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Heteromeric TRPV4-C1 channels contribute to store-operated Ca^{2+} entry in vascular endothelial cells

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ABSTRACT

There is controversy as to whether TRP channels participate in mediating store-operated current (I_{SOC}) and store-operated Ca^{2+} entry (SOCE). Our recent study has demonstrated that TRPC1 forms heteromeric channels with TRPV4 in vascular endothelial cells and that Ca^{2+} store depletion enhances the vesicle trafficking of heteromeric TRPV4-C1 channels, causing insertion of more channels into the plasma membrane in vascular endothelial cells. In the present study, we determined whether the enhanced TRPV4-C1 insertion to the plasma membrane could contribute to SOCE and I_{SOC}. We found that thapsigargin-induced SOCE was much lower in aortic endothelial cells derived from $trpv4^{-/-}$ or $trpc1^{-/-}$ knockout mice when compared to that of wild-type mice. In human umbilical vein endothelial cells (HUVECs), thapsigargin-induced SOCE was markedly reduced by knocking down the expression of TRPC1 and/or TRPV4 with respective siRNAs. Brefeldin A, a blocker of vesicular translocation, inhibited the SOCE. These results suggest that an enhanced vesicular trafficking of heteromeric TRPV4-C1 channels contributes to SOCE in vascular endothelial cells. Vascular tension studies suggest that such an enhanced trafficking of TRPV4-C1 channels may play a role in thapsigargin-induced vascular relaxation in rat small mesenteric arteries.

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1. Introduction

Store-operated Ca^{2+} entry (SOCE) is a major mechanism for Ca^{2+} influx in many cell types including vascular endothelial cells [1,2]. For SOCE, Ca^{2+} entry is activated in response to depletion of intracellular Ca^{2+} stores [1]. SOCE serves to replenish intracellular Ca^{2+} stores, and more importantly functions as a key signal to regulate diverse cellular processes [1]. In vascular endothelial cells, SOCE is involved in the control of vascular tone, vascular permeability and many other functions [3,4].

The best characterized store-operated current is the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}), and recent studies have identified Orai proteins as the pore forming subunits that mediate I_{CRAC} [5]. In addition, Ca^{2+} may enter cells through other store-operated channels (I_{SOC}) which have much greater conductance than that of I_{CRAC}. Although it is still under heavy debate [1,6,7], TRPC channels have been suggested to be the candidates that mediate I_{SOC} and SOCE [1,6,7]. In vascular endothelial cells, several studies have provided evidence that TRPC channels, including TRPC1 and -C4, may contribute to SOCE [3,4,8]. However, a recent study challenged the role of TRPC in endothelial I_{SOC} and SOCE [9].The mechanism of SOCE is the subject of intensive research. It has been found that the Ca^{2+} store depletion is sensed by a Ca^{2+} sensor STIM1 in endoplasmic reticulum. In response to a decrease in [Ca^{2+}] in the endoplasmic reticulum, STIM1 aggregates and translocates to regions immediately underneath the plasma membrane, where it can be detected as puncta. The location of these puncta represents the site where STIM1 regulates the plasma membrane channels involved in SOCE [10]. A series of recent reports suggest that STIM1 may directly and/or indirectly gate TRPC channels to induce appearance of SOCE [11,12]. In addition to direct and/or indirect channel gating, SOCE could presumably be promoted by enhanced trafficking of channel-containing vesicles to the plasma membrane, resulting in more channel insertion into the plasma membrane [13]. However, the latter mechanism has received much less attention [13]. There is still a lack of a general model for the role of

vesicular trafficking and plasma membrane insertion of TRP channels in SOCE [13].

Recently, we have demonstrated that TRPC1 forms heteromeric channels with TRPV4 in vascular endothelial cells [14], and that Ca^{2+} store depletion enhances the vesicle trafficking of heteromeric TRPV4-C1 channels, causing insertion of more channels into the plasma membrane in these cells [15]. In the present study, we hypothesized that the enhanced TRPV4-C1 trafficking to the plasma membrane may contribute to SOCE and I_{SOC} in the endothelial cells. We tested our hypothesis by multiple approaches including patch–clamp electrophysiology, fluorescence measurement of cytosolic Ca^{2+} and vascular tension measurement.

2. Materials and methods

2.1. Materials

Human embryonic kidney cell line HEK293 was from ATCC, USA. Human umbilical vein endothelial cells (HUVECs, CC-2517), endothelial cell growth medium (EGM), endothelial cell basal medium (EBM) and bovine brain extract (BBE) were from Lonza, USA. Fura-2/AM and pluronic F127 were from Molecular Probes Inc. (+)-Brefeldin A and thapsigargin were from Calbiochem. N,N,N′,N′-Tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) were from Invitrogen. RN1734 was from Menai Organics Ltd.

2.2. Cell isolation and culture

Primary aortic endothelial cells were isolated and cultured as described elsewhere with modification [16]. Briefly, male $trpc1^{-/-}$, $trpv4^{-/-}$ and wild-type 129SV mice were killed with CO_2. The thoracic aortas were removed, cut into small segments, and then treated with 0.25% collagenase in serum free RPMI 1640 medium for 15 min at 37 °C. The suspension after the enzyme digestion was centrifuged at 1200 rpm for 5 min. The precipitated endothelial cells were re-suspended and then cultured in 90% RPMI 1640 and 10% FBS in a 35 mm collagen Type-I coated dish. To remove SMC, after 2 h incubation at 37 °C, the medium was removed, the cells were washed with warmed PBS, and then re-cultured in RPMI 1640 medium supplemented with 20% FBS, 100 μg/ml penicillin and 100 U/ml streptomycin [16]. Confluent cell monolayers were passaged using 0.25% trypsin containing 2.5 mM EDTA. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin and 100 U/ml streptomycin. HUVECs were cultured in EGM medium supplemented with 1% BBE, 100 μg/ml penicillin and 100 U/ml streptomycin. For HUVECs and the primarily isolated aortic endothelial cells, only cells from first four passages were used for experiments. The identity of the primary cultured aortic endothelial cells was confirmed by immunostaining using an antibody against PECAM-1 (platelet/endothelial cell adhesion molecule-1). The results showed that >95% of the cells were positively stained, indicating that they were of endothelial origin.

2.3. Cloning and transfection

Human TRPC1 cDNA (NM_003304) was obtained by RT-PCR from human coronary endothelial cells CC2585 (BioWhittaker). TRPV4^{M680D} and TRPC1$^{Mut-pore}$, each has mutation at channel pore region, was described elsewhere [17,18]. The nucleotide sequences of all constructs were verified with DNA sequencing. TRPC1-siRNA, TRPV4-siRNA, STIM1-siRNA and scramble control were from Ambion [15].

Transfection condition was as described elsewhere [15]. Briefly, HEK293 cells were transfected with various constructs using Lipofectamine 2000. HUVECs were transfected with siRNAs by electroporation using Nucleofector II following the procedure following

the manufacturer's instruction manual. Functional studies were performed 2–3 days post-transfection.

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

$[Ca^{2+}]_i$ in cultured cells was measured as described elsewhere [14]. Briefly, cultured HEK cells or HUVECs were loaded with 10 μM Fura-2/AM and 0.02% pluronic F-127 for 1 h in dark at 37 °C in normal physiological saline solution (NPSS). In experiments studying SOCE, Ba^{2+} was used as surrogate ion to avoid potential artifact caused by thapsigargin inhibition on endoplasmic reticulum Ca^{2+}-pump (SERCA)[19]. If Ca^{2+} was used as the influx ion, the reduced SERCA activity by thapsigargin would prevent Ca^{2+} sequestration into intracellular Ca^{2+} stores, creating an additional Fura-2 fluorescence rise that is independent of Ca^{2+} store depletion [19]. In these experiments, cells were pre-treated with thapsigargin (4 μM, 20 min) or TPEN (1 mM, 20 min) to deplete intracellular Ca^{2+} stores, followed by changing extracellular medium from a Ca^{2+}-free ($0Ca^{2+}$-PSS) to a Ba^{2+}-containing solution ($1Ba^{2+}$-PSS). $0Ca^{2+}$-PSS contained in mM: 140 NaCl, 5 KCl, 1 MgCl$_2$, 10 glucose, 2 EGTA and 5 HEPES, pH 7.4; $1Ba^{2+}$-PSS contained in mM: 140 NaCl, 5 KCl, 1 BaCl$_2$, 1 MgCl$_2$, 10 glucose, 5 HEPES, pH 7.4. Fura-2 fluorescence signals were measured at room temperature using dual excitation wavelengths at 340 and 380 nm using an Olympus fluorescence imaging system. SOCE was determined by Fura-2 ratio change and was then converted to $[Ba^{2+}]_i$ based on the calibration using standard solutions of different concentrations of Ba^{2+}. If needed, brefeldin A (5 μM) was introduced 30 min before application of thapsigargin or TPEN. In hypotonicity series of experiments, $[Ca^{2+}]_i$ was measured using 10 μM Fura-2/AM. Isotonic solution contained in mM: 65 Na-aspartate, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, 140 mannitol, pH 7.4 with NaOH, ~300 mOsm. Hypotonic solution contained in mM: 65 Na-aspartate, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH, and calibrated with mannitol, ~210 mOsm.

2.5. Whole-cell patch clamp

Whole cell current was measured with an EPC-9 patch clamp amplifier as described elsewhere [18]. Cells were clamped at 0 mV. Whole cell current density (pA/pF) was recorded in response to successive voltage pulses of +80 mV and −80 mV for 100 ms duration. These whole cell current values were then plotted vs. time. The store-dependent cation current was recorded with pipette solution containing in mM: 140 CsCl, 2 MgCl$_2$, 1 ATP, 5 EGTA, 10 HEPES, pH 7.2, and the bath solution containing in mM: 140 NaCl, 5 KCl, 0.5 EGTA, 10 HEPES, pH 7.4. The bath solution was then was replaced by a solution containing 140 NMDG-Cl, 5 KCl, 0.5 EGTA, 10 HEPES, pH 7.4. If needed, brefeldin A (5 μM) was introduced 30 min before the application of thapsigargin.

2.6. Biotinylation of cell surface proteins and immunoblots

Biotinylation of cell surface proteins was carried out using a cell surface protein isolation kit following the manufacturer's instructions (catalog no. 89881; Pierce) as described elsewhere [15]. Proteins were resolved by SDS-PAGE and analyzed by immunoblots using anti-TRPV4 (1:200) or anti-TRPC1 (1:200) antibodies.

2.7. Arterial tension measurement

Segments of the third-order branches of rat mesenteric artery (2–3 mm long) were dissected and mounted in a DMT myograph (model 610 M) under a normalized tension as described elsewhere [20]. Cumulative concentration–response relationships for the relaxant effect of thapsigargin were determined in rings
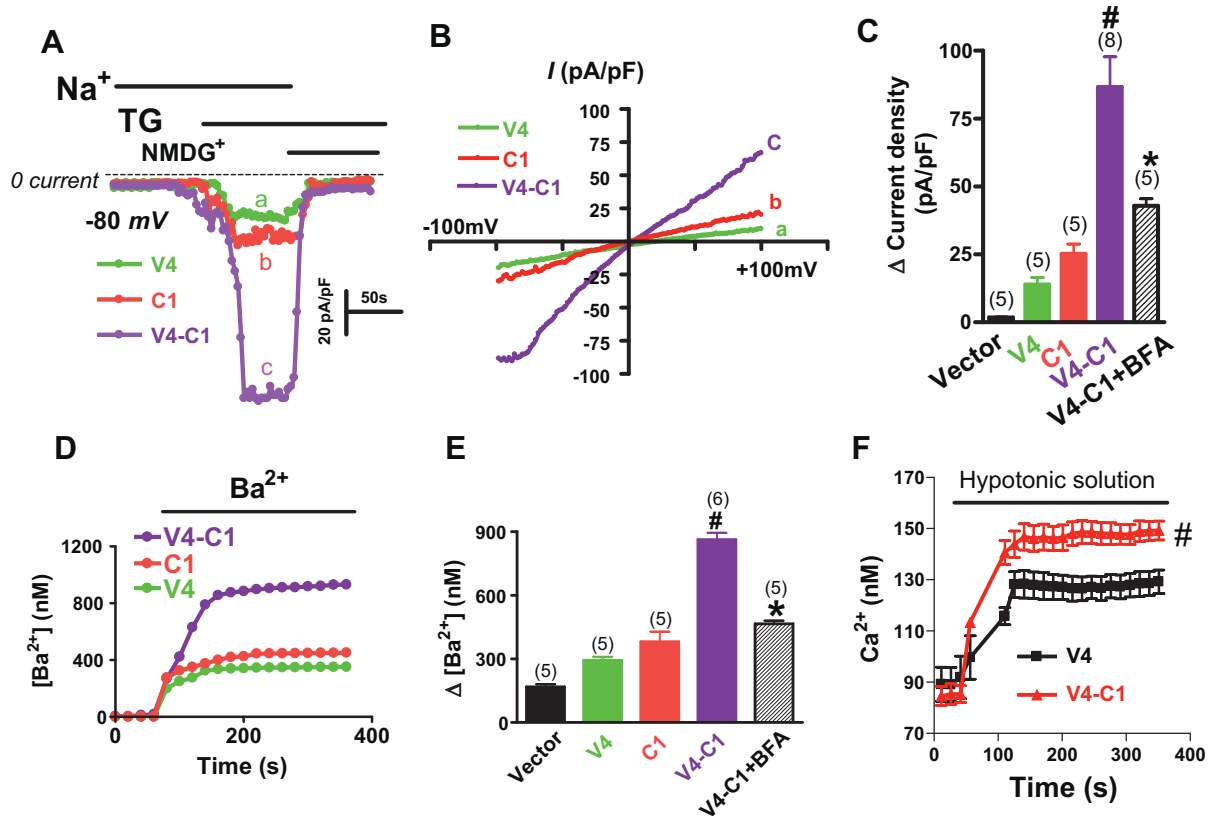
Fig. 1. Formation of store depletion-sensitive Ca^{2+} entry channels in HEK cells that were co-transfected with TRPC1 and TRPV4. A-E, shown are the store-operated cation current and Ba^{2+} influx in transfected HEK cells. HEK cells were transfected with TRPV4 or TRPC1 or TRPV4 plus TRPC1. The experimental protocol involves incubation of cells in media containing 150 mM Na^+ to measure the monovalent current before and after cell stimulation with thapsigargin (TG, 4 µM). Na^+ was then replaced with $NMDG^+$. For cation currents (A–C), shown are representative current traces at −80 mV (A), the corresponding I–V curves (B) and summary data for the maximal change in current in response to thapsigargin (C). For store-operated Ba^{2+} influx (D and E), shown are representative traces (D) and summary data for the maximal change in $[Ba^{2+}]_i$ (E). F, Time-course of $[Ca^{2+}]_i$ change in response to hypotonicity in HEK cells expressing TRPV4 alone or co-expressing TRPV4 plus TRPC1. Mean ± SE (n = 4–6 experiments). #, $P < 0.05$ compared to the cells transfected with either TRPV4 or TRPC1 alone. *$P < 0.05$ compared to V4-C1 without BFA.

after steady contraction with phenylephrine. The concentration of phenylephrine varied from 3 to 10 µM to achieve similar degree of constriction in different arteries. The artery segments were preincubated with T1E3 (1:100) or preimmune IgG (1:100) at 4 °C overnight, or with RN1734 (5 µM) or brefeldin A (5 µM) for 10–30 min at 37 °C.

2.8. Statistics

Student's t-test was used for statistical comparison. For the comparison of multiple groups, one-way ANOVA with Newman–Keuls was used. Significances were set as $p < 0.05$.

3. Results

3.1. Formation of store depletion-sensitive Ca^{2+} entry channel upon co-expression of TRPV4 and TRPC1 in HEK cells

In our previous report, surface biotinylation method showed that endogenous expression of TRPV4 and TRPC1 were low and less than detection level [15], and that co-expression of TRPV4 and TRPC1 in HEK cells results in an increased amount of both proteins at cell surface [15]. Here we measured whole-cell currents. Changes in whole-cell currents in response to 4 µM thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), were taken as I_{SOC}. As shown in Fig. 1A, thapsigargin-induced inward current at −80 mV was much larger in HEK cells co-expressing TRPV4-C1 than those cells over-expressing TRPC1 or

TRPV4 alone. In cells co-expressing TRPV4 and TRPC1, substitution of TRPC1 by a corresponding pore-mutant construct TRPC1^Mut-pore markedly reduced the I_{SOC}. Interestingly, brefeldin A (5 µM), an agent that inhibits vesicular translocation, also markedly reduced I_{SOC} (Fig. 1C). SOCE was measured by pre-treating the cells with thapsigargin (4 µM, 20 min), followed by changing to extracellular medium containing 1 mM Ba^{2+} (1Ba^{2+}-PSS). Ba^{2+} was used as the surrogate ion for Ca^{2+} influx [19]. In agreement with the results of I_{SOC}, the magnitude of SOCE was much larger in TRPV4-C1 co-expressing HEK cells than the cells over-expressing TRPC1 or TRPV4 alone (Fig. 1D and E). Again, SOCE induced by thapsigargin was markedly suppressed by brefeldin A treatment (5 µM) and by the replacement of TRPC1 with TRPC1^Mut-pore (Fig. 1E). Previously, we have shown that brefeldin A treatment suppressed the TG-induced insertion of heteromeric TRPV4-C1 channels to the plasma membrane [15]. Here we showed that brefeldin A suppressed the TG-induced I_{SOC} and SOCE. These data support the notion that overexpressed heteromeric TRPV4-C1 channels contribute to I_{SOC} and SOCE, and that the mechanism involves an enhanced vesicular trafficking of TRPV4-C1 channels to the plasma membrane.

TRPV4 is known to be activated by hypotonicity-induced cell swelling [21]. Here the effect of hypotonicity on heteromeric TRPV4-C1 channels was examined. Similar to the cells expressing TRPV4 alone, hypotonicity also elicited cytosolic Ca^{2+} rises in cells that were co-expressed with TRPV4 and TRPC1 channels (Fig. 1F), suggesting that heteromeric TRPV4-C1 channels could also respond to hypotonicity.
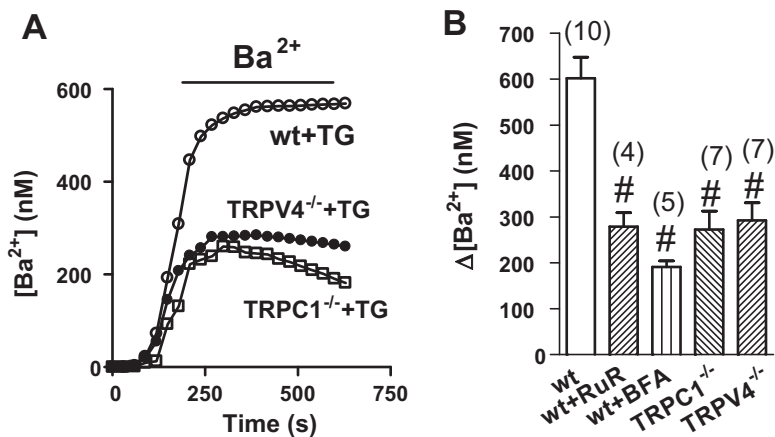
Fig. 2. SOCE in mouse aortic endothelial cells. (A) Representative traces of store-operated Ba^{2+} entry in aortic endothelial cells derived from wild-type, *trpc1$^{-/-}$* and *trpv4$^{-/-}$* mice. (B) Summary for the maximal Ba^{2+} entry in A. TG, 4 μM; BFA, 5 μM; RuR, 5 μM. Mean ± SE (n = 4–10). $^{\#}P < 0.05$ compared to endothelial cells from wild-type mice.
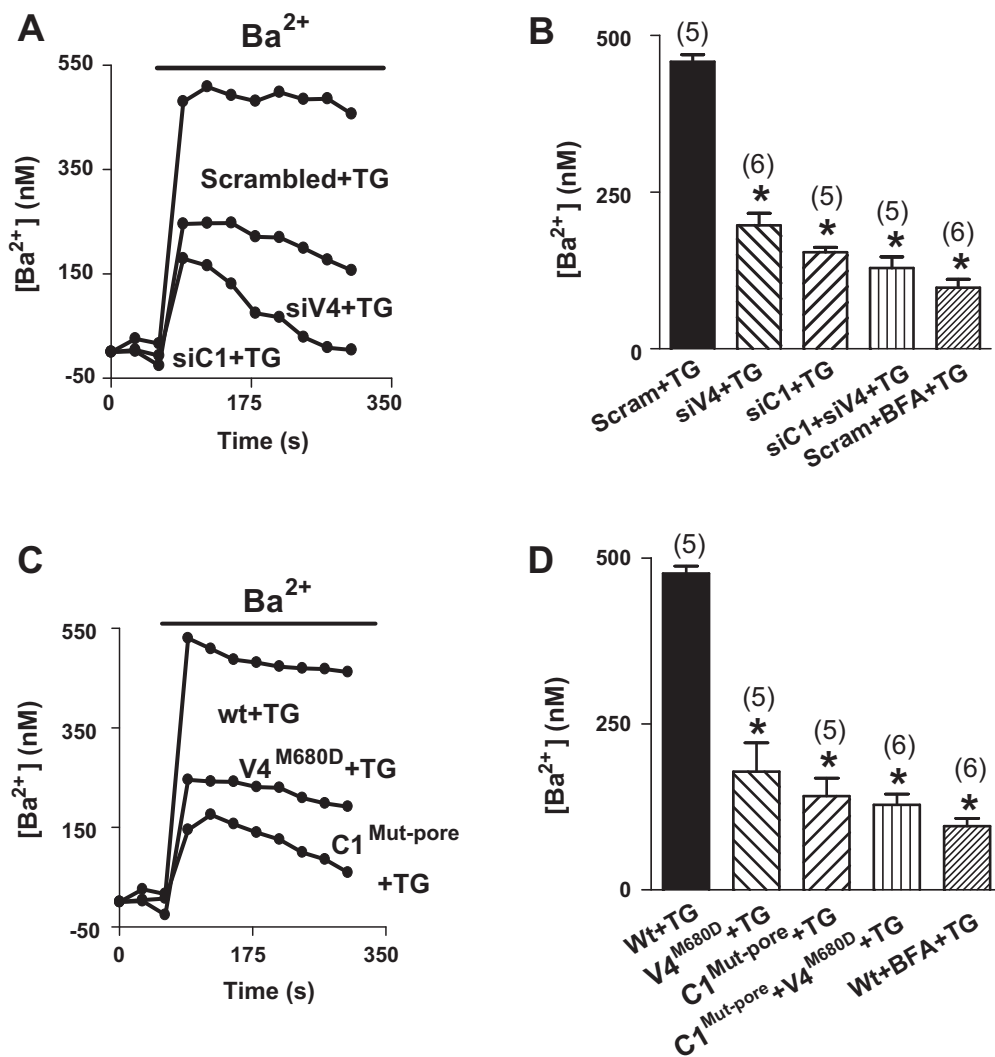

Fig. 3. SOCE in HUVECs. (A and C) Representative time course of store-operated Ba^{2+} entry in endothelial cells treated with siRNAs (A) or dominant negative constructs (C), followed by thapsigargin treatment (TG, 4 μM). (B and D) Summary data for the maximal change in $[Ba^{2+}]_i$ in A and C. BFA, 5 μM; Mean ± SE (n = 5–6). $^{*}P < 0.05$ compared to scrambled-siRNA (in B), or to TG-treatment cells without pore mutant (in D). TRPV4^{M680D}, a pore mutant of TRPV4; TRPC1$^{Mut-pore}$, a pore-dead mutant of TRPC1; Scram, scrambled-siRNA; siV4, TRPV4-siRNA; siC1, TRPC1-siRNA.
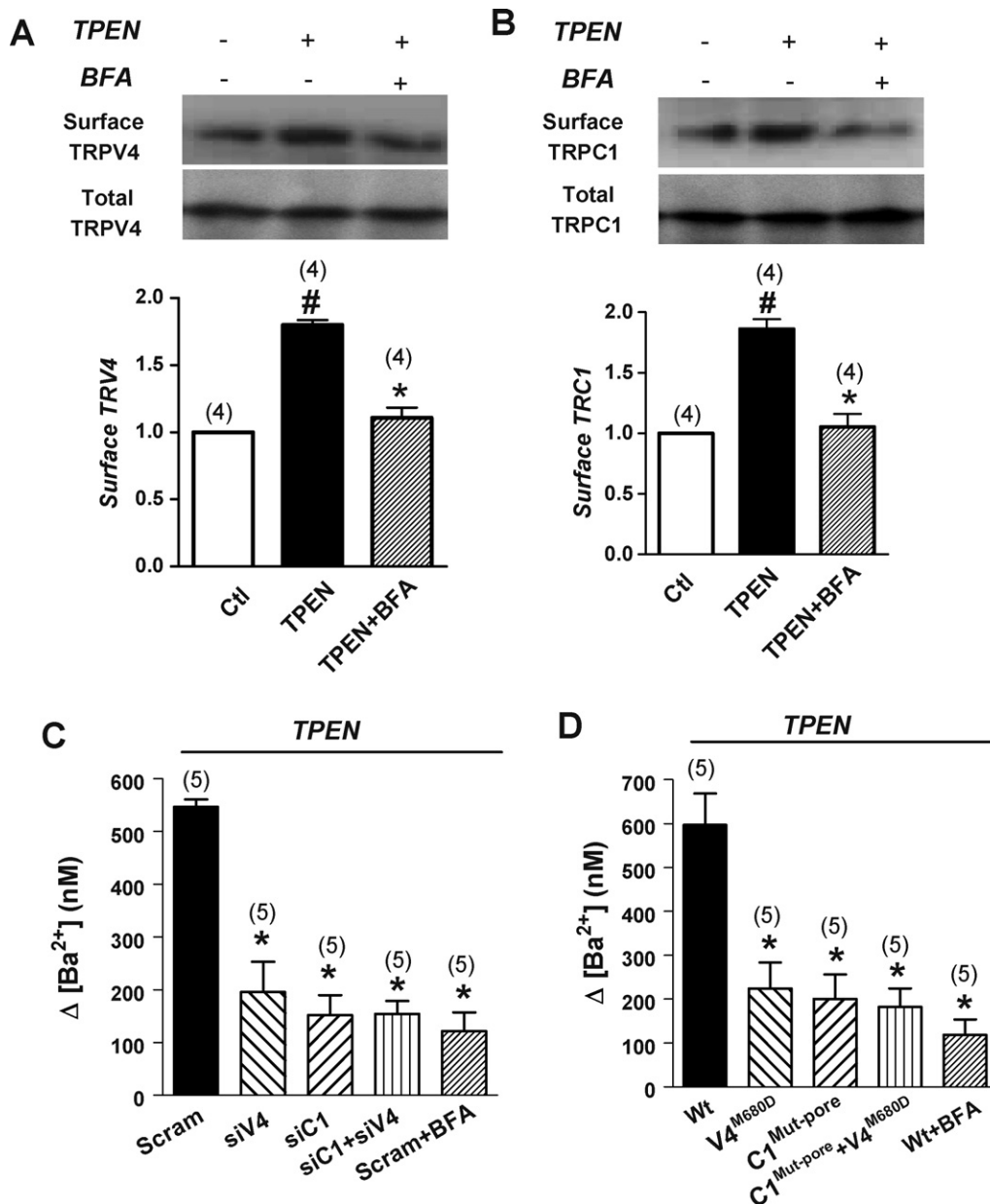
Fig. 4. TPEN-induced TRPV4-C1 trafficking to the plasma membrane and its associated SOCE in HUVECs. A and B, Representative images (upper panel of A and B) and summary data (lower panel of A and B) of cell surface biotinylation experiments showing TPEN-induced translocation of TRPV4-C1 to the plasma membrane. The cells were treated with or without TPEN, 1 mM for 20 min, followed by cell surface biotinylation assay. If necessary, 5 μM BFA was introduced 30 min before TPEN application. Total cellular contents of TRPV4 or TRPC1 were used as control. C and D, summary data for the TPEN-induced maximal change in $[Ba^{2+}]_i$. TPEN, 1 mM; mean $\pm$ SE ($n = 5$). Data are given as the mean $\pm$ SE (the number of experiments is labeled on top of the bars). $^{\#}P < 0.05$ vs. control without TPEN (Ctl), and $^{*}P < 0.05$ vs the TPEN-treated cells without BFA (A and B), or vs. scrambled-siRNA without BFA (C), or vs. Wt without BFA (D). TRPV4^{M680D}, a pore mutant of TRPV4; TRPC1$^{Mut-pore}$, a pore-dead mutant of TRPC1; Scram, scrambled-siRNA; siV4, TRPV4-siRNA; siC1, TRPC1-siRNA.

3.2. SOCE in vascular endothelial cells

We next examined the SOCE in aortic endothelial cells derived from wild-type, $trpc1^{-/-}$ and $trpv4^{-/-}$ knockout mice. Compared to SOCE in aortic endothelial cells derived from wild-type mice, the SOCE in cells derived from $trpc1^{-/-}$ or $trpv4^{-/-}$ mice was much smaller in magnitude, suggesting a contribution of both TRPC1 and TRPV4 to SOCE (Fig. 2). SOCE was also reduced by ruthenium red (RuR), which is a commonly used inhibitor of TRPVs [22]. Again, SOCE induced by thapsigargin was markedly reduced by brefeldin A (5 μM) (Fig. 2).

Another endothelial cell type, HUVEC, was also examined for the potential role of TRPC1 and TRPV4 in SOCE. SOCE was induced

either by thapsigargin (Fig. 3) or TPEN (Fig. 4). Thapsigargin was previously shown to enhance the trafficking of heteromeric TRPV4-C1 channels to the plasma membrane [15]. Here TPEN was also found to increase the amount of TRPV4 and TRPC1 proteins in the plasma membrane by cell surface biotinylation method (Fig. 4A and B). In Ca^{2+} study, thapsigargin- or TPEN-induced SOCE was greatly reduced in cells that were treated with TRPC1- and/or TRPV4-specific siRNAs (Figs. 3A,B and 4C), as compared to the controls that were transfected with scrambled siRNA (Figs. 3A,B and 4C). The specificity and effectiveness of these TRPC1- and TRPV4-specific siRNAs have been previously demonstrated by us in another report [14]. In that report, we demonstrated that TRPC1-siRNA only knocked-down the protein level of TRPC1 but had no effect on
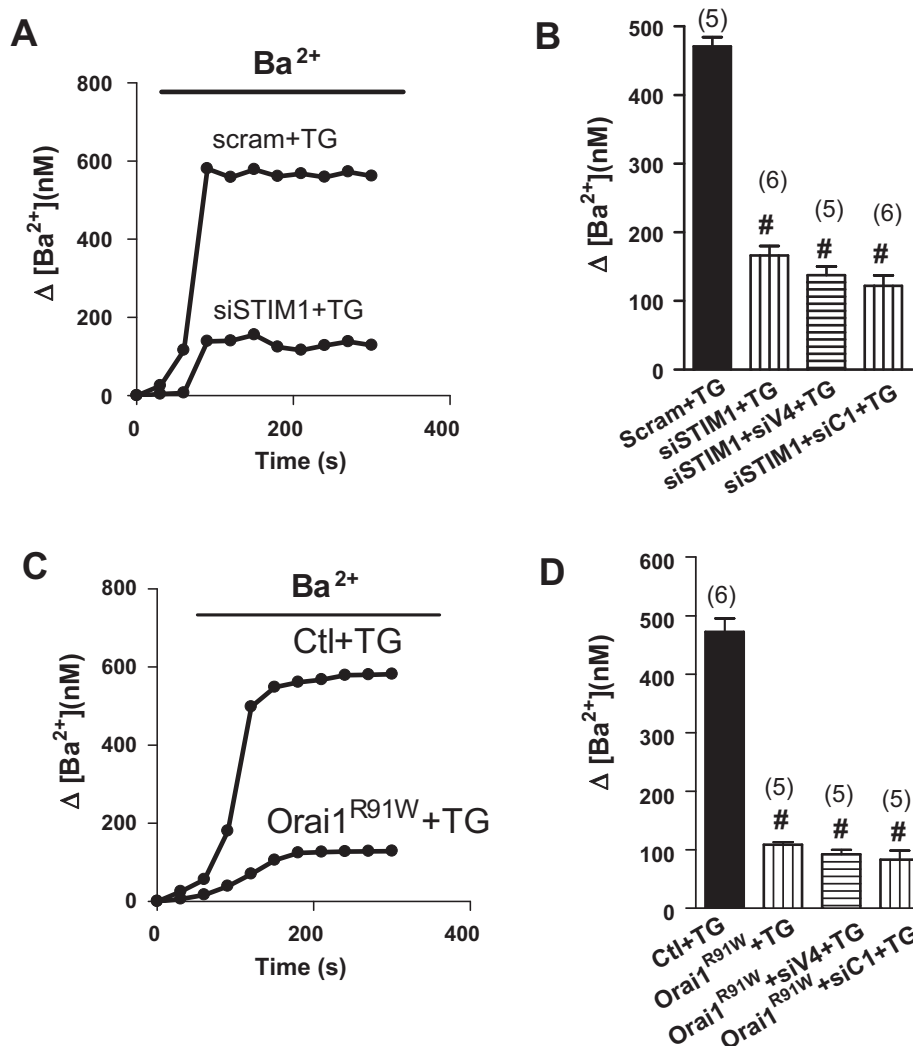
Fig. 5. Role of STIM1 and Orai1 in SOCE in HUVECs. (A and C) Representative traces of store-operated Ba^{2+} entry in endothelial cells treated with STIM1-siRNA (A) or dominant negative construct $Orai^{R91W}$ (C), followed by thapsigargin treatment (TG, 4 μM). (B and D) Summary data for the maximal store-operated $[Ba^{2+}]_i$ change as in A and C. $^{\#}P < 0.05$ compared to the cells transfected with scrambled siRNA. Scram, scrambled-siRNA; siV4, TRPV4-siRNA; siC1, TRPC1-siRNA; siSTIM1, STIM1-siRNA; $Orai1^{R91W}$, a dominant-negative mutant of Orai1.

that of TRPV4, and *vice versa* [14]. Pore-dead mutants of TRPC1 (TRPC1$^{Mut-pore}$) and/or TRPV4 (TRPV4^{M680D}) [17,18] were also used, and both constructs reduced the thapsigargin- or TPEN-induced SOCE (Figs. 3C,D and 4D). Again, brefeldin A (5 μM) treatment reduced the thapsigargin- or TPEN-induced SOCE in HUVECs (Figs. 3B,D and 4C,D).

3.3. Role of STIM1 and Orai1 in SOCE of HUVECs

The role of STIM1 an Orai1 in thapsigargin-induced SOCE of HUVECs was examined. Transfection of HUVECs with STIM1-specific siRNA significantly reduced SOCE (Fig. 5A and B). In cells where the expression of TRPC1 or TRPV4 was already knocked-down by respective siRNAs, STIM1-siRNA had no additional effect on SOCE. The effectiveness of STIM1-specific siRNA has been previously shown by us in another report [15]. Dominant-negative mutant construct Orai1^{R91W} [23] was used to examine the possibility of Orai1 involvement. This construct has previously been shown to inhibit endogenous Orai1 and CRAC channel function [23,24]. The results showed that Orai1^{R91W} reduced SOCE in HUVECs (Fig. 5C and D). Furthermore, in cells where the expression of TRPC1 or

TRPV4 was already knocked-down by respective siRNAs, Orai1^{R91W} had no additional effect on SOCE (Fig. 5D).

3.4. Role of TRPV4 and TRPC1 in thapsigargin-induced vascular relaxation

We explored the functional role of the present scheme in vascular tone modulation. It is known that SERCA inhibitors may induce endothelial cells to release vasodilators, causing endothelium-dependent vascular relaxation [25]. Wire myography studies showed that thapsigargin (0.3–30 μM) induced concentration-dependent relaxation in the third-order of rat mesenteric arterial segments that were pre-contracted with phenylephrine (Fig. 6A). The relaxation was significantly inhibited in the arterial segments that had been treated with a TRPV4 blocker RN1734 (5 μM) or a TRPC1-specific blocking antibody T1E3 (1:100 dilution) (Fig. 6C). Combined treatment with RN1734 (5 μM) plus T1E3 (1:100) has no additional effect (Fig. 6B and C). BFA also significantly reduced the thapsigargin-induced vascular relaxation but have no additional effect in arteries that were pretreated with RN1734 (5 μM) and/or T1E3 (1:100) (Fig. 6C). These data support the functional role of TRPV4-C1 trafficking in thapsigargin-induced vascular relaxation.
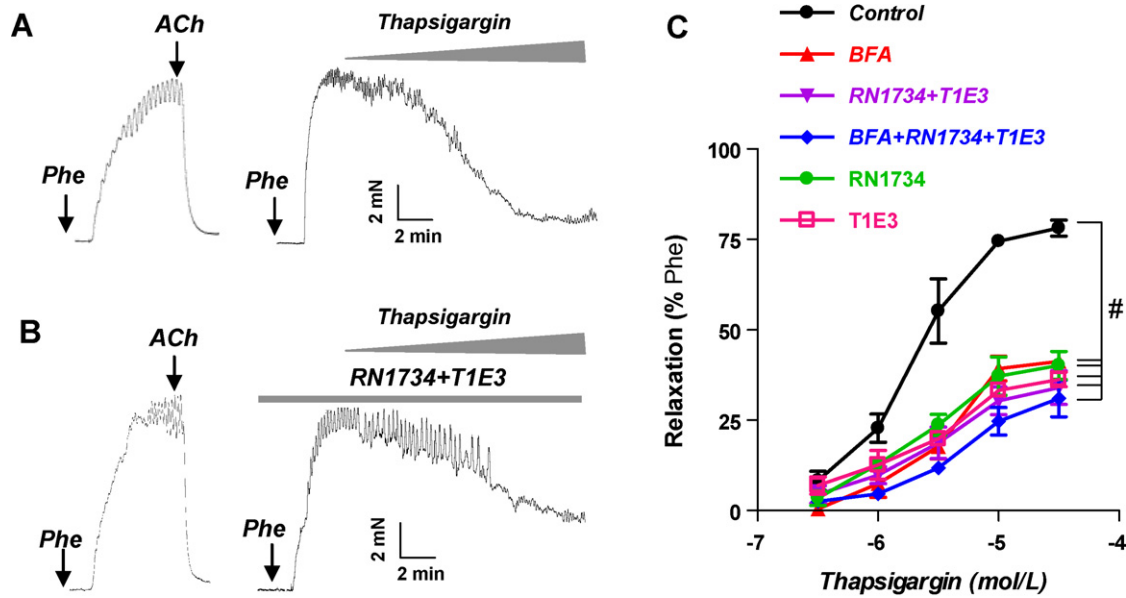
Fig. 6. Effect of T1E3, RN1734 and BFA on thapsigargin-induced relaxation in rat small mesenteric arteries. (A and B) Representative traces for thapsigargin-induced relaxation in arteries pre-treated with RN1734 plus T1E3 (B) or preimmune IgG as control (A). (C) Summary of data showing the effect of RN1734 plus T1E3 and BFA on dose-dependent vascular relaxation to thapsigargin. Values are mean ± SE ($n = 4$). $^{\#}P < 0.05$ compared to control. Thapsigargin, 0.3–30 μM; RN1734, 5 μM; T1E3, 1:100; preimmune IgG, 1:100.

4. Discussion

In the present study, we found that both TRPC1 and TRPV4 contribute to SOCE in vascular endothelial cells. This is the first report suggesting an involvement of TRPV4 in SOCE and I_{SOC}. We recently reported that Ca^{2+} store depletion enhances the vesicular trafficking of exogenously expressed TRPV4 and TRPC1 channels to the plasma membrane and suggested that these two TRP proteins constitute heteromeric channels. Here we report that delivery of heteromeric TRPV4-C1 channels to the plasma membrane following internal Ca^{2+} store depletion accounts for a significant part of the observed SOCE and I_{SOC}. We show that brefeldin A, a blocker of vesicular translocation, drastically reduced SOCE in vascular endothelial cells. We also show that SOCE was reduced in vascular endothelial cells derived from $trpc1^{-/-}$ and $trpv4^{-/-}$ mice. In addition, vascular tension studies suggested an important role of TRPV4-C1 trafficking in thapsigargin-induced vascular relaxation in rat small mesenteric arteries.

Presently, there is heated debate on whether TRPC1 channels contribute to SOCE. Over the years, many studies using a variety of different methods have reported that TRPC1 contributes to SOCE and I_{SOC} in many cell types [1]. However, controversy arises when SOCE and/or I_{SOC} were examined in cells derived from TRPC1 knockout mice [6,26–28]. On the one hand, two studies reported an reduced SOCE and I_{SOC} in submandibular gland acinar cells and pancreatic acinar cells derived from $trpc1^{-/-}$ mice when compared to that of control mice ($trpc1^{+/+}$), suggesting a role of TRPC1 in SOCE and I_{SOC} [26,28]. On the other hand, two other studies detected no difference in SOCE and/or I_{SOC} in platelets cerebral artery smooth muscle cells when control mice and $trpc1^{-/-}$ mice were compared, arguing against the contribution of TRPC1 to SOCE and I_{SOC} [6,27]. There is similar controversy in vascular endothelial cells. Substantial amount of data suggest a contribution of TRPC1 to SOCE and I_{SOC} in vascular endothelial cells including human umbilical vein endothelial cells (HUVECs) [29–32]. Evidence appears to be strong, not only because the data were generated from different groups but also because a variety of different methods were used in these studies. These methods include: TRPC1-specific antisense oligonucleotides [29], TRPC1-specific blocking antibodies [31], and overexpression of TRPC1 in endothelial cells [30,32]. However, a recent study argued against the involvement of TRPC1 or TRPC4 in SOCE and I_{SOC} in HUVECs [9]. Although we cannot explain the reason for the discrepancy, the present study, by using endothelia cells from $trpc1^{-/-}$ mice or those treated with TRPC1-specific siRNA, demonstrates a strong correlation between TRPC1 expression level and SOCE in vascular endothelial cells including HUVECs. While methodological differences could contribute to the differences in results regarding the role of TRPC1 in SOCE, other possible explanations include; (1) the contribution of TRPC1 in SOCE and I_{SOC} could be different in different cell types; (2) source of cells, cell passage number, and/or culture conditions may alter the role of TRPC1 in SOCE and/or I_{SOC}. Along these lines, studies have shown that TRPC1 may switch between store-operated and non-operated modes dependent on expression level [33] and protein ratio of TRPC1 vs. STIM1 [34]. Furthermore, the expression of TRPC1 might be regulated by a number of other factors [4,35], the difference of which could also contribute to the discrepancy in results obtained from various studies.

This is the first report suggesting an involvement of TRPV4 in SOCE and I_{SOC}. TRPV4 is expressed in a variety of tissues, performing diverse functional roles including osmoregulation, bone formation, flow sensation in circulation and renal systems [36]. The activity of the channels is known to be stimulated by heat, hypotonicity, flow shear force, and natural agonists such as anandamide and epoxyeicosatrienoic acids [36]. The present study shows that depletion of intracellular Ca^{2+} stores enhances the vesicular trafficking of TRPV4-C1 channels to the plasma membrane, contributing to SOCE and I_{SOC} in the endothelial cells. An enhanced Ca^{2+} entry would be expected to stimulate vascular endothelial cells to release vasodilators including nitric oxide, endothelium-derived hyperpolarization factors and prostacyclin, resulting in subsequent vascular relaxation [3]. This was indeed confirmed by vascular tension studies, in which thapsigargin-induced vascular relaxation was shown to be reduced by BFA, TRPV4-siRNA and TRPC1-siRNA.

STIM1 and Orai1 are important proteins for SOCE [5,10]. STIM1 is the Ca^{2+} sensor in endoplasmic reticulum that has been associated with regulation of Orais and TRPCs, two candidates that mediate SOCE [5,10.]. In addition, Orai1 could also interact with

TRPCs to influence SOCE [23]. Recently, we showed that STIM1 is required for thapsigargin-induced translocation of TRPV4-C1 heteromeric channels to the plasma membrane [15]. The present data demonstrates that STIM1 regulates SOCE in HUVECs, constituent with previous studies [9,15]. We also showed a crucial role of Orai1 in regulating SOCE in HUVECs. Interestingly, STIM1-siRNA and dominant-negative Orai construct did not induce further decrease in SOCE in cells where TRPV4 or TRPC1 expression were reduced. These data suggest that STIM1 and Orai1 act through heteromeric TRPV4-C1 channels to exert its effect on SOCE. Taken together, our findings reported herein strongly demonstrate a role for TRPC1 and TRPV4 in SOCE in vascular endothelial cells. We suggest that Ca^{2+} store depletion enhances trafficking of heteromeric TRPV4-C1 channels to the plasma membrane where these channels contribute to SOCE and I_{SOC} in a STIM1- and Orai1-dependent manner.

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