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Epoxyeicosatrienoic acids act through TRPV4–TRPC1–K_{Ca}1.1 complex to induce smooth muscle membrane hyperpolarization and relaxation in human internal mammary arteries

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ABSTRACT

Human left internal mammary arteries (LIMAs) are commonly used as donor grafts for coronary bypass surgery. Previous reports suggested that 11,12-epoxyeicosatrienoic acid (11,12-EET) is an important endothelial-derived hyperpolarizing factor (EDHF) in human LIMAs and that EETs act through large conductance Ca^{2+}-activated K^+ channels (K_{Ca}1.1) to induce smooth muscle cell hyperpolarization and relaxation in these tissues. In this study, we aimed to explore the role of vanilloid transient receptor potential channel 4 (TRPV4) and canonical transient receptor potential channel 1 (TRPC1) channels in the EET-induced smooth muscle hyperpolarization and vascular relaxation in human LIMAs. Co-immunoprecipitation studies demonstrated that TRPV4, TRPC1, and K_{Ca}1.1 physically interacted with each other to form a complex. Sharp microelectrode and vascular tension studies demonstrated that 11,12-EET (300 nmol/L) and 4α-phorbol 12,13-didecanoate (5 μmol/L) were able to induce smooth muscle membrane hyperpolarization and vascular relaxation in isolated human LIMA segments. The hyperpolarizing and relaxant effects were markedly reduced by treatments that could suppress the expression/activity of TRPV4, TRPC1, or K_{Ca}1.1. With the use of human embryonic kidney 293 cells that overexpressed with TRPV4, TRPC1 and K_{Ca}1.1, we found that TRPC1 is the linker through which TRPV4 and K_{Ca}1.1(α) can interact. The present study revealed that 11,12-EET targets the TRPV4–TRPC1–K_{Ca}1.1 complex to induce smooth muscle cell hyperpolarization and vascular relaxation in human LIMAs. This finding provides novel mechanistic insights for the EET action in human LIMAs.

1. Introduction

EETs are cytochrome P_{450} epoxygenase metabolites of arachidonic acid that are released from vascular endothelial cells in response to bradykinin, pulsatile stretch and shear stress [1,2]. In vasculature, P_{450} 2C and 2J epoxygenases are the most important families to modulate vascular tone. To date, the molecular targets of EETs are still fully unresolved. One potential target for 5,6-EET and 11,12-EET is vanilloid transient receptor potential channel 4 (TRPV4) [1]. TRPV4 contains a putative arachidonate recognition site in its N-terminal cytoplasmic domain, to which EETs could potentially bind [3]. Functionally, in rat cerebral arteries EETs activate TRPV4 in vascular smooth muscle cells (VSMCs) to induce Ca^{2+} influx, which stimulates ryanodine receptors (RyR), causing subsequent activation of K_{Ca}1.1. Activity of K_{Ca}1.1 leads to smooth muscle cell hyperpolarization and consequent vascular relaxation [4]. In addition, EETs may also activate TRPV4 channels in endothelial cells, resulting in endothelial cell hyperpolarization via K_{Ca}3.1 and K_{Ca}2.3 [5,6]. Besides their action on TRPV4, EETs are suggested to stimulate vesicular translocation of canonical transient receptor potential channel 6 (TRPC6) towards the plasma membrane [7] and to inhibit volume-activated Cl^- channels via a cGMP-dependent pathway [8].

TRPC1 is another Ca^{2+}-permeable channel that has been reported to play an important functional role in vascular tone control [9–13].

Abbreviations: LIMAs, human left internal mammary arteries; EETs, epoxyeicosatrienoic acids; 11,12-EET, 11,12-epoxyeicosatrienoic acid; K_{Ca}1.1, large conductance Ca^{2+}-activated K^+ channels; TRPV4, vanilloid transient receptor potential channel 4; TRPC1, canonical transient receptor potential channel 1; 4α-PDD, 4α-phorbol 12,13-didecanoate; HEK293, human embryonic kidney 293; VSMCs, vascular smooth muscle cells; EDHFs, endothelium-derived hyperpolarizing factors; HC, HC-067047; RyR, ryanodine receptor

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However, there are controversies as to whether the activity of TRPC1 has pro-contractile or anti-contractile function in VSMCs [9–13]. On the one hand, hypoxic treatment was reported to enhance TRPC1 protein expression with a parallel increase in vascular contractility in pulmonary arteries [9–11]. Furthermore, inhibiting TRPC1 activity by an anti-TRPC1 antibody T1E3 attenuated the endothelin-1-evoked contraction in rat basilar artery [9]. These studies suggested a pro-contractile function of TRPC1 in VSMCs. On the other hand, several recent reports suggested that TRPC1 may have an anti-contractile function. We previously reported that TRPC1 and $K_{Ca}1.1$ are physically coupled in VSMCs and that Ca^{2+} entry through TRPC1 activates $K_{Ca}1.1$ to hyperpolarize VSMCs, resulting in smooth muscle relaxation in rat aortic and mesenteric arteries [12]. This hypothesis of anti-contractile function of TRPC1 was recently confirmed by Marrelli's group with the use of TRPC1 knockout mice [13]. The reason for the discrepancy in the results regarding the pro- or anti-contractile function of TRPC1 is unclear, but it could be related to the difference in artery bed types and/or physiological conditions.

In the present study, we utilized human left internal mammary arteries (LIMAs) as the model to investigate the role of TRPV4 and TRPC1 in EET-induced vascular relaxation. Human LIMAs are often used as donor vessels in coronary bypass surgery due to their relative resistance to atherosclerosis and superior endothelial function [14,15]. Thus it is of clinical importance to understand the underlying mechanism of vascular tone control in these arteries. Previous studies demonstrated that endothelial cells of LIMAs not only release large amounts of nitric oxide and prostacyclin [14,16], but also produce endothelium-derived hyperpolarizing factors (EDHFs). The predominate EDHF in human LIMAs is 11,12-EET [17]. 11,12-EET activates the $K_{Ca}1.1$ current to induce smooth muscle cell hyperpolarization, consequently causing vascular relaxation of human LIMAs [17]. However, up to the present it is still unknown whether TRPV4 and/or TRPC1 play any role in EET-induced vascular relaxation in human LIMAs. Here, we employed electrophysiological and molecular biological techniques to explore the involvement of TRPV4 and TRPC1 in EET-induced smooth muscle hyperpolarization and relaxation in human LIMAs.

2. Materials and methods

2.1. Materials

HEK293 was from ATCC, USA. The primary rabbit antibodies against $K_{Ca}1.1(\alpha)$ (APC-021), TRPC1 (ACC-010), and TRPV4 (ACC-034) were from Alomone Labs. T1E3 was raised by us from rabbits. Protein A-agarose and protease inhibitor cocktail tablets were from Roche. Lipofectamine 2000, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), medium 231, smooth muscle growth supplement, phosphate buffered saline (PBS), and dithiothreitol were from Invitrogen. Fluo-4/AM and pluronic F127 were from Molecular Probes Inc. 4α-PDD was from Calbiochem. HC-067047 and GSK1016790A were from Sigma. (±)11,12-EET was from Cayman Chemical. Papain was from Fluka. Nonidet P-40, iberiotoxin, trypsin, albumin bovine serum (BSA), collagenase and poly-L-lysine were from Sigma.

2.2. Cell and tissue preparation and culture

Human LIMA segments were from discarded tissues at the time of coronary artery bypass grafting with the written consent of family members and the approval of the Clinical Research Ethics Committee, Chinese University of Hong Kong. The investigation conforms with the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects.

To isolate the primary VSMCs, LIMAs were dissected and the lumen of the artery was cut open. Endothelial layer was mechanically denuded by rubbing the lumen with cotton wool. Smooth muscle layers were peeled off from adventitial layers with forceps, and were digested in a Ca^{2+}-free PBS containing 0.2% collagenase type 1A, 0.9% papain, 0.5% BSA and 10 μmol/L dithiothreitol at 37 °C for 1 h. The dispersed VSMCs were washed with PBS and then cultured for 3–4 days in medium 231 with smooth muscle growth supplement, 100 μg/mL penicillin, and 100 U/mL streptomycin. These cells were used for co-immunoprecipitation experiments without further passage. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin and 100 U/mL streptomycin.

2.3. Cloning and transfection

Human $K_{Ca}1.1(\alpha)$ 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 cells CC2585 (BioWhittaker). Mouse TRPV4 gene (NM_022017) was a gift from Dr. Nilius B, Belgium. $TRPC1^{mut\text{-}pore}$ was a gift from Dr. Ambudkar I, NIH, USA. TRPC1 and $K_{Ca}1.1(\alpha)$ genes were cloned into pcDNA6 vector, and TRPV4 gene was cloned into pcAGGS vector for expression. The nucleotide sequences of all constructs were verified with DNA sequencing. TRPV4-siRNAs and scrambled controls were from Ambion, Texas, USA. The sequences for human TRPV4-siRNA were GUCUUCAACCGGCCUA UCCuu (sense sequence) and GGAUAGGCCGGUUGAAGACuu (antisense sequence) as described by others [18].

Transfection condition was as described elsewhere [19]. 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 μg plasmid and 6 μL Lipofectamine 2000 in 200 μL Opti-MEM reduced serum medium in 6-well plates. VSMCs were transfected by electroporation using Nucleofector II following the procedure in the manufacturer's instruction manual. If needed, LIMA segments were transfected with TRPV4 (human)-siRNA using Lipofectamine RNAiMAX following the protocol used elsewhere [6,20]. Briefly, LIMA was cut into ~2 mm segments and cultured in a 24-well plate. Transfection was performed with 50 nmol/L siRNA and 1.5 μL Lipofectamine RNAiMAX in 500 μL Opti-MEM reduced serum medium. After 6-hour incubation, the transfection medium was replaced by DMEM. Tissues were ready for functional studies after 24–48 h post-transfection. The culture medium was changed every day. About 80% of HEK293 cells were successfully transfected by respective protocols as indicated by control transfection using a GFP-expressing pCAGGS vector. All genes were transiently transfected into targeted cells. Functional studies were performed 2–3 days post-transfection.

2.4. Preparation of T1E3 and preimmune IgG

T1E3 antibody was raised in rabbits using the strategy developed by Xu et al. [12,21]. Briefly, a peptide corresponding to the E3 region near the ion permeation pore, between transmembrane regions S5 and S6, of TRPC1 (CVGIFCEQQSNDTFHSFIGT) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) at Alpha Diagnostic International (USA). The coupled T1E3 peptide was injected to the back of a rabbit (day 0), followed by two boost doses at day 21 and day 42, respectively. Antiserum was collected four weeks after the second boost. Immunoglobulin G was purified from the T1E3 antiserum using a HiTrap Protein G column (GE Healthcare). For control, pre-immune serum was purified with a HiTrap Protein G column to obtain the immunoglobulin G, which was then used in the experiments.

2.5. Immunoprecipitation and immunoblots

Immunoprecipitation and immunoblots were described previously [12]. Proteins were extracted with a detergent-containing extraction buffer, which contained 1% (vol/vol) Nonidet P-40, 150 mmol/L NaCl, and 20 mmol/L Tris–HCl, pH 8.0, with the addition of protease inhibitor cocktail tablets. 800 μg of extracted proteins was then incubated with 7 μg of anti-TRPC1 (ACC-010; Alomone Laboratory), anti-TRPV4 (ACC-

034; Alomone Laboratory) or anti-$K_{Ca}1.1(\alpha)$ (APC-107; Alomone Laboratory) antibody on a rocking platform overnight at 4 °C. Protein A agarose was then added, followed by further incubation at 4 °C for 3 h. The immunoprecipitates were washed with saline 3 times and resolved on 8% SDS-PAGE gel. The proteins were then transferred onto a polyvinylidene difluoride membrane using a semi-dry transfer system (Bio-Rad). The membrane carrying the transferred proteins was incubated at 4 °C overnight with the primary antibody at 1:200 dilution in PBST buffer containing 0.1% Tween 20 and 5% nonfat dry milk. Immunodetection was accomplished using horseradish peroxidase-conjugated secondary antibody. Antibody binding was detected by the ECL system, followed by exposure to an X-ray film [12].

2.6. Membrane potential measurement of VSMCs in LIMA segments using sharp microelectrode

Sharp microelectrode methods were more appropriate for recording cells in blood vessels [22]. The distal 10 mm of LIMAs was obtained from discarded tissues at coronary artery bypass grafting surgery, immediately placed in iced physiological saline. Segments of LIMAs were dissected into about 3 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 potentials were measured using glass microelectrodes filled with 3 mol/L KCl (resistance: 60–100 MΩ) as described elsewhere [12,23]. The arterial segments were opened longitudinally and the electrodes were inserted into the VSMCs from the lumen side. Successful impalement was indicated by an abrupt drop in voltage, followed by a sharp return to baseline on exit. Hyperpolarization of VSMCs to 11,12-EET, GSK1016790A or 4α-PDD was recorded. In some experiments, human LIMA segments were mounted on a wire myograph and sharp microelectrodes were impaled from the adventitial side. In these experiments, the artery segments were pre-constricted with phenylephrine, followed by 300 nmol/L 11,12-EET. If needed, the arteries were pre-incubated at room temperature for 1 h with T1E3 (1:100) or pre-immune IgG (1:100), or at 37 °C for 30 min with iberiotoxin (50 nmol/L), HC-067047 (10 μmol/L) or 14,15-EEZE (10 μmol/L). Electrical signals were monitored continuously by an EPC9 amplifier (HEKA) with Pulse software. The currents were sampled at 5 kHz and filtered at 1 kHz. Data were analyzed with PulseFit (HEKA). We determined the effect of 14,15-EEZE, HC-067047, T1E3, or iberiotoxin pretreatment on basal resting membrane potentials of the smooth muscle cells in phenylephrine-preconstricted LIMA segments. The results showed that these treatments (30 min pretreatment with iberiotoxin or 14,15-EEZE or HC-067047; or 1 h treatment with T1E3) had no significant effect on the basal resting membrane potentials.

2.7. Arterial tension measurement

We followed the method used by others, as described elsewhere [17]. The LIMAs were removed of adhering connective tissues and cut into 3-mm ring segments. If needed, the endothelium layer was denuded by gently passing a knotted suture through the lumen. The successful removal of the endothelial cell layer was verified by the lack of any relaxant response to acetylcholine (1 μmol/L). LIMA segments were equilibrated for 45 min in 10 mL Krebs–Henseleit solution oxygenated with a gas mixture of 95% O_2 and 5% CO_2 at 37 °C before experiments. The samples were excluded if they were not received in iced saline within 1 h of harvest. Each ring was suspended between two stainless wire hooks in a 10 mL organ chamber filled with Krebs solution that contained in mmol/L: 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.2 KH_2PO_4, and 11 D-glucose. The upper wire was connected to a force–displacement transducer (Grass Instruments, RI, USA) and the lower one fixed to the bottom of the organ bath. Rings were placed under an

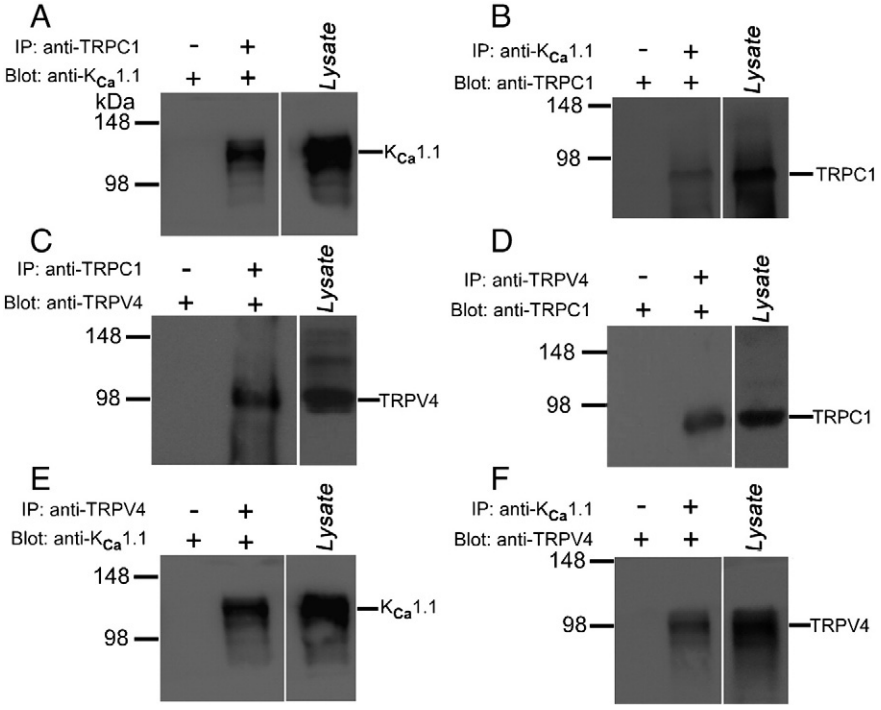

Fig. 1. Co-immunoprecipitation of TRPV4, TRPC1 and $K_{Ca}1.1(\alpha)$ in the lysates of human LIMA VSMCs. A and B, TRPC1 with $K_{Ca}1.1(\alpha)$. C and D, TRPC1 with TRPV4. E and F, TRPV4 with $K_{Ca}1.1(\alpha)$. The data are representative of 3 to 4 experiments. The pulling antibody and the blotting antibody were indicated (+). Control immunoprecipitation (−) was performed using the preimmune IgG. IP, immunoprecipitation; Blot, immunoblot.

optimal resting tension of 2 g for 3 mm long segments and allowed to equilibrate for 90 min. The optimal tension was equivalent to that generated at 0.9 times the diameter of the vessels at 100 mm Hg [24].

The vessels were preconstricted with phenylephrine to the contraction force of ~1.4 g for each 3 mm artery segment. Cumulative concentration of 11,12-EET or 5 μmol/L 4α-PDD was then added to evoke relaxations. In experiments examining the role of TRPV4, $K_{Ca}1.1$ and TRPC1, the vascular segments were first exposed to iberiotoxin (50 nmol/L, 30 min), T1E3 (1:50, 1 h), or HC-067047 (10 μmol/L, 30 min), before contracting them with phenylephrine. The concentration of phenylephrine varied (3–10 μmol/L) in order to achieve a similar degree of contraction (1.4 g/3 mm segment) for different pretreatments. 11,12-EET or 4α-PDD was then applied. The effect of preimmune IgG (1:50, 1 h) and 14,15-EEZE (10 μmol/L, 30 min) was also tested.

2.8. Statistics

For comparison of multiple groups, one-way ANOVA with the Newman–Keuls post hoc test was used. For comparison of two groups, Student's *t*-test was used for statistical comparison. For comparison of two or more curves, two-way ANOVA followed by the Bonferroni post hoc test was used. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Physical interaction between TRPV4, TRPC1 and $K_{Ca}1.1(\alpha)$ in VSMCs of human LIMAs

Co-immunoprecipitation was used to determine the physical interaction of $K_{Ca}1.1(\alpha)$, TRPC1 and TRPV4. We found that an anti-TRPC1 antibody could pull down $K_{Ca}1.1(\alpha)$ (Fig. 1A) and TRPV4 (Fig. 1C) in cell lysates freshly prepared from the primary cultured VSMCs derived from human LIMAs. Furthermore, an anti-$K_{Ca}1.1(\alpha)$ antibody or an anti-TRPV4 antibody could reciprocally pull down TRPC1 (Fig. 1B and D). Moreover, the anti-TRPV4 antibody could pull down anti-$K_{Ca}1.1(\alpha)$ (Fig. 1E), and the anti-$K_{Ca}1.1(\alpha)$ could reciprocally pull down TRPV4 (Fig. 1F). For controls, the IgG purified from preimmune serum failed to pull down TRPV4 or TRPC1 or anti-$K_{Ca}1.1(\alpha)$ (left lane in Fig. 1A–F). All three antibodies used for co-immunoprecipitation were previously shown by us and others to be highly specific to their respective targets [12,19,25,26].

3.2. Role of TRPV4–TRPC1–$K_{Ca}1.1$ complex in 11,12-EET-, GSK1016790A- and 4α-PDD-induced vascular smooth muscle cell hyperpolarization in human LIMAs

We next directly measured the membrane potentials of VSMCs in the endothelium-denuded human LIMA segments using sharp

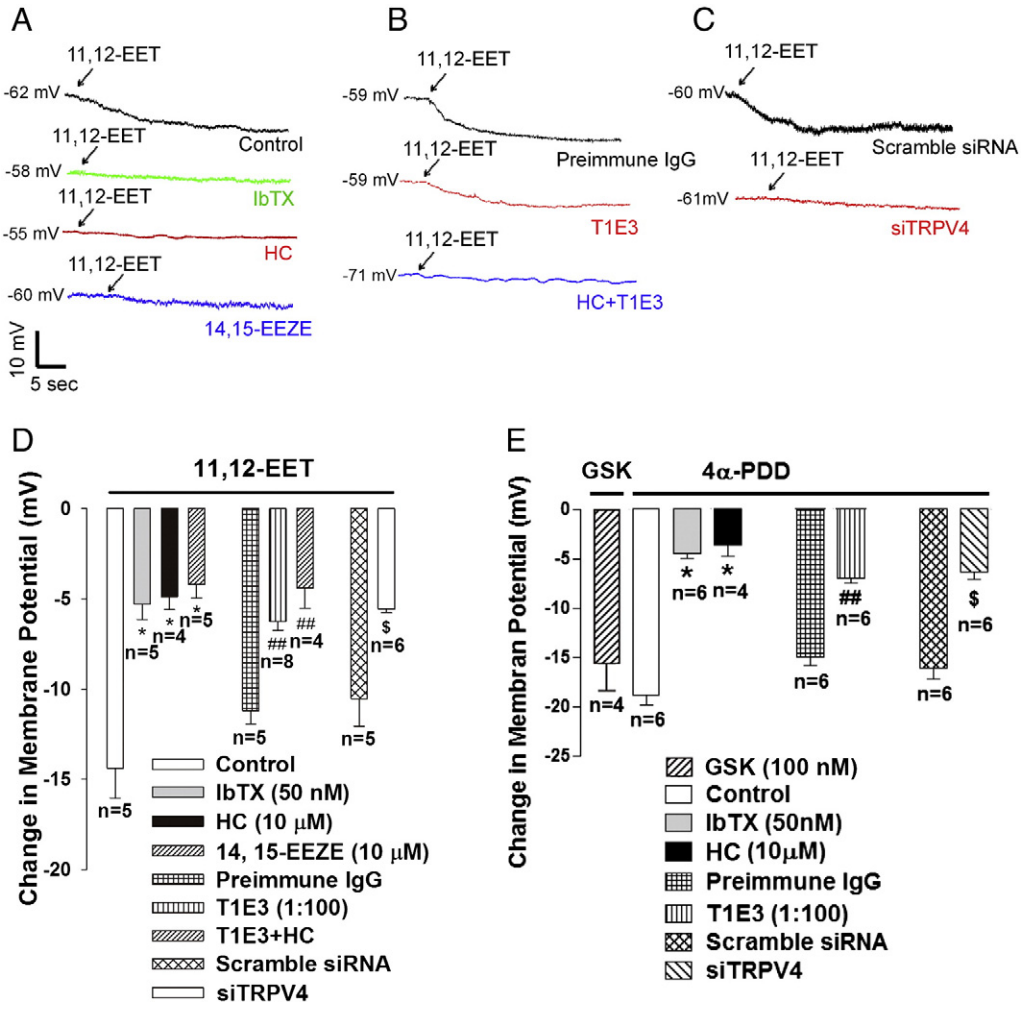

Fig. 2. Sharp microelectrode measurement of VSMC membrane potentials in endothelium-denuded human LIMA segments. A through C, representative traces illustrating the membrane potential changes in response to 300 nmol/L 11,12-EET. D and E, summary of data showing the maximal changes of membrane potentials in response to 300 nmol/L 11,12-EET (D) and 100 nmol/L GSK1016790A or 5 μmol/L 4α-PDD (E). Values are means ± SE ($n = 5$ to 8). GSK, GSK1016790A; IbTX, iberiotoxin; siTRPV4, TRPV4-siRNA; HC, HC-067047. *, $P < 0.05$ compared to control (without inhibitors); ##, $P < 0.01$ compared to preimmune IgG. $, $P < 0.05$ compared to scrambled siRNA.

microelectrodes. Microelectrodes were impaled from the lumen side into longitudinally opened human LIMA segments [12,23]. Application of 11,12-EET (300 nmol/L) or GSK1016790A (a TRPV4 specific agonist, 100 nmol/L) or 4α-PDD (a TRPV4 agonist, 5 μmol/L) each induced smooth muscle hyperpolarization in human LIMA segments (Fig. 2). The hyperpolarization was inhibited by a TRPV antagonist HC-067047 (10 μmol/L), a TRPV4 (human)-specific siRNA, a highly selective $K_{Ca}1.1(\alpha)$ inhibitor iberiotoxin (50 nmol/L), a TRPC1-specific blocking antibody T1E3 (1:100, 1 h pre-incubation) and T1E3 + HC-067047 (Fig. 2) [12,21]. In addition, if we increased IbTX concentration from 50 nM to 500 nM, 11,12-EET-induced hyperpolarization was completely abolished (Supplementary Fig. 1). These data suggest the involvement of TRPV4, TRPC1 and $K_{Ca}1.1$ in the hyperpolarizing responses to 11,12-EET and 4α-PDD. The action of 11,12-EET was also inhibited by 14,15-EEZE (10 μmol/L) (Fig. 2A and D), which is a selective EET antagonist. For control experiments, the effectiveness of TRPV4-siRNA was demonstrated in the western blots, in which TRPV4-siRNA was found to reduce the TRPV4 protein expression by ~60% (Supplementary Fig. 2). Furthermore, TRPV4-siRNA had no effect on the expression of $K_{Ca}1.1(\alpha)$ or TRPC1 (Supplementary Fig. 2).

Control experiments were also performed using microelectrode impalement from the adventitial side in intact arterial segments that were pre-constricted with phenylephrine. The results were similar to those obtained using microelectrode impalement from the lumen side in longitudinally opened human LIMA segments (Supplementary Fig. 3). In another control, the smooth muscle hyperpolarization of human LIMAs to cromakalim (a K_{ATP} channel agonist, 1 μmol/L) was not affected by iberiotoxin, HC-067047 or T1E3 (Supplementary Fig. 4).

3.3. Role of TRPV4–TRPC1–$K_{Ca}1.1(\alpha)$ complex in 11,12-EET- and 4α-PDD-induced vascular relaxation in human LIMAs

Human LIMA segments were preconstricted with varying concentrations of phenylephrine (3–10 μM) to induce similar degrees of contraction (~1.4 g/3 mm segment). Subsequent application of 11,12-EET induced vascular relaxation dose-dependently in the arteries that

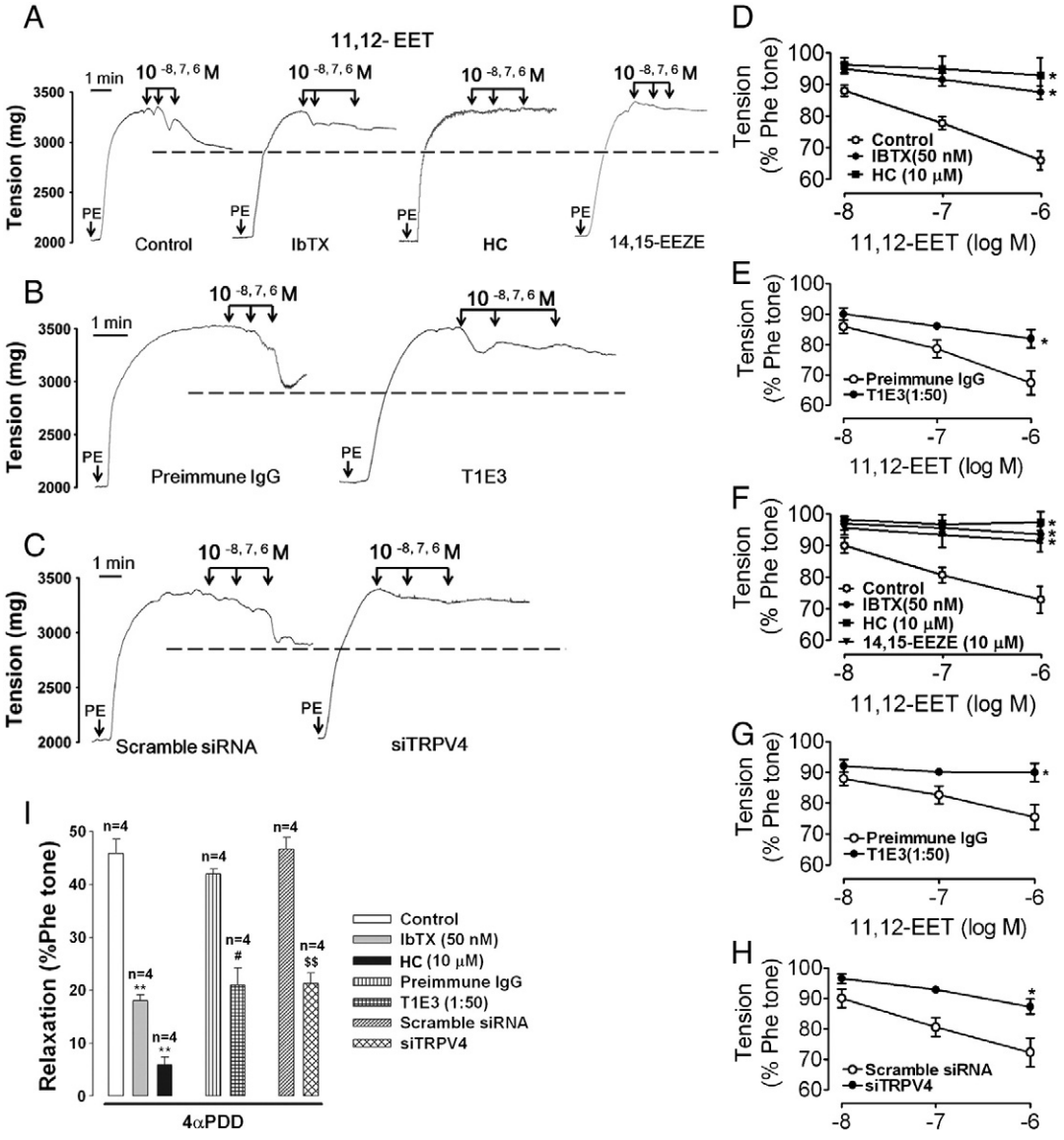

Fig. 3. Effects of T1E3, HC-067047, iberiotoxin, TRPV4-siRNA and 14,15-EEZE on 11,12-EET- and 4α-PDD-induced vascular relaxation in human LIMA segments. A through C, representative traces of dose-dependent vascular relaxation to 11,12-EET. D through H, summary of data showing the effects of HC-067047, iberiotoxin (IbTX), 14,15-EEZE, TRPV4-siRNA (siTRPV4) and T1E3 on vascular relaxation to 11,12-EET in endothelium-intact LIMA segments (D and E) and endothelium-denuded human LIMA segments (F–H). Values are means ± SE ($n = 4$ to 10). *, $P < 0.05$ compared to control (without inhibitors in D and F) or preimmune IgG (in E and G) or scrambled siRNA (in H). I, the data summary is shown. Human LIMA segments were endothelium-denuded, precontracted with phenylephrine, followed by 5 μmol/L 4α-PDD for relaxation. IbTX, iberiotoxin; siTRPV4, TRPV4-siRNA. Values are means ± SE ($n = 4$). **, $P < 0.05$ compared to control (without inhibitors); #, $P < 0.01$ compared to preimmune. $$, $P < 0.05$ compared to scrambled siRNA.

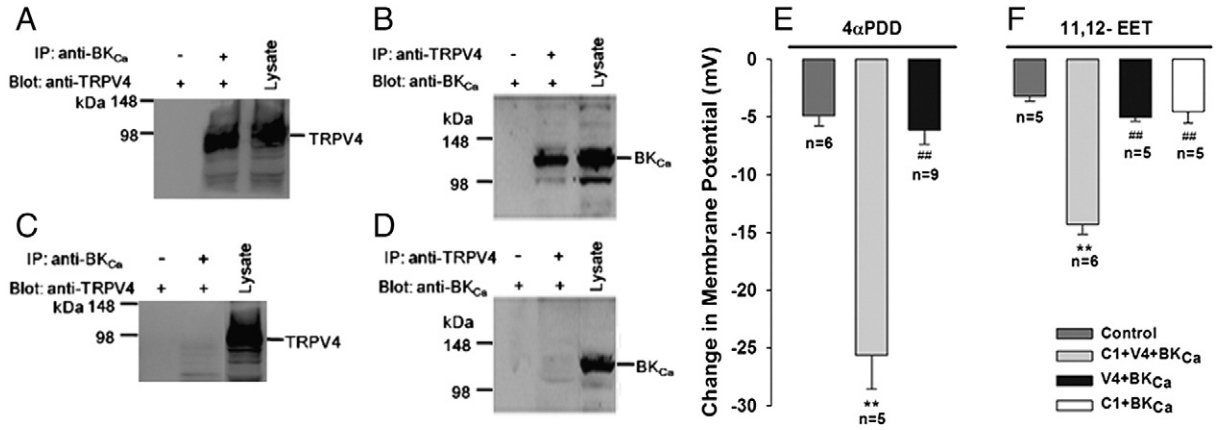

Fig. 4. Requirement of TRPC1 for TRPV4 and $K_{Ca}1.1(\alpha)$ interaction. A–D, co-immunoprecipitation of TRPV4 and $K_{Ca}1.1(\alpha)$ in the lysates of HEK293 cells. A and B, HEK293 cells that over-expressed with TRPV4, TRPC1 and $K_{Ca}1.1$. C and D, HEK293 cells that over-expressed with TRPV4 and $K_{Ca}1.1$ but without TRPC1. The right lanes in C and D were immunoblots using cell lysates without immunoprecipitation. The pulling antibody and the blotting antibody were indicated (+). Control immunoprecipitation (−) was performed using the preimmune IgG. IP, immunoprecipitation; Blot, immunoblot. $n = 3$ to 4 experiments. E–F, the maximal hyperpolarization in response to 300 nmol/L 11,12-EET (E) and 5 μmol/L 4α-PDD (F) in HEK293 cells that were transfected with indicated constructs. C1 stands for TRPC1. V4 stands for TRPV4. Values are means ± SE ($n = 5$ to 9). $^{**}P < 0.01$ compared to the controls (non-transfected), $^{\#\#}P < 0.01$ compared to C1 + V4 + $K_{Ca}1.1$.

were either endothelium-intact or endothelium-denuded (Fig. 3). Similarly, 4α-PDD also induced vascular relaxation (Fig. 3). The relaxations to 11,12-EET and 4α-PDD were inhibited by HC-067047 (10 μmol/L), TRPV4-siRNA, iberiotoxin (50 nmol/L) and T1E3 (1:50) (Fig. 3). 14,15-EEZE (10 μmol/L) inhibited the relaxation to 11,12-EET (Fig. 3).

3.4. Role of TRPV4–TRPC1–$K_{Ca}1.1(\alpha)$ complex in 11,12-EET-, GSK1016790A- and 4α-PDD-induced hyperpolarization in HEK293 overexpression system

HEK293 overexpression system was then used to explore the detailed mechanisms of interaction between TRPV4, TRPC1 and $K_{Ca}1.1(\alpha)$. Previously, we showed that TRPC1 co-immunoprecipitated with $K_{Ca}1.1(\alpha)$ and with TRPV4 [12,19], when they were co-expressed in HEK293 cells. Here, we found that TRPV4 and $K_{Ca}1.1(\alpha)$ failed to pull down each other when they were co-expressed (Fig. 4C and D). However, when a third gene TRPC1 was also over-expressed, TRPV4 and $K_{Ca}1.1(\alpha)$ could pull down each other (Fig. 4A and B).

In the functional study, 11,12-EET, 4α-PDD and GSK1016790A could induce membrane hyperpolarization in HEK293 cells that were over-expressed with all three constructs, i.e. TRPV4, TRPC1 and $K_{Ca}1.1(\alpha)$ (Fig. 4E and F, Fig. 5C and F), but they had little effect in cells that were only co-transfected with $K_{Ca}1.1(\alpha)$ and TRPV4 without TRPC1 or $K_{Ca}1.1(\alpha)$ and TRPC1 without TRPV4 (Fig. 4E and F). Again, the hyperpolarization to 11,12-EET, 4α-PDD and GSK1016790A was inhibited

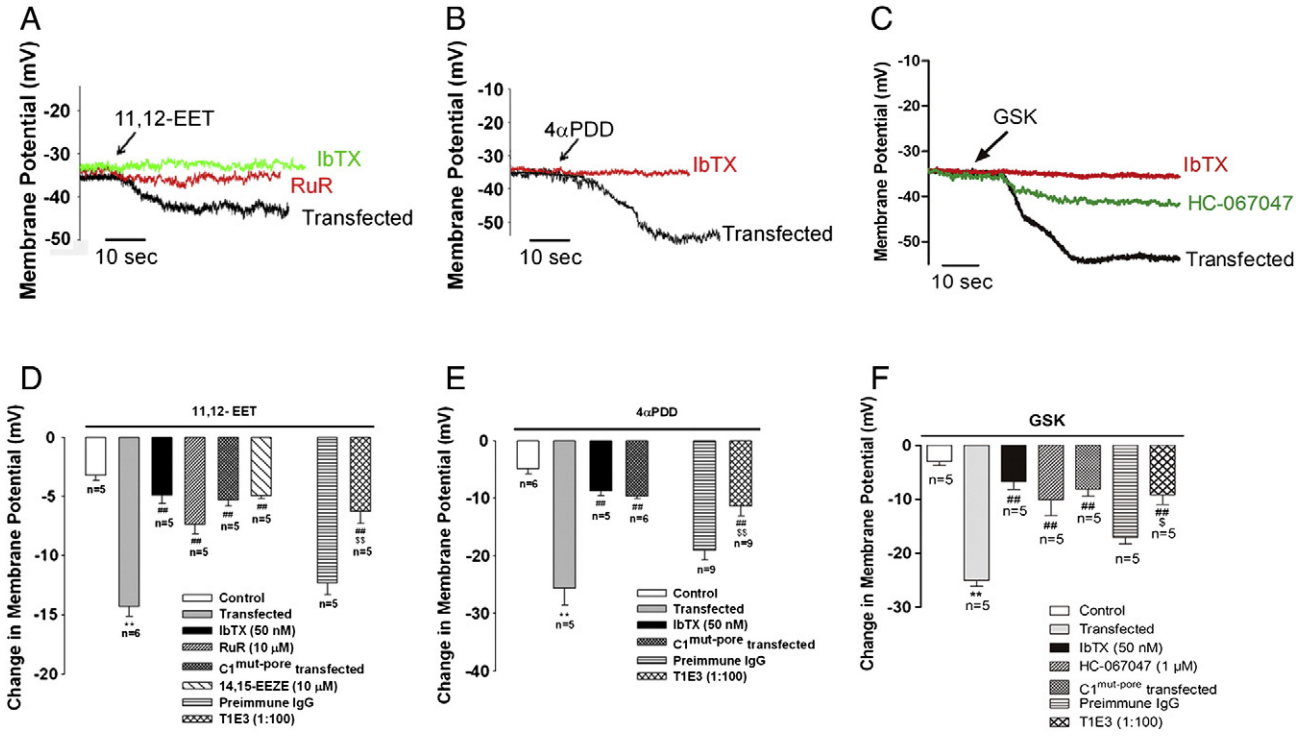

Fig. 5. Membrane potential changes in HEK293 cells that over-expressed with TRPV4, TRPC1 and $K_{Ca}1.1$. A and B, representative traces illustrating the hyperpolarizing responses to 300 nmol/L 11,12-EET (A), 5 μmol/L 4a-PDD (B) and 100 nmol/L GSK1016790A (C). D–F, summary of data showing the maximal hyperpolarization to 300 nmol/L 11,12-EET (D), 5 μmol/L 4a-PDD (E) and 100 nmol/L GSK1016790A (F) under different conditions. $C1^{mut\text{-}pore}$, TRPC1 were replaced by a TRPC1 pore-dead mutant $C1^{mut\text{-}pore}$. Values are means ± SE ($n = 5$ to 9). **, $P < 0.01$ compared to the controls (non-transfected); ##, $P < 0.01$ compared to the transfected cells without inhibitors; $$, $P < 0.01$ compared to preimmune IgG.

by HC-067047 (10 μmol/L), iberiotoxin (50 nmol/L) and T1E3 (1:100) (Fig. 5). The hyperpolarization to 11,12-EET was inhibited by 14,15-EEZE (10 μmol/L) (Fig. 5). We also utilized a TRPC1 pore-dead mutant $TRPC1^{mut\text{-}pore}$, which carries the point mutations at the pore region of TRPC1 [27]. Replacement of TRPC1 with $TRPC1^{mut\text{-}pore}$ also markedly reduced the hyperpolarizing responses to 11,12-EET, 4α-PDD and GSK1016790A (Fig. 5).

4. Discussion

The major novel findings of this study are as follows: (1) co-immunoprecipitation experiments demonstrated that TRPV4, TRPC1 and $K_{Ca}1.1$ form a physical complex in human LIMA VSMCs and TRPV4–TRPC1–$K_{Ca}1.1$ co-expressing HEK293 cells; (2) electrophysiological recordings showed that 11,12-EET and 4α-PDD act through the TRPV4–TRPC1–$K_{Ca}1.1$ complex to induce membrane hyperpolarization; and (3) arterial tension measurement showed that 11,12-EET acts through the TRPV4–TRPC1–$K_{Ca}1.1$ complex to induce vascular relaxation in human LIMAs. Together, these data suggest that EETs act through the TRPV4–TRPC1–$K_{Ca}1.1$ complex to induce smooth muscle cell hyperpolarization and vascular relaxation.

For coronary artery bypass surgery, human LIMA grafts have superior long-term patency rates and improved survival as compared to saphenous vein grafts [16]. The benefit of LIMAs over saphenous veins on mortality has been consistently observed irrespective of age and gender [28]. This could be attributed to several reasons: 1) LIMA grafts rarely develop atherosclerosis [16]; and 2) LIMAs release more protective vasodilators including nitric oxide and EDHFs. Reports showed that 11,12-EET, as a major EDHF in human LIMAs, activates $K_{Ca}1.1$ current to induce smooth muscle cell hyperpolarization and vascular relaxation [17]. However, the detailed molecular mechanisms of how EET could activate $K_{Ca}1.1$ to induce vascular relaxation in human LIMAs remain obscure. In the present study, we found that the 11,12-EET-induced smooth muscle cell hyperpolarization and relaxation in human LIMAs were inhibited by iberiotoxin, confirming the involvement of $K_{Ca}1.1$. Furthermore, HC-067047, TRPV4-siRNA, and T1E3 markedly inhibited the EET-induced membrane hyperpolarization and/or relaxation in human LIMAs, suggesting an important role of TRPV4 and TRPC1 in the processes.

Interestingly, co-immunoprecipitation experiments demonstrated that TRPV4, TRPC1 and $K_{Ca}1.1$ physically associated with each other to form a complex in human LIMA smooth muscle cells. Such a complex should allow efficient signal transduction delivered to $K_{Ca}1.1$, the activity of which is expected to induce smooth muscle hyperpolarization and vascular relaxation.

As for signaling pathway, presumably 11,12-EET is expected to first act on TRPV4, because TRPV4 contains an EET binding site [3–6]. This idea was supported by a series of experiments in which we found that the actions of 11,12-EET resembled those of 4α-PDD, which is a selective agonist of TRPV4. Similar to 11,12-EET, 4α-PDD could also induce smooth muscle hyperpolarization and vascular relaxation in human LIMAs. Furthermore, the actions of 4α-PDD were inhibited by iberiotoxin and T1E3, suggesting that TRPC1 and $K_{Ca}1.1$ are downstream of TRPV4. Alternatively, TRPV4 may heteromerize with TRPC1 to form a heteromeric channel, the activity of which may then activate $K_{Ca}1.1$. Such heteromeric TRPV4–TRPC1 channels are previously known to be present in vascular endothelial cells [19]. To further exclude the possibility that TRPC1 and TRPV4 may activate $K_{Ca}1.1$ via two independently pathways, we examined the combined effect of HC-067047 + T1E3 on 11,12-EET-induced smooth muscle hyperpolarization. The results showed that the combined application of HC-067047 + T1E3 had no additional effect compared to that of HC-067047, confirming that TRPC1 and TRPV4 share a single signaling pathway.

HEK293 cells are a commonly used system for gene overexpression. The system offers the advantage of low expression level of endogenous proteins, thus the function of exogenously expressed proteins can be studied with less ambiguity. In membrane potential recording experiments, 11,12-EET, GSK1016790A and 4α-PDD were able to induce membrane hyperpolarization in HEK293 cells that were overexpressed with TRPV4, TRPC1 and $K_{Ca}1.1$. Co-immunoprecipitation experiments demonstrated that TRPV4, TRPC1 and $K_{Ca}1.1$ also form a physical complex in HEK293 overexpression system (Fig. 4 and Ref. [12,19]). Interestingly, TRPV4 and $K_{Ca}1.1(\alpha)$ failed to pull down each other in the absence of TRPC1, indicating that TRPV4 and $K_{Ca}1.1(\alpha)$ are not capable of forming a physical complex on their own. The physical association between TRPV4 and $K_{Ca}1.1(\alpha)$ only occurred in the presence of TRPC1 (Fig. 4). In agreement, 4α-PDD and 11,12-EET failed to induce membrane hyperpolarization in HEK293 cells that over-expressed with TRPV4 and $K_{Ca}1.1(\alpha)$. In contrast, a marked hyperpolarization occurred when the HEK293 cells were also transfected with a third gene, TRPC1. These results suggest that TRPC1 serves as a linker through which TRPV4 and $K_{Ca}1.1(\alpha)$ can interact physically and functionally, thus enabling EET- and 4α-PDD-induced membrane hyperpolarization.

Our present model of EET action on TRPV4–TRPC1–$K_{Ca}1.1$ complex differed from an early model by Earley et al. [4] in two aspects: 1) in our model, an additional component TRPC1 is added. TRPC1 appears to act as a linker through which TRPV4 and $K_{Ca}1.1(\alpha)$ can interact and 2) in our model, TRPV4, TRPC1 and $K_{Ca}1.1(\alpha)$ are physically associated, whereas no physical interaction is suggested in Earley's model.

5. Conclusions

We demonstrated that TRPV4, TRPC1 and $K_{Ca}1.1$ physically associate with each other to form a signaling complex in human LIMA vascular smooth muscle cells. 11,12-EET acts on the TRPV4–TRPC1–$K_{Ca}1.1$ complex to induce VSMC hyperpolarization and vascular relaxation in human LIMAs. This finding is of clinical importance, because human LIMAs are important donor tissue in coronary bypass surgery, and provides the physiological relevance of endothelium-derived hyperpolarizing factors in LIMAs.

Disclosures

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.12.010.

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