TRPC1 Associates With BK_{Ca} Channel to Form a Signal Complex in Vascular Smooth Muscle Cells

Hiu-Yee Kwan,* Bing Shen,* Xin Ma, Yuk-Chi Kwok, Yu Huang, Yu-Bun Man, Shan Yu, Xiaoqiang Yao

Abstract—TRPC1 (transient receptor potential canonical 1) is a Ca^{2+}-permeable cation channel involved in diverse physiological function. TRPC1 may associate with other proteins to form a signaling complex, which is crucial for channel function. In the present study, we investigated the interaction between TRPC1 and large conductance Ca^{2+}-sensitive K^+ channel (BK_{Ca}). With the use of potentiometric fluorescence dye $DiBAC_4(3)$, we found that store-operated Ca^{2+} influx resulted in membrane hyperpolarization of vascular smooth muscle cells (VSMCs). The hyperpolarization was inhibited by an anti-TRPC1 blocking antibody T1E3 and 2 BK_{Ca} channel blockers, charybdotoxin and iberiotoxin. These data were confirmed by sharp microelectrode measurement of membrane potential in VSMCs of intact arteries. Furthermore, T1E3 treatment markedly enhanced the membrane depolarization and contraction of VSMCs in response to several contractile agonists including phenylephrine, endothelin-1, and U-46619. In coimmunoprecipitation experiments, an antibody against BK_{Ca} α-subunit [$BK_{Ca}(\alpha)$] could pull down TRPC1, and moreover an anti-TRPC1 antibody could reciprocally pull down $BK_{Ca}(\alpha)$. Double-labeling immunocytochemistry showed that TRPC1 and BK_{Ca} were colocalized in the same subcellular regions, mainly on the plasma membrane, in VSMCs. These data suggest that, TRPC1 physically associates with BK_{Ca} in VSMCs and that Ca^{2+} influx through TRPC1 activates BK_{Ca} to induce membrane hyperpolarization. The hyperpolarizing effect of TRPC1-BK_{Ca} coupling could serve to reduce agonist-induced membrane depolarization, thereby preventing excessive contraction of VSMCs to contractile agonists. (*Circ Res*. 2009;104:670-678.)

Key Words: TRPC1 ■ BK_{Ca} ■ physical coupling ■ hyperpolarization ■ vascular smooth muscle cells

TRPC1 (transient receptor potential canonical 1) is among the most-studied TRP channels.[1] The channel is widely expressed in many cell types,[1] and its activity is stimulated by depletion of intracellular Ca^{2+} stores,[1–4] receptor activation,[5–6] and mechanical stretch.[7] In VSMCs, TRPC1 is believed to be the main channel responsible for store-operated Ca^{2+} (SOC),[1–2] a type of Ca^{2+} influx that is activated by depletion of intracellular Ca^{2+} stores.[8] TRPC1 may coassemble with other TRP isoforms, including TRPC3, -P2, and -C4/-C5, to form heteromultimeric channels.[1,4] TRPC1 may also interact with other proteins to form a signaling complex. For an example, TRPC1 interacts with a Ca^{2+}-binding protein STIM1, and this interaction is crucial for TRPC1-mediated SOC.[3]

Functionally, TRPC1 activity is positively associated with VSMC proliferation[1] and neointimal hyperplasia.[9] However, there are conflicting reports regarding the role of TRPC1 in VSMC contraction.[10–12] Some chronic treatments, such as hypoxia and organ cultures, were found to enhance TRPC1

protein expression with a parallel increase in vascular contractility.[11–13] However, 2 other studies argued against any linkage between SOC and an increased vascular contractility in freshly isolated arteries.[10,14] In yet another study, blockage of TRPC1 activity by an anti-TRPC1 antibody T1E3 was found to reduce endothelin-1–induced contraction in rat basilar artery but not in rat caudal artery.[15]

TRP channels allow influx of positive ions such as Na^+ and Ca^{2+}. Such a cation influx is expected to result in membrane depolarization.[16] Indeed, it has been shown that activity of TRPC3, -C6, and -M4 results in membrane depolarization,[17] and this depolarization subsequently activates voltage-gated Ca^{2+} channels in VSMCs, leading to vascular contraction.[17] Interestingly, a recent study found that activation of another TRP channel, TRPV4, results in membrane hyperpolarization. In this instance, TRPV4 forms a Ca^{2+} signaling complex with ryanodine receptor and BK_{Ca}. Ca^{2+} influx through TRPV4 channel preferentially stimulates ryanodine receptor in the sarcoplasmic reticulum, generating

Ca^{2+} sparks that signal adjacent BK$_{Ca}$ to open and cause membrane hyperpolarization.[18] Such a coupling between BK$_{Ca}$-TRPV4-ryanodine receptor is believed to be the underlying mechanism for epoxyeicosatrienoic acid-mediated endothelium-derived hyperpolarizing factor activity in some vascular beds.[18]

In the present study, we investigated possible interaction between TRPC1 and BK$_{Ca}$ in VSMCs. Our results show that TRPC1 physically associates with BK$_{Ca}$ to form a signaling complex and that Ca^{2+} influx through TRPC1 activates BK$_{Ca}$ to induce membrane hyperpolarization in VSMCs. This hyperpolarizing effect of TRPC1-BK$_{Ca}$ coupling may serve to prevent excessive contraction of VSMCs to contractile agonists.

Materials and Methods

Cell Culture

All animal experiments were conducted in accordance with NIH publication no. 8523. Primary cultured VSMCs were isolated from male Sprague–Dawley rats. Briefly, thoracic aorta was dissected. After rubbing off endothelial layer, smooth muscle layers were peeled off and then digested with 0.2% collagenase type 1A and 0.9% papain for 1 hour. The dispersed VSMCs were cultured for 5 to 7 days before experimental use. VSMCs and HEK293 cells were both cultured in DMEM supplemented with 10% FBS.

$[Ca^{2+}]_i$ Measurement

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was measured as described elsewhere.[19] Briefly, cells were loaded with 10 μmol/L Fluo-3/AM. Ca^{2+} stores was depleted by treating VSMCs with 4 μmol/L thapsigargin for 6 to 8 minutes in a Ca^{2+}-free physiological saline (0Ca^{2+}-PSS), which contained (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl$_2$, 10 glucose, 0.2 EGTA, 5 Hepes, pH 7.4. Ca^{2+} influx was initiated by applying 1 mmol/L extracellular Ca^{2+}. The cells were pretreated with/without T1E3 (1:200) or antigen-preabsorbed T1E3 for 1 hour before experiments. Fluorescence signal was recorded by Fluoview FV1000 confocal laser scanning system. Changes in $[Ca^{2+}]_i$ were displayed as a ratio of fluorescence relative to the intensity before the application of extracellular Ca^{2+} (F1/F0).

Membrane Potential Measurement

For membrane potential measurement using potentiometric fluorescence dye bis-oxonol [DiBAC$_4$(3)], primary cultured VSMCs or endothelium-denuded aortic strips (3 mm wide by 5 mm long) were loaded with 100 nmol/L DiBAC$_4$(3) at 37°C for 10 minutes. The tissues or cells were treated with/without iberiotoxin (50 nmol/L) at 37°C for 10 minutes or with/without T1E3 (1:200) or antigen-preabsorbed T1E3 overnight at 4°C for tissues or 1 hour at room temperature for cultured cells. Change in fluorescence was measured by FV1000 confocal system. A quantitative relationship between changes in DiBAC$_4$(3) fluorescence versus membrane potential was established using Na$^+$ ionophore gramicidin in Na$^+$-free media.[20]

The methods for sharp microelectrode measurement was as described elsewhere.[21] Briefly, segments of mesenteric arteries was cut open. After rubbing off endothelial layer, a conventional sharp microelectrode filled with 3 mol/L KCl (tip resistance 40 to 80 MΩ) was inserted into smooth muscle cells from the lumen side. The artery segments were preincubated with T1E3 (1:50) or preimmune IgG (1:50) at 4°C overnight or with/without iberiotoxin (50 nmol/L) at 37°C for 10 minutes.

Arterial Tension Measurement

Segments of the secondary and tertiary branches of rat mesenteric artery ($\approx$2 to 3 mm long) were dissected, and endothelial layer was rubbed off. The segments were mounted in a DMT myograph (model 610M) under a normalized tension as previously described.[22] Contractile agonists were added in a cumulative fashion to the bath to obtain concentration–response curves. The contractions were expressed as active wall tension (WT)=$F/2x$, where F stands for the force in millinewtons and x for the longitudinal length of the vessels in millimeters. The artery segments were preincubated with T1E3 (1:50), antigen-preabsorbed T1E3, or preimmune IgG (1:50) at 4°C overnight. Iberiotoxin incubation (50 nmol/L) was at 37°C for 10 minutes. T1E3 and preimmune IgG were applied in similar quantities to balance possible osmotic effect.

Preparation of T1E3 and Preimmune IgG

T1E3 antibody was raised in rabbits using the strategy developed by Xu et al[23] Briefly, a peptide corresponding to TRPC1 putative pore region (CVGIFCEQQSNDTFHSFIGT) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) at Alpha Diagnostic International. The coupled T1E3 peptide was injected into the tail vein of a rabbit followed by two boost doses. T1E3 antiserum was collected 4 weeks after the second boost. IgG was purified from T1E3 antiserum and preimmune serum using a protein G column.

In antigen preabsorption control, T1E3 was preabsorbed with excessive amount of peptides (1:16 weight ratio) for 2.5 hours at room temperature.

Cloning and Transfection

Human BK$_{Ca}$ cDNA (NM_002247) was a gift from Dr Desir GV (Yale University). Human TRPC1 cDNA (NM_003304) was obtained by RT-PCR from human coronary endothelial cells. Both genes were cloned into pcDNA6 vector.

Transfection condition was as described elsewhere.[24] Briefly, HEK293 cells were transfected with constructs containing TRPC1 and/or BK$_{Ca}$ using Lipofectamine 2000 in 6-well plates. Approximately 80% of HEK293 cells were successfully transfected as determined by a control transfection using a GFP-expressing pcDNA6. Functional studies were performed 3 days posttransfection.

Immunoprecipitation and Immunoblots

Immunoprecipitation and immunoblots were as described elsewhere.[24] To prepare smooth muscle cell lysates, smooth muscle layers were obtained by peeling off from the adventitial layers with forceps, followed by homogenization. The proteins were extracted from the lysates of smooth muscle cells or HEK293 cells with detergent extracted buffer, which contained 1% Nonidet P-40, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, with addition of protease inhibitor cocktail tablets. Extracted proteins (800 μg) were then incubated with 7 μg of anti-TRPC1 (Alomone Laboratory) or anti-BK$_{Ca}$ (Alomone Laboratory) on a rocking platform overnight at 4°C. Protein A agarose was then added, followed by a further incubation at 4°C for 3 hours. The immunoprecipitates were washed and resolved on an 8% SDS-PAGE gel.

For immunoblots, the poly(vinylidene difluoride) membrane carrying transferred proteins was incubated at 4°C overnight with the primary anti-TRPC1 (1:200), anti-BK$_{Ca}$ (1:200), or T1E3 (1:200). Immunodetection was accomplished using horseradish peroxidase–conjugated secondary antibody, followed by ECL detection system.

Double-Labeling Immunofluorescence Assay

Double immunofluorescence assay was performed as described elsewhere.[25] Briefly, freshly dispersed rat aortic VSMCs were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Nonspecific immunostaining was blocked by preincubating the cells with 2% BSA. The cells were incubated with a mixture of T1E3 (1:50, raised in rabbit) and anti-BK$_{Ca}$ (1:50, raised in goat) at 4°C overnight, followed by incubation with a mixture of secondary goat anti-rabbit IgG-conjugated to Alexa Fluor 488 (1:200) and rabbit anti-goat IgG-conjugated to Alexa Fluor 546 (1:100). Immunofluorescence was detected by FV1000 confocal system. A quantitative
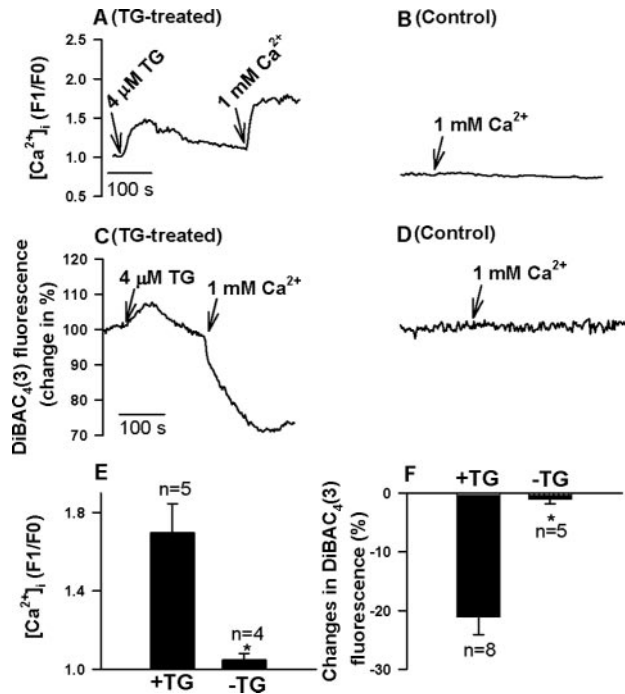
Figure 1. SOC and its associated membrane hyperpolarization in the primary cultured rat aortic VSMCs. A through D, Representative traces for changes in $[Ca^{2+}]_i$ (A and B) and membrane potential (C and D) in response to thapsigargin (TG) and extracellular Ca^{2+}. E and F, summary of data showing changes in $[Ca^{2+}]_i$ (E for A and B) and membrane potential (F for B and D) in response to extracellular Ca^{2+}. Values are mean±SE (n=4 to 8). *$P<0.05$ compared to the cells without TG treatment.

analysis of the colocalization was carried out through pixel-by-pixel correlation of red and green images using FV10-ASW 1.5 software.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

SOC and Its Associated Membrane Hyperpolarization in Primary Cultured VSMCs

We first studied SOC in primary cultured rat aortic VSMCs. The cells were treated with thapsigargin (4 μmol/L) for 6 to 8 minutes in $0Ca^{2+}$-PSS to deplete intracellular Ca^{2+} stores, which resulted in a rise in $[Ca^{2+}]_i$ (Figure 1A). Subsequent application of extracellular Ca^{2+} (1 mmol/L) elicited the SOC (Figure 1A and 1E). In control cells without thapsigargin treatment, application of extracellular Ca^{2+} had no effect on $[Ca^{2+}]_i$ level (Figure 1B and 1E).

The role of SOC in modulating membrane potential was examined using a potentiometric fluorescence dye DiBAC$_4$(3).[26] Initiation of SOC by applying extracellular Ca^{2+} caused a marked membrane hyperpolarization in VSMCs as indicated by 21±3% (n=8) decrease in DiBAC$_4$(3) fluorescence (Figure 1C and 1F). In control cells without store depletion, addition of extracellular Ca^{2+} had no effect on membrane potential (Figure 1D and 1F). These data suggest that SOC induces membrane hyperpolarization in VSMCs.

To calibrate the changes of DiBAC$_4$(3) fluorescence versus the membrane potential, a standard curve was established (Figure I in the online data supplement). Based on this

standard curve, a 21% decrease in DiBAC$_4$(3) fluorescence was equivalent to a hyperpolarization of 24 mV (n=14). Whole cell patch clamp was used to verify this hyperpolarization. Under the same conditions as in DiBAC$_4$(3) experiments, SOC-induced membrane hyperpolarization measured by whole cell patch clamp was 28±4 mV (n=14) (supplemental Figure II), which was close to the value estimated by DiBAC$_4$(3) method.

Role of TRPC1 in SOC and Its Associated Membrane Hyperpolarization in Primary Cultured VSMCs

We used a polyclonal antibody T1E3 that can plug the pore of TRPC1.[2] Preincubation of cells with T1E3 (1:200) for 1 hour completely abolished SOC (Figure 2A, 2B, and 2H). This blocking effect was absent after T1E3 was preabsorbed by excessive amount of peptide antigen (Figure 2C and 2H), confirming that the blockage was attributable to the specific action of T1E3 on TRPC1. These data agreed well with the results from other labs,[1] supporting the notion that TRPC1 is the main component of SOC in VSMCs.
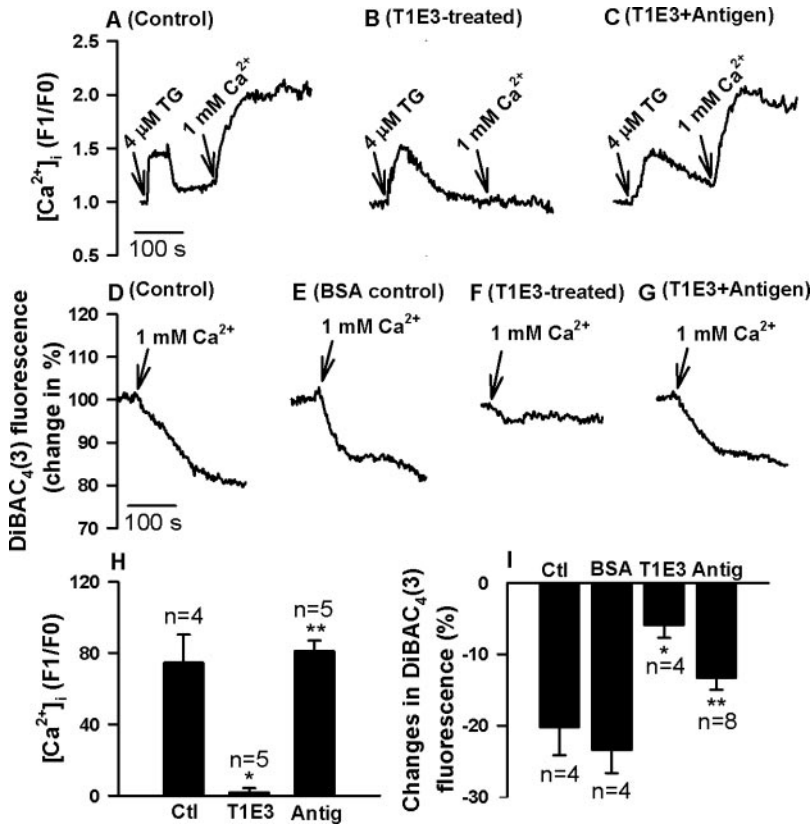
Effect of T1E3 on the SOC-induced membrane hyperpolarization was then tested. Incubation of VSMCs with T1E3 (1:200) for 1 hour diminished this hyperpolarization (Figure 2D, 2F, and 2I). For controls, incubation with antigen-preabsorbed T1E3 or bovine serum albumin (BSA) for 1 hour had no effect on the hyperpolarization (Figure 2E, 2G, and 2I). Furthermore, verapamil (10 μmol/L) had no effect on this hyperpolarization, indicating that voltage-gated Ca^{2+} channels were not involved (supplemental Figure III).

Role of BK$_{Ca}$ in SOC-Induced Membrane Hyperpolarization of Primary Cultured VSMCs

Presumably, cation influx through TRPC1 should result in membrane depolarization instead of hyperpolarization. Therefore, we hypothesized that Ca^{2+} influx through TRPC1 may activate BK$_{Ca}$, resulting in membrane hyperpolarization. Two selective BK$_{Ca}$ blockers, iberiotoxin and charybdotoxin, were used to test this hypothesis. Both agents at 50 nmol/L completely abolished the SOC-induced membrane hyperpolarization (Figure 3A through 3D), supporting a functional association of TRPC1 with BK$_{Ca}$.

In the present study, T1E3 was used as the main tool to determine the functional involvement of TRPC1. Therefore, we needed to exclude the possibility of a direct T1E3 action on BK$_{Ca}$ activity. In whole-cell patch clamp experiments, we recorded a large iberiotoxin-sensitive current in VSMCs, which could be attributed to BK$_{Ca}$ (supplemental Figure IV). T1E3 had no effect on this current, indicating that T1E3 did not directly inhibit BK$_{Ca}$ activity (supplemental Figure IV). Another concern is whether TRPC1 is coupled to intermediate conductance Ca^{2+}-sensitive K^+ channel (I_{KCa}), the expression of which was reported to be upregulated during VSMC culture.[27] Presumably, such a coupling could also contribute to the SOC-induced membrane hyperpolarization. In experiments, a low level of I_{KCa} expression was indeed detected in the primary cultured VSMCs (supplemental Fig-

Figure 2. Effect of T1E3 on SOC and its associated membrane hyperpolarization in the primary cultured VSMCs. A through G, Representative traces for changes in [Ca^{2+}]$_i$ (A through C) and membrane potential (D through G). Cells were pretreated with T1E3 (B and F) or antigen-preabsorbed T1E3 (C and G) or bathed with 1% BSA (E). Controls (A and D) had no relevant treatment. H and I, Summary of data showing changes in [Ca^{2+}]$_i$ (H) and membrane potential (I) in response to extracellular Ca^{2+}. Ctl indicates control; Antig, antigen-preabsorbed T1E3. Values are mean±SE ($n=4$ to 8). *$P<0.05$ compared to control, **$P<0.05$ compared to T1E3-pretreated cells.

ure V). However, inhibition of I_{KCa} activity with TRAM-34 (10 μmol/L) had no effect on the SOC-induced membrane hyperpolarization (Figure 3D), suggesting that I_{KCa} was not involved.
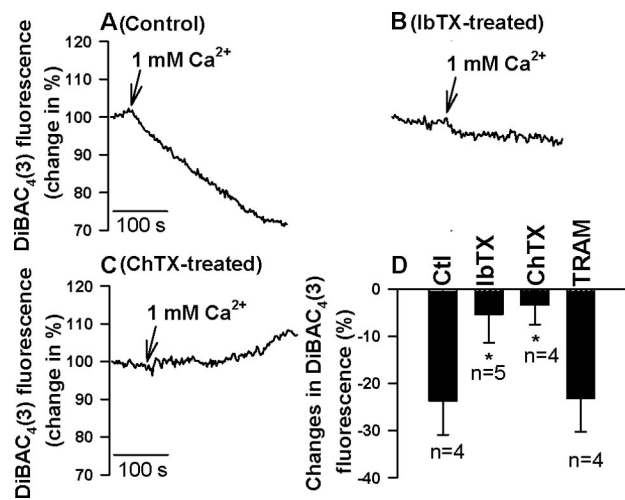

Figure 3. Effect of K$^+$ channel inhibitors on the SOC-induced membrane hyperpolarization in primary cultured VSMCs. A through C, Representative traces for changes in membrane potential in response to 1 mmol/L extracellular Ca^{2+} in the absence (A) or the presence of iberiotoxin (IbTX) (50 nmol/L) (B) or charybdotoxin (ChTX) (50 nmol/L) (C). D, Summary of data showing the magnitude of SOC-induced membrane hyperpolarization. TRAM indicates 10 μmol/L TRAM-34. Values are mean±SE ($n=4$ to 5). *$P<0.05$ compared to control.

Role of BK$_{Ca}$ and TRPC1 in SOC-Induced Membrane Hyperpolarization of VSMCs in Intact Vascular Tissues

Primary cultured VSMCs may not fully represent the cells in vivo, because cell isolation and culture procedures could cause phenotypic drift. Therefore, intact vascular tissues were used to verify the above findings. In one series of experiments, endothelium-denuded aortic strips were loaded with DiBAC$_4$(3). The strips bathed in 0Ca^{2+}-PSS were treated with thapsigargin (4 μmol/L, 20 minutes) to deplete Ca^{2+} stores. Subsequent application of extracellular Ca^{2+} (1 mmol/L) induced membrane hyperpolarization in VSMCs within the strips (Figure 4A and 4E). The hyperpolarization was inhibited by iberiotoxin (50 nmol/L, 10 minutes) (Figure 4D and 4E) and T1E3 (1:200, overnight) (Figure 4B and 4E). Antigen-preabsorbed T1E3 had no blocking effect (Figure 4C and 4E). Similar results were also obtained by sharp microelectrode methods, in which the membrane potential of VSMCs in intact rat mesenteric arteries was measured directly (Figure 5A through 5C). Taken together, these data strongly suggest that TRPC1 is indeed functionally coupled to BK$_{Ca}$ in VSMCs in vivo.

Note that, in the absence of extracellular Ca^{2+}, application of thapsigargin (4 μmol/L) caused a small depolarization in VSMCs (Figure 1C and 5A). The reason for this depolarization was unclear, but it could result from Na$^+$ influx through store-operated cation channels, mainly TRPC1. In experiments, T1E3 treatment reduced this depolarization (Figure 5B and 5C), supporting the involvement of TRPC1 in this depolarization.
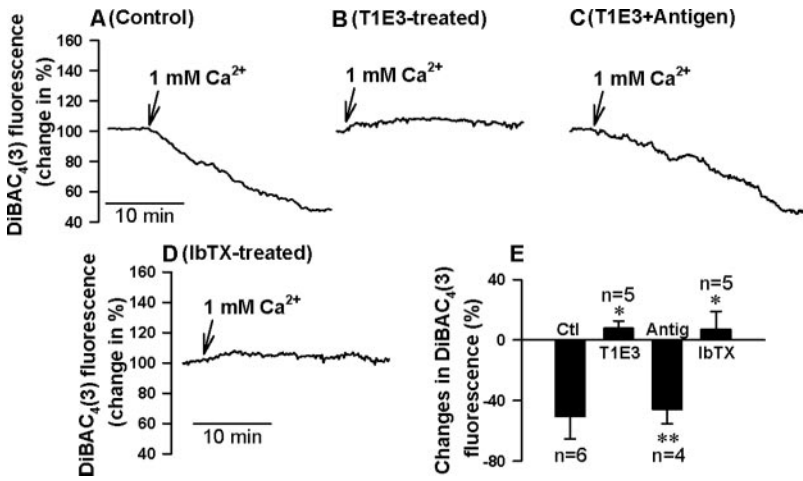
Figure 4. Effect of T1E3 and iberiotoxin on SOC-induced membrane hyperpolarization in VSMCs within endothelium-denuded rat aortic strips. A through D, Representative traces for the SOC-induced membrane hyperpolarization in cells pretreated with T1E3 (B) or antigen-preabsorbed T1E3 (C) or iberiotoxin (IbTX, 50 nmol/L) (D). Control (A) had no relevant treatment. E, Summary of data showing the magnitude of membrane hyperpolarization as in A through D. Values are mean±SE (n=4 to 5). *$P<0.05$ compared to control, **$P<0.05$ compared to T1E3-pretreated cells.

Effect of T1E3 and Iberiotoxin on Phenylephrine-, Endothelin-1–, and U46619-Induced Membrane Depolarization and Contraction in Rat Mesenteric Arteries

We then tested whether TRPC1-BK_{Ca} coupling has any role in modulating agonist-induced membrane depolarization and vascular contraction. Several physiologically relevant agents, including an $\alpha1$-adrenoceptor agonist phenylephrine, endothelin-1, and a thromboxane mimetic, U46619, were used. Sharp microelectrode and isometric tension studies showed that application of these agonists caused dose-dependent membrane depolarization (Figure 5D through 5F) and contraction (Figure 6) of VSMCs in isolated rat mesenteric arteries. Importantly, a preincubation of the vessels with T1E3 (1:50, overnight) or iberiotoxin (50 nmol/L, 10 minutes) enhanced the depolarization (Figure 5D through 5F) and contraction (Figure 6) to all 3 agonists. Antigen-preabsorbed T1E3 had no effect on the contraction (Figure 6). These data support a role of TRPC1-BK_{Ca} in agonist-induced membrane depolarization and vascular contraction.

Note that in the presence of iberiotoxin, T1E3 had no additional effect on agonist-induced vascular contraction (supplemental Figure VI). This is consistent with the notion that TRPC1 exerts its effect through BK_{Ca}. However, it

appeared that iberiotoxin was more effective than T1E3 in enhancing the agonist-induced depolarization (Figure 5D through 5F) and contraction (Figure 6). This could either be attributable to a poor permeability of T1E3 in vascular tissues or attributable to existence of an alternative TRPC1-independent BK_{Ca} activation pathway.

Physical Association of TRPC1 With $BK_{Ca}(\alpha)$

The above results indicate that TRPC1 and BK_{Ca} are functionally linked. We next used coimmunoprecipitation method to determine whether these two proteins are physically associated. Two antibodies used for coimmunoprecipitation, anti-TRPC1 and anti-$BK_{Ca}(\alpha)$ (both from Alomone Laboratory), were previously reported to be highly specific to their targets.[7,28] Our immunoblot experiments confirmed that 2 antibodies recognized a single band in TRPC1- and BK_{Ca}-transfected HEK cells respectively (Figure 7A and 7D). The specificity of T1E3 was also verified by immunoblots, in which T1E3 recognized the expected band whereas antigen-preabsorbed T1E3 had no band (Figure 7A). Importantly, coimmunoprecipitation experiments demonstrated that anti-$BK_{Ca}(\alpha)$ antibody was able to pull down TRPC1 in the proteins lysates freshly prepared from rat aortic smooth muscle layers (Figure 7B). Furthermore, anti-TRPC1 anti-
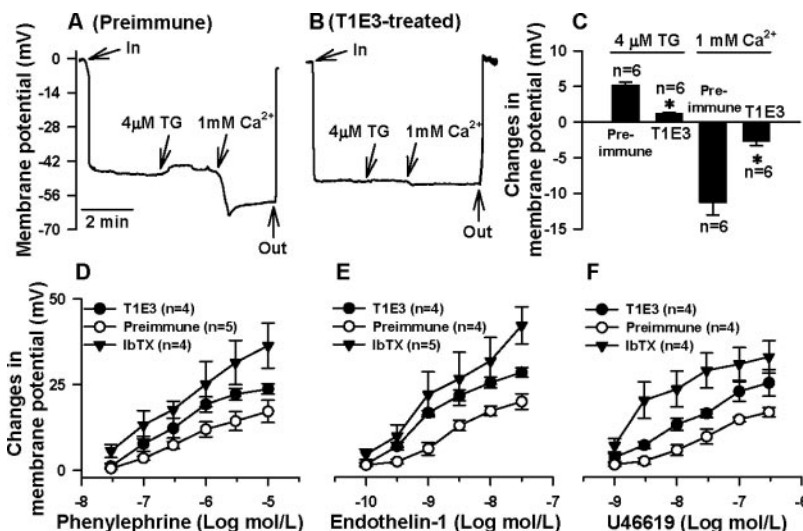
Figure 5. Sharp microelectrode measurement of the VSMC membrane potential in endothelium-denuded rat mesenteric arteries. A through C, Representative traces (A, treated with preimmune IgG; B, treated with T1E3) and summary of data (C) for changes in membrane potential in response to thapsigargin and extracellular Ca^{2+}. The vessels bathed in $0Ca^{2+}$-PSS were treated with thapsigargin (4 μmol/L), followed by 1 mmol/L extracellular Ca^{2+}. In indicates electrode impalement; Out, electrode withdrawal. *$P<0.05$ compared to preimmune IgG. D through F, Summary of data showing the effect of T1E3 and iberiotoxin (50 nmol/L) on dose-dependent VSMC depolarization to phenylephrine (D), endothelin-1 (E), and U46619 (F). For D through F, the vessels were bathed in Krebs solution. Values are mean±SE (n=4 to 6). $P<0.05$ between the concentration response curves of T1E3 (or IbTX)-treated groups and that of the preimmune IgG group using two-way ANOVA.
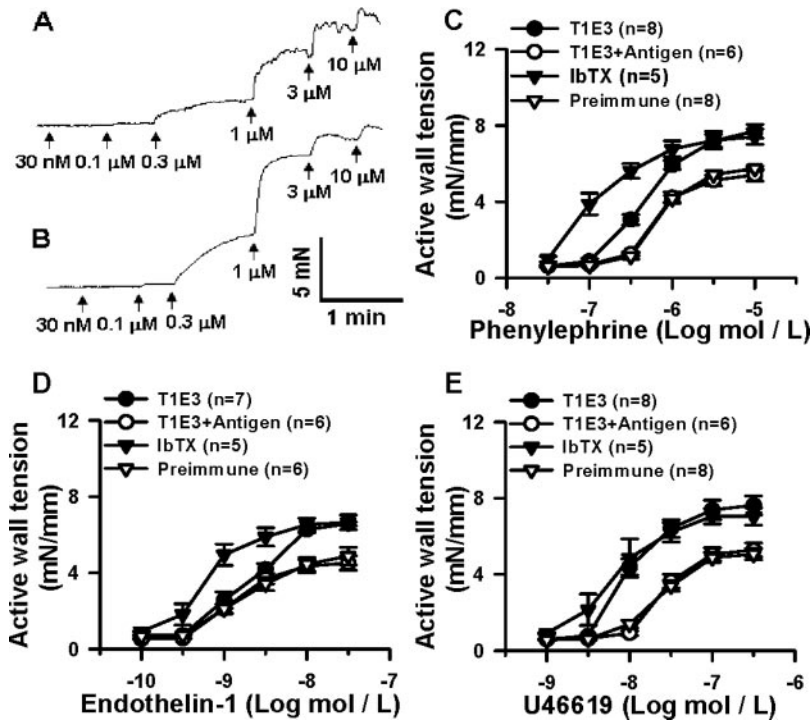
Figure 6. Effect of T1E3 and iberiotoxin on phenylephrine-, endothelin-1–, and U46619-induced contraction in endothelium-denuded rat small mesenteric arteries. A and B, Representative traces for phenylephrine-induced contraction in arteries pretreated with T1E3 (B) or preimmune IgG as control (A). C through E, Summary of data showing the effect of T1E3, antigen-preabsorbed T1E3, and iberiotoxin on dose-dependent vascular contraction to phenylephrine (C), endothelin-1 (D), and U46619 (E). Values are mean±SE (n=5 to 8). $P<0.05$ between the concentration response curves of T1E3 (or IbTX)-treated groups and that of the preimmune IgG group using 2-way ANOVA.

body was able to reciprocally pull down BK$_{Ca}(\alpha)$ (Figure 7C). In control experiments [labeled as IP(-) in Figure 7B and 7C], the pull-down experiments were performed using preimmune IgG. As expected, no band could be observed [IP(-) in Figure 7B and 7C]. Taken together, these data suggest that TRPC1 physically associates with BK$_{Ca}(\alpha)$, forming a signaling complex.

To further confirm that TRPC1 and BK$_{Ca}(\alpha)$ indeed have physical interaction, we next used the HEK293 cells that

overexpress both TRPC1 and BK$_{Ca}$. The results showed that anti-BK$_{Ca}(\alpha)$ antibody could pull down TRPC1, and anti-TRPC1 antibody could reciprocally pull down BK$_{Ca}(\alpha)$ (supplemental Figure VII).
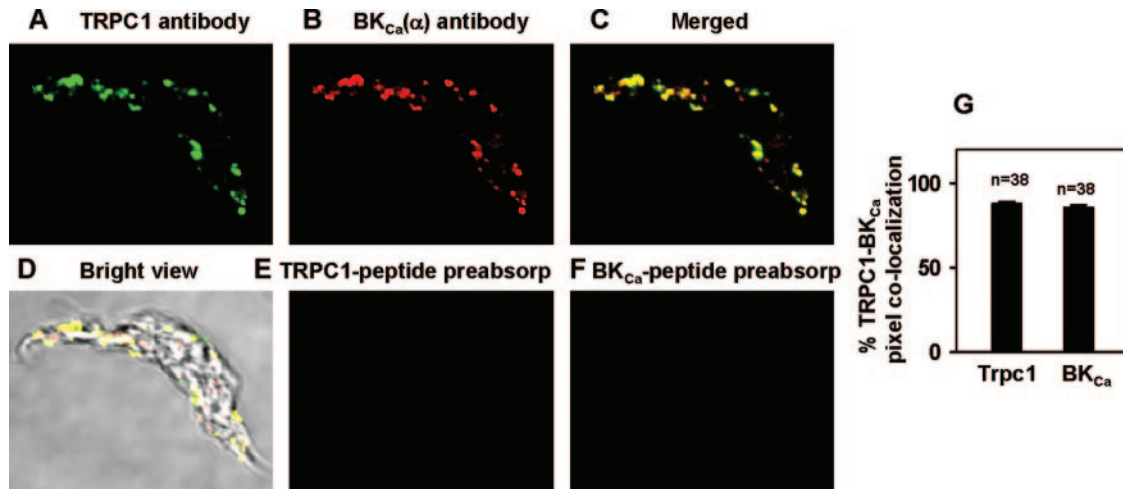
Colocalization of TRPC1 and BK$_{Ca}(\alpha)$ at Subcellular Level

Double-labeling immunofluorescence experiments were performed to determine subcellular localization of TRPC1 and
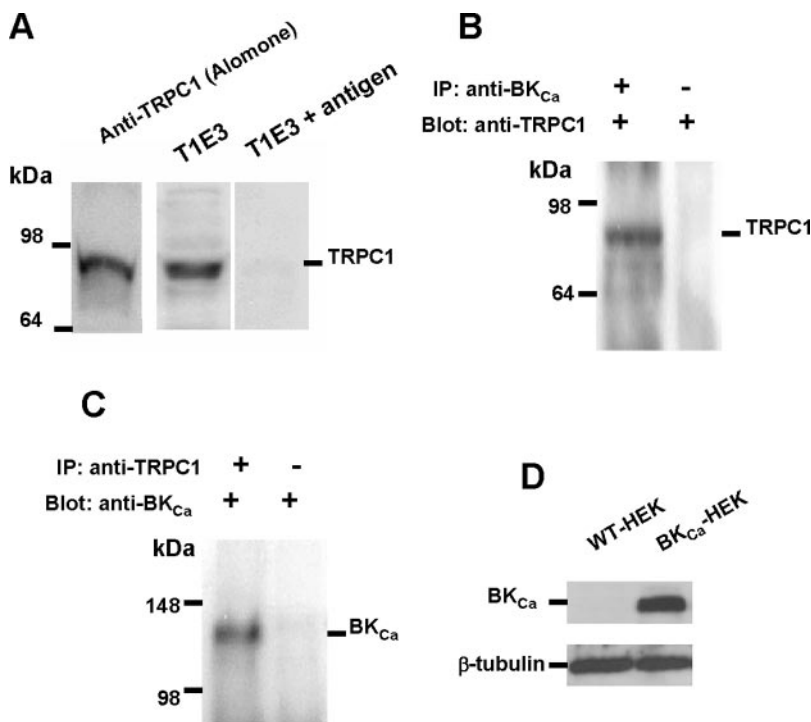
Figure 7. Coimmunoprecipitation of TRPC1 and BK$_{Ca}(\alpha)$ in the lysates of rat aortic smooth muscle layers. A, An immunoblot showing that anti-TRPC1 (Alomone Laboratory), T1E3, but not antigen-preabsorbed T1E3 recognized TRPC1 proteins in TRPC1-transfected HEK293 cells. B and C, Representative images showing coimmunoprecipitation followed by immunoblots [B, immunoblot with anti-TRPC1; C, immunoblot with anti-BK$_{Ca}(\alpha)$]. Proteins from aortic smooth muscle layers were immunoprecipitated with indicated antibody (+) or preimmune IgG (−). D, Immunoblot showing that anti-BK$_{Ca}$ (Alomone Laboratory) recognized BK$_{Ca}(\alpha)$ proteins in BK$_{Ca}(\alpha)$-transfected HEK293 cells but not in wild-type HEK. Immunoblots with anti–β-tubulin antibody showed that an equal amount of proteins was loaded onto each lane. n=4 to 5 experiments.

Figure 8. Immunolocalization of TRPC1 and $BK_{Ca}(\alpha)$ in VSMCs. A through F, Representative images from a freshly dispersed rat aortic VSMC. Alexa 488−conjugated IgG was used to detect TRPC1 (green) (A). Alexa 546−conjugated IgG was used to detect BK_{Ca} (red) (B). When green and red images are superimposed, colocalization of antibodies is indicated by a yellow color (C). D, The bright field image of the cell. E, TRPC1 antibody was preabsorbed with excessive TRPC1 peptide. F, $BK_{Ca}(\alpha)$ antibody was preabsorbed with excessive $BK_{Ca}(\alpha)$ peptide. G, Summary of data showing the percentage of TRPC1-$BK_{Ca}(\alpha)$ pixel colocalization. n=38 cells from 4 experiments.

$BK_{Ca}(\alpha)$ in freshly dispersed rat aortic VSMCs. Cells were stained for TRPC1 with Alexa fluor 488 (green) and for $BK_{Ca}(\alpha)$ with Alexa fluor 546 (red). As shown in Figure 8, both TRPC1 and $BK_{Ca}(\alpha)$ were mostly localized on the plasma membrane. On merged images, there was very strong overlapping of TRPC1 and $BK_{Ca}(\alpha)$ fluorescence (yellow) (Figure 8C). Quantitative colocalization analysis showed that $88\pm1\%$ (n=38) of TRPC1 labeling was colocalized with $BK_{Ca}(\alpha)$, and conversely $85\pm2\%$ (n=38) of $BK_{Ca}(\alpha)$ labeling was colocalized with TRPC1 (Figure 8G). In control experiments, there was no staining if the primary antibodies were preabsorbed with excessive amounts of respective antigens (Figure 8E and 8F). These data suggest that TRPC1 is colocalized with $BK_{Ca}(\alpha)$ in rat aortic VSMCs.

Discussion

The major findings of this study are as follows: (1) in the primary cultured VSMCs, SOC was mediated by TRPC1, and furthermore this Ca^{2+} influx induced membrane hyperpolarization; (2) this hyperpolarization was diminished in the presence of BK_{Ca} channel blockers or an anti-TRPC1 blocking antibody T1E3; (3) the above functional results were confirmed in VSMCs of intact arteries using both potentiometric fluorescence dye and sharp microelectrode methods; (4) Inhibition of TRPC1 activity by T1E3 markedly enhanced the membrane depolarization and contraction of VSMCs in response to several physiologically-relevant contractile agonists; (5) coimmunoprecipitation experiments showed that an anti-$BK_{Ca}(\alpha)$ antibody could pull down TRPC1, and furthermore an anti-TRPC1 antibody could reciprocally pull down $BK_{Ca}(\alpha)$; and (6) double-labeling immunocytochemistry showed that TRPC1 and BK_{Ca} were colocalized in the same subcellular regions, mainly on the plasma membrane, in VSMCs. Taken together, these data suggest that TRPC1 and BK_{Ca} are physically associated with each other in VSMCs, and that Ca^{2+} influx through TRPC1 activates BK_{Ca}, leading to membrane hyperpolarization. The hyperpolarizing effect of

TRPC1-BK_{Ca} coupling could serve to reduce agonist-induced membrane depolarization, thereby preventing excessive contraction of VSMCs to contractile agonists.

Mounting evidence suggests that TRPC1 associates with other proteins to form a signaling complex that can include inositol trisphosphate receptor, homer, calmodulin, caveolin-1, and myxovirus-resistance protein A.[1] In the present study, we found that TRPC1 physically associates with $BK_{Ca}(\alpha)$, and this interaction links TRPC1 to membrane hyperpolarization of VSMCs. These results add a new dimension to the role of TRPC1 channel. The physical coupling between BK_{Ca} and TRPC1 would allow an efficient signal transduction between TRPC1 and BK_{Ca}. Furthermore, because TRPC1 coassembles with other TRP channels to form heterotetramers in vivo, our model also allows an interaction of BK_{Ca} with other assembly partners of TRPC1, including TRPC4, -C5, and -P2. The presence of such a signaling complex, which contains BK_{Ca}, TRPC1 and its assembly partners, would allow diverse signals, including store-depletion, receptor activation, hypoosmotic cell swelling and oxidative stress, to converge on this signaling complex to activate BK_{Ca}, thereafter inducing hyperpolarization in VSMCs.[1,29]

Both BK_{Ca} and TRPC1 are abundantly expressed in almost all types of smooth muscle cells.[1,30] We expect that the membrane hyperpolarization caused by TRPC1-BK_{Ca} coupling would inactivate voltage-gated Ca^{2+} channels, which are the dominant Ca^{2+} influx channels in VSMCs, thus serving to reduce vascular tone. We subsequently tested this hypothesis using rat mesenteric arteries. Several physiologically relevant agents, including phenylephrine, endothelin-1, and U46619, were used to depolarize VSMCs and to contract the vessels. It is well documented that these contractile agents induce vascular contraction at least partly by stimulating Ca^{2+} release from intracellular Ca^{2+} stores.[31–33] These agonists also cause membrane depolarization,[31,34] which would increase Ca^{2+} influx through voltage-gated Ca^{2+} channels. In addition, the store Ca^{2+} release is expected to result in an

enhanced SOC, which is known to be mediated by TRPC1 in VSMCs.[1] Based on our present model of TRPC1-BK$_{Ca}$ coupling, we reasoned that this enhanced SOC would tend to have a hyperpolarizing effect and might thus reduce agonist-induced membrane depolarization and contraction in VSMCs. Our prediction was indeed confirmed. Sharp microelectrode study showed that blockage of TRPC1 by T1E3 antibody caused a marked increase in membrane depolarization in response to phenylephrine, endothelin-1, and U46619. Iso-metric tone studies showed that T1E3 enhanced vascular contraction in response to these three agonists. These results suggest that TRPC1-BK$_{Ca}$ coupling has an important functional role in modulating agonist-induced vascular contraction.

Our results appear to conflict with a previous report by Bergdahl et al,[15] who demonstrated that T1E3 treatment reduced endothelin-1-induced contraction in rat basilar artery but not in caudal artery. The reason for this discrepancy is not clear. One possibility is the variations in vessel types used in two studies. TRPC1 and/or BK$_{Ca}$ could be expressed at different level in different vascular beds, and their coupling could also vary. These may contribute to the altered vascular responses. Several previous studies also showed that some chronic treatments such as hypoxia and organ cultures enhanced TRPC1 protein expression in arteries with a parallel increase in vascular contractility.[11–13] However, these data are not in direct conflict with our model, because the overexpressed TRPC1 under chronic treatments might not be physically coupled to BK$_{Ca}$. Without TRPC1-BK$_{Ca}$ coupling, Ca^{2+} influx through overexpressed TRPC1 is indeed expected to cause vascular contraction instead of relaxation.

The physiological roles of TRPC1-BK$_{Ca}$ coupling may not be limited to vascular tone control. TRPC1 and BK$_{Ca}$ channels are expressed across many cell types,[1,35] and both are suggested to play a key role in a variety of other body function including VSMC proliferation,[36] neointimal hyperplasia,[9] neurotransmission,[1,35] neuronal growth cone formation,[1,37] and cell volume regulation.[1,38] Thus, it is possible that TRPC1-BK$_{Ca}$ signal complex may play a more general role in other body function. Consistent with this notion, our coimmunoprecipitation data showed that, when overexpressed in HEK293 cells, BK$_{Ca}$ and TRPC1 also bind to each other to form a protein complex (supplemental Figure VII). Furthermore, a physical coupling between TRPC1 and BK$_{Ca}$ was also found in vascular endothelial cells (data not shown).

In conclusion, we demonstrated that TRPC1 and BK$_{Ca}$ are physically associated with each other. Ca^{2+} influx through TRPC1 activates BK$_{Ca}$, leading to membrane hyperpolarization in VSMCs. This hyperpolarizing effect of TRPC1-BKCa coupling could serve to prevent excessive contraction of VSMCs to contractile agonists.

Sources of Funding

Disclosures

None.

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Correction

TRPC1 Associates With BK_{Ca} Channel to Form a Signal Complex in Vascular Smooth Muscle Cells: Correction

In the article that appears on page 670 of the March 13, 2009, issue, Figure 8 was published with errors. Incorrect secondary antibodies were used for immunostaining. The experiments are now repeated with corrected secondary antibodies (donkey anti-rabbit immunoglobulin G conjugated to Alexa Fluor 488 and donkey anti-goat immunoglobulin G conjugated to Alexa Fluor 546). The corrected Figure 8 is printed below. These corrections do not affect the conclusions presented in the article. The authors regret the error.
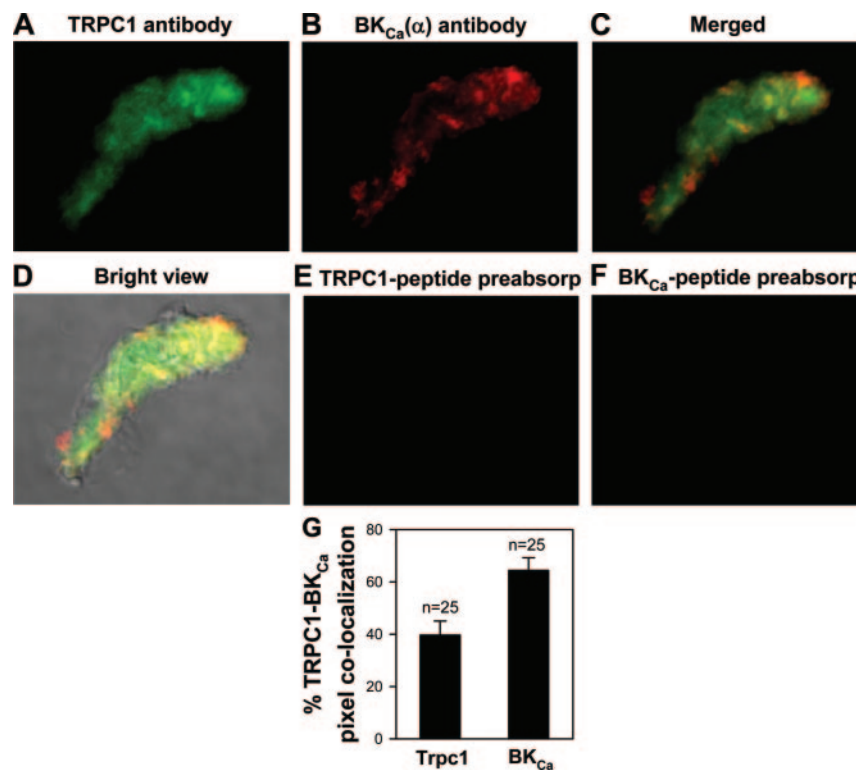
This error has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/104/5/670

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Supplement Material

Expanded methods

Materials

Human embryonic kidney cell line HEK293 was from ATCC, USA. The primary rabbit antibodies against $BK_{Ca}(\alpha)$ (APC-021), TRPC1 (ACC-010), and IK_{Ca} (APC-064) were from Alomone Labs. The primary goat anti-$BK_{Ca}(\alpha)$ antibody was from Santa Cruz Biotech. TIE3 was raised by us from rabbits. Protein A-agarose was from Roche. $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid) trimethine oxonol] was from Biotium, USA. Fluo-3/acetoxymethyl ester (Fluo-3/AM), pluronic F127, lipofectamine 2000, soybean trypsin inhibitor, PMSF (phenylmethylsulfonyl fluoride), fetal bovine serum (FBS), DMEM, and dithiothreitol were from Invitrogen. Thapsigargin, amphotericin, aprotinin, and leupeptin were from Calbiochem. Nonidet P-40, EGTA, iberiotoxin, charybdotoxin, TRAM-34, gramicidin, trypsin, albumin bovine serum (BSA), collagenase, and phenylephrine were from Sigma. Papain was from Fluka. U46619 was from Cayman. Endothelin-1 was from Tocris.

Cell preparation and culture

All animal experiments were conducted in accordance with the regulation of the U.S. National Institute of Health (NIH publication No.8523). Rats were killed by overdose of CO_2. VSMCs were isolated from male Sprague-Dawley rats of approximately 250-300g. Briefly, thoracic aorta was dissected, and the lumen of the artery was cut open. Endothelial layer was mechanically removed by rubbing the lumen with cotton wool. Smooth muscle layers were obtained by peeling off from the adventitial layers with forceps, and were then incubated in a Ca^{2+}-free PBS containing 0.2% collagenase type 1A, 0.9% papain, 0.5% BSA, and 10 mmol/L dithiothreitol at 37°C for 60 min. The dispersed VSMCs were then washed with PBS, and cultured for 5-7 days before experimental use. These cells were used for experiments without further cell passage. The VSMCs and HEK293 cells were both cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin, and 100 U/ml streptomycin.

$[Ca^{2+}]_i$ measurement

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was measured as described elsewhere.[1] Briefly, the cells were loaded with 10 μmol/L Fluo-3/AM and 0.02% pluronic F-127 at 37°C for 1 hr in dark in a normal physiological saline solution (NPSS) that contained in mmol/L: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 Hepes, pH 7.4. Store depletion was achieved by treating VSMCs with 4

μmol/L thapsigargin for 6-8 min in Ca^{2+}-free physiological saline ($0Ca^{2+}$-PSS), which contained in mmol/L: 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, 5 Hepes, pH 7.4. Ca^{2+} influx was initiated by applying 1 mmol/L extracellular Ca^{2+}. Unless stated otherwise, cells were pretreated with/without TIE3 (1:200) or antigen-preabsorbed T1E3 for 1 hr at room temperature. The fluorescence signal was recorded and analyzed by Fluoview FV1000 confocal laser scanning system. Changes in $[Ca^{2+}]_i$ were displayed as a ratio of fluorescence relative to the intensity before the application of extracellular Ca^{2+} (F1/F0).

Membrane potential measurement using DiBAC$_4$(3) and whole cell patch clamp

DiBAC$_4$(3) is a membrane potential-sensitive dye, the fluorescence of which is decreased when the dye exits the cell membrane as a result of membrane hyperpolarization.[2] To prepare the aortic strips, rat thoracic aorta was dissected and was cut into a small strip (3 mm width x 5 mm long). Endothelial layer was mechanically rubbed off. The strip was mounted onto an experimental chamber with lumen facing the microscope objectives. 100 nmol/L DiBAC$_4$(3) was loaded into the primary cultured rat aortic VSMCs or the endothelium-denuded aortic strips in NPSS at 37^oC for 10 min. Ca^{2+} stores were depleted by treating with 4 μmol/L thapsigargin (20 min for tissue, 6-8 min for the primary cultured cells) in $0Ca^{2+}$-PSS. The SOC-induced membrane hyperpolarization was initiated by applying 1 mmol/L extracellular Ca^{2+}. Unless stated otherwise, the tissues were treated with/without iberiotoxin (50 nmol/L) or charybdotoxin (50 nmol/L) for 10 min; or with/without TIE3 (1:200) or antigen-preabsorbed T1E3 (1 hr at room temperature for the primary cells, or 24 hr overnight for the tissues).

Calibration of DiBAC$_4$(3) fluorescence versus membrane potential was performed using Na^+ ionophore gramicidin (2 μg/ml) in Na^+-free physiological saline solution.[3,4] In this method, in the presence of gramicidin, the transmembrane Na^+ concentration gradient becomes zero, and the membrane potential is approximately equal to the K^+ equilibrium potential, which is determined by Nernst equation.[3,4] The composition of the Na^+ free physiological saline solution was (in mmol/L): 118-135 NMDG-Cl, 1 $CaCl_2$, 2-19 KCl, 1 $MgCl_2$, 10 glucose, 5 Hepes, pH 7.4 plus 2 μg/ml gramicidin. DiBAC$_4$(3) fluorescence was measured following a stepwise change in extracellular K^+ (2, 3, 5, 8, 13 and 19 mmol/L). The osmolarity was maintained constant by reduction of NMDG. Membrane potential at each extracellular K^+ concentration was calculated based on the intracellular K^+ concentration of 137 mmol/L.[3]

An EPC-9 patch clamp amplifier was used to measure the membrane potential of the primary cultured rat aortic VSMCs in whole-cell perforated patch mode. The pipette solution mimicked those described by others[5,6] with slight modification in order to balance the osmotic pressure between the pipette and bath solutions. The pipette solution contained (in mmol/L): 105 K^+-gluconate, 30 KCl, 1 $MgCl_2$, 10 NaCl, 10 HEPES, pH 7.2, with 250 µg/ml amphotericin. The cells were treated with 4 µmol/L thapsigargin for 6-8 min in $0Ca^{2+}$-PSS, followed by application of 1 mmol/L extracellular Ca^{2+}.

Whole cell patch clamp was also used to study BK_{Ca} current in the primary cultured rat aortic VSMCs (Online Figure IV). The pipette solution contained (in mmol/L): 105 K^+-gluconate, 30 KCl, 1 $MgCl_2$, 2.1 $CaCl_2$, 5 Na_2ATP, 5 EGTA, 10 HEPES, pH 7.2. The free Ca^{2+} was calculated to be ~200 nmol/L. The bath solution was NPSS.

Membrane potential measurement using sharp microelectrode

Segments of rat mesenteric arteries were dissected into about 2 mm rings, and periadventitial fat was removed. Endothelial layer was mechanically rubbed off. The arterial segments were opened longitudinally and equilibrated for 60 min in Krebs solution oxygenated with a gas mixture of 95% O_2 and 5% CO_2. Membrane potential was measured using glass microelectrodes filled with 3 mol/L KCl (resistance: 50-80 MΩ). Electrodes were inserted into the smooth muscle cells from the lumen side. Successful impalement was indicated by an abrupt drop in voltage, followed by a sharp return to baseline on exit. Electrical signals were monitored continuously by EPC9 amplifier (HEKA) with Pulse software. The artery segments were preincubated with T1E3 (1:50) or pre-immune IgG (1:50) at 4°C overnight, or with/without iberiotoxin (50 nmol/L) at 37°C for 10 min.

Arterial tension measurement

Tension in rat aortic segments was measured as described elsewhere.[7] Briefly, rat aorta was cut into 3-mm long ring segments and mounted between two stainless wire hooks in a 10-ml organ bath. The upper wire was connected to a force-displacement transducer (Grass Instruments, USA) and the lower one fixed at the bottom of the organ bath. The segments were placed under an optimal resting tension of 0.5 g, which had been determined in length–tension relationship experiments. Sustained contraction of endothelium-denuded arteries in response to 60 mmol/L K^+ was induced in segments pretreated with or without 10 µmol/L

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verapamil for 15 min. In these experiments, Na^+ ions were replaced with an equimolar concentration of K^+ to maintain the same ionic strength.

For tension measurement in rat small mesenteric arteries, the arteries were dissected into about 2 mm ring segments. The segments were mounted in a myograph (model 610M, DMT, Denmark) under a normalized tension. Contractile agonists were added in a cumulative fashion to the bath to obtain the concentration–response curves. The contractions were expressed as active wall tension WT=F/2x, where F stands for the force (in millinewtons) measured by the transducer and x for the longitudinal length (in millimeters) of the vessels. The artery segments were preincubated with T1E3 (1:50), antigen-preabsorbed T1E3, or preimmune IgG purified from pre-immune serum (1:50) at 4°C overnight. Iberiotoxin incubation (50 nmol/L) was at 37°C for 10 min. T1E3 and preimmune IgG were applied in similar quantities in order to balance possible osmotic effect.

Before the commencement of all tension experiments, artery segments were allowed to equilibrate for about 60 min at 37^oC in Krebs solution bubbled with 95% O_2 and 5% CO_2. The endothelium was mechanically rubbed off with a small piece of plastic tubing. The successful removal of the endothelial cell layer was verified by the lack of any relaxant response to acetylcholine (1 μmol/L).

Preparation of T1E3 and preimmune IgG

T1E3 antibody was raised in rabbits using the strategy developed by Xu *et al.*[8] Briefly, a peptide corresponding to TRPC1 putative pore-region (CVGIFCEQQSNDTFHSFIGT) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) at Alpha Diagnostic International (USA). The coupled T1E3 peptide (0.5 mg) was mixed with an equal volume of complete Freund's adjuvant and then injected into the tail vein of a rabbit (day 0). Two boost doses were injected into the same rabbit at day 21 and day 42 respectively. Antiserum was collected four weeks after the second boost. IgG was purified from the T1E3 antiserum and the preimmune serum using a HiTrap protein G column (GE Healthcare).

In antigen preabsorption control, T1E3 was preabsorbed with excessive amount of peptides (16 μg peptide per μg T1E3) for 2.5 hr at room temperature in PBS with 1% BSA.

Cloning and transfection

Human BK_{Ca} cDNA (NM_002247) is a gift from Dr. Desir GV, Yale University. Human TRPC1 cDNA (NM_003304) was obtained by RT-PCR from human coronary endothelial

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cells. Both genes were cloned into pcDNA6 vector. All clones were auto-sequenced by ABI310 autosequencer to verify the authenticity of the genes.

Transfection condition was as described elsewhere.[9] Briefly, HEK293 cells were transfected with constructs containing TRPC1 and/or BK_{Ca} using lipofectamine 2000. Abound 6×10^4 HEK293 cells were grown in each well of the 6-well plates. Transfection was done with 4 μg plasmid and 6 μl lipofectamine 2000 in 200 μl Opti-MEM reduced serum medium in 6-well plates. About 80% of HEK293 cells were successfully transfected under this transfection condition as determined by a control transfection using a GFP-expressing pcDNA6. Functional studies were performed 3 days post-transfection.

Immunoprecipitation and Immunoblots

Immunoprecipitation and immunoblots were as described elsewhere.[9] To prepare the smooth muscle cell lysates, the smooth muscle layers were obtained by peeling off from the adventitial layers with forceps. The tissues were homogenized and the proteins were extracted with a detergent extraction buffer, which contained 1% (vol/vol) Nonidet P-40, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, with addition of protease inhibitor cocktail tablets. The whole cell lysates from HEK293 cells were also extracted with the same buffer. TRPC1 or BK_{Ca} proteins were immunoprecipitated by incubating 800 μg of the extracted proteins with 7 μg of anti-TRPC1 or anti-BK_{Ca} on a rocking platform overnight at 4^oC. Protein A agarose was then added and incubated for further 3 hr at 4^oC. The immunoprecipitates were washed with saline for 3 times and were resolved on an 8% SDS/PAGE gel.

Proteins were then transferred to a PVDF membrane. The membrane was incubated at 4^oC overnight with the primary anti-TRPC1 (1:200) or anti-BK_{Ca} (1:200) antibody or T1E3 (1:200) in TBS buffer containing 0.1% Tween 20 and 5% nonfat dry milk. Immunodetection was accomplished using horseradish peroxidase-conjugated secondary antibody, followed by ECL detection system.

Double immunofluorescence assay

Freshly dispersed rat aortic smooth muscle cells were seeded on glass coverslips, rinsed with PBS three times, fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 15 min. Nonspecific immunostaining was blocked by incubating the cells in 2% BSA in PBS for 1 hr at room temperature. Cells were incubated with a mixture of T1E3

(1:50, raised in rabbit) and anti-BK$_{Ca}$ (1:50, raised in goat) at 4°C overnight. After three washes with PBS, the cells were incubated with a mixture of secondary goat anti-rabbit IgG (1:200) conjugated to Alexa Fluor 488 and rabbit anti-goat IgG (1:200) conjugated to Alexa Fluor 546 for 1 hr at room temperature. After washing and mounting, immunofluorescence of the cells was observed using Fluoview FV1000 confocal system.

Supplemental results:

Calibration of DiBAC$_4$(3) fluorescence versus membrane potential

A standard curve was constructed to calibrate the changes of DiBAC$_4$(3) fluorescence versus the membrane potential using Na$^+$ ionophore gramicidin (2 μg/ml) in Na$^+$-free physiological saline solution[3,4] (Online Figure I). Based on this standard curve, a 21% decrease in DiBAC$_4$(3) fluorescence was equivalent to a hyperpolarization of 24 mV ($n = 14$).

Membrane hyperpolarization in the primary cultured rat aortic VSMCs as measured by whole cell perforated patch clamp

DiBAC$_4$(3) provides an easy and non-invasive means to measure the changes in membrane potential, but the method is indirect.[2] The changes in membrane potential were therefore verified using the whole cell perforated patch clamp. Under the same conditions as in DiBAC$_4$(3) experiments, the magnitude of SOC-induced membrane hyperpolarization measured by patch clamp was 28 ± 4 mV ($n = 14$) (Online Figure II), which is close to the value (~24 mV) estimated by DiBAC$_4$(3) method. Thapsigargin (4 μmol/L)-induced depolarization was measured to be 7 ± 1 mV ($n = 11$).

Role of voltage-gated Ca^{2+} channels on SOC-induced change in membrane potentials

It is well documented that voltage-gated Ca^{2+} channels are the dominant Ca^{2+} influx channels in vascular smooth muscle cells.[10,11] We therefore tested possible role of voltage-gated Ca^{2+} channels in SOC-induced membrane hyperpolarization in primary cultured rat aortic VSMCs. A well-known voltage-gated Ca^{2+} channel inhibitor verapamil was used.[10,11] In control experiments (Online Figure III A), 10 μmol/L verapamil completely abolished high K$^+$-induced contraction in endothelium-denuded rat aortic segments. Because high K$^+$-induced contraction of rat aortic smooth muscle cells is well known to be caused by the opening of voltage-gated Ca^{2+} channels,[10,11] these control experiments indicated that 10 μmol/L verapamil was sufficient for complete blockage of voltage-gated Ca^{2+} channels. However, 10

μmol/L verapamil had no effect on SOC-induced membrane hyperpolarization in aortic VSMCs (Online Figure III B), indicating that voltage-gated Ca^{2+} channels do not contribute significantly to the SOC-induced membrane hyperpolarization. These results are not unexpected, because store-operated Ca^{2+} influx (following thapsigargin) is believed to be mediated by TRPC channels but not voltage-gated Ca^{2+} channels.

Direct effect of T1E3 on BK_{Ca} activity?

In the present study, T1E3 was used as the main tool to determine the functional involvement of TRPC1. We therefore need to exclude the possibility of a direct T1E3 action on BK_{Ca} activity. Whole cell currents were recorded in the primary cultured rat aortic VSMCs in response to successive voltage pulses of 500 ms duration increased in 20 mV increments from -60 to 100 mV. A large iberiotoxin-sensitive current (attributed to BK_{Ca}) was recorded (Online Figure IV). T1E3 had no effect on this current, indicating that T1E3 did not directly inhibit BK_{Ca} (Online Figure IV).

Expression of IK_{Ca} in the primary cultured rat aortic VSMCs

One concern is whether TRPC1 may also be coupled to the intermediate conductance Ca^{2+}-sensitive K^+ channel (IK_{Ca}), the expression of which was reported to be up-regulated during VSMC culture.[12] In immunoblot experiments, IK_{Ca} expression was indeed detected in the primary cultured VSMCs (Online Figure V). However, its expression level appeared to be much lower than that in vascular endothelial cells (Online Figure V). Functional inhibition of IK_{Ca} with TRAM-34 (10 μM) had no effect on the SOC-induced membrane hyperpolarization (Figure 3D), suggesting that IK_{Ca} was not involved in the SOC-induced membrane hyperpolarization.

Effect of T1E3 on phenylepherine-, endothelin-1, and U46619-induced contraction in the presence of iberiotoxin

Both T1E3 (1:50, overnight) and iberiotoxin (50 nmol/L) caused an enhanced artery contraction (Figure 6 in the manuscript) to phenylepherine, endothelin-1, and U46619. Furthermore, in the presence of iberiotoxin, T1E3 had no additional effect on the agonist-induced contraction (Online Figure VI). This is consistent with the notion that TRPC1 exerts its effect through BK_{Ca}.

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**Physical association of TRPC1 with BK_{Ca} in HEK293 cells co-expressing $BK_{Ca}(\alpha)$ and TRPC1**

To further confirm that TRPC1 and $BK_{Ca}(\alpha)$ indeed have physical interaction, we utilized the HEK293 cells that were co-transfected with BK_{Ca} and TRPC1 cDNA to do co-immunoprecipitation. The results showed that anti-$BK_{Ca}(\alpha)$ antibody could pull down TRPC1 (Online Figure VII A, left lane). Reciprocal co-immunoprecipitation was also performed, in which anti-TRPC1 antibody (Alomone Lab) was able to pull down $BK_{Ca}(\alpha)$ (Online Figure VII B). In control experiments (labeled as IP(-) in Online Figure VII A and VII B), the pull-down experiments were performed using preimmune IgG. As expected, no band was observed. These data confirm that $BK_{Ca}(\alpha)$ and TRPC1 are physically coupled in the HEK cells that co-express $BK_{Ca}(\alpha)$ and TRPC1.

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Online Figure I.
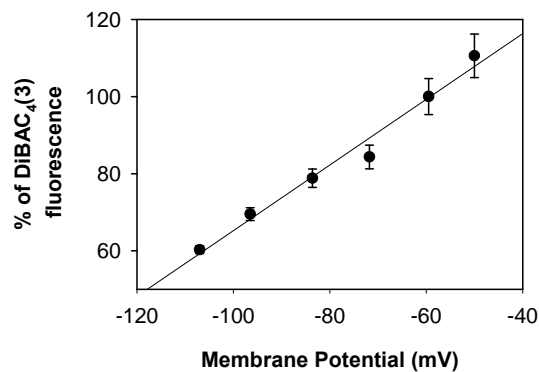
Online Figure I. Relationship between DiBAC$_4$(3) fluorescence change in response to the changes of membrane potential. Extracellular K^{+} concentration was varied to induce change in membrane potential, which was calculated from Nernst equation. The value of DiBAC$_4$(3) fluorescence at the membrane potential of -60 mV was normalized to 100%. Values are mean ± S.E. ($n = 14$).
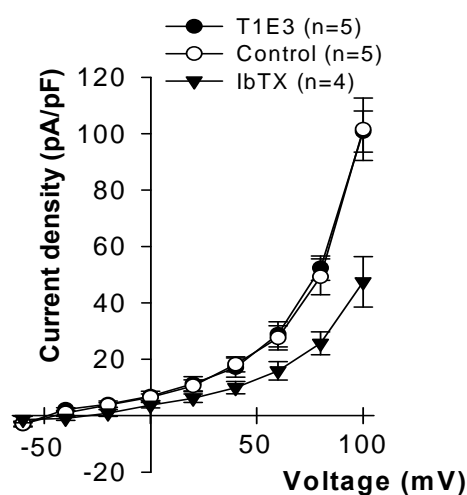
Online Figure II.
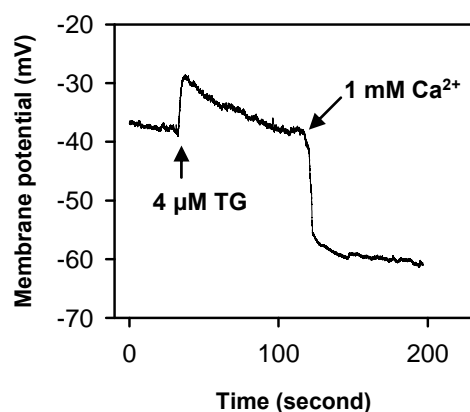
Online Figure II. A representative trace of SOC-induced membrane hyperpolarization in a primary cultured VSMC as measured by whole cell patch clamp. The cell bathed in a Ca^{2+}-free physiological saline was treated with thapsigargin (TG, 4 μmol/L), followed by addition of extracellular Ca^{2+} (1 mmol/L), which caused membrane hyperpolarization. The value of hyperpolarization was 28 ± 4 mV ($n = 14$ experiments).
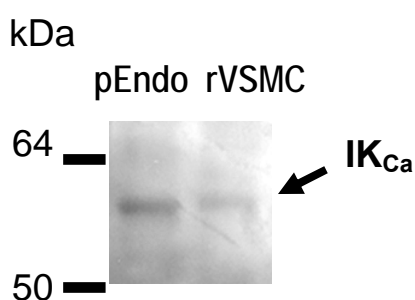
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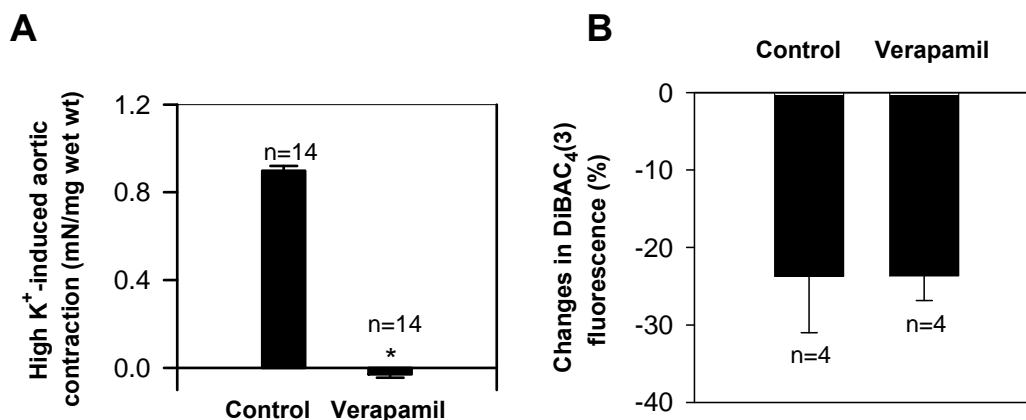
Online Figure III: Effect of verapamil on high K^+-induced contraction of rat aortic segments (A) and on SOC-induced membrane hyperpolarization in the primary cultured rat aortic VSMCs (B). In A, the magnitude of sustained contraction in response to 60 mmol/L K^+ in endothelium-denuded aortic segments in the absence (control) or presence of verapamil (10 μmol/L). (B) SOC-induced membrane hyperpolarization in the primary cultured rat aortic VSMCs in the absence (control) or presence of verapamil (10 μmol/L). The cells bathed in $0Ca^{2+}$-PSS were treated with thapsigargin for 6-8 min (4 μmol/L), followed by re-addition of extracellular Ca^{2+} (1 mmol/L), which initiated SOC-induced membrane hyperpolarization. Values are mean ± S.E. ($n = 4$-14).
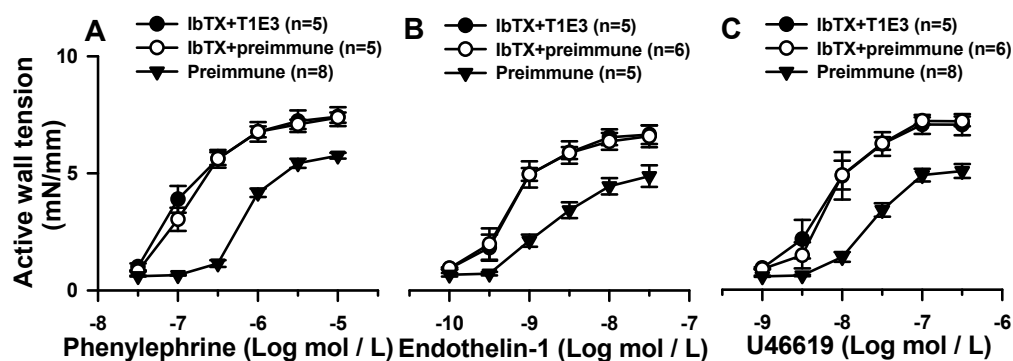
Online Figure IV.

Online Figure IV. Current-voltage relationship of the primary cultured rat aortic VSMCs. Cells were treated with/without T1E3 (1 hr, 1:50) or iberiotoxin (50 nmol/L, 10 min). The cells were held at -60 mV. Whole cell currents were recorded in response to successive voltage pulses of 500 ms duration increased in 20 mV increments from -60 to 100 mV. Values are mean $\pm$ S.E. (n = 4-5).
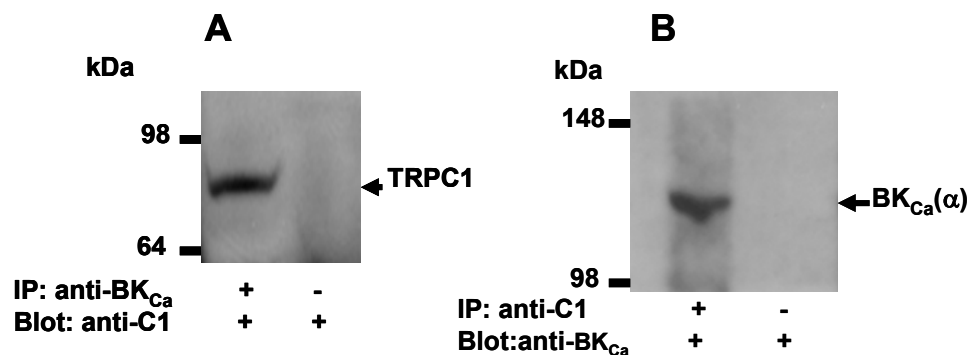
Online Figure V.

Online Figure V. Representative immunoblot showing the expression of intermediate conductance Ca^{2+}-sensitive K^+ channel (IK_{Ca}). Protein lysates were prepared either from freshly isolated porcine aortic endothelial cells (pEndo) or primary cultured rat aortic VSMCs (rVSMC). 20 μg of proteins were loaded onto each lane. The blot was incubated with the primary anti-IK_{Ca} antibody (1:200). (n = 3).

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Online Figure VI

Online Figure VI. Effect of T1E3 on phenylepherine-, endothelin-1-, and U46619-induced contraction. Rat small mesenteric arteries segments were preincubated with T1E3 or preimmune IgG overnight at 37°C. Dose-dependent vascular contractions to phenylepherine (A), endothelin-1 (B) and U46619 (C) were shown. Iberiotoxin (50 nmol/L) enhanced the contraction. The addition of iberiotoxin on top of T1E3 had no additional effect on agonist-induced contraction. Values are mean ± S.E. (n = 5-8)

Online Figure VII

Online Figure VII. Co-immunoprecipitation of TRPC1 and BK_{Ca} in the lysates of HEK293 cells that were co-transfected with TRPC1 and $BK_{Ca}(\alpha)$. Shown were representative pictures of co-immunoprecipitation followed by immunoblots. [A, immunoblot with anti-TRPC1; B, immunoblot with anti-$BK_{Ca}(\alpha)$]. Loaded proteins were the immunoprecipitates pulled with indicated antibody (+) or preimmune IgG (-). n = 3-4 experiments.