Facilitated hyperpolarization signaling in vascular smooth muscle overexpressing TRIC-A channels*

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*Running title: Tric-a-overexpressing vascular smooth muscle

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Keywords: Blood pressure, Ca^{2+}-dependent K^+ channel, Ca^{2+} spark, inositol trisphosphate receptor, intracellular Ca^{2+} store, ryanodine receptor, TRIC channel, vascular smooth muscle

Background: TRIC channels likely mediate counter-K^+ movements during store Ca^{2+} release, but their roles are unknown in many cell types.

Results: Smooth muscle-specific Tric-a-transgenic mice developed hypotension due to facilitated hyperpolarization signaling in vascular muscle.

Conclusion: TRIC-A channels facilitate Ca^{2+} spark generation in vascular muscle.

Significance: TRIC-A channels may have unexplained roles in store Ca^{2+} distribution but provide a potential pharmaceutical target for vasomodulation.

ABSTRACT

The TRIC channel subtypes, namely TRIC-A and TRIC-B, are intracellular monovalent cation-specific channels and likely mediate counter-ion movements to support efficient Ca^{2+} release from the sarco/endoplasmic reticulum. Vascular smooth muscle cells (VSMCs) contain both TRIC subtypes and two Ca^{2+} release mechanisms; incidental opening of ryanodine receptors (RyRs) generates local Ca^{2+} sparks to induce hyperpolarization and relaxation, whereas agonist-induced activation of inositol trisphosphate (IP_3) receptors produces global Ca^{2+} transients causing contraction.
Tric-a-knockout mice develop hypertension due to insufficient RyR-mediated Ca\(^{2+}\) sparks in VSMCs. Here we describe transgenic mice overexpressing TRIC-A channels under the control of an SMC-specific promoter. The transgenic mice developed congenital hypotension. In Tric-a-overexpressing VSMCs from the transgenic mice, the resting membrane potential decreased because RyR-mediated Ca\(^{2+}\) sparks were facilitated and cell-surface Ca\(^{2+}\)-dependent K\(^{+}\) channels were hyperactivated. Under such hyperpolarized conditions, L-type Ca\(^{2+}\) channels were inactivated, and thus, the resting intracellular Ca\(^{2+}\) levels were reduced in Tric-a-overexpressing VSMCs. Moreover, Tric-a overexpression impaired IP\(_3\)-sensitive stores to diminish agonist-induced Ca\(^{2+}\) signaling in VSMCs. These altered features likely reduced vascular tonus leading to the hypotensive phenotype. Our Tric-a-transgenic mice, together with Tric-a-knockout mice, indicate that TRIC-A channel density in VSMCs is responsible for controlling basal blood pressure at the whole-animal level.

INTRODUCTION

Two major classes of intracellular Ca\(^{2+}\) channels, inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) and ryanodine receptors (RyRs), provide the fundamental pathway for Ca\(^{2+}\) release from internal stores into the cytoplasm (1-4). IP\(_3\)R-mediated Ca\(^{2+}\) release from IP\(_3\)-sensitive stores in the sarco/endoplasmic reticulum (SR/ER) plays a central role in cellular signaling systems. RyR-mediated Ca\(^{2+}\) release from caffeine-sensitive stores also regulates important cellular functions, such as excitation-contraction coupling, secretion/exocytosis and gene expression, primarily in excitable cells. When the divalent cation Ca\(^{2+}\) is released from the SR/ER, a negative potential is likely generated on the luminal side and may inhibit subsequent Ca\(^{2+}\) release. Therefore, counter-ion movements may balance SR/ER membrane potential to establish efficient Ca\(^{2+}\) release mechanisms in various cell types (5, 6). Indeed, previous electrophysiological and fluorometric analyses detected several ionic currents, such as K\(^{+}\), Cl\(^{-}\) and H\(^{+}\) fluxes, across the SR/ER membrane (7-10). However, the channel/transporter components mediating such ionic flows have not been identified, and thus, their contributions to the proposed counter-ion movements are largely unknown.

Recently, we identified two TRIC (trimeric intracellular cation) channel subtypes, TRIC-A and TRIC-B, which assemble into bullet-shaped homo-trimers to form monovalent cation-selective channels in the SR/ER and nuclear membranes (11-13). Double-knockout mice lacking both the Tric-a and Tric-b genes show embryonic cardiac failure, and the mutant cardiomyocytes display weak RyR-mediated Ca\(^{2+}\) release from the SR (14). Tric-a-knockout mice develop hypertension associated with vascular hypertonicity, and the mutant vascular smooth muscle cells (VSMCs) show insufficient Ca\(^{2+}\) sparks for inducing hyperpolarization (15). Moreover, mutant skeletal muscle from Tric-a-knockout mice occasionally
exhibits alternan contraction responses, likely due to destabilized RyR-mediated Ca\(^{2+}\) release (16). On the other hand, Tric-b-knockout mice develop respiratory failure at birth, and the mutant alveolar epithelial cells exhibit compromised IP\(_3\)R-mediated Ca\(^{2+}\) release and insufficient handling of surfactant lipids (17). These defects observed in the knockout mice support our hypothesis that TRIC channel subtypes partly mediate counter-ion movements to facilitate SR/ER Ca\(^{2+}\) release.

VSMCs possess both caffeine- and IP\(_3\)-sensitive stores and also contain both TRIC-A and TRIC-B channels. Therefore, VSMCs are an ideal model system to examine the physiological functions of TRIC channel subtypes. We recently detected not only insufficient RyR-mediated Ca\(^{2+}\) sparks but also facilitated IP\(_3\)-R-mediated Ca\(^{2+}\) transients in Tric-a-knockout VSMCs (15). These observations led to the hypothesis that TRIC-A channels preferentially support RyR-mediated Ca\(^{2+}\) release and also regulate Ca\(^{2+}\) distribution between the caffeine- and IP\(_3\)-sensitive stores. To further verify this hypothesis, we planned to generate mutant mice carrying the SMC-specific Tric-a transgene and to examine altered features in Tric-a-overexpressing VSMCs.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice and Blood Pressure Monitoring** - Transgenic mice overexpressing TRIC-A channels under the control of the human \(\alpha\)-smooth muscle actin promoter were generated and genotyped as previously described (15). The transgenic and wild-type littermates (8-12 weeks old) were examined physiologically and biochemically in this study. All experiments were conducted with the approval of the Animal Research Committee at Kyoto University according to the regulations on animal experimentation at Kyoto University. To telemetrically monitor arterial blood pressure, PA-C10 transmitters (Data Sciences International, New Brighton, MN, US) were implanted into mice, and the pressure-sensing catheters were inserted into the aortic arch under anesthesia. Radio signals from the implanted transmitter were captured using the Physiotel RPC-1 receiver (Data Sciences International), and the data were stored online using the Dataquest ART data acquisition system (Data Sciences International). Blood pressure and heart rate in the conscious state were also monitored by tail-cuff plethysmography (Model BP-98A-L, Softron, Japan) during the daytime.

**Anatomical Analysis** - Histological and ultrastructural analyses were carried out as described previously (18). Briefly, mesenteric arteries from adult mice were fixed in 3% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.4). After the tissues were dehydrated and embedded in Epon, ultrathin sections (~80-nm thick) were prepared and stained with toluidine blue for histological observation or uranyl acetate and lead citrate for electron-microscopic analysis (JEM-200CX, JEOL).

**Imaging Analysis** - Single VSMCs were enzymatically isolated from mesenteric arteries and subjected to Ca\(^{2+}\) spark and membrane potential
monitoring as described previously (15, 19). Briefly, single VSMCs were seeded onto glass-bottomed dishes and incubated with 5 μM Fluo-4AM (Dojindo, Japan) in HEPES-buffered saline (HBS, in mM: 137 NaCl, 5.9 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 14 glucose and 10 HEPES, pH 7.4 with NaOH). The Ca²⁺ spark images with excitation at 488 nm and emission at >520 nm were captured using a total internal reflection fluorescence microscopy system (TE-2000U, Nikon), and the data were analyzed using the custom software Aquacosmos (Hamamatsu Photonics). For membrane potential monitoring, single VSMCs on glass-bottomed dishes were perfused with HBS containing 200 nM oxonol VI (Fluka). Fluorescence images with excitation at 559 nm and emission at >606 nm were captured at a sampling rate of ~1.6 s using a confocal microscope system (FV1000, Olympus).

For monitoring intracellular Ca²⁺ concentration ([Ca²⁺]i) in VSMCs, the inner surface of the artery was gently wiped with gauze to remove endothelial cells as described previously (20). The resulting arterial muscle segments were incubated with 5 μM Fura-PE3AM (Santa Cruz) and 0.02% cremophor EL (Sigma-Aldrich) and tightly fixed with fine steel pins onto a silicone rubber sheet, which was placed on a glass-bottomed dish. A CCD camera (ImagEM, Hamamatsu Photonics) mounted on the microscope (DMI 4000B, Leica) equipped with a polychromatic illumination system (MetaFluor ver7.7r3, Molecular Device) was used to capture fluorescence images with excitation at 340 and 380 nm and emission at >510 nm at room temperature (~23 °C).

**Patch-Clamp and Membrane Potential Recording** - Whole-cell voltage-clamp recording was carried out using the Axopatch 200B amplifier (Molecular Device) essentially as described previously (21). For spontaneous transient outward current (STOC) monitoring in single VSMCs, the STOC pipette solution (in mM: 140 KCl, 4 MgCl₂, 5 Na₂ATP, 0.05 EGTA and 10 HEPES, pH 7.2 with KOH) and HBS as a bathing solution were utilized. For the recording of total Ca²⁺-dependent K⁺ currents, the BK pipette solution (in mM: 140 KCl, 1 MgCl₂, 6.1 CaCl₂, 1 Na₂ATP, 10 EGTA and 10 HEPES, pH 7.2 with KOH, pCa 6.5) and HBS containing 100 μM CdCl₂ as a bathing solution were utilized. All physiological measurements were carried out at room temperature.

The arterial muscle segments were subjected to transmembrane potential monitoring with high-resistance glass microelectrodes (30–50 MΩ) filled with 3 M KCl solution as described previously (22). Membrane potential was determined as an average of the potentials obtained from stable impalements longer than 1 min from different areas in the muscle segment. The transmembrane potential was amplified by a high-input impedance amplifier with capacitance neutralization (MEZ-7200, Nihon Kohden, Japan), and the electrical signals were digitized and analyzed using a Power Lab system (AD Instruments, New Zealand). The bath solution used was modified Krebs solution bubbled with 95% O₂-5% CO₂ and maintained at 37 °C at pH ~7.4 (in mM: 112 NaCl, 4.7 KCl, 2.2 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, and 14 glucose).

**RT-PCR and Immunochemical Analysis** - For quantitative PCR analysis, total RNA preparations
from various tissues (at least three mice of each genotype) were used as templates for cDNA synthesis (ReverTra ACE qPCR-RT kit, Toyobo, Japan) and analyzed using a real-time PCR system according to the manufacturer’s instructions (Thermal Cycler TP800, Takara, Japan). The cycle threshold was determined from the cDNA amplification curve as an index for relative mRNA content in each reaction. The PCR primer sets used in this study were listed previously (15). For immunochemical analysis, tissue lysates and fixed VSMCs were examined using primary antibodies to TRIC channel subtypes and calnexin (Santa Cruz) as described previously (14, 18).

RESULTS

Hypotension in SMC-Specific Tric-a-Transgenic Mice – We previously generated transgenic mice with SMC-specific overexpression of TRIC-A channels using the α-smooth muscle actin promoter (23). Although the constructed Tric-a-transgene rescues the hypertensive phenotype developed in Tric-a-knockout mice (15), the effects of the transgene have not been examined in a wild-type genetic background. Therefore, we generated two Tric-a-transgenic mouse lines in wild-type backgrounds, which we designated as TgA3 and TgA20 mice. Both transgenic mouse lines grew and reproduced normally (Supplementary Fig. S1A). RT-PCR analysis demonstrated that the transgene was highly expressed in blood vessels and other tissues containing SMCs, and its expression was faintly detected in the liver and skeletal muscle (Supplementary Fig. S1B). In accordance with our previous observation that the transgene dosage in the TgA20 mouse line is higher than in the TgA3 line (15), TgA20 mice showed higher transgene expression in SMC-containing tissues than TgA3 mice in RT-PCR analysis (Supplementary Fig. S1C).

By telemetric blood pressure monitoring, both TgA20 and TgA3 mice, even at a young-adult age, showed low blood pressure levels throughout the entire day (Fig. 1A). This hypotensive phenotype was further confirmed by tail-cuff monitoring (Fig. 1B). Most likely reflecting the difference in transgene expression between the mouse lines, TgA20 mice developed slightly lower blood pressure than TgA3 mice. Elevated blood pressure stimulates the baroreceptor reflex and effectively decreases heart rate in normal animals (24). However, in both transgenic mice, a slow heart rate was clearly observed under hypotensive conditions. The blockage of cardiac autonomic controls by coapplication of the muscarinic blocker atropine and the β-adrenoceptor blocker metoprolol ameliorated the bradycardic phenotype, whereas low blood pressure was clearly maintained in the transgenic mice (Fig. 1B). Therefore, ectopic Tric-a expression in baroreceptors and/or autonomic neurons likely produced vagal-dominant states in both transgenic mice. Moreover, the α1-adrenoceptor blocker prazosin induced similar vasodilating effects in both wild-type and transgenic mice (Supplementary Fig. S1E). These observations suggest that impaired sympathetic stimuli to the heart and vascular smooth muscle were not significant contributors to hypotension in the transgenic mice. It may be
reasonable that altered vascular functions underlie the hypotensive phenotype in both transgenic mice because Tric-a-knockout mice develop hypertension due to increased vascular tonus (15).

**Tric-a-Overexpressing VSMCs from the Transgenic Mice** - Resistance arteries distributed to peripheral tissues are primarily responsible for blood pressure maintenance. The mesenteric artery is a typical resistance vessel and was examined in our studies below. By RT-PCR analysis of mesenteric arteries, the Tric-a mRNA levels in TgA20 and TgA3 mice were at least 400-fold greater than those of wild-type controls (Fig. 2A). Western blot analysis confirmed Tric-a overexpression, and enhanced immunoreactivities provided estimates that the TRIC-A protein levels in the arteries from the transgenic mice were increased more than 500-fold compared with wild-type arteries (Fig. 2B). However, neither the heart nor skeletal muscle from the transgenic mice showed an obvious enhancement of TRIC-A immunoreactivity (Supplementary Fig. S1D). Such SMC-specific overexpression of TRIC-A channels may not cause serious damage to the fundamental functions of resistance arteries because the transgenic mice retained circadian blood pressure variation (Fig. 1A) and regular blood pressure fluctuation in response to typical vasodilators targeting VSMCs (Supplementary Fig. S1E).

Histological observations revealed no obvious abnormalities in mesenteric arteries from TgA20 and TgA3 mice (Fig. 2C). For example, under relaxation conditions after incubation in a Ca^{2+}-free bathing solution, similar passive diameters and VSMC layer thicknesses of the arteries were observed in the transgenic and wild-type mice (Supplementary Fig. S2A). These observations suggest that the hypotensive phenotype was mainly caused by insufficient myogenic tonus of resistance arteries under *in vivo* conditions in the transgenic mouse.

Electron microscopic observation detected irregular membranous ultrastructures in mesenteric arteries from the transgenic mice. In Tric-a-overexpressing VSMCs, stacked rough ER elements accumulated around cell-interior regions (Fig. 2D, left panels). The generation of stacked rough ER has been repeatedly reported in various cell types overexpressing integral SR/ER membrane proteins (25, 26), and such irregular ER elements were not detected in wild-type VSMCs. Indeed, dense immunofluorescence signals of ectopic TRIC-A channels were predominantly observed in the cell-interior regions of VSMCs from transgenic mice (Supplementary Fig. S2B), suggesting that the ER stacks were generated by nonspecific effects of excess TRIC-A protein. In SMCs, IP_{3}Rs are distributed uniformly over the nuclear and ER/SR membranes (27, 28), whereas RyRs are predominantly localized in peripheral SR elements (29, 30). Therefore, it is possible that the formation of the stacked rough ER may have a functional impact on IP_{3}-sensitive stores. However, such a morphological abnormality may minimally affect caffeine-sensitive stores.

In VSMCs, free-floating small vesicles, originally referred to as “surface vesicles” (31), were abundantly arranged underneath the cell
membrane (Fig. 2D, right panels). Accompanied by the surface vesicles, larger-sized vacuoles with myelin figures (multilamellar lipid deposits) or without electron-dense material in the lumen were frequently observed in Tric-a-overexpressing VSMCs. Such vacuoles were rarely detected in wild-type VSMCs. In order to resolve the stacked ER elements, the large vacuoles may be aberrantly generated in Tric-a-overexpressing VSMCs. Alternatively, the excess amounts of TRIC-A protein may have promoted the swelling and fusion of normal small vesicles, generating the vacuoles. Swelling of SR elements is frequently accompanied by stored Ca$^{2+}$ overloading in skeletal and cardiac muscle (16, 32, 33), and RyR-mediated Ca$^{2+}$ sparks are predominantly generated underneath the cell membrane in VSMCs (3, 4). The facilitated formation of the vacuoles may imply altered Ca$^{2+}$-handling characteristics in caffeine-sensitive stores of Tric-a-overexpressing VSMCs.

Facilitated Hyperpolarization Signaling in Tric-a-Overexpressing VSMCs - In VSMCs, incidental activation of RyRs generates Ca$^{2+}$ sparks and then activates big-conductance Ca$^{2+}$-dependent K$^+$ (BK) channels to evoke STOCs, leading to hyperpolarization (3, 4). To identify the direct cause of the hypotensive phenotype, we investigated Ca$^{2+}$ spark and STOC generation in VSMCs from the transgenic mice because hyperpolarization signaling is impaired in Tric-a-knockout VSMCs (15). Our Ca$^{2+}$ spark imaging captured hyperactivated sparks at specified intracellular hotspots in single VSMCs prepared from the transgenic mice (Fig. 3A). Statistical analysis of the imaging data detected no difference in Ca$^{2+}$ spark amplitude between the genotypes. However, Ca$^{2+}$ spark frequency was substantially elevated and the number of spark hotspots was significantly increased in Tric-a-overexpressing VSMCs from both TgA20 and TgA3 mice (Fig. 3B-D). RT-PCR analysis detected no altered gene expression of the key components in intracellular Ca$^{2+}$ signaling and G protein-coupled receptor signaling, such as RyR, IP$_3$R, Ca$^{2+}$-pump protein, \( \alpha \)-adrenoceptor and phospholipase C, in mesenteric arteries and thoracic aorta from the transgenic mice (Supplementary Fig. S3A). Therefore, Tric-a overexpression appears to hyperactivate Ca$^{2+}$ spark generation without affecting the cellular levels of major Ca$^{2+}$-handling proteins in VSMCs.

For monitoring BK channel-mediated STOCs, single VSMCs were examined by patch-clamp recording using the nominally Ca$^{2+}$-free STOC pipette solution. VSMCs from the transgenic mice showed highly activated STOCs (Fig. 4A). In particular, both STOC frequency and amplitude were markedly increased at a holding potential of -60 mV near the resting potential in the Tric-a-overexpressing cells (Fig. 4B). We also monitored total K$^+$ currents using the Ca$^{2+}$-containing BK pipette solution and examined the effects of the BK channel inhibitor iberiotoxin on VSMCs. VSMCs from the transgenic mice showed normal levels of total K$^+$ currents both sensitive and insensitive to iberiotoxin (Supplementary Fig. S3B). Therefore, despite normal cell-surface densities of BK channels, STOC generation was likely facilitated under physiological
conditions in Tric-a-overexpressing VSMCs. The enhanced STOCs were fully consistent with the hyperactivated Ca\(^{2+}\) sparks observed (Fig. 3), suggesting that hyperpolarization signaling generated by functional crosstalk between the RyR and BK channels was stimulated in VSMCs from the transgenic mice.

**Deepened Resting Potential in Tric-a-Overexpressing VSMCs** - In SMCs, BK channel-mediated STOCs contribute to the maintenance of resting membrane potential and the fine-tuning of cellular excitability (3, 4). Based on the facilitated STOCs observed, we next focused on membrane potential in Tric-a-overexpressing VSMCs and leveraged confocal microscopic imaging using the voltage-dependent dye oxonol VI. Imaging analysis showed that fractional fluorescence intensity was clearly decreased in VSMCs from both TgA20 and TgA3 mice at basal conditions (Fig. 5A). The decreased intensities suggested that Tric-a-overexpressing VSMCs were deeply hyperpolarized during the resting state (Fig. 5B). We previously prepared the calibration plot representing the relationship between the fluorescence intensity and membrane potential using the reported resting potential of -59.9 mV in wild-type VSMCs (15, 34). The calibration plot estimated that the resting membrane potentials of TgA3, TgA20 and Tric-a-knockout VSMCs were -63.6, -63.9 and -54.6 mV, respectively. The estimated potentials agreed closely with actual measurement values from direct monitoring using high-resistance microcapillary electrodes; the detected resting potentials of wild-type, TgA20 and Tric-a-knockout VSMCs were -59.4 ± 0.6, -64.8 ± 1.0 and -53.7 ± 0.7 mV, respectively (Supplementary Fig. S3C). Moreover, from oxonol VI imaging, enlarged intensity shifts were evoked by iberiotoxin in VSMCs from the transgenic mice (Fig. 5C). The enhanced responses directly reflected the hyperactivation of BK channels at resting conditions in Tric-a-overexpressing VSMCs. However, while being exposed to iberiotoxin, similar fractional intensities were observed in wild-type and Tric-a-overexpressing VSMCs. Because many channels and transporters, in addition to BK channels, contribute to generating resting potential, the iberiotoxin-induced realignment of resting intensities suggested that the components other than BK channels functioned normally on the cell-surface of Tric-a-overexpressing VSMCs. In summary, these results suggest that specific hyperactivation of BK channels lowered resting membrane potential in VSMCs from the transgenic mice.

**Altered Ca\(^{2+}\) Handling in Tric-a-Overexpressing VSMCs** - Of the excitable cell types, SMCs have relatively shallow resting membrane potentials. In VSMCs at basal conditions, L-type voltage-gated Ca\(^{2+}\) channels generate persistent Ca\(^{2+}\) influx and maintain both resting [Ca\(^{2+}\)]\text{_{i}}\) and intrinsic tonus (3, 4). We examined cellular Ca\(^{2+}\) handling in VSMCs from the transgenic mice and found several abnormal features. First, our ratiometric Fura-PE3 imaging detected that resting [Ca\(^{2+}\)]\text{_{i}}\) was markedly decreased in VSMCs from the transgenic mouse (Fig. 6A and B). In accordance with the detected membrane potentials, TgA20 cells exhibited slightly lower
[Ca$^{2+}$]$_i$ than TgA3 cells. In a Ca$^{2+}$-free extracellular solution, similar resting [Ca$^{2+}$]$_i$ levels were observed in wild-type and Tric-a-overexpressing VSMCs. Moreover, the downward-adjusted resting [Ca$^{2+}$]$_i$ was clearly rescued by the L-type Ca$^{2+}$ channel inhibitor verapamil and the activator BayK8644 in Tric-a-overexpressing VSMCs (Fig. 6C). Therefore, L-type Ca$^{2+}$ channels were likely inactivated at basal conditions, and resting [Ca$^{2+}$]$_i$ was thus decreased in Tric-a-overexpressing VSMCs.

VSMCs contain both IP$\gamma$- and caffeine-sensitive stores, and they are functionally interconnected (3, 4). The SR/ER Ca$^{2+}$-pump inhibitor cyclopiazonic acid (CPA) causes Ca$^{2+}$ leakage from both stores, and the total stored Ca$^{2+}$ contents can be estimated from CPA-induced responses. CPA-induced Ca$^{2+}$ depletion from intracellular stores generally triggers store-operated Ca$^{2+}$ entry (SOCE) (35). VSMCs from the transgenic mice exhibited normal CPA-induced and SOCE responses (Fig. 6D and E). However, Tric-a-overexpressing VSMCs showed weak IP$_\gamma$R-mediated Ca$^{2+}$ release in response to the $\alpha$-adrenoceptor agonist phenylephrine (PE) (Fig. 6F and G). We also observed that PE-induced responses were diminished after normal caffeine-evoked transients in Tric-a-overexpressing VSMCs (Supplementary Fig. S4A). Furthermore, in response to sequential applications of PE and caffeine under Ca$^{2+}$-free conditions, Tric-a-overexpressing VSMCs exhibited poor IP$_\gamma$R-mediated transients immediately followed by enhanced RyR-mediated transients (Fig. 6H and I). It is unlikely that poor PE-induced transients were due to compromised phosphoinositide turnover signaling because RT-PCR analysis suggested normal expression of major components of adrenoceptor-mediated signaling in Tric-a-overexpressing VSMCs (Supplementary Fig. S3A). Therefore, in addition to facilitating Ca$^{2+}$ spark generation as described above, Tric-a overexpression appeared to affect IP$_\gamma$-sensitive stores in VSMCs.

**DISCUSSION**

Single-channel recording in planar lipid bilayers demonstrated that purified TRIC-A and TRIC-B proteins form monovalent cation-specific channels (12-14). Therefore, TRIC channel subtypes likely function as SR/ER K$^+$ channels under intracellular conditions. Although TRIC channels exhibit marked voltage dependence, they are likely to contribute to persistent K$^+$ flux when membrane potential and osmotic pressure fluctuate in the SR/ER (11). In our previous study, Tric-a-knockout mice displayed high vascular tonus and developed hypertension because the hyperpolarization signaling triggered by Ca$^{2+}$ sparks was compromised in the knockout VSMCs (15). In the present study, as a polar opposite of the TRIC-A-null model, SMC-specific Tric-a-transgenic mice developed hypotension, and Ca$^{2+}$ spark generation was facilitated in Tric-a-overexpressing VSMCs. Therefore, we reasonably conclude that TRIC-A channels support RyR-mediated Ca$^{2+}$ spark generation and contribute to setting the basal tonus in VSMCs. From a biophysical point of view, the density-dependent contribution of TRIC-A channels to Ca$^{2+}$ spark generation can be explained by an oversimplified scheme (Supplementary Fig. S4B).
Several lines of evidence indicate that RyR subtypes are nonselective cation channels equipped with large-diameter pores and can bidirectionally conduct Ca\(^{2+}\) and K\(^+\) (36). Our model tentatively assumes that only K\(^+\) provided by TRIC-A or RyR channels acts as a counter-ion for stabilizing SR membrane potential during Ca\(^{2+}\) release. In wild-type VSMCs, both TRIC-A and RyR channels may conduct counter-K\(^+\) to support Ca\(^{2+}\) release. However, the SR K\(^+\) currents are likely insufficient to stabilize membrane potential during vital Ca\(^{2+}\) release, and many events of incidental RyR opening may fail to grow to the Ca\(^{2+}\) sparks visualized in Ca\(^{2+}\)-imaging analysis. Even lower SR K\(^+\) currents are proposed for Tric-a-knockout VSMCs, and thus, the growth to detectable Ca\(^{2+}\) sparks is further attenuated by the TRIC-A-null conditions. In contrast, in Tric-a-overexpressing VSMCs, enhanced SR K\(^+\) currents may no longer attenuate the growth of Ca\(^{2+}\) sparks. Additionally, the excess K\(^+\) currents mediated by overexpressed TRIC-A channels may persistently prevent negative-shifting of the membrane potential toward the Ca\(^{2+}\) reversal potential and could effectively maintain Ca\(^{2+}\) release from partially depleted stores. Such biophysical mechanisms likely underlie the facilitated Ca\(^{2+}\) sparks observed in Tric-a-overexpressing VSMCs.

In the present study, several altered functions linked to the hypotensive phenotype were characterized in Tric-a-overexpressing VSMCs (Fig. 7). Excess TRIC-A channels facilitate Ca\(^{2+}\) spark (Fig. 3) and STOC generation (Fig. 4) and decrease resting membrane potential in VSMCs (Fig. 5). Under such hyperpolarized conditions, L-type Ca\(^{2+}\) channels are relatively inactivated, and resting [Ca\(^{2+}\)]\(_i\) is suppressed in Tric-a-overexpressing VSMCs (Fig. 6). Decreased [Ca\(^{2+}\)]\(_i\) in VSMCs likely reduces the intrinsic tonus of resistance arteries, leading to hypotension in the Tric-a-transgenic mice (Fig. 1).

VSMCs possess both caffeine- and IP\(_3\)-sensitive stores and contain both TRIC-A and TRIC-B channels with similar estimated expression levels (15). In accordance with previous studies (37-39), our Ca\(^{2+}\) imaging also detected that caffeine-induced responses were larger than agonist-induced responses in wild-type VSMCs from mesenteric arteries (Fig. 6K). In addition to the altered features directly linked to hypotension, Tric-a-overexpressing VSMCs exhibited an unexplainable abnormality in SR Ca\(^{2+}\) handling; PE-induced Ca\(^{2+}\) release was attenuated despite unchanged total Ca\(^{2+}\) content in the SR (Fig. 6D). In contrast, Tric-a-knockout VSMCs exhibit facilitated PE-induced Ca\(^{2+}\) release from the Ca\(^{2+}\)-overloaded SR (15). The alterations in PE-induced response appear to display a mirror image between Tric-a-overexpressing and knockout VSMCs (Supplementary Fig. S4A). Low Ca\(^{2+}\) contents of IP\(_3\)-sensitive stores may contribute to impaired PE-induced Ca\(^{2+}\) release in Tric-a-overexpressing cells because the sequential application of PE and caffeine implied poor loading in IP\(_3\)-sensitive stores (Fig. 6H). In this case, overexpressed TRIC-A channels may selectively gather Ca\(^{2+}\) into caffeine-sensitive stores to relatively deplete IP\(_3\)-sensitive stores in VSMCs. Because IP\(_3\)-R channels are activated by both high luminal and cytoplasmic Ca\(^{2+}\) (1, 38), lowered luminal and
cytoplasmic Ca$^{2+}$ levels may synergistically inhibit IP$_3$R channels in Tric-a-overexpressing VSMCs, whereas elevated Ca$^{2+}$ levels on both sides could facilitate IP$_3$Rs in Tric-a-knockout VSMCs. Alternatively, altered PE-induced responses could be explained by differentially modulated IP$_3$Rs in Tric-a-overexpressing and knockout VSMCs. In addition to generating counter-currents favorable to RyR activation, the TRIC-A protein might inhibit IP$_3$R channels in VSMCs. These postulated inhibitory effects might be enhanced to remarkably depress PE-induced Ca$^{2+}$ release in Tric-a-overexpressing VSMCs but might be removed to facilitate PE-evoked responses in Tric-a-knockout VSMCs. Furthermore, other molecular mechanisms can also be hypothesized for the altered PE-induced Ca$^{2+}$ release depending upon TRIC-A contents. To further investigate the mysterious role of TRIC-A channels in IP$_3$-sensitive stores, physiological measurements of the mutant VSMCs under membrane-permeable “skinned” conditions, together with comprehensive proteomic survey for its binding partners in Ca$^{2+}$ stores, would be required in future studies. From another point of view, it can be presumed that TRIC-A and TRIC-B channels may have distinct subtype-specific roles in SR Ca$^{2+}$ handling. To verify this possibility, Tric-b-overexpressing VSMCs, producible with the use of similar transgenic technology, will provide an important model system.

In our clinical study, the TRIC-A gene polymorphisms in the Japanese population were associated with both hypertension susceptibility and sensitivity to antihypertensive medications (15). As discussed above, TRIC-A channel density in VSMCs is an important determinant of basal blood pressure at the whole animal level. Therefore, the human hypertensive risk allele is likely associated with relatively low Tric-a expression in VSMCs. Hypertension represents a major health problem with an appalling annual toll, and various antihypertensive drugs have been developed. However, hypertension still remains resistant to multiple antihypertensive medications in a considerable number of patients. Although renal sympathetic denervation has recently come into use in patients with malignant hypertension, novel target proteins are still required for creating new medication strategies (40). Our transgenic mice, together with the knockout mice, suggest that TRIC-A channel openers may potentially be drugs with benefits for malignant hypertension.

Acknowledgments - We thank Miss Yuki Mizobe for her technical assistance and Dr. Masamitsu Iino for valuable comments on the experimental data.

REFERENCES

1. Bezprozvanny, I., and Ehrlich, B.E. (1995) The inositol 1,4,5-trisphosphate (InsP3) receptor. J. Membr. Biol. 145, 205–216

2. Meissner, G. (1994) Ryanodine receptor/Ca$^{2+}$ release channels and their regulation by endogenous
effectors. *Annu. Rev. Physiol.* **56**, 485–508

3. Berridge, M.J. (2008) Smooth muscle cell calcium activation mechanisms. *J. Physiol.* **586**, 5047–5061

4. Wellman, G.C., and Nelson, M.T. (2003) Signaling between SR and plasmalemma in smooth muscle: sparks and the activation of Ca\(^{2+}\)-sensitive ion channels. *Cell Calcium* **34**, 211–229

5. Somlyo, A.V., Gonzalez-Serratos, H.G., Shuman, H., McClellan, G., and Somlyo, A.P. (1981) Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron-probe study. *J. Cell Biol.* **90**, 577-594

6. Fink, R.H., and Veigel, C. (1996) Calcium uptake and release modulated by counter-ion conductances in the sarcoplasmic reticulum of skeletal muscle. *Acta Physiol. Scand.* **156**, 387–396

7. Coronado, R., and Miller, C. (1980) Decamethonium and hexamethonium block K\(^+\) channels of sarcoplasmic reticulum. *Nature* **288**, 495-497

8. Meissner, G. (1983) Monovalent ion and calcium ion fluxes in sarcoplasmic reticulum. *Mol. Cell. Biochem.* **55**, 65–82

9. Ide, T., Sakamoto, H., Morita, T., Taguchi, T., and Kasai, M. (1991) Purification of a Cl\(^-\)-channel protein of sarcoplasmic reticulum by assaying the channel activity in the planar lipid bilayer system. *Biochem. Biophys. Res. Commun.* **176**, 38-44

10. Kamp, F., Donoso, P., and Hidalgo, C. (1998) Changes in luminal pH caused by calcium release in sarcoplasmic reticulum vesicles. *Biophys. J.* **74**, 290-296

11. Venturi, E., Sitsapesan, R., Yamazaki, D., and Takeshima, H. (2013) TRIC channels supporting efficient Ca\(^{2+}\) release from intracellular stores. *Pflugers Arch.* **465**, 187-195

12. Pitt, S.J., Park, K-H., Nishi, M., Urashima, T., Aoki, S., Yamazaki, D., Ma, J., Takeshima, H., and Sitsapesan, R. (2010) Charade of the SR K\(^+\)-channel: two ion-channels, TRIC-A and TRIC-B, masquerade as a single K\(^+\)-channel. *Biophys. J.* **99**, 417–426

13. Venturi, E., Matyjaszkiewicz, A., Pitt, S.J., Tsaneva-Atanasova, K., Nishi, M., Yamazaki, D., Takeshima, H., and Sitsapesan, R. (2013) TRIC-B channels display labile gating: evidence from the TRIC-A knockout mouse model. *Pflugers Arch.* in press.

14. Yazawa, M., Ferrante, C., Feng, J., Mio, K., Ogura, T., Zhang, M., Lin, P-H., Pan, Z., Komazaki, S., Kato, K., Nishi, M., Zhao, X., Weisleder, N., Sato, C., Ma, J., and Takeshima, H. (2007) TRIC channels are essential for Ca\(^{2+}\) handling in intracellular stores. *Nature* **448**, 78–82

15. Yamazaki, D., Tabara, Y., Kita, S., Hanada, H., Komazaki, S., Naitou, D., Mishima, A., Nishi, M., Yamamura, H., Yamamoto, S., Kakizawa, S., Miyachi, H., Yamamoto, S., Miyata, T., Kawano, Y., Kamide, K., Ogihara, T., Hata, A., Umemura, S., Soma, M., Takahashi, N., Imaizumi, Y., Miki, T., Iwamoto, T., and Takeshima, H. (2011) TRIC-A channels in vascular smooth muscle contribute to blood pressure maintenance. *Cell Metab.* **14**, 231–241

16. Zhao, X., Yamazaki, D., Park, K-H., Komazaki, S., Tjondrokoesoemo, A., Nishi, M., Lin, P., Hirata, Y.,
Brotto, M., Takeshima, H., and Ma, J. (2010) Ca\(^{2+}\) overload and sarcoplasmic reticulum instability in tric-a null skeletal muscle. J. Biol. Chem. 285, 37370–37376

17. Yamazaki, D., Komazaki, S., Nakanishi, H., Mishima, A., Nishi, M., Yazawa, M., Yamazaki, T., Taguchi, R., and Takeshima, H. (2009) Essential role of the TRIC-B channel in Ca\(^{2+}\) handling of alveolar epithelial cells and in perinatal lung maturation. Development 136, 2355–2361

18. Takeshima, H., Komazaki, S., Nishi, M., Iino, M., and Kangawa, K. (2000) Junctophilins: a novel family of junctional membrane complex proteins. Mol. Cell 6, 11-22

19. Ohi, Y., Yamamura, H., Nagano, N., Ohya, S., Muraki, K., Watanabe, M., and Imaizumi, Y. (2001) Local Ca\(^{2+}\) transients and distribution of BK channels and ryanodine receptors in smooth muscle cells of guinea-pig vas deferens and urinary bladder. J. Physiol. 534, 313–326

20. Iwamoto, T., Kita, S., Zhang, J., Blaustein, M.P., Arai, Y., Yoshida, S., Wakimoto, K., Komuro, I., and Katsuragi, T. (2004) Salt-sensitive hypertension is triggered by Ca\(^{2+}\) entry via Na\(^+\)/Ca\(^{2+}\) exchanger type-1 in vascular smooth muscle. Nat. Med. 10, 1193–1199

21. Hotta, S., Morimura, K., Ohya, S., Muraki, K., Takeshima, H., and Imaizumi, Y. (2007) Ryanodine receptor type 2 deficiency changes excitation-contraction coupling and membrane potential in urinary bladder smooth muscle. J. Physiol. 582, 489-506

22. Kiyoshi, H., Yamazaki, D., Ohya, S., Kitsukawa, M., Muraki, K., Saito, S.Y., Ohizumi, Y., and Imaizumi, Y. (2006) Molecular and electrophysiological characteristics of K\(^+\) conductance sensitive to acidic pH in aortic smooth muscle cells of WKY and SHR. Am. J. Physiol. Heart Circ. Physiol. 291, H2723-2734

23. Nakano, Y., Nishihara, T., Sasayama, S., Miwa, T., Kamada, S., and Kakunaga, T. (1991) Transcriptional regulatory elements in the 5' upstream and first intron regions of the human smooth muscle (aortic type) alpha-actin-encoding gene. Gene 99, 285–289

24. Smith, O.A. (1974) Reflex and central mechanisms involved in the control of the heart and circulation. Annu. Rev. Physiol. 36, 93-123

25. Pathak, R.K., Luskey, K.L., and Anderson, R.G. (1986) Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum emerges from the nuclear envelope. J. Cell Biol. 102, 2158-2168

26. Snapp, E.L., Hegde, R.S., Francolini, M., Lombardo, F., Colombo, S., Pedrazzini, E., Borgese, N., and Lippincott-Schwartz, J. (2003) Formation of stacked ER cisternae by low affinity protein interactions. J. Cell Biol. 163, 257-269

27. Tasker, P.N., Michelangei, F., and Nixon, G.F. (1999) Expression and distribution of the type 1 and type 3 inositol 1,4,5-trisphosphate receptor in developing vascular smooth muscle. Circ. Res. 84, 536-542

28. Vermassen, E., Van Acker, K., Annaert, W.G., Himpens, B., Callewaert, G., Missaen, L., De Smedt, H., and Parys, J.B. (2003) Microtubule-dependent redistribution of the type-1 inositol 1,4,5-trisphosphate receptor in A7r5 smooth muscle cells. J. Cell Sci. 116, 1269-1277
29. Lesh, R.E., Nixon, G.F., Fleischer, S., Airey, J. A., Somlyo, A.P., and Somlyo, A.V. (1998) Localization of ryanodine receptors in smooth muscle. *Circ. Res.* **82**, 175-185

30. Moore, E.D., Voigt, T., Kobayashi, Y.M., Isenberg, G., Fay, F.S., Gallitelli, M.F., and Franzini-Armstrong, C. (2004) Organization of Ca\(^{2+}\) release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophys. J.* **87**, 1836-1847

31. Devine, C.E., Somlyo, A.V., and Somlyo, A.P. (1972) Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *J. Cell Biol.* **52**, 690-718

32. Ikemoto, T., Takeshima, H., Iino, M., and Endo, M. (1998) Effect of calmodulin on Ca\(^{2+}\)-induced Ca\(^{2+}\) release of skeletal muscle from mutant mice expressing either ryanodine receptor type 1 or type 3. *Pflugers Arch.* **437**, 43-48

33. Takeshima, H., Komazaki, S., Hirose, K., Nishi, M., Noda, T., and Iino, M. (1998) Embryonic lethality and abnormal cardiac myocytes in mice lacking ryanodine receptor type 2. *EMBO J.* **17**, 3309-3316

34. Koshita, M., Hidaka, K., Ueno, H., Yamamoto, Y., and Suzuki, H. (2007) Properties of acetylcholine-induced hyperpolarization in smooth muscle cells of the mouse mesenteric artery. *J. Smooth Muscle Res.* **43**, 219-227

35. Smyth, J.T., Hwang, S.Y., Tomita, T., DeHaven, W.I., Mercer, J.C., and Putney, J.W. (2010) Activation and regulation of store-operated calcium entry. *J. Cell. Mol. Med.* **14**, 2337-2349

36. Gillespie, D., Chen, H., and Fill, M. (2012) Is ryanodine receptor a calcium or magnesium channel? Roles of K\(^{+}\) and Mg\(^{2+}\) during Ca\(^{2+}\) release. *Cell Calcium* **51**, 427-433

37. Iino, M. (1990) Calcium release mechanisms in smooth muscle. *Jpn. J. Pharmacol.* **54**, 345-354

38. Iino, M. (2000) Molecular basis of spatio-temporal dynamics in inositol 1,4,5-trisphosphate-mediated Ca\(^{2+}\) signalling. *Jpn. J. Pharmacol.* **82**, 15-20

39. Bolton, T.B., Prestwich, S.A., Zholos, A.V., and Gordienko, D.V. (1999) Excitation-contraction coupling in gastrointestinal and other smooth muscles. *Annu. Rev. Physiol.* **61**, 85-115

40. Laurent, S., Schlaich, M., and Esler, M. (2012) New drugs, procedures, and devices for hypertension. *Lancet* **380**, 591-600

**FOOTNOTES**

This work was supported in part by grants from the MEXT (Grants-in-Aid for Scientific Research and Platform for Drug Design, Discovery and Development, the MHLW, the Vehicle Racing Commemorative Foundation, the Astellas Foundation, the Suzuken Memorial Foundation, the Kanae Foundation, the Japan Foundation for Applied Enzymology, the Takeda Science Foundation, the Ono Medical Research Foundation and the Kowa Life Science Foundation.
The abbreviations used are as follows: BK channel, big-conductance Ca\(^{2+}\)-dependent K\(^+\) channel; [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; HBS, HEPES-buffered saline; IP\(_3\)R, inositol trisphosphate receptor; PE, phenylephrine; RyR, ryanodine receptor; SOCE, store-operated Ca\(^{2+}\) entry; STOC, spontaneous transient outward current; SR, sarcoplasmic reticulum; VSMC, vascular smooth muscle cell.

**Figure legends**

**FIGURE 1.** Hypotension in SMC-specific Tric-a-transgenic mice. (A) Telemetric blood pressure monitoring. Circadian fluctuations in systolic blood pressure (SBP) were monitored, and the data were averaged over each 2-hr interval during a 24-hr period. (B) SBP and heart rate (HR) monitoring by tail-cuff plethysmography during the daytime. After the baseline monitoring (Ctrl), autonomic controls were blocked using an intraperitoneal injection of the muscarinic antagonist atropine (Atr, 4 mg/kg) and the β-antagonist metoprolol (Met, 4 mg/kg). Upon drug application, HR was quickly attenuated, but SBP changed in a time-dependent manner (see Supplementary Fig. S1E). The data represent the mean ± SEM, and the numbers of mice examined are shown in parentheses. Significant differences between the genotypes are marked with asterisks (*p<0.05, **p<0.01 by Student’s t-test).

**FIGURE 2.** Irregular membranous features of Tric-a-overexpressing VSMCs. (A) Quantitative detection of Tric-a mRNA in mesenteric artery (MA) and aorta (right panels). Total tissue RNA preparations from transgenic (#3 and #20) and wild-type mice (n=3) were examined by quantitative RT-PCR using a primer set for amplifying Tric-a mRNA from both the endogenous gene and the transgene. The cDNA fragments amplified by 33 PCR cycles were analyzed by agarose gel electrophoresis. RT-PCR data obtained are summarized in the left graph. The cycle threshold (C\(_t\)) indicates the cycle number at which the amount of amplified cDNA reached a fixed threshold in each reaction. (B) Western blot analysis of TRIC-A protein in MA and aorta (right panels). The vessels dissected from the Tric-a-transgenic (#3 and #20), Tric-a-knockout (A-/-) and wild-type mice were homogenized to prepare total cell lysates (post-nuclear fractions). Wild-type lysates (10 μg protein), with or without lysates from the transgenic mice (0.1 μg), were analyzed with an antibody against the TRIC-A protein. Lysates from the Tric-a-knockout mice (10 μg) served as negative controls. Arrowheads indicate TRIC-A protein bands. Immunoreactive signals were digitalized and statistically analyzed to estimate the relative contents of TRIC-A protein in the different genotypes as shown in the left graph. (C) Normal histology in MA from Tric-a-transgenic mice. MA preparations were incubated in a Ca\(^{2+}\)-free solution for ~20 min and then fixed for anatomical analysis. Thin sections were stained with toluidine blue for photomicroscopic observation. IVL, intravascular lumen. Scale bar, 10 μm. (D) Formation of stacked ER elements and vacuoles in Tric-a-overexpressing VSMCs. Rough ER elements at the
cell-interior portion (left panels: scale bar, 500 nm). Stacks of rough ER elements were frequently observed in VSMCs from transgenic mice (approximately 27% of TgA3 cells and 80% of TgA20 cells), whereas such stacked ER was not detected in wild-type controls (n = 45-97 cells from 3-6 mice in each genotype). Surface vesicles and vacuoles were located at the cell periphery (right panels: scale bar, 200 nm). TRIC-A overexpression appeared to promote the formation of large-sized vacuoles containing myelin figures (arrows) or no electron-dense materials (arrowheads). Such vacuoles were frequently observed in more than 50% of VSMCs from TgA3 and TgA20 mice, but only detected in 9.5% of wild-type VSMCs (n = 53-89 cells from 6 mice in each genotype).

FIGURE 3. Facilitated Ca\(^{2+}\) sparks in Tric-a-overexpressing VSMCs. Single VSMCs were prepared from mesenteric arteries and loaded with Fluo-4 for total internal reflection fluorescence imaging. (A) Representative Ca\(^{2+}\)-spark monitoring data in a normal bathing solution. The fluorescence intensity was normalized to the baseline intensity to yield the relative intensity (F/F\(_0\)), and time courses of the intensity changes at the subcellular hotspots (see colored circles in the F\(_0\) cell images) are illustrated in the traces (scale bar, 5 \(\mu\)m). The F/F\(_0\) images were color coded as indicated by the bar to prepare the Ca\(^{2+}\)-spark images at the numbered time points. The data on spark amplitude (B), frequency (C) and spot number (D) are summarized for each genotype. Significant differences between the genotypes are indicated by asterisks (**p<0.01 by t-test).

FIGURE 4. Facilitated STOCs in Tric-a-overexpressing VSMCs. The membrane potential of single VSMCs was controlled by the whole-cell patch-clamp technique using the STOC pipette to monitor membrane currents. Representative STOC recording data are illustrated in (A). The data for STOC frequency and amplitude are summarized in (B). The data represent the mean ± SEM, and the numbers of cells examined from at least 3 mice are shown in parentheses. Significant differences between the genotypes are indicated by asterisks (*p<0.05, **p<0.01 by t-test).

FIGURE 5. Decreased resting membrane potential in Tric-a-overexpressing VSMCs. Single VSMCs were examined by confocal microscopic imaging using the voltage-dependent dye oxonol VI. Cellular fluorescence intensities were normalized to the maximum value to yield the fractional intensity (F/F\(_{140K}\)). (A) Representative imaging data. (B) The summarized data of the resting intensity. (C) The summarized data of the intensity shift by iberiotoxin (IBTX). The data represent the mean ± SEM, and the numbers of cells examined from at least 3 mice are shown in parentheses. Significant differences between the genotypes are indicated by asterisks (**p<0.01 by t-test).

FIGURE 6. Abnormal Ca\(^{2+}\) handling in Tric-a-overexpressing VSMCs. VSMC segments were examined
by Fura-PE3 Ca$^{2+}$ imaging. The change in [Ca$^{2+}$]$_i$ was expressed as ratio of the fluorescence intensity (F$_{340}$/F$_{380}$). (A-C) Decreased resting [Ca$^{2+}$]$_i$ in Tric-a-overexpressing VSMCs. (A) Representative traces under normal conditions and responses to Ca$^{2+}$ removal and L-type Ca$^{2+}$ channel modulators are illustrated. Resting [Ca$^{2+}$]$_i$ data are summarized in (B), and [Ca$^{2+}$]$_i$ responses are analyzed in (C). (D-E) Normal Ca$^{2+}$ store contents in Tric-a-overexpressing VSMCs. Representative CPA-induced responses are shown in (D), and the data are summarized in (E). (F-G) Impaired IP$_3$-mediated Ca$^{2+}$ release in Tric-a-overexpressing VSMCs. Representative phenylephrine (PE)-induced responses are shown in (F), and the data are summarized in (G). (H-I) Altered Ca$^{2+}$ distribution to store compartments in Tric-a-overexpressing VSMCs. Sequential responses to PE and caffeine (Caf) under Ca$^{2+}$-free conditions are shown in (H), and the data are summarized in (I). The data represent the mean ± SEM, and the numbers of mice examined are shown in parentheses. Significant differences between the genotypes are indicated by asterisks (*$p<0.05$, **$p<0.01$ by t-test).

FIGURE 7. Facilitated hyperpolarization signaling in Tric-a-overexpressing VSMCs. Tric-a overexpression appears to activate Ca$^{2+}$ spark generation directly, thus facilitating the hyperpolarization signaling produced by functional coupling between RyR and BK channels and decreasing the resting membrane potential in VSMCs. In this situation, L-type Ca$^{2+}$ channels (Cav) are inactivated, and resting [Ca$^{2+}$]$_i$ is decreased to develop insufficient spontaneous tonus in resistance arteries and hypotension in the transgenic mice. Upon sympathetic stimulation, the $\alpha$1-adrenoceptor ($\alpha$1AR), trimeric GTP-binding protein G$q$ and phospholipase C (PLC) are coordinately activated to trigger IP$_3$R-mediated Ca$^{2+}$ release. In Tric-a-overexpressing VSMCs, IP$_3$R-mediated Ca$^{2+}$ release is unexpectedly impaired. The poor agonist-induced Ca$^{2+}$ release may be due to partial depletion of IP$_3$-sensitive stores or TRIC-A-mediated inhibition of IP$_3$Rs. In contrast, hyperpolarization signaling is impaired, and IP$_3$-sensitive stores are likely overloaded in Tric-a-knockout VSMCs (15).
Figure 1
Figure 2
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Figure 5
Figure 6
Figure 7
Facilitated hyperpolarization signaling in vascular smooth muscle overexpressing TRIC-A channels
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J. Biol. Chem. published online April 16, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.435396

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