

Depletion of Intracellular Ca²⁺ Stores Stimulates the Translocation of Vanilloid Transient Receptor Potential 4-C1 Heteromeric Channels to the Plasma Membrane

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Objective—To examine the effect of Ca²⁺ store depletion on the translocation of vanilloid transient receptor potential (TRPV) 4-C1 heteromeric channels to the plasma membrane.

Methods and Results—Vesicular trafficking is a key mechanism for controlling the surface expression of TRP channels in the plasma membrane, where they perform their function. TRP channels in vivo are often composed of heteromeric subunits. Experiments using total internal fluorescence reflection microscopy and biotin surface labeling show that Ca²⁺ store depletion enhanced TRPV4-C1 translocation into the plasma membrane in human embryonic kidney 293 cells that were coexpressed with TRPV4 and canonical transient receptor potential 1 (TRPC1). Fluorescent Ca²⁺ measurement and patch clamp studies demonstrated that Ca²⁺ store depletion enhanced 4 α -PDD-stimulated Ca²⁺ influx and cation current. The translocation required stromal interacting molecule 1 (STIM1). TRPV4-C1 heteromeric channels were more favorably translocated to the plasma membrane than TRPC1 or TRPV4 homomeric channels. Similar results were obtained in native vascular endothelial cells.

Conclusion—Ca²⁺ store depletion stimulates the insertion of TRPV4-C1 heteromeric channels into the plasma membrane, resulting in an augmented Ca²⁺ influx in response to flow in the human embryonic kidney cell overexpression system and native endothelial cells. (*Arterioscler Thromb Vasc Biol.* 2010;30:2249-2255.)

Key Words: TRPV4-C1 heteromeric channel ■ depletion intracellular Ca²⁺ stores ■ endothelial cell ■ flow ■ translocation

An important means to regulate TRP channel function is to modulate the translocation of channel proteins to the plasma membrane.¹ Hormones, growth factors, and agonists for Gq protein-coupled receptors, such as carbachol, can stimulate the translocation of several TRP channels, including TRPC3–6 and TRPV-1 to the plasma membrane.¹ Multiple cellular factors, including soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Homer, and small G proteins, may participate in the translocation processes.¹ Ca²⁺ store depletion may also stimulate the translocation of TRPC3 and TRPC6 to the plasma membrane.^{2,3} However, the exact molecular mechanism by which TRP channels insert into the plasma membrane is unknown. There is still a lack of a general model for TRP trafficking.¹

TRP channels in vivo are often composed of heteromeric subunits. Heteromeric assembly usually occurs between the members within the same TRP subfamily, such as between TRPC1 and TRPC3.⁴ However, coassembly could also hap-

pen between the subunits from a different TRP subfamily, such as between TRPC1 and polycystic transient receptor potential 2 (TRPP2) (PKD2)^{5,6} and between TRPV4 and TRPP2.⁷ These heteromultimeric channels may display properties different from those of homomultimeric channels.^{8,9} To our knowledge, there is still no report about the vesicular translocation of heteromeric TRP channels. Recent findings indicate that TRPC1 can coassemble with TRPV4 to form heteromeric channels.¹⁰ In vascular tissues, the TRPV4-C1 heteromeric channels are the main channels responsible for flow-induced endothelial Ca²⁺ influx and subsequent vascular relaxation.¹⁰ In separate studies, we also found that Ca²⁺ store depletion potentiates flow-induced Ca²⁺ influx¹¹ and subsequent vascular relaxation.¹² In the present study, we tested the hypothesis that Ca²⁺ store depletion may enhance the translocation of TRPV4-C1 heteromeric channels to the plasma membrane. Our results show that Ca²⁺ store depletion triggers the translocation of TRPV4-C1 heteromers to the

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plasma membrane, resulting in an enhanced Ca^{2+} influx in response to flow. This scheme provides a mechanistic explanation for the potentiated Ca^{2+} influx to flow under the condition of Ca^{2+} store depletion in vascular endothelial cells.

Methods

Cell Culture, Clones, and Transfection

Human embryonic kidney (HEK) 293 cells and human umbilical vein endothelial cells (HUVECs) were cultured using standard protocol. The human TRPC1 gene (NM_003304) and the mouse TRPV4 gene (NM_022017) were cloned into pcDNA6 or pCAGGS vector for expression. Channel pore mutants TRPV4^{M680D} and TRPC1^{Mut-pore} were as described elsewhere.^{13,14} For total internal fluorescence reflection microscopy (TIRFM) experiments, TRPV4 was tagged with cyan fluorescent protein (CFP) at its C-terminus and TRPC1 was tagged with yellow fluorescent protein (YFP) at its N-terminus. Small interfering RNAs (siRNAs) and scramble control were from Ambion, Austin, Tex. HEK cells were transfected using Lipofectamine 2000. HUVECs were transfected by electroporation using Nucleofector II. Functional studies were performed 2 to 3 days after transfection.

TIRFM and Biotinylation of Cell Surface Proteins and Immunoblots

Briefly, the evanescent field fluorescence (EFF) intensity of single cells or vesicles was measured by TIRFM (Olympus, Tokyo, Japan); samples were excited by a 440-nm laser for enhanced cyan fluorescent protein (ECFP) and a 513-nm laser for enhanced yellow fluorescent protein (EYFP). The EFF values before thapsigargin (TG) (or tetrakis-(2-pyridylmethyl)ethylenediamine [TPEN], ATP, or bradykinin) treatment were normalized to 1. Most results were expressed as maximal change in EFF intensity ($\text{Max}\Delta\text{EFF}$). A $\text{Max}\Delta\text{EFF}$ value of 1 represents an increase in EFF value by 1-fold (or 100%). Occasionally, raw EFF values were also presented.

Biotinylation of cell surface proteins was performed using a cell surface protein isolation kit based on the manufacturer's instructions. Proteins were resolved by SDS-PAGE and analyzed by immunoblots using anti-TRPV4 (1:200), anti-TRPC1 (1:200), or anti-STIM1 (1:200) antibodies.

Double Immunolabeling, Fluorescence Resonance Energy Transfer, and $[\text{Ca}^{2+}]_i$ Measurement

Double immunolabeling in HUVECs was performed by incubating the cells with a mixture of anti-TRPC1 plus anti-caveolin 1 or anti-TRPV4 plus anti-caveolin 1 antibodies, followed by fluorescence-labeled secondary antibodies. Fluorescence signals were detected by an FV1000 laser scanning confocal system.

For fluorescence resonance energy transfer (FRET), CFP (or YFP)-tagged TRPV4 and YFP (or CFP)-tagged TRPC1 were cotransfected into HEK cells. An inverted microscope equipped with 3-cube FRET filters and a charge-coupled device camera was used to measure the FRET ratio.

$[\text{Ca}^{2+}]_i$ in cultured cells was measured as described elsewhere.¹⁰ In flow experiments, flow was initiated by pumping normal physiological saline solution into a specially-designed parallel plate flow chamber,¹⁰ in which the cells were adhered to the bottom. We used a flow rate with shear stress of approximately 5 dyne per square centimeter. If necessary, brefeldin A (BFA), 5 $\mu\text{mol/L}$, was introduced 30 minutes before the application of TG.

Whole Cell Patch Clamp

Whole cell current was measured with an EPC-9 patch clamp amplifier, as described elsewhere.¹⁴ Cells were pretreated with or without TG for 15 minutes. If necessary, BFA, 5 $\mu\text{mol/L}$, was introduced 30 minutes before the application of TG.

The *t* test was used for statistical comparison, with $P < 0.05$ considered significant. For comparison of multiple groups, a 1-way ANOVA with a Newman-Keuls test was used.

For expanded experimental procedures, please see the supplemental information (available online at <http://atvb.ahajournals.org>).

Results

Effect of Ca^{2+} Store Depletion on Translocation of TRPV4-C1 Heteromeric Channels to the Plasma Membrane in the Overexpression System

TIRFM uses an evanescent wave to illuminate fluorophores within 250 nm of the plasma membrane.¹⁵ It is a valuable method for observing protein movements within the periplasmic space. In these experiments, HEK293 cells were first cotransfected with CFP-tagged TRPV4 and YFP-tagged TRPC1 to allow the formation of TRPV4-C1 heteromers.¹⁰ Treatment of the cells with TG, TPEN, or physiological agonists (ATP and bradykinin), each of which depletes intracellular Ca^{2+} stores, induced a time-dependent increase of TRPV4 and TRPC1 fluorescence in the periplasmic space (Figure 1A–D, supplemental Figure I, and supplemental Figure IIA [raw data]). The appearance of TRPV4 and TRPC1 fluorescence matched well temporally and spatially (Figure 1A and C, supplemental Figure I, and supplemental Figure IIA [raw data]), which is even more evident when single-vesicle fluorescence was examined (Figure 1C and supplemental Figure IC). A time-series plot shows that the total fluorescence of the cell surface peaked at approximately 2 minutes after TG treatment, then gradually and slowly decreased (Figure 1B and supplemental Figure IIA [raw data]). There were still significant fluorescence signals 15 minutes after TG treatment. BFA, a blocker of vesicular translocation, abrogated the fluorescence increase (Figure 1B and C). These data suggest that Ca^{2+} store depletion stimulates the translocation of TRPV4-C1-containing vesicles close to the plasma membrane. As a control, TG treatment had no effect on the fluorescence of yellow fluorescent protein tagged fluorescent plasma membrane marker (Pmem-YFP) (supplemental Figure IIIA and B), which is the fluorescent marker of the plasma membrane. This control excluded the possibility of plasma membrane movement in the axial direction during the experiments. There was no fluorescence signal in mock-transfected cells (supplemental Figure IIIC).

More careful studies were performed on single vesicles. The fluorescence of single vesicles dissipated relatively faster than that of the whole cell surface. The average residency time of single vesicle fluorescence was 309 ± 11 seconds ($n = 62$ vesicles) (Figure 1D and supplemental Figure IC). The time-series images of single-vesicle fluorescence (Figure 1D and supplemental Figure IC) reflect the movement of a TRPV4-C1-containing vesicle toward the plasma membrane (shown as the gradual increase in vesicle fluorescence), followed by free diffusion of TRPV4-C1 proteins toward another area of the plasma membrane after vesicle fusion with the plasma membrane (shown as a decrease in vesicle fluorescence). Our confocal system could only resolve the structures that contain a cluster of fluorophores, such as TRPV4-C1-containing vesicles, but could not resolve a single fluorophore-containing molecule.

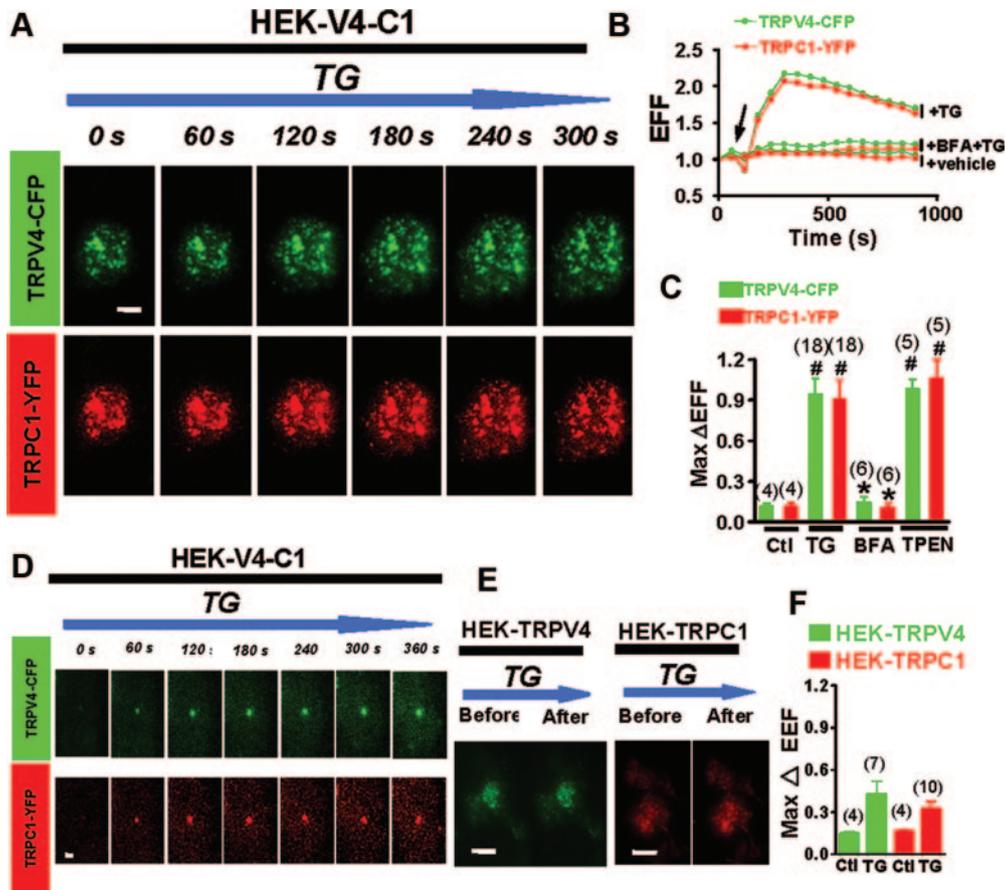


Figure 1. Effect of Ca^{2+} store depletion on the translocation of TRPV4–C1 heteromeric channels to the plasma membrane, as measured by TIRFM. A through D, HEK cells were coexpressed with TRPV4–CFP plus TRPC1–YFP and were incubated with TG, 4 μ mol/L. A and D, Time-series images of EFF for a representative single HEK cell (A) or a single vesicle (D). The bar indicates 5 μ m in A and 1 μ m in D. B, Time-series plot of EFF in representative cells for experiments similar to A. C, Summary data in experiments similar to A, showing the maximal change in EFF in response to TG or TPEN, 1 mmol/L. If necessary, BFA, 5 μ mol/L, was introduced 30 minutes before TG application. E and F, HEK cells were expressed with either TRPV4–CFP or TRPC1–YFP alone. EFF images of a representative single cell (E) and summary data (F) before and after TG stimulation were shown. Controls in C and F were subjected to vehicle treatment (1% dimethyl sulfoxide). Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). # P <0.05 vs control, and * P <0.05 vs TG-treated cells without BFA.

FRET was used to further confirm the interaction of TRPV4 and TRPC1 in the plasma membrane. Previously, we found the interaction of TRPV4 and TRPC1 by FRET at the whole cell level.¹⁰ Herein, we limited the FRET detection to the plasma membrane region only, and the results confirmed a physical interaction of CFP-tagged TRPV4 and YFP-tagged TRPC1 in the plasma membrane (supplemental Figure IV).

TIRFM detects the protein movement within the periplasmic space, but it does not necessarily define surface expression itself. Thus, cell surface biotinylation methods were used. TG treatment increased the amount of biotinylated TRPV4 and TRPC1 by $70 \pm 11\%$ ($n=4$) and $64 \pm 14\%$ ($n=3$), respectively, indicating an increase in cell surface TRPV4 and TRPC1 (Figure 2A and B). BFA abolished the stimulation effect of TG (Figure 2A and B). In HEK cells that were overexpressed with only 1 construct (TRPV4 or TRPC1 alone), TG treatment had little or no effect on the trafficking of these proteins, as determined by biotinylation (Figure 2C and D) and TIRFM (Figure 1E and F and supplemental Figure IIB–D), suggesting that TG enhances the translocation of TRPV4–C1 heteromeric channels more favorably than that

of TRPV4 or TRPC1 homomeric channels. Supplemental Figure IID also shows that, under basal conditions without TG treatment, there was more surface fluorescence of TRPV4 and TRPC1 in cells that are coexpressed with TRPV4 plus TRPC1 when compared with the cells that were expressed with only 1 protein (either TRPV4 or TRPC1) (supplemental Figure IID).

The observed biotinylated TRPV4 and TRPC1 only reflected the overexpressed TRPV4 and TRPC1 but not the endogenous ones, because biotinylated signals were absent in nontransfected HEK cells (supplemental Figure V). Cell surface expression of endogenous TRPC1 and TRPV4 in nontransfected HEK cells, if any, was low and less than the detection level (supplemental Figure V). Two antibodies for immunoblots (anti-TRPC1 and anti-TRPV4) were previously shown to be highly specific to their respective targets.^{10,16,17}

Functional Study of TRPV4–C1 Heteromeric Channels

4 α -PDD is a synthetic phorbol ester that can activate TRPV4 homomeric channels¹⁸ and TRPV4–C1 heteromeric chan-

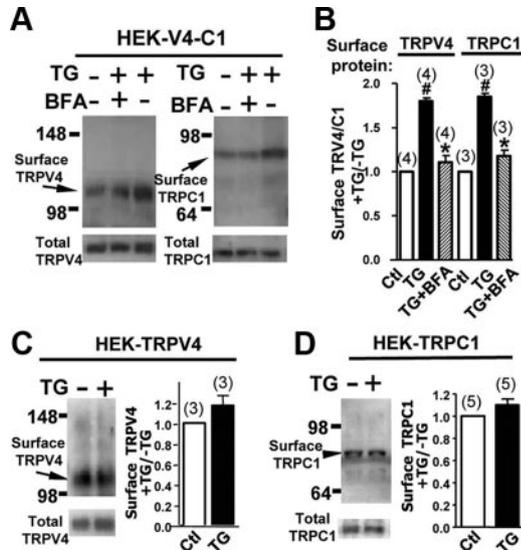


Figure 2. TG-induced translocation of TRPC1, TRPV4, and TRPV4-C1, as measured by cell surface biotinylation. A through D, HEK cells were transfected with TRPV4 plus TRPC1 (A and B), TRPV4 alone (C), or TRPC1 alone (D). The cells were then treated with or without TG, 4 μ mol/L for 15 minutes, followed by a biotinylation assay. Representative images (A and left panel of C and D) and summary data (B and right panel of C and D) of cell surface biotinylation experiments were shown. If necessary, BFA, 5 μ mol/L, was introduced 30 minutes before TG application. Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). # P <0.05 vs control (Ctl), and * P <0.05 vs the TG-treated cells without BFA.

nels.¹⁰ However, the inactivation kinetics of the 4 α -PDD-stimulated current differ greatly between TRPV4 homomeric and TRPV4-C1 heteromeric channels. For TRPV4 homomeric channels, the 4 α -PDD-stimulated current is more transient and decays quickly,¹⁰ whereas for TRPV4-C1 heteromeric channels, this current is much more prolonged (Figure 3A).¹⁰ In whole cell recording, no apparent decay in current was observed within the duration of the experiments, which lasted for 6 to 10 minutes (Figure 3A). TG treatment, 4 μ mol/L, for 15 minutes potentiated the 4 α -PDD-stimulated cation current (Figure 3C); it also augmented the 4 α -phorbol 12,13-didecanoate (4 α -PDD)-stimulated Ca²⁺ influx (Figure 3E). These data match well with those from TIRFM (Figure 1C) and the cell surface biotinylation assay (Figure 2B), both of which show an increased surface expression of TRPV4-C1 heteromers 15 minutes after TG treatment. BFA abolished the potentiation effect of TG (Figure 3C-E).

Participation of STIM1

An STIM1-specific siRNA¹⁹ could effectively and selectively “knock down” the expression level of STIM1 proteins (supplemental Figure VI). TIRFM experiments show that this STIM1-siRNA was markedly reduced in TG-induced translocation of TRPV4-C1 heteromers in HEK cells that were coexpressed with TRPV4 and TRPC1 (Figure 4A and B). In functional studies, STIM1-siRNA reduced the 4 α -phorbol 12,13-didecanoate (4 α -PDD)-stimulated Ca²⁺ influx (Figure 4C). Immunoblots show that the anti-STIM1 antibody was highly specific to STIM1 (supplemental Figure VII).

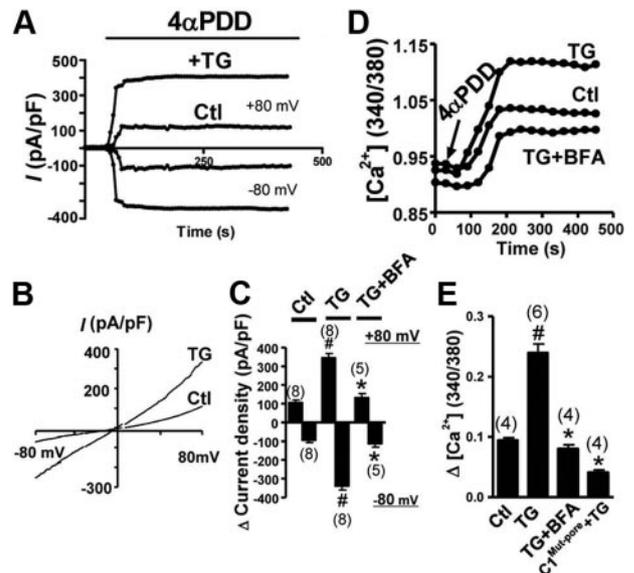


Figure 3. 4 α -PDD-stimulated cation current and [Ca²⁺]_i increase. The HEK cells were cotransfected with TRPV4 and TRPC1. A through C, For cation currents, representative current traces were shown at \pm 80 mV (A), corresponding I-V curves (B), and summary data for the maximal change in current in response to 4 α -PDD at \pm 80 mV (C). D and E, For [Ca²⁺]_i measurement, representative traces (D) and summary data (E) for the maximal change in [Ca²⁺]_i to 4 α -PDD were shown. C1^{Multi-pore} was used to replace TRPC1 in some experiments as labeled. TG, 4 μ mol/L, was given for 15 minutes; 4 α -PDD, 5 μ mol/L; BFA, 5 μ mol/L, was given 30 minutes before TG. Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). Ctl indicates control. # P <0.05 vs the cells without TG pretreatment, and * P <0.05 vs the bar labeled with # in the same panel.

Effect of TG on Translocation of TRPV4-C1 Heteromeric Channels in HUVECs

Next, we explored whether Ca²⁺ store depletion can facilitate the translocation of TRPV4-C1 heteromeric channels to the plasma membrane in HUVECs. Biotinylation

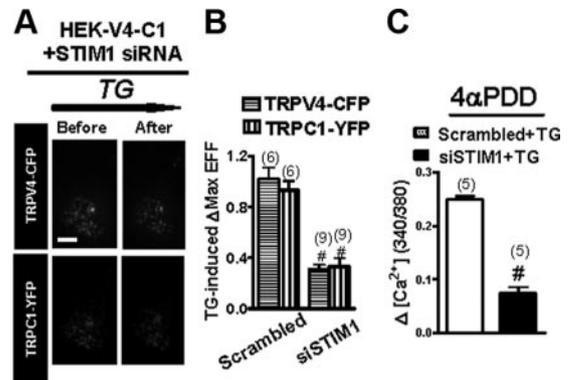


Figure 4. Involvement of STIM1 in the translocation of TRPV4-C1 heteromeric channels in response to Ca²⁺ store depletion. TRPV4-C1-coexpressing HEK cells were treated with STIM1-siRNA or scrambled siRNA, followed by TG treatment, 4 μ mol/L. A, EFF images of a representative single cell in the presence of STIM1-siRNA before and after TG stimulation. B and C, Summary data showing that STIM1-siRNA diminished the TG-induced increase in EFF intensity (B) and 4 α -PDD-stimulated Ca²⁺ influx (C). Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). # P <0.05 vs the scrambled siRNA.

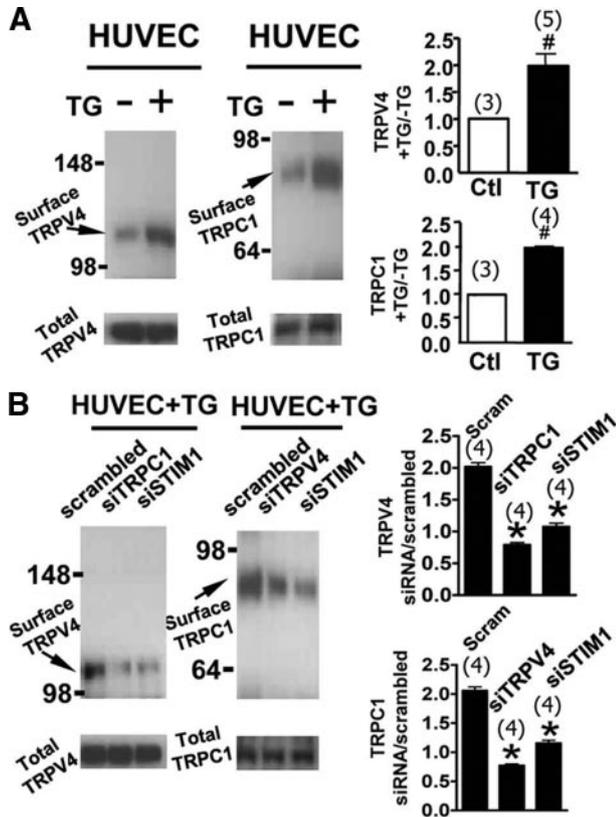


Figure 5. TG-induced translocation of TRPV4–C1 heteromeric channels to the plasma membrane in HUVECs. A, Representative images (left and middle) and summary (right) of TG, 4 $\mu\text{mol/L}$ -induced increases in cell surface TRPV4 (left) and TRPC1 (middle) proteins, as measured by a biotinylation assay. B, Similar to A, but showing the effect of TRPC1-siRNA, TRPV4-siRNA, or STIM1-siRNA. TG, 4 $\mu\text{mol/L}$, was given for 15 minutes. Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). The values in the absence of TG were normalized to 1. Ctl indicates control. # $P < 0.05$ vs cells without TG, and * $P < 0.05$ vs scrambled siRNA.

tion experiments demonstrated that TG treatment, 4 $\mu\text{mol/L}$ for 15 minutes, markedly increased the TRPV4 and TRPC1 proteins in the plasma membrane (Figure 5A); it had no effect on the total amount of cellular TRPV4 and TRPC1 proteins (Figure 5A). TRPC1-siRNA and TRPV4-siRNA were used, each of which was capable of “knock down” the expression of its targeted genes in HUVECs (supplemental Figure VIII). Intriguingly, suppressing the TRPC1 protein level by TRPC1-siRNA reduced the TRPV4 translocation to the plasma membrane (Figure 5B). Similarly, TRPV4-siRNA reduced the TRPC1 translocation to the plasma membrane (Figure 5B). STIM1-siRNA reduced the translocation of both TRPV4 and TRPC1 (Figure 5B). These results in HUVECs are consistent with those obtained in TRPV4-C1-coexpressing HEK cells, supporting the notion that Ca^{2+} store depletion stimulates the translocation of TRPV4-C1 heteromeric channels to the plasma membrane and that TRPV4-C1 heteromeric channels are more favorably delivered to the plasma membrane than TRPV4 or TRPC1 homomeric channels.

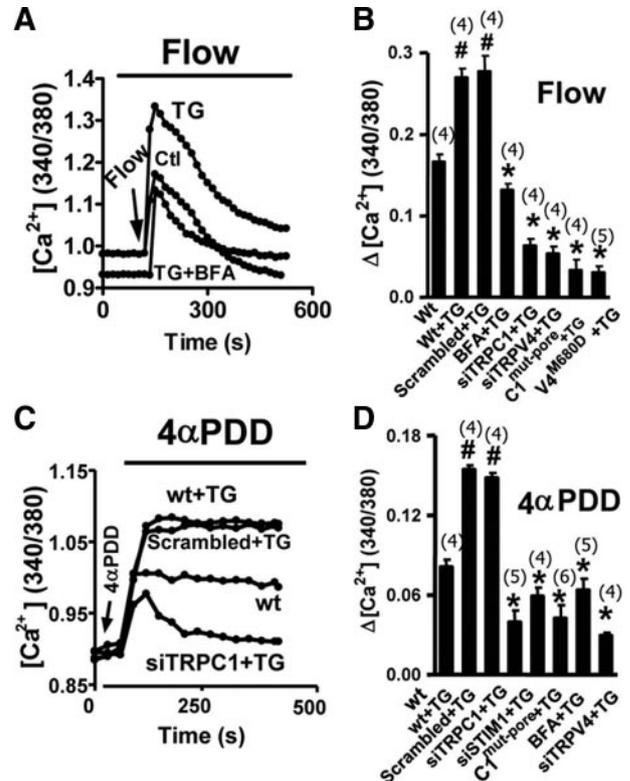


Figure 6. Potentiation of flow-induced Ca^{2+} influx and 4 α -PDD-stimulated $[\text{Ca}^{2+}]_i$ increase by TG in HUVECs. A through D, Representative traces (A and C) and summary data (B and D) for the maximal change in $[\text{Ca}^{2+}]_i$ in response to flow (A and B) or 4 α -PDD (C and D) were shown. TG, 4 $\mu\text{mol/L}$, was given for 15 minutes; BFA, 5 $\mu\text{mol/L}$, was given for 30 minutes before TG. Data are given as the mean \pm SE (the number of experiments is labeled on top of the bars). # $P < 0.05$ vs cells without TG, and * $P < 0.05$ vs scrambled siRNA (for siRNA experiments) or wild type+TG (in BFA or C1^{Multi-pore} experiments).

Role of TRPV4-C1 Heteromer Translocation in Flow-Induced Ca^{2+} Influx in HUVECs

An important function of TRPV4-C1 heteromeric channels is to mediate flow-induced Ca^{2+} influx in vascular endothelial cells.¹⁰ Previously, Ca^{2+} store depletion by TG potentiated the flow-induced Ca^{2+} influx in vascular endothelial cells.¹¹ This was confirmed (Figure 6A). In addition, we found that this potentiated $[\text{Ca}^{2+}]_i$ response to flow was abolished by BFA (Figure 6B), suggesting an involvement of vesicular trafficking. In the absence of TG treatment, BFA had no effect on flow-induced $[\text{Ca}^{2+}]_i$ increase (supplemental Figure IX). The potentiated $[\text{Ca}^{2+}]_i$ response to flow was also inhibited by TRPV4-siRNA, a pore mutant of TRPV4 (TRPV4^{M680D}),¹⁴ TRPC1-siRNA, and a pore mutant of TRPC1 (TRPC1^{Multi-pore}),¹³ suggesting an involvement of TRPV4-C1 heteromeric channels in the response (Figure 6B).

The possible caveolar localization of TRPV4-C1 heteromeric channels was explored. In double-labeling immunofluorescence experiments, we found strong overlapping of TRPC1 and TRPV4 fluorescence with that of caveolin-1 (supplemental Figure X), suggesting the localization of TRPC1 and TRPV4 in the caveolar compartment in HUVECs.

4 α -PDD-stimulated Ca^{2+} influx and cation current were also studied in HUVECs (Figure 6C and D and supplemental

Figure XI). TG treatment potentiated 4α -PDD-stimulated Ca^{2+} influx, the effect of which was abolished by BFA, TRPV4-siRNA, STIM1-siRNA, TRPC1-siRNA, and TRPC1^{Multi-pore} (Figure 6C and D). In whole cell patch clamp recordings, TG potentiated the 4α -PDD-stimulated cation current, the effect of which was inhibited by BFA and TRPC1^{Multi-pore} (supplemental Figure XI). These data support an involvement of TRPV4-C1 heteromeric channels and vesicular trafficking in the TG potentiation of 4α -PDD-stimulated Ca^{2+} and cation current responses.

Discussion

The major findings of this study are as follows: (1) TIRFM, cell surface biotinylation, and functional studies show that Ca^{2+} store depletion enhances the vesicular trafficking and insertion of TRPV4-C1 heteromeric channels into the plasma membrane in the HEK cell overexpression system, (2) STIM1 is important in controlling the vesicular trafficking and insertion of TRPV4-C1 heteromeric channels into the plasma membrane, (3) TRPV4 and TRPC1 are colocalized with caveolin-1 in the caveolar compartment of the plasma membrane in HUVECs, and (4) Ca^{2+} store depletion causes an increased delivery of TRPV4-C1 heteromeric channels to the plasma membrane in HUVECs, resulting in an augmented Ca^{2+} influx in endothelial cells in response to shear flow.

Regulated translocation of channel proteins to the plasma membrane is an important means to control the plasma membrane expression of TRP channels and their function.¹ In the present study, we used the unique feature of our TIRFM system to simultaneously track the forward trafficking of CFP-tagged TRPV4 and YFP-tagged TRPC1 to the plasma membrane in the HEK cell overexpression system. The results show that Ca^{2+} store depletion by TG, TPEN, or physiological agonists (bradykinin and ATP) caused an enhanced translocation of TRPV4-C1 heteromeric channels to the plasma membrane. The increased TRPV4-C1 translocation is related to vesicular trafficking because BFA abolished it. These results were confirmed by cell surface biotinylation experiments and functional studies, in which 4α -PDD-stimulated cation current and Ca^{2+} influx were examined. The appearance of TRPV4 and TRPC1 fluorescence on the plasma membrane matched well temporally and spatially in TIRFM, suggesting that TRPC1 and TRPV4 are inserted into the plasma membrane as TRPV4-C1 heteromers.

Heteromultimerization of different TRP subunits could affect their translocation to the plasma membrane.⁸ TRPC4, TRPM7, and TRPP1 may facilitate the translocation of TRPC1, TRPM6, and TRPP2, respectively, to the plasma membrane.^{8,20,21} In the present study, TIRFM experiments show that there was more surface fluorescence of TRPV4 and TRPC1 in cells that are coexpressed with TRPV4 plus TRPC1 than those expressed with only 1 protein alone (either TRPV4 or TRPC1) (supplemental Figure IID). These data seem consistent with previous reports,^{8,20,21} suggesting that coexpression of heteromers facilitates their translocation to the plasma membrane. Interestingly, we found that Ca^{2+} store depletion preferentially stimulated the translocation of TRPV4-C1 heteromeric channels to the plasma membrane but had little or no effect on that of TRPV4 or TRPC1

homomeric channels. The reason for this preferential effect of TG is not clear. One speculation could be that TRPV4-C1 heteromers are preferentially packaged into vesicles; thus, their trafficking is more subjected to regulation by Ca^{2+} store depletion.

Substantial evidence indicates that STIM1 is the Ca^{2+} sensor in the endoplasmic reticulum and that it serves to sense Ca^{2+} store depletion.²² In the present study, we found that knocking down of STIM1 using STIM1-siRNA markedly suppressed the TG-stimulated delivery of TRPV4-C1 heteromers to the plasma membrane, suggesting an important role of STIM1 in TG-induced translocation of TRPV4-C1 heteromeric channels to the plasma membrane.

Recent studies have found that TRPV4-C1 heteromeric channels mediate flow-induced endothelial $[\text{Ca}^{2+}]_i$ influx and subsequent vascular relaxation.¹⁰ In vascular endothelial cells, Ca^{2+} store depletion can potentiate flow-induced Ca^{2+} influx.¹¹ Such an interaction between Ca^{2+} store filling status and flow-induced Ca^{2+} influx has profound physiological importance. Vascular endothelial cells are exposed to circulating blood that contains metabolites, hormones, growth factors, and cytokines, many of which can induce Ca^{2+} release from intracellular Ca^{2+} stores, resulting in Ca^{2+} store depletion. The depletion of Ca^{2+} stores would then augment flow-induced endothelial Ca^{2+} influx¹¹ and subsequent vascular dilation.¹² However, the mechanism of how Ca^{2+} store depletion could enhance flow-induced Ca^{2+} influx remains elusive. In the present study, a cell surface biotinylation assay shows that Ca^{2+} store depletion increases the cell surface expression of TRPV4-C1 heteromeric channels in HUVECs. In agreement, functional studies also show that Ca^{2+} store depletion increases 4α -PDD-stimulated Ca^{2+} influx and cation current in HUVECs. More important, after measuring the flow-induced $[\text{Ca}^{2+}]_i$ increase, we found that TG potentiates the increase in HUVECs. This potentiation effect of TG was abolished by BFA, TRPV4-siRNA, TRPV1-siRNA, and TRPV4 and TRPC1 pore mutants, suggesting the involvement of vesicular trafficking and TRPV4-C1 heteromeric channels. Our working scheme is that Ca^{2+} store depletion causes an increased delivery of TRPV4-C1 heteromeric channels to the plasma membrane in HUVECs, resulting in an augmented Ca^{2+} influx in endothelial cells in response to shear flow. This scheme provides a mechanistic explanation for the potentiation effect of Ca^{2+} store depletion on flow-induced endothelial Ca^{2+} influx and vascular relaxation.^{11,12}

Caveolae in endothelial cells have been implicated as plasma membrane microdomains that sense or transduce hemodynamic changes into biochemical signals that regulate vascular function.²³ Previously, TRPV4 was reported to be colocalized with caveolin-1 in the caveolar compartment of the plasma membrane in HUVECs.²⁴ In the present study, we found that both TRPV4 and TRPC1 are localized with caveolin-1 in the caveolar compartment. The functional implication of this colocalization in flow-induced Ca^{2+} influx in endothelial cells should be resolved.

In conclusion, we demonstrated that depletion of intracellular Ca^{2+} stores induced a rapid insertion of TRPV4-C1 heteromeric channels into the plasma membrane in TRPV4-C1-overexpressing HEK cells and native endothelial cells.

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Disclosures

None.

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Supplemental Materials

Materials

Human embryonic kidney cell line HEK293 was from ATCC, USA. Human umbilical vein endothelial cells (HUVEC, CC-2517), endothelial cell growth medium (EGM), endothelial cell basal medium (EBM) and bovine brain extract (BBE) were from Lonza, USA. Anti-TRPC1 (ACC-010), anti-TRPV4 (ACC-034), anti-STIM1 (ACC-063) were from Alomone Labs. Anti-caveolin1 (610407) was from BD Transduction Laboratories. Fura-2/AM and pluronic F127 were from Molecular Probes Inc. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Phosphate Buffered Saline (PBS), N,N,N',N'-Tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) were from Invitrogen. Brefeldin A, 4 α -phorbol 12,13-didecanoate (4 α -PDD) were from Calbiochem. Nonidet P-40, trypsin, albumin bovine serum (BSA), collagenase and poly-L-lysine were from Sigma. Cell surface protein isolation kit was from Pierce.

Cell culture

HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 μ g/ml penicillin and 100 U/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) was cultured in EGM medium supplemented with 1% BBE, 100 μ g/ml penicillin and 100 U/ml streptomycin.

Cloning and transfection

Human TRPC1 cDNA (NM_003304) was obtained by RT-PCR from human coronary endothelial cells CC2585 (BioWhittaker). Channel pore mutants TRPV4^{M680D} and TRPC1^{Mut-pore} were as described elsewhere.^{1,2} For TIRFM, TRPV4 was tagged with CFP at its C-terminus, and TRPC1 was tagged with YFP at its N-terminus. For biotinylation experiments, non-tagged version of TRPV4 and/or TRPC1 were transfected into HEK cells. All genes were cloned into pcDNA6 vector for expression. The nucleotide sequences of all constructs were verified with DNA sequencing. TRPC1-siRNA and scramble control were from Ambion. The sequences for human TRPC1-siRNA were GGAUGUGCGGGAGGUGAAGtt (sense strand) and CUUCACCUCCCGCACAUCctt

(antisense strand) as described by others.³ The sequences for human TRPV4-siRNA were GUCUUCAACCGGCCUAUCCuu (sense strand) and GGAUAGGCCGGUUGAAGACuu (antisense strand) as described by others.⁴ The sequences for human STIM1-siRNA were GCCUAUAUCCAGAACCGUUt (sense strand) and AACGGUUCUGGAUAUAGGCaa (antisense strand) as described by others.⁵

Transfection condition was as described elsewhere.⁶ Briefly, HEK293 cells were transfected with various constructs using Lipofectamine 2000. About 6×10^4 HEK293 cells were grown in each well of the 6-well plates. Transfection was done with 4 μ g plasmid and 6 μ l Lipofectamine 2000 in 200 μ l Opti-MEM reduced serum medium in 6-well plates. HUVECs were transfected with TRPC1-siRNA, TRPV4-siRNA, STIM1-siRNA or a scrambled-siRNA as control by electroporation using Nucleofector II following the procedure in manufacturer's instruction manual. About 80% of HEK293 cells and about 70% of HUVECs were successfully transfected by respective protocols as indicated by control transfection using a GFP-expressing pCAGGS vector. Functional studies were performed 2-3 days post-transfection.

Total internal fluorescence reflection microscopy (TIRFM)

TIRFM generates an evanescent field that declines exponentially with increasing distance from the interface between the coverglass and the cytoplasm, illuminating only a thin section (250 nm) of the cell in contact with the coverglass, including the plasma membrane.⁷ Briefly, measurements of single cell evanescent field fluorescence (EFF) intensity were performed using TIRFM, with a 60x 1.45 N.A. objective (Olympus, Japan), where samples were excited using a 440 nm laser for ECFP and a 513nm laser for EYFP. In this configuration, the microscope uses only a dual-band pass dichroic mirror to separate the excitation and emission light, with no excitation filters used. ECFP and EYFP emission light was simultaneously harvested using the DualView splitter (Photometrics), equipped with a filter cube containing S470/30nm and S535/30nm emission filters for ECFP and EYFP emission, respectively, and a 505nm dichroic mirror for separation of emission wavelengths. Fluorescence images were collected with a back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (Photometric QuantEM 512SC). MetaMorph software was used to control the

protocol of acquisition and to perform data analysis. The EFF values before thapsigargin (or TPEN or ATP or bradykinin) treatment were normalized to 1. Most results were expressed as maximal change in EFF intensity (Max Δ EFF). A Max Δ EFF value of 1 represents an increase in EFF value by 1 fold (or 100%). Occasionally, raw EFF values were presented (Suppl Fig. IC).

Biotinylation of surface proteins and immunoblots

Biotinylation of surface proteins was carried out using a cell surface protein isolation kit (catalog no. 89881; Pierce) as the manufacturer's instructions. In brief, cells in T75 cm² flasks of 90–95% confluent were treated with 4 μ M thapsigargin for 15 min in normal physiological saline solution (NPSS). NPSS contained in mM: 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 5 HEPES, pH 7.4. After stimulation, cells were washed with ice-cold phosphate buffered saline (PBS) and then Sulfo-NHS-SS linked biotin was added in PBS and incubated on ice for 10 min at 4°C. Quenching solution was added to quench the reaction and the cells were washed with PBS to remove free biotin. Lysates were prepared in standard lysis buffer, which contained 1.5% (vol/vol) Nonidet P-40, 150 mM NaCl, 50 mM NaF, 50 mM Tris-HCl, pH 8.0, with addition of protease inhibitor cocktail tablets.⁸ Cell lysate was cleared by centrifugation, and biotinylated proteins were then precipitated by incubation with immobilized NeutAvidin Gel (agarose beads; Pierce) 60 min at room temperature. The beads were washed with a washing buffer including protease inhibitors. The bound proteins were released by incubation with sample buffer at room temperature and then resolved by SDS-PAGE sample buffer containing 50 mM DTT for immunoblot analysis. Immunoblots were as described elsewhere.⁸ Proteins from transfected HEK cells or HUVECs were then transferred to a PVDF membrane. The membrane was incubated at 4°C overnight with the primary anti-TRPV4 (1:200), anti-TRPC1 (1:200), or anti-STIM1 (1:200) in PBST 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.

Fluorescence Resonance Energy Transfer (FRET) detection

CFP (or YFP)-tagged TRPV4 and YFP (or CFP)-tagged TRPC1 were co-transfected into HEK cells. FRET signals were detected as described elsewhere.⁹ Briefly, an inverted microscope equipped with three-cube FRET filters and CCD camera was used to measure FRET. Three-cube FRET filter cubes were listed as follows (excitation; dichroic; emission): YFP (S500/20 nm; Q515lp; S535/30 nm); FRET (S430/25 nm; 455dclp; S535/30 nm); and CFP (S430/25 nm; 455dclp; S470/30 nm). Average background signal was subtracted. FRET ratio (FR) was calculated by the following equation:

$$FR = \frac{F_{AD}}{F_A} = \frac{[S_{FRET}(DA) - R_{D1} * S_{CFP}(DA)]}{R_{A1} * [S_{YFP}(DA) - R_{D2} * S_{CFP}(DA)]}$$

in which F_{AD} represents the total YFP emission with 430/25-nm excitation, and F_A represents the direct YFP emission with 500/20-nm excitation. In $S_{CUBE}(SPECIMEN)$, CUBE indicates the filter cube (CFP, YFP, or FRET), and SPECIMEN indicates whether the cell is expressing donor (D, CFP), acceptor (A, YFP), or both (DA). $R_{D1} = S_{FRET}(D)/S_{CFP}(D)$, $R_{D2} = S_{YFP}(D)/S_{CFP}(D)$, and $R_{A1} = S_{FRET}(A)/S_{YFP}(A)$ are predetermined constants that require measurement of the bleed-through of the emission of only CFP- or YFP-tagged molecules into the FRET channel and the emission of only CFP-tagged molecules into the YFP channel.

Double immunolabeling

Briefly, HUVECs were seeded on glass coverslips. The cells were rinsed with PBS three times, then fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Nonspecific immunostaining was blocked by incubating the cells with 2% BSA in PBS. The cells were then incubated with a mixture of anti-TRPC1 (rabbit polyclonal antibody, ACC-010, Alomone Labs) plus anti-caveolin-1 (mouse polyclonal antibody, 610407, BD Transduction Laboratories), or anti-TRPV4 (rabbit polyclonal antibody, ACC-034, Santa Cruz Biotechnology) plus anti-caveolin-1 (mouse polyclonal antibody, 610407, BD Transduction Laboratories) antibodies per well overnight. After three washes with PBS, cells were incubated for 1 hr with a mixture of secondary donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (1:200) and donkey anti-mouse IgG-conjugated to Alexa Fluor 546 (1:100). After washing and mounting, immunofluorescence of the cells was detected using FV1000 confocal system. Quantitative analysis of the colocalization was carried out by pixel-by-pixel correlation of red and green images using FV10-ASW 1.5 software.

[Ca²⁺]_i measurement

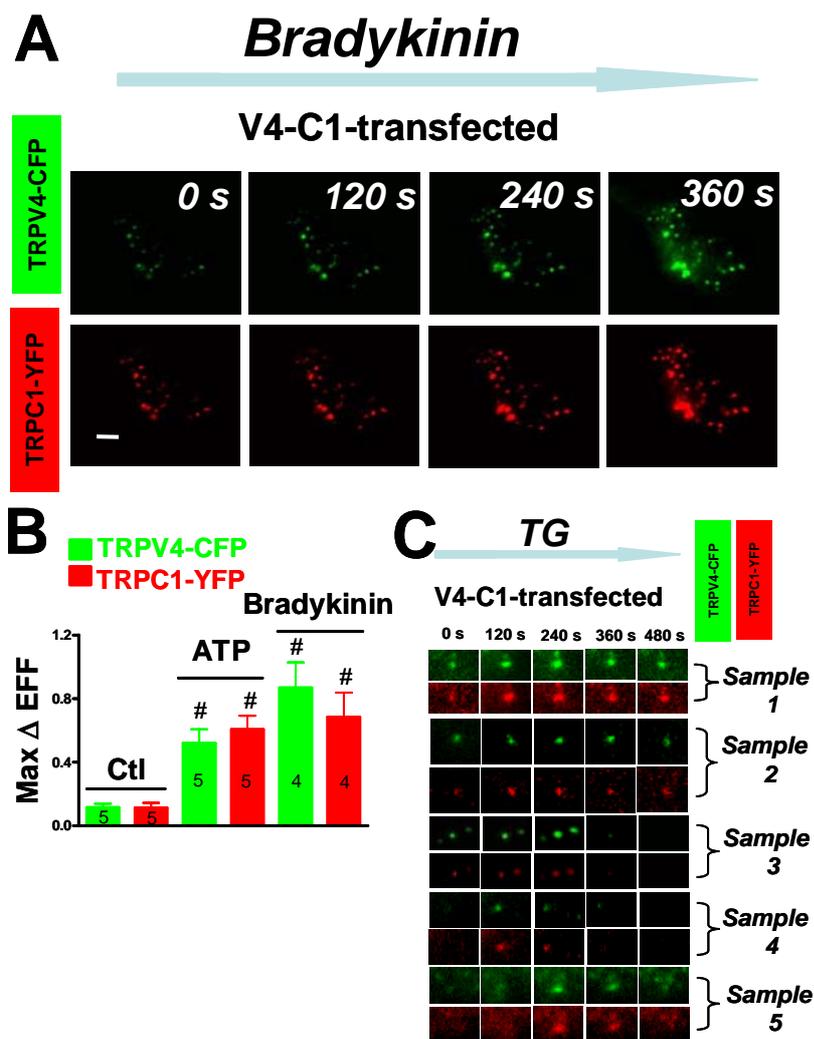
[Ca²⁺]_i in cultured cells was measured as described elsewhere.⁸ Briefly, cultured HEK cells or HUVECs were loaded with 10 μM Fura-2/AM and 0.02% pluronic F-127 for 1 hour in dark at 37°C in NPSS. The cells were pre-treated with 4 μM thapsigargin for 15 min in NPSS. [Ca²⁺]_i change in response to 4α-PDD (5 μM) was then measured. In flow experiments, flow was initiated by pumping NPSS to a specially-designed parallel plate flow chamber,⁸ in which the cells were adhered to the bottom. We used a flow rate with shear stress of ~5 dyne/cm². Fura-2 fluorescence signals were measured using dual excitation wavelengths at 340 and 380 nm using an Olympus fluorescence imaging system. Change in [Ca²⁺]_i was displayed as change in Fura-2 ratio. 8 to 20 cells were analyzed in each experiment. If needed, brefeldin A (BFA, 5 μM) was introduced 30 min before application of thapsigargin.

Whole-cell patch clamp

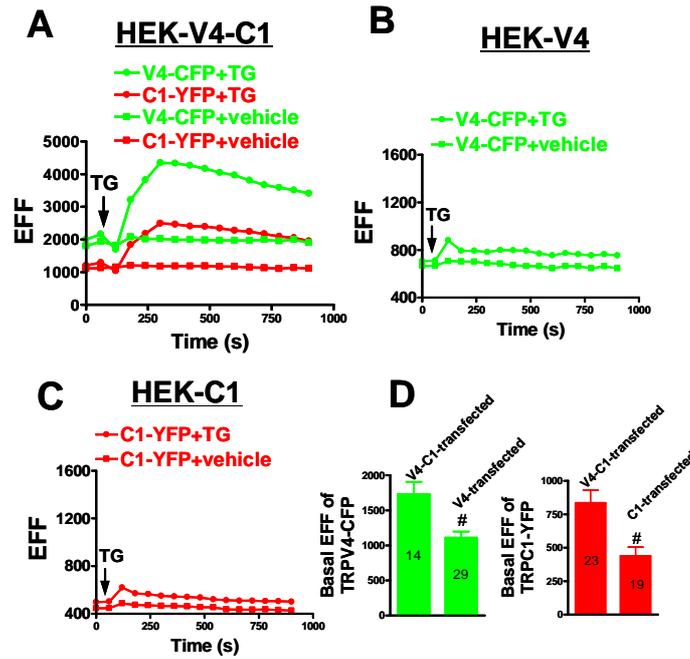
Whole cell current was measured with an EPC-9 (HEKA Elektronik, Lambrecht, Germany) patch clamp amplifier as described elsewhere.² Patch electrodes had a resistance 3~4 MΩ. An Ag-AgCl wire was used as a reference electrode. Glass coverslips with cells were mounted in a home-built perfusion chamber and solutions are changed by slow perfusion. 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 recordings were made before and after 4α-PDD (5 μM) application. The pipette solution contained in mM: 20 CsCl, 100 Cs⁺-aspartate, 1 MgCl₂, 4 ATP, 4.5 CaCl₂, 10 BAPTA, 10 HEPES, pH 7.2. Free Ca²⁺ in pipette solution was 100 nM (calculated with "webmaxc" available at <http://www.stanford.edu/~cpatton/maxc.html>).¹⁰ Bath solution contained in mM: 150 NaCl, 6 CsCl, 1 MgCl₂, 1.5 CaCl₂, 10 glucose, 10 HEPES, pH 7.4. If needed, brefeldin A (BFA, 5 μM) was introduced 30 min before application of thapsigargin. All currents were sampled at 50 kHz and filtered at 5 kHz, and the data were analyzed with PulseFit. HEK cells have an average capacitance of ~20 pF. The calculated liquid junction is ~14 mV. The current-voltage relations are not corrected for liquid junction potential.

Statistics

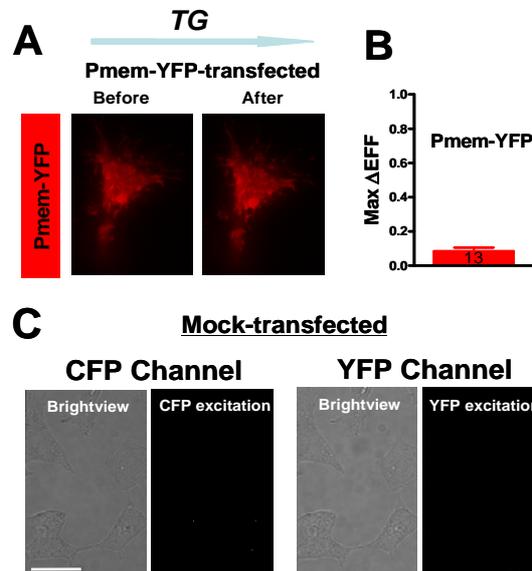
Student's t-test was used for statistical comparison, with probability $p < 0.05$ as a significant difference. For comparison of multiple groups, One-way ANOVA with Newman-keuls was used.



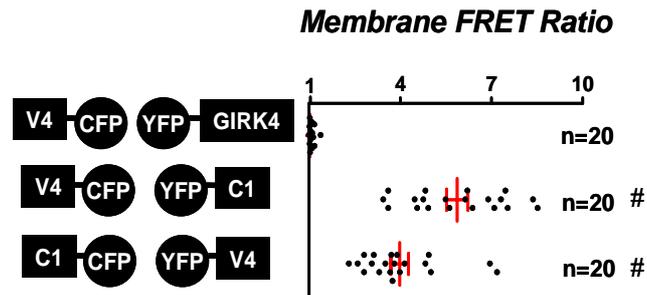
Supplemental Figure I. Effect of bradykinin and ATP on the translocation of TRPV4-C1 complex to the plasma membrane as measured by TIRFM. HEK cells were co-expressed with TRPV4-CFP plus TRPC1-YFP. A-B, The cells were incubated with bradykinin (100 nM) or ATP (100 μ M). A, Time series EFF images a representative single HEK cell. Scale bar is 5 μ m. B, Summary data in experiments similar to A, showing the maximal change in EFF in response to ATP and bradykinin. Mean \pm SE (number of experiments is labeled inside of the bars). #, $P < 0.05$ compared to the control. C, More examples of time series EFF images for single vesicles after TG stimulation, similar to Fig. 1D. Scale bar is 1 μ m. Time interval between each consecutive image is 120 s.



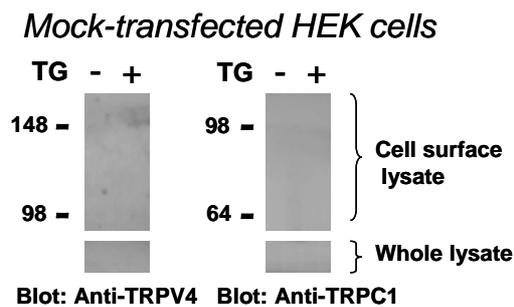
Supplemental Figure II. Time series plot of raw EFF values in representative HEK cells for experiments similar to Fig 1A. Cells were transfected with TRPV4-CFP plus TRPC1-YFP (A), or TRPV4-CFP alone (B), or TRPC1-YFP alone (C). Shown was the effect of thapsigargin (TG, 4 μ M) on the translocation of TRPV4 and/or TRPC1 to the plasma membrane as measured by TIRFM. D, Summary data showing the basal EFF of TRPV4-CFP or TRPC1-YFP in HEK cells were transfected with TRPV4 plus TRPC1 or with TRPV4 alone or with TRPC1 alone. Mean \pm SE ($n = 4-18$ experiments, number of analyzed cells is labeled inside of the bars).



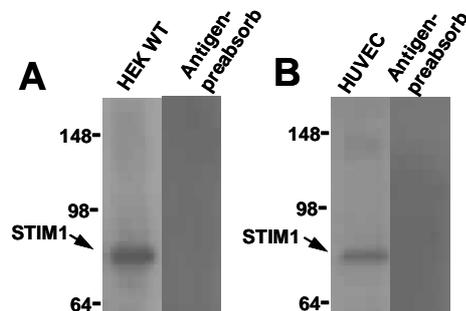
Supplemental Figure III. Effect of thapsigargin on EFF intensity in control as determined by TIRFM. A, Representative EFF images of a single HEK cell expressing Pmem-YFP before (left) and 2.5 min after TG (right). B, Summary data as in A after stimulation with thapsigargin (TG, 4 μ M). C, Representative EFF images of mock-transfected HEK cells. No CFP or YFP fluorescence could be detected either in CFP or in YFP channel. Scale bar is 5 μ m. Mean \pm SE ($n = 3$ experiments, number of analyzed cells is labeled inside of the bar).



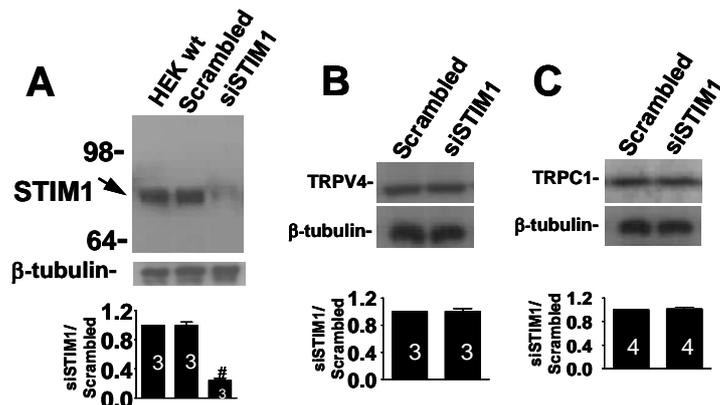
Supplemental Figure IV. Plasma membrane fluorescence resonance energy transfer (FRET) detection by three-cube FRET system in HEK cells co-transfected with CFP-tagged TRPV4 plus YFP-tagged TRPC1, or CFP-tagged TRPC1 plus YFP-tagged TRPV4. HEK cells co-transfected with CFP-tagged TRPV4 plus YFP-tagged GIRK4 was taken as negative control. GIRK4 belongs to inwardly rectifying K⁺ channels bearing no similarity to TRP channels, serving as control for plasma membrane protein. Horizontal axes indicate FRET ratio of living cells expressing indicated constructs. Each point represents the FRET ratio of a single cell. The red lines and error bars indicate the average FR values and standard error. FR = 1, no FRET. FR > 1, having FRET. Mean ± SE (*n* = 4-5 experiments, number of analyzed cells is labeled in the Figure). #, *P* < 0.001 compared to negative controls.



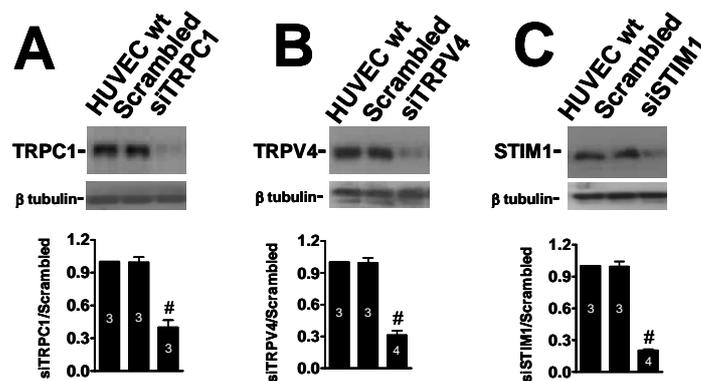
Supplemental Figure V. Representative images of cell surface biotinylation experiments in mock-transfected HEK cells. Cells were treated with or without thapsigargin (TG, 4 μM, 15 min), followed by biotinylation assay. Left panel, immunoblotted with anti-TRPV4 antibody; Right panel, immunoblotted with anti-TRPC1 antibody. *n* = 3 experiments.



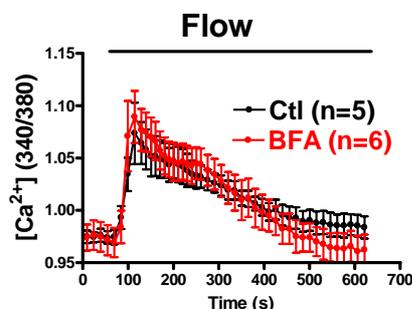
Supplemental Figure VI. Immunoblots showing the specificity of STIM1 antibody to its target in HEK cells (A) and HUVECs (B). The extracted proteins were immunoblotted with an anti-STIM1 antibody (Alomone Lab). The antibody recognized the expected band (indicated by a arrow) in both HEK cells and HUVECs. No band was observed if this antibody was preabsorbed by excessive amount of peptide antigen. *n* = 3 experiments.



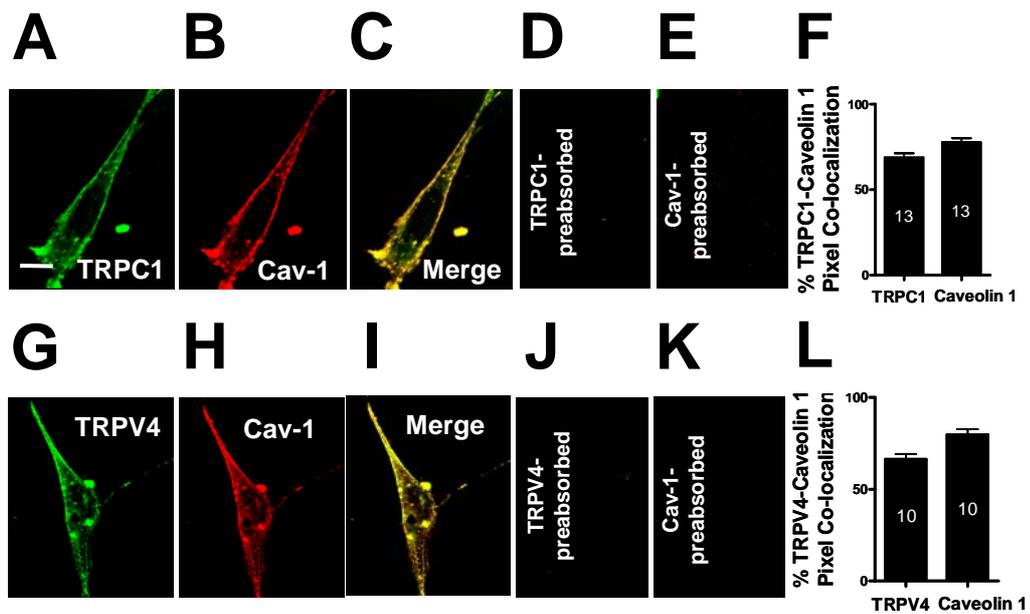
Supplemental Figure VII. Representative images (top) and summary (bottom) of immunoblot experiments showing the effectiveness (A) and selectivity (B and C) of STIM1-siRNA in HEK cell overexpression system. HEK cells were co-expressed with TRPV4 and TRPC1. STIM1-siRNA reduced the expression of STIM1 proteins but had no effect on that of TRPV4 (B) and TRPC1 (C). Control siRNA with scrambled sequence had no effect on STIM1 protein level. Immunoblots with anti- β -tubulin antibody showed that equal amount of proteins was loaded onto each lane. Mean \pm SE (number of experiments is labeled inside of the bars). #, $P < 0.05$ compared to the scrambled siRNA.



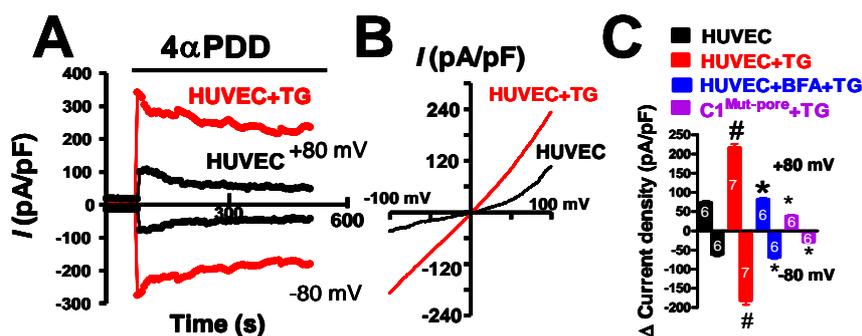
Supplemental Figure VIII. Effectiveness of siRNAs against their respective targets in HUVECs. Shown were representative images (top) and summary (bottom) of immunoblot experiments. A, TRPC1-siRNA series; B, TRPV4-siRNA series; C, STIM1-siRNA series. Control siRNA with scrambled sequence had no effect. Immunoblots with anti- β -tubulin antibody showed that equal amount of proteins was loaded onto each lane. Mean \pm SE (number of experiments is labeled inside of the bars). #, $P < 0.05$ compared to the scrambled siRNA.



Supplemental Figure IX. Effect of brefeldin A on flow-induced Ca^{2+} influx in HUVECs. Brefeldin A (BFA, 5 μM) was introduced 30 min before application of flow. Mean \pm SE ($n = 5-6$ experiments as labeled).



Supplemental Figure X. Double immunolabeling experiments showing subcellular co-localization of caveolin-1 (Cav-1) vs. TRPC1 (A-F), and caveolin-1 vs. TRPV4 (G-L) in HUVECs. A to B, representative images of single cell showing TRPC1 (A, green) and caveolin-1 (B, red); C, overlay image of A and B; F, Summary of data showing the percentage of Cav-1-TRPC1 pixel colocalization ($n = 3$ experiments, number of analyzed cells is labeled inside of the bars). G to H, representative images of single cell showing TRPV4 (G, green) and caveolin-1 (H, red); I, overlay image of G and H. L, Summary of data showing the percentage of Cav-1-TRPC1 pixel colocalization ($n = 3$ experiments, number of analyzed cells is labeled inside of the bars). D and J, TRPC1 antibody was preabsorbed with excessive TRPC1 peptide. E and K, TRPV4 antibody was preabsorbed with excessive TRPV4 peptide.



Supplemental Figure XI. 4α -PDD-stimulated cation current in HUVECs. Shown were representative current traces at ± 80 mV (A), corresponding I-V curves (B), and summary data for the maximal change in current in response to 4α -PDD (C). TG, 4 μ M; Brefeldin A (BFA), 5 μ M, 30 min before TG; 4α -PDD, 5 μ M. C1^{Multi-pore}, cells transfected with a pore mutant of TRPC1 (C1^{Multi-pore}). Mean \pm SE (number of experiments is labeled inside of the bars). #, $P < 0.05$ compared to the cells without TG pretreatment. *, $P < 0.05$ compared to the bar labeled with # in the same panel.

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