK$_{Ca}$ currents were 1.7 ± 0.3 pA/pF (n = 5) and 0.3 ± 0.3 pA/pF (n = 5), respectively (p < 0.01, Fig. 2D). Furthermore, the components in KO were 0.8 ± 0.2 pA/pF (n = 6) and 0.3 ± 0.2 pA/pF (n = 8), respectively (p > 0.05) (Fig. 2D). It is notable that the amplitude of the EGTA-resistant component in the WT was significantly larger than that in the KO myocytes (p < 0.05).

In the next series of experiments, the functional expression of LVDCC and BK$_{Ca}$ in mesenteric arterial SMCs was compared between WT and KO. In mesenteric arterial SMCs, Cav1.2 was expressed so abundantly that substantial inward currents through LVDCC were detected at positive potentials to −20 mV and peaked at +10 mV under the conditions, where outward currents were blocked. The LVDCC currents were detected as the component blocked by the addition of 50 μM Cd$^{2+}$. The peak LVDCC currents were recorded at +10 or +20 mV, and neither cell capacitance nor the peak LVDCC currents density at +10 mV were changed by the deletion of caveolin-1 (WT: 25.0 ± 1.0 pF; 2.9 ± 0.5 pA/pF, n = 5; KO: 26.8 ± 2.5 pF, 3.0 ± 0.6 pA/pF, n = 7; p > 0.05). The BK$_{Ca}$ current density at +40 mV was not changed by caveolin-1 deficiency (WT: 27.1 ± 1.7 pF, 1.8 ± 0.3 pA/pF, n = 5; KO: 27.4 ± 1.8 pF, 1.7 ± 0.2 pA/pF, n = 8, p > 0.05). These results indicate that the functional expression levels of LVDCC and BK$_{Ca}$ in KO are comparable with those in WT.

Similar experiments were performed in aortic myocytes. The current density of BK$_{Ca}$ (WT: 1.4 ± 0.2 pA/pF, n = 7; KO: 1.2 ± 0.2 pA/pF, n = 8; at +40 mV; p > 0.05) and Cav1.2 (WT: 0.34 ± 0.06 pA/pF, n = 3; KO: 0.36 ± 0.10 pA/pF, n = 6; at +10 mV; p > 0.05) in the aortic myocytes from KO mice was similar to that from WT mice, respectively. EGTA-resistant current was detected in the WT but not KO in aortic myocytes (in WT, 1.3 ± 0.3 pA/pF with EGTA (n = 7), 0.3 ± 0.1 pA/pF with BAPTA (n = 7), p < 0.05; in KO, 0.3 ± 0.1 pA/pF with EGTA (n = 8), 0.4 ± 0.2 pA/pF with BAPTA (n = 8), p > 0.05). Taken together, it can be suggested that caveolin-1 deficiency diminished the tight-coupling between BK$_{Ca}$ and Cav1.2 in freshly isolated arterial SMCs regardless of the vessel diameter.

BK$_{Ca}$ and Cav1.2 Form Molecular Complex in Caveolae of VSMCs—To visualize BK$_{Ca}$-Cav1.2 molecular complex in VSMCs and investigate the contribution of caveolin-1 to the complex formation, we performed TIRF microscopy molecular imaging (30).

First, BK$_{Ca}$ and LVDCC in freshly isolated mesenteric SMCs were stained with an anti-BKα antibody and DM-BODIPY (−)-dihydropriodine, respectively. In TIRF microscope images, fluorescent particles were detected by the Alexa405 and BODIPY dyes binding to these channels in the plasma membrane (Fig. 3A). A part of the particles from the BK$_{Ca}$ were co-localized with those from LVDCC in both WT and KO myocytes, but the ratio of co-localized particles against total BK$_{Ca}$ particles was significantly lower in KO than WT myocytes (WT: 45.8 ± 1.9%, n = 8; KO: 24.1 ± 8.0%, n = 7; p < 0.05; Fig. 3B).
Next, BKα and VDCCα1C, which were labeled with YFP and CFP at the C termini (BKα-YFP and Cav1.2-CFP, respectively), were transiently co-expressed in SMCs isolated from mesenteric artery (Fig. 3, 4) and aorta, which were then primary-cultured. The transient expression of the labeled molecules was carefully performed by regulating the amount of cDNA to minimize the artifacts due to overexpression (see "Experimental Procedures"). Thus, the influence of transient expression of BKα-YFP on the functional expression of BKCa was examined in aortic myocytes. BKα-YFP or YFP alone were transiently expressed in aortic myocytes from WT mice, and the functional expression of BKCa was measured by the whole cell patch clamp recording. The pipette filling the solution contained 10 mM BAPTA to minimize the influence by changes in intracellular Ca\(^{2+}\) concentration by Ca\(^{2+}\) influx and release. The density of Pax-sensitive currents was 1.4 ± 0.3 pA/pF at +40 mV (n = 4) in the control myocytes (i.e. expressing YFP alone; mean cell capacitance of 15.3 ± 0.3 pF) and 1.1 ± 0.2 pA/pF (n = 6, p > 0.05 versus control) in BKα-YFP-expressing myocytes (mean cell capacitance of 17.3 ± 1.1 pF). The density of BKCa current in aortic myocytes was not significantly changed by the expression of BKα-YFP.

The detection of single molecule or the cluster of BKα-YFP alone, Cav1.2-CFP alone, and BKα-YFP/Cav1.2-CFP co-localization was performed as shown by the green, red, and yellow dots in the TIRF images, respectively (Fig. 3C and supplemental Movies 1 and 2). The ratio of the co-localization against BKα-YFP alone was compared between mesenteric arterial SMCs from KO and WT (Fig. 3D). The ratio of co-localization was significantly smaller in KO than WT (WT: 4.61 ± 0.91, n = 10; KO: 2.09 ± 0.7, n = 9; p < 0.05).

To detect protein-protein interactions between BKCa and Cav1.2, FRET analyses were performed using the acceptor photobleaching method (31) (Fig. 4). The validity of the system was confirmed with HEK293 cells co-expressing BKα-YFP and BKCa-CFP as a positive control. We also co-expressed BKα-YFP and CFP-labeled Nav1.5 (CFP-Nav1.5) or expressed Cav1.2-CFP alone as a negative control (BKα-YFP+BKCa-CFP: 18.2 ± 1.8%, n = 5; BKα-YFP+CFP-Nav1.5: 2.5 ± 2.7%, n = 8; Cav1.2-CFP: −0.7 ± 2.2%, n = 14).

In WT myocytes expressing BKα-YFP and Cav1.2-CFP, the fluorescence intensity of Cav1.2-CFP was increased after the bleaching of BKα-YFP. The increase in Cav1.2-CFP fluorescence intensity was attenuated in KO myocytes (Fig. 4A). The E\(_{\text{FRET}}\) values shown in Fig. 4A were 15.3% in the WT and 0% in the KO. As summarized in Fig. 4B, E\(_{\text{FRET}}\) was significantly smaller in the KO than WT myocytes (WT: 9.6 ± 1.5%, n = 34; KO: 3.3 ± 1.8%, n = 26; p < 0.05). Similar data were obtained in aortic myocytes (WT: 8.3 ± 1.2%, n = 23; KO: 0.0 ± 2.0%, n = 29; p < 0.01). In addition, BKα, which was labeled with YFP at the extracellular N terminus (YFP-BKα), was also used for FRET analyses. No FRET interaction was detected between YFP-BKα and Cav1.2-CFP (WT: −4.0 ± 1.0%, n = 10; KO: 0.1 ± 1.1%, n = 4). These data indicate that BKCa directly interacts with Cav1.2 in VSMCs, and caveolin-1 facilitates the formation of the complex.
The direct couplings between caveolin-1 and BK<sub>Ca</sub>, and caveolin-1 and LVDC in mesenteric SMCs were also examined by FRET analyses (Fig. 4C). Myocytes expressing only CFP-caveolin-1 were used as a negative control (1.6 ± 2.0%, n = 11). FRET interaction was observed in cells expressing CFP-caveolin-1 + BK<sub>α</sub>-YFP (12.0 ± 3.0%, n = 9, p < 0.01) and CFP-caveolin-1 + Cav1.2-YFP (8.8 ± 1.6%, n = 13, p < 0.05).

**Molecular Assembly of Cav1.2 and BK<sub>Ca</sub> Complex and the Contribution of Caveolin-1**—Furthermore, we applied single-molecule fluorescence bleaching analyses (30, 32, 37) to clarify the number of GFP-tagged BKα and VDCCα1C in the single fluorescent particles. Again, to minimize the artifacts of overexpression, the expression of GFP labeled BK<sub>Ca</sub> and/or Cav1.2 was kept relatively low.

At first the usefulness and accuracy of the system for single-molecule fluorescence bleaching analyses were verified using HEK293 cells expressing Cav1.2-GFP. Single Cav1.2 as an α1C-subunit can form a functional channel on its own without other Ca<sup>2+</sup> channel subunits (37) (Fig. 5A). The majority of fluorescent particles displayed a single bleaching step (1 step, 79.3 ± 5.3%; 2 steps, 18.4 ± 5.2%; 3 steps, 2.4 ± 2.4%; 4 steps, 0%, analyzed from 46 spots from 6 cells; Fig. 5B). A small part of spots bleached in two or three steps, and these probably arose from the rare co-localization of channels within a diffraction-limited area (37).

In the WT and KO myocytes, the fluorescent particles of BK<sub>α</sub>-GFP exhibited mainly 1–4 bleaching steps (1 step, 35.7 ± 8.8 and 41.6 ± 9.2%; 2 steps, 23.8 ± 5.1 and 26.2 ± 8.6%; 3 steps, 25.3 ± 9.4 and 19.3 ± 6.4%; 4 steps, 15.2 ± 4.4 and 12.9 ± 8.4%, analyzed from 39 and 22 spots in 5 and 4 myocytes from WT and KO mice, respectively) (Fig. 5, C–E). These results confirmed our previous observation that only a part of BK<sub>Ca</sub>s contains BKα-GFP as components of the tetramer with 1–3 native BKα molecules in mesenteric arterial myocytes under the experimental conditions in this study (30).

A single Cav1.2 molecule is thought to form the LVDCC channel pore as the α1C subunit as shown in Fig. 5A. Thus, it was a rather unexpected result that many of the fluorescent particles of Cav1.2-GFP exhibited more than 1 bleaching step (1 step, 32.0 ± 7.5 and 50.4 ± 6.9%; 2 steps, 27.9 ± 8.5 and 30.9 ± 4.7%; 3 steps, 20.5 ± 5.3 and 12.4 ± 3.3%; 4 steps, 19.4 ± 3.8 and 6.3 ± 2.9%, p < 0.05), analyzed from 57 and 77 spots in 7 and 9 myocytes from WT and KO mice, respectively (Fig. 5, C–E). Notably, the proportion of the particles exhibiting four steps of Cav1.2-GFP bleaching in KO myocytes was significantly smaller than that in WT myocytes. Overall the number of bleaching steps in KO myocytes tended to be smaller than WT myocytes. These data may suggest that Cav1.2 preferentially formed homo-clusters. However, based on the finding that BK<sub>Ca</sub>s forms a complex with Cav1.2 in caveolae, it is more likely that each BK<sub>α</sub> molecule of the tetramer formation in a BK<sub>Ca</sub>s interacts with a single Cav1.2 to form hetero-clusters as a consequence.

**Smaller STOC Frequency in KO Than in WT Myocytes**—To examine the contribution of caveolae to the loose coupling between BK<sub>Ca</sub> and RyR, we performed simultaneous recordings of Ca<sup>2+</sup> sparks and STOCs at −40 mV in mesenteric arterial SMCs freshly isolated from WT and KO mice. Typical TIRF images are shown in supplemental Movies 3 and 4. Changes in the membrane currents and cytosolic Ca<sup>2+</sup> levels at the sites indicated by circles in the images were shown in Fig. 6A. The STOC frequency in the KO myocytes (1.1 ± 0.2 Hz, n = 10) was significantly smaller than the WT (2.4 ± 0.4 Hz, n = 5; p < 0.01) (Fig. 6B). The averaged STOC amplitude in the WT myocytes (25.1 ± 1.7 pA, n = 5) was similar to that in KO (29.7 ± 3.5 pA, n = 10; p > 0.05) (Fig. 6B). On the other hand, the Ca<sup>2+</sup> spark frequency (1.22 ± 0.06 Hz, n = 5) and amplitude (1.3 ± 0.2%, n = 5) in WT myocytes were comparable with those in KO (0.99 ± 0.10 Hz, n = 10; 1.8 ± 0.3%, n = 10, respectively; p > 0.05, Fig. 6C). These results suggest that the lack of caveolae attenuates the coupling between BK<sub>Ca</sub> and RyR and thereby reduces the efficacy to translate Ca<sup>2+</sup> spark signals to electrical STOC signals.

**Attenuated Negative Feedback of Ca<sup>2+</sup> Regulation via BK<sub>Ca</sub> Activation in KO Myocytes**—Next, we examined the contribution of caveolin-1 to the regulation of SMC contraction via BK<sub>Ca</sub> activity. When 40 KCl was applied to freshly isolated mesenteric arterial SMCs, the contraction in the KO myocytes (−10.3 ± 1.6%, n = 19) was larger in WT myocytes (−5.0 ± 0.9%, n = 21; p < 0.01) (Fig. 7, A and B). Ca<sup>2+</sup> imaging showed that this difference was attributable to the enhanced Ca<sup>2+</sup> rise in KO myocytes (the increase in fluorescent intensity in WT, 6.2 ± 1.4% n = 8; KO, 12.8 ± 2.2%, n = 9, p < 0.05) (Fig. 7, C and D). The application of 1 μM Pax induced a smaller contraction in the KO myocytes (−2.6 ± 0.3%, n = 20) than in the WT.
An additional application of 40 KCl generated a comparable contraction in the WT myocytes (11.5 ± 2.3%, n = 12; p < 0.01) (Fig. 7E). An additional application of 40 KCl generated a comparable contraction in the WT myocytes (11.5 ± 2.3%, n = 12) and KO (−11.1 ± 2.0%, n = 20; p > 0.05) (Fig. 7E). These results suggest that depolarization-induced cell contraction in KO myocytes is larger than that in WT myocytes, because the negative feedback mechanism by BKCa activation to membrane excitation was attenuated in KO myocytes.

**DISCUSSION**

This report is, to our knowledge, the first to demonstrate that caveolin-1 facilitates molecular interaction between Cav1.2 and BKCa and their accumulation as a molecular complex in caveolae in living VSMCs. It is suggested that each of tetrameric BKα subunits, which form a functional BKCa, can directly interact with Cav1.2 molecule to promote Cav1.2 clustering in the molecular complex in caveolae.

Several technologies, including analyses using patch clamp, biochemical procedures, mass spectrometry, and morphological analyses, have been applied to determine the molecular components and their molecular and/or functional couplings in Ca2+ microdomains (1, 2, 20). To date, molecular interaction between two molecules among BKCa, Cav1.2, and caveolin-1 has been reported in several types of cells including expression systems. We also showed that caveolin-1 directly interacts with BKCa or Cav1.2 with FRET analysis. The caveolin-1 N terminus contains a caveolin-1 scaffolding domain (9) that interacts with various types of signaling molecules containing the caveolin-1 scaffolding domain.
binding motif (10, 38). Caveolin-1 binding motifs are characterized as ΦXΦXXXΦ and ΦXXXΦXXΦ, where Φ is an aromatic amino acid, and X is any amino acid (38). It has been reported that BKCa has a caveolin-1 binding motif (1042YNML1047) within its C terminus and presumably accumulates in caveolae (10, 11). Caveolin-3, which is mainly expressed in skeletal muscle, also directly interacts with BKCa at the same binding site and presumably contributes to form the dystrophin microdomain (23).

VDCCα1C also contains the motif (1479FDYLTRDW1486) at the C terminus, and its association with caveolin-1 and caveolin-3 has been reported in several different tissues (39, 40). Although the binding sites remain to be determined, the functional tight-coupling between BKCa and Cav1.2 and their co-immunoprecipitation have been demonstrated in neurons, oocytes, and CHO cells (18). In this study the direct interaction between BKCa and Cav1.2 was first visualized by FRET analyses in living cells. In addition, their functional coupling was demonstrated in mesenteric and aortic SMCs using two different Ca2+ chelators, EGTA and BAPTA, in combination with the whole cell patch clamp recordings as has been shown in neurons (1, 18, 20). Because caveolin-1 deficiency resulted in caveolae disruption, a significant decrease in EGTA-resistant BKCa current density, co-localization ratio, and FRET efficiency between BKα-YFP and Cav1.2-CFP, it is clear that caveolin-1 facilitates the BKCa-Cav1.2 interaction in VSMCs. It was, however, also suggested that caveolin-1 is not an essential factor for BKCa-Cav1.2 tight coupling, because a small but nonetheless significant amount of coupling was observed in caveolin-1 KO. Caveolin-1 may indirectly promote BKCa-Cav1.2 complex formation by offering a platform for both BKCa and Cav1.2 to be accumulated in caveolae and prove higher probability to BKCa-Cav1.2 complex formation in caveolae than in other cell membrane areas.

Single-molecule bleaching analyses revealed that single fluorescent spots derived from BKα-GFP included 1–4 bleaching steps in VSMCs. These findings confirm the previous observation that a tetrameric BKα assembly included 1–4 fluorescent protein-labeled BKαs in VSMCs (30). On the other hand, a portion of the fluorescent particles of Cav1.2-GFP also exhibited more than one bleaching step in VSMCs, although only one VDCCα1C subunit can act as a functional Ca2+ channel in HEK293 cells. The possibility that the BKCa-Cav1.2 complex may consist of tetrameric BKα assembly and four Cav1.2 channels has been speculated from the consequence of two-dimensional gel electrophoresis (18) and in a review (20). The result reported here that single fluorescent spots from Cav1.2-GFP exhibited multistep bleaching in mesenteric arterial myocytes provides the first direct evidence supporting this hypothesis (Fig. 8A). It has been also demonstrated that Cav1.2 can interact with nearby Cav1.2 channels via their C termini, and this interaction enables coupled gating of these channels in arterial myocytes (41). In the present study, however, the number of Cav1.2-GFP within single fluorescent particles analyzed by the step-bleaching in mesenterial arterial myocytes of KO appeared to be apparently smaller than WT. There are two limitations in this approach (37). (i) When a single fluorescent spot consists of a substantial number of multimers (>10), the detection of discrete steps becomes more difficult. In this case, the number of multimers can be estimated from the size of a single bleaching step and the total fluorescence, but the accuracy of this estimation may be limited. (ii) The other is that the GFP fluorescence emission occurs at the probability of ~80% in distinct fusion proteins when being excited continuously. This may result in the underestimation of the number.

In addition, SMCs from animal tissues, which expressed endogenous channels, were used in this study. In the preparation, fluorescent spots contained native subunits without GFP labeling that led to the underestimation of the subunit number within a single spot. This meant that the distribution histogram shown in Fig. 5E may be shifted to the left by the included native subunit (i.e. fluorescent spots contain more number of subunits than that estimated by bleaching steps counting). Taken together, it can be strongly suggested that caveolin-1 localizes/accumulates Cav1.2 and BKCa in caveolae and promotes effective coupling between these channels and their clustering, particularly that of Cav1.2 (Fig. 8B).

The tight-coupling between Cav1.2 and BKCa and their accumulation in the caveolae of VSMCs may be much more signif-
BKCa-Cav1.2 Complex in Caveolae

![Diagram of BKCa-Cav1.2 complex in caveolae](image)

**FIGURE 8.** BKCa-Cav1.2 complex in VSMCs. A, BKCa-Cav1.2 complex consists of one BKCa (a tetrameric BKα assembly) and four Cav1.2 channels. In caveolin-1-deficient (KO) myocytes, the interaction between BKCa and Cav1.2 is attenuated by following reasons. The BKCa-Cav1.2 complex, which is facilitated by the accumulation of these channels in caveolae by the interaction with caveolin-1, is reduced in KO myocytes. The number of Cav1.2 within the BKCa-Cav1.2 complex is also reduced in KO myocytes. B, BKCa can be activated by three steps of Ca2+ increase. The first is Ca2+ influx from tightly coupled Cav1.2. The second is Ca2+ release from loosely coupled RyR, i.e., CICR. The third step is global Ca2+ level elevation evoked by the Ca2+ wave. We propose that large portions of the BKCa-Cav1.2 complexes are localized in caveolae and that the complex of 1 BKCa with 4 Cav1.2 cluster may contribute to initiate Ca2+ local events in caveolae as the key membrane structure in Ca2+ microdomain, which, however, includes the effective negative feedback mechanism for cytosolic Ca2+ regulation.

...that in the KO myocytes. It is thus strongly suggested that the enhancement of BKCa-Cav1.2 tight-coupling by caveolin-1 significantly contributes to the negative feedback regulation of membrane excitability and contraction in mesenteric arterial SMCs. RhoA and Rho kinase cause Ca2+-induced Ca2+ sensitization in response to depolarization (43). Because caveolin-1 attenuates RhoA activity (44, 45), caveolin-1 deficiency may activate RhoA and induce synergistic enhancement of tonic contraction. Thus, the augmentation of two pathways, (i) CICR and (ii) Ca2+-induced Ca2+-sensitization in KO cells, may enhance smooth muscle cell contraction.

So far the information about the relation between caveolae and STOC generation is controversial; both an increase (36) and decrease (14) in STOC frequency have been demonstrated upon caveolae disruption. In the present study data from a simultaneous recording of Ca2+ sparks and STOCs revealed that the characteristics of the Ca2+ sparks were unchanged, but the frequency of STOCs was decreased. In single myocytes, Pax induced a smaller contraction in KO than WT myocytes. This finding strongly suggests that cell excitability and contractility in the resting state are higher in KO than WT myocytes. It can be speculated that caveola deficiency makes BKCa more inaccessible to the RyR (13), and this leads to the lower frequency of detectable STOCs. Thus, caveolae and caveolin-1 play obligatory roles in both tight and loose coupling in the Ca2+ microdomain of SMCs. In addition to Ca2+ sparks, Ca2+-sparklets have been known as a quantal factor in Ca2+ elevation by single LVDCC opening at rest (46, 47). In cells where BKCa-Cav1.2 tight coupling is present, STOCs due to Ca2+-sparklets may concomitantly occur.

Guia et al. (48) have revealed that in coronary myocytes, Ca2+ influx through LVDCC directly activated BKCa, at −30 mV in the presence of BayK8644, ryanodine, and cyclopiazonic acid using the cell-attached patch clamp technique. This means that the Ca2+ sparklet may activate the tight-coupled BKCa around the resting membrane potential and induce STOCs. However, these experiments were performed under limited conditions, where LVDCCs were well activated by BayK8644. Therefore, it is unclear whether Ca2+ sparklets evoke or potentiate STOCs under physiological conditions. The major contributor to the STOCs generation in the resting state may be Ca2+ sparks rather than Ca2+-sparklets, because the amplitude of the BKCa current due to a sparklet may be too small to explain the STOC amplitude in arterial myocytes.

In urinary bladder SMCs (49), Ca2+-sparklets often trigger CICR and subsequent BKCa current activation mainly via loose coupling, but a smaller component that is due to direct BKCa activation is also included. In the present study, however, the simultaneous measurement of Ca2+ sparklet and STOCs was not systematically performed, and this remains to be determined.

Caveolin-1 acts as an endothelial nitric-oxide synthase inhibitor; accordingly, caveolin-1 ablation induces an elevation of the NO level (14). On the other hand, caveolin-1 deficiency causes remodeling of resistant vessels (50) and increases the...
responsiveness to adrenergic stimulation by an elevation in protein kinase C activity (44). Furthermore the production of endothelin-derived hyperpolarization factor is impaired in the KO artery because the activities of connexin 43 and transient receptor potential vanilloid 4 (TRPV4) in the endothelium are attenuated (51). It has been also reported that the number of caveolae is reduced in endothelial cells of hypertensive rat aorta (52) and that the ratio of caveolin-1 dimer is decreased in mesenteric arterial myocytes from spontaneously hypertensive rats (53), i.e. hypertension disassembles caveolae. Our data from single isolated myocytes suggest that the lack of caveolae reduced the BKCa-Cav1.2 complex and BKCa activation by Ca\({}^{2+}\) influx via Cav1.2. The negative feedback mechanism for Cav1.2 activity regulation may, therefore, be reduced in KO myocytes. It is apparent that the effects of caveolin-1 deficiency on systemic arterial pressure are much more complex.

In conclusion, BKCa activation upon depolarization takes place by three distinct steps of Ca\(^{2+}\) increase (Fig. 8B). The first step is Ca\(^{2+}\) influx through tightly coupled Cav1.2. The second step is local CICR via loosely coupled RyR in discrete sarcoplasmic reticula. The third step is global Ca\(^{2+}\) level elevation due to CICR conduction in whole myocytes. It was demonstrated that large portions of the BKCa-Cav1.2 complexes are accumulated in caveolae by the interaction with caveolin-1 serving as a microdomain that plays an obligatory role in the control of Ca\(^{2+}\) signaling, excitability, and contractility in VSMCs.

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