

Original Article

Translocation of PKG1 α acts on TRPV4-C1 heteromeric channels to inhibit endothelial Ca²⁺ entry

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Aim: TRPV4-C1 heteromeric channels contribute to store-operated Ca²⁺ entry in vascular endothelial cells. However, the negative regulation of these channels is not fully understood. This study was conducted to investigate the inhibitory effect of PKG1 α on TRPV4-C1 heteromeric channels.

Methods: Immuno-fluorescence resonance energy transfer (FRET) was used to explore the spatial proximity of PKG1 α and TRPC1. Phosphorylation of endogenous TRPC1 was tested by phosphorylation assay. [Ca²⁺]_i transients and cation current in MAECs were assessed with Fura-2 fluorescence and whole-cell recording, respectively. In addition, rat mesenteric arteries segments were prepared, and vascular relaxation was examined with wire myography.

Results: In immuno-FRET experiments, after exposure of these cells to 8-Br-cGMP, more PKG1 α was observed in the plasma membrane, and PKG1 α and TRPC1 were observed to be in closer proximity. TAT-TRPC1^{S172} and TAT-TRPC1^{T313} peptide fragments, which contain the PKG targeted residues Ser172 and Thr313, respectively, were introduced into isolated endothelial cells to abrogate the translocation of PKG1 α . Furthermore, a phosphorylation assay demonstrated that PKG directly phosphorylates TRPC1 at Ser172 and Thr313 in endothelial cells. In addition, PKG activator 8-Br-cGMP markedly reduced the magnitude of the 4 α PDD-induced and 11,12-EET-induced [Ca²⁺]_i transients, the cation current and vascular relaxation.

Conclusion: This study uncovers a novel mechanism by which PKG negatively regulates endothelial heteromeric TRPV4-C1 channels through increasing the spatial proximity of TRPV4-C1 to PKG1 α via translocation and through phosphorylating Ser172 and Thr313 of TRPC1.

Keywords: vascular endothelial cells; store-operated Ca²⁺ entry; TRPV4-C1 heteromeric channels; PKG; 8-Br-cGMP; 4 α PDD; 11,12-EET; vascular relaxation

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Introduction

Vascular endothelial cells provide a crucial interface between circulating blood and vascular tissue. They respond to numerous humoral and physical stimuli by secreting relaxing and contracting factors, which modulate the contractility of vascular smooth muscle cells^[1]. In many cases, one of the early responses of endothelial cells to these diverse signals involves changes in the endothelial cytosolic Ca²⁺ concentration

([Ca²⁺]_i). Endothelial Ca²⁺ homeostasis depends on both intracellular Ca²⁺-store release and Ca²⁺ influx across the plasma membrane. One of the major Ca²⁺ influx pathways is store-operated Ca²⁺ entry, which is triggered by the depletion of the intracellular Ca²⁺ store^[2].

Store-operated Ca²⁺ entry in vascular endothelial cells not only serves to refill the intracellular Ca²⁺ stores but also acts to stimulate the synthesis of nitric oxide (NO), a key vasodilatory factor^[3–5]. Previously, we have examined the role of cGMP in regulating store-operated Ca²⁺ entry in aortic endothelial cells and demonstrated that cGMP, via a protein kinase G (PKG)-dependent mechanism, plays a key role in the regulation of store-operated Ca²⁺ entry^[6]. However, the mechanism related

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to the inhibitory effects of PKG-sensitive store-operated Ca^{2+} channel remains unclear.

Recently, our group showed that TRPC1 can co-assemble with TRPV4 to form TRPV4-C1 heteromeric channels^[7]. In vascular endothelial cells, heteromeric TRPV4-C1 channels contribute to store-operated Ca^{2+} entry and flow-induced Ca^{2+} influx^[6,7]. In the present study, we studied the regulation of this channel by the NO/cGMP pathway and the underlying mechanism. By blocking the PKG-mediated phosphorylation of TRPC1 with TAT-S172/T313-fragment peptides, we demonstrated that the function of the TRPV4-C1 channel can be negatively modulated by PKG-mediated phosphorylation on Ser172 and Thr313 of TRPC1. Taken together, our findings indicate that the TRPV4-C1 heteromeric channel serves as a PKG-inhibitable Ca^{2+} entry channel through increasing the spatial proximity of TRPV4-C1 to PKG1 α via translocation and through phosphorylating Ser172 and Thr313 of TRPC1 in endothelial cells.

Materials and methods

Animals

All of the animal experiments were conducted in conformity with the Guide for Animal Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No 8523), and the procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Sprague-Dawley rats or mice were placed in a chamber and were killed by exposure to carbon dioxide.

Antibodies and chemicals

Endothelial cell growth medium (EGM), endothelial cell basal medium, and bovine brain extract (BBE) were from Lonza. Anti-TRPC1 antibody (ACC-010) was from Alomone Labs. Anti-TRPC1 (sc-133076) and anti-TRPV4 (sc-47527) antibodies were from Santa Cruz Biotechnology. Anti-PKG1 α antibody (KAS-PK005) was from Stressgen. Anti-pSer/Thr antibody (ab17464) was from Abcam. Fura-2/AM, Fluo-4 AM and pluronic F127 were from Molecular Probes Inc. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium, and phosphate-buffered saline (PBS) were from Invitrogen. 4 α -Phorbol 12,13-didecanoate (4 α -PDD), KT5823 and Ruthenium red were from Calbiochem. 11,12-EET was from Cayman. Protein A agarose was from Roche. Nonidet P-40, trypsin, bovine serum albumin (BSA), collagenase, and poly-L-lysine were from Sigma.

Cell culture

Primary mesenteric artery endothelial cells (MAECs) were isolated from TRPC1 knock-out and wild-type (WT) 129/Sv mice from Prof Lutz BIRNBAUMER (NIH/NIEHS) (8–12 weeks of age). Briefly, the abdomen was opened, the small intestine was dissected out, and the mesenteric bed containing blood vessels along the small intestine was completely and carefully excised. The remaining arterial branches were digested with 0.02% collagenase in EBM for 45 min at 37°C. After

centrifugation at 3000 r/min for 5 min, the pelleted cells were resuspended in ECM medium supplemented with 5% FBS and 1% ECGS and were cultured in a flask at 37°C with 5% CO_2 . Nonadherent cells were removed 2 h later. The adherent endothelial cells were cultured with ECM medium at 37°C with 5% CO_2 for 1–2 d. These cells were used for experiments without further cell passage. The identity of endothelial cells was verified by immunostaining with an antibody against von Willebrand Factor^[7,8].

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

$[\text{Ca}^{2+}]_i$ in cultured cells was measured as described elsewhere^[7]. Briefly, primary cultured MAECs were loaded with 10 $\mu\text{mol/L}$ Fura-2/AM and 0.02% pluronic F-127 for 0.5 h in the dark at 37°C in normal physiological saline solution (NPSS). In 4 α PDD-stimulated Ca^{2+} -influx experiments, the $[\text{Ca}^{2+}]_i$ change in response to 4 α PDD (5 $\mu\text{mol/L}$) was measured. NPSS contained the following: 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 10 mmol/L glucose, and 5 mmol/L HEPES, pH 7.4. Fura-2 fluorescence was measured using dual excitation at 340 and 380 nm using an Olympus fluorescence imaging system. Changes in $[\text{Ca}^{2+}]_i$ were calculated as the change in the Fura-2 ratio.

Endothelial $[\text{Ca}^{2+}]_i$ was also measured *in situ* in freshly isolated mouse thoracic aortas. An arterial segment was cut open along its longitudinal axis and pinned onto a Sylgard-coated dish with the lumen side upward. Vessels were incubated with 10 $\mu\text{mol/L}$ Fluo-4 AM at room temperature for 60 min, followed by the Ca^{2+} assay in modified Krebs solution containing the following: 119 mmol/L NaCl, 4.7 mmol/L KCl, 25 mmol/L NaHCO_3 , 2.5 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 1.2 mmol/L KH_2PO_4 , and 11 mmol/L *D*-glucose. The experiments were performed at 37°C unless otherwise indicated. Fluo-4 fluorescence was measured using an excitation at 488 nm with a LSM 5 Pascal confocal microscope (Zeiss). Changes in $[\text{Ca}^{2+}]_i$ were calculated as the change in the Fluo-4 ratio.

Whole-cell patch clamp

Whole-cell current was measured with an EPC-9 patch-clamp amplifier as described elsewhere^[9]. Cells were clamped at 0 mV. The whole-cell current density (pA/pF) was recorded in response to successive voltage pulses of +80 mV and -80 mV for 100-ms duration. The whole-cell current values were plotted versus time. The recordings were made before and after 4 α -PDD (5 $\mu\text{mol/L}$) application. For the 4 α PDD-operated current, the pipette solution contained the following: 20 mmol/L CsCl, 100 mmol/L Cs⁺-aspartate, 1 mmol/L MgCl_2 , 4 mmol/L ATP, 0.08 mmol/L CaCl_2 , 10 mmol/L BAPTA, and 10 mmol/L HEPES, pH 7.2. The bath solution contained the following: 150 mmol/L NaCl, 6 mmol/L CsCl, 1 mmol/L MgCl_2 , 1.5 mmol/L CaCl_2 , 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.4.

TAT-mediated peptide transduction

Small peptides that contain the PKG phosphorylation sites on TRPC1 (TAT-TRPC1^{S172}, NKKDSLRLHS; TAT-TRPC1^{T313},

RRKPTCKKI) or the corresponding scrambled peptide (TAT-scrambled, NHRLDKSKS) were conjugated to an NH₂-terminal 11-amino acid HIV Tat protein transduction domain (YGRKKRRQRRR) (Alpha Diagnostic International, USA; China-Peptides, PRC)^[7,10]. In some experiments, after the transduction process, the arterial segments were washed and cut open, and the intimal surfaces of the segments were placed upside down between two coverslips on the microscope. Images were captured using excitation at 488 nm with a LSM 5 Pascal confocal microscope (Zeiss).

Arterial tension measurement

Two-millimeter segments of the third or fourth branches of the rat mesenteric arteries were mounted in a DMT myograph (model 610M; Danish Myo Technology, Aarhus, Denmark), and the changes in the isometric tension of arteries were measured. The rings were stretched to an optimal baseline force of 2 mN for 2-mm artery segments. The force and wall tension relationship is defined as follows: tension=force/2×segment length. The optimal force is equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. After equilibration, the segments were pre-treated with phenylephrine (3–10 μmol/L). When appropriate, arterial segments were pre-incubated with 8-Br-cGMP (2 mmol/L) at 37 °C for 40 min. In the experiments intended to reverse the action of 8-Br-cGMP, S172A/T313A (TAT-TRPC1^{S172A} and TAT-TRPC1^{T313A}) was added at least 20 min before the addition of 8-Br-cGMP. The bath solution is the modified Krebs solution.

TRPC1 phosphorylation assay

TRPC1 phosphorylation was measured as described elsewhere^[11,12]. Briefly, primary cultured MAECs were treated with 8-Br-cGMP (2 mmol/L) for 30 min. If needed, KT5823 (2 μmol/L), a protein kinase G (PKG) inhibitor^[12], was included 20 min before the addition of 8-Br-cGMP (2 mmol/L). TAT-scrambled (600 nmol/L) or TAT-TRPC1 (600 nmol/L) was included 30 min before the addition of 8-Br-cGMP (2 mmol/L). Two milligrams of isolated proteins was incubated overnight with 7 μg of anti-TRPC1 (Alomone Labs) antibody and 100 μL of Protein A agarose. The phosphorylation levels were detected using an anti-pSer/Thr antibody (Abcam).

Immunofluorescence resonance energy transfer (FRET)

MAECs were treated with 8-Br-cGMP for 10 min. If needed, TAT-scrambled (control) or TAT-TRPC1^{S172} (S172A) or TAT-TRPC1^{T313} (T313A) or TAT-TRPC1^{S172}+TAT-TRPC1^{T313} (S172/T313A) was incubated 30 min before the addition of 8-Br-cGMP (2 mmol/L), and then the MAECs were fixed. Paraformaldehyde-fixed MAECs were incubated with primary anti-TRPC1 and anti-PKG1 antibodies. Cells were then labeled with the secondary antibodies Cy2-conjugated for TRPC1 or TRPV4 and Cy3-conjugated for PKG1α. Following washing and mounting, fluorescence images were acquired using a Leica TCS SP5 laser-scanning confocal microscope. Cy2 and Cy3 were excited at 488 and 543 nm, respectively, with emissions at 505–530 and >560 nm, respectively. First, images of

the donor and acceptor distributions were taken. The acceptor dyes were then bleached by repetitive scans with the 514-nm laser at maximal laser power. After photobleaching, a donor image was recorded again. The LSM data acquisition software (Leica TCS SP5 LAS AF Version 1.7.0) was used to define ROIs and calculate the mean cellular fluorescence intensities in each channel^[13,14].

Preparation of T1E3 and preimmune IgG

The T1E3 antibody was raised in rabbits using a strategy developed by Xu *et al*^[7]. Briefly, a peptide corresponding to the E3 region near the ion permeation pore, between the transmembrane regions S5 and S6, of TRPC1 (CVGIFCEQQSNDT-FHSFIGT) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) at Alpha Diagnostic International (USA). The coupled T1E3 peptide was injected into the back of a rabbit (d 0), and this was followed by two booster doses, at d 21 and 42. 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). As a control, pre-immune serum was purified with a HiTrap Protein G column to obtain immunoglobulin G, which was then used in the experiments.

Statistics

Data are expressed as the mean±SEM. Statistical analysis was performed using repeated measures one-way or two-way ANOVA followed by Newman-Keuls or Bonferroni's test for multiple-group comparisons, respectively, using GraphPad Prism software. Differences were considered statistically significant when $P < 0.05$.

Results

PKG phosphorylation of TRPC1 due to translocation-induced spatial proximity

First, we examined the subcellular localization of PKG1α and TRPC1 with or without 8-Br-cGMP treatment. In double-labeled immunofluorescence experiments, primary MAECs isolated from TRPC1 WT mice were stained for TRPC1 and PKG1α. TRPC1 was mainly distributed on the plasma membrane, while PKG1α was abundant intracellularly. An increase in the proportion of PKG1α in the plasma membrane was observed after exposure to 8-Br-cGMP, indicating translocation to the plasma membrane (Figure 1A). To further explore the spatial proximity of PKG1α and TRPC1, we used FRET in primary MAECs. As shown in Figure 1A, an immuno-FRET signal between PKG1α and TRPC1 was detected in MAECs. 8-Br-cGMP exposure markedly increased this signal. PKG phosphorylates specific residues on TRPC3 and TRPC6 and thereby modulates channel function^[15,16]. These phosphorylation sites are conserved at Ser172 and Thr313 in TRPC1^[7]. To directly investigate the molecular mechanism of the negative regulation of the TRPC1 channel by PKG in primary MAECs, two fusion peptides were synthesized by fusing TRPC1 peptide fragments that contain these two sites with the membrane translocation signals from the HIV-1 tat protein, resulting in

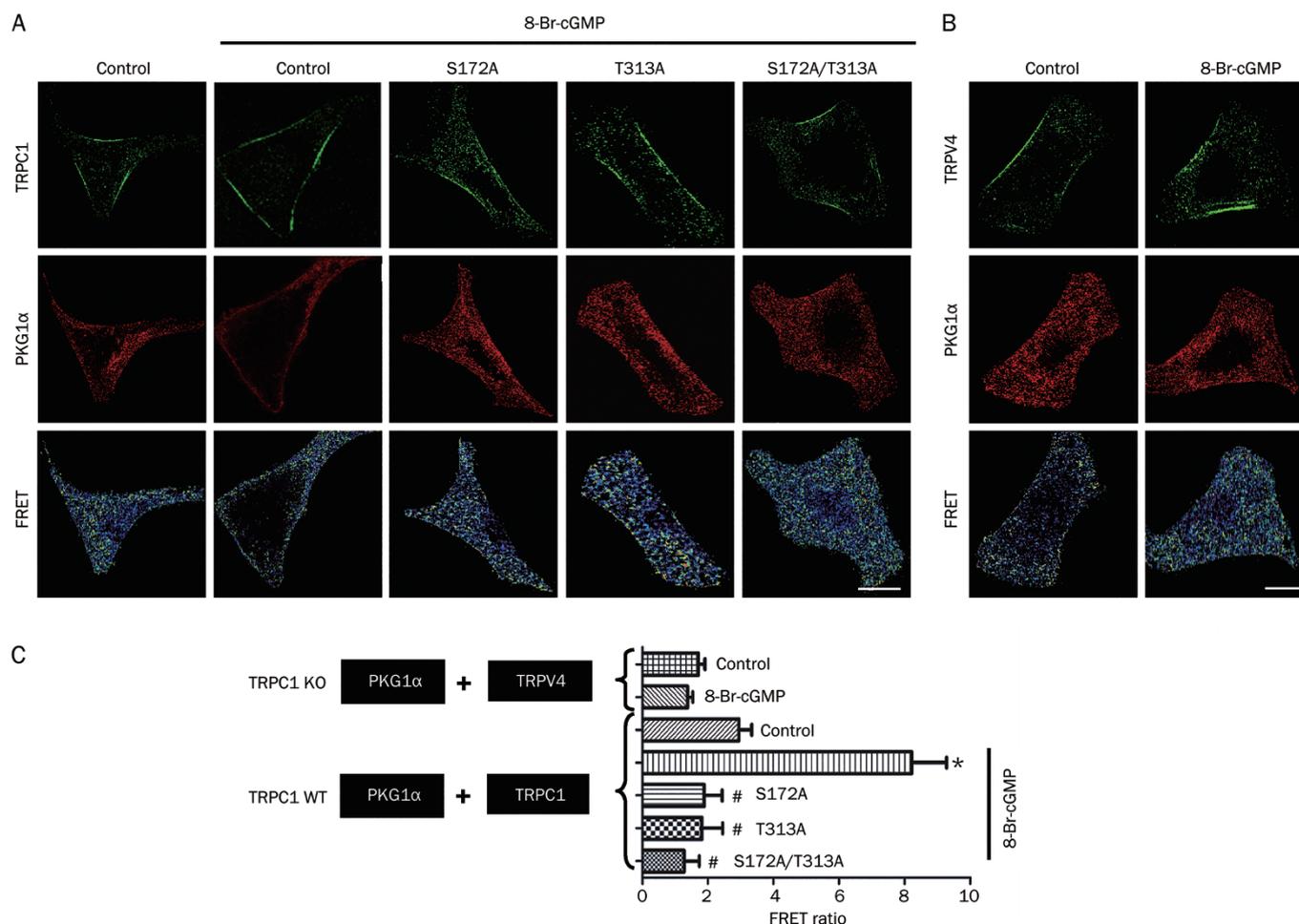


Figure 1. PKG-mediated phosphorylation of TRPC1 due to translocation-induced spatial proximity. (A) Representative images of immuno-FRET in TRPC1 WT endothelial cells. (B) Representative images of immuno-FRET in TRPC1 KO endothelial cells. (C) Summary data for immuno-FRET. The horizontal axis indicates the FRET ratio for endothelial cells. Data are mean \pm SEM. $n=10$ experiments from 4–5 animals. * $P<0.05$ vs without 8-Br-cGMP 2 mmol/L. # $P<0.05$ vs with 8-Br-cGMP. Scale bar, 5 μ m.

S172A (TAT-TRPC1^{S172}) and T313A (TAT-TRPC1^{T313}); TAT-scrambled was used as a control^[17]. This allowed efficient and abundant intracellular delivery of the exogenous PKG substrates S172A/T313A (TAT-TRPC1^{S172} and TAT-TRPC1^{T313}) and blocked the PKG-mediated phosphorylation of endogenous TRPC1 channels. Application of S172A (TAT-TRPC1^{S172}) or T313A (TAT-TRPC1^{T313}) alone, or S172A/T313A (TAT-TRPC1^{S172} plus TAT-TRPC1^{T313}), abolished the enhancing effect of 8-Br-cGMP. No immuno-FRET signal between PKG1 α and TRPV4 was detectable in primary MAECs isolated from TRPC1 KO mice (Figure 1B). We next assessed the direct PKG-mediated phosphorylation of TRPC1 in primary cultured MAECs. The activation of PKG by 8-Br-cGMP enhanced TRPC1 phosphorylation, and KT5823 inhibited TRPC1 phosphorylation in MAECs (Figure 2A). In addition, S172A/T313A (TAT-TRPC1^{S172} plus TAT-TRPC1^{T313}) suppressed the PKG-mediated phosphorylation of TRPC1 proteins in MAECs (Figure 2B). As controls, KT5823 and control (TAT-scrambled) alone had no effect on the PKG-mediated phosphorylation of

TRPC1 proteins in MAECs (Figure 2). These data suggest that 8-Br-cGMP induces the translocation of PKG1 α , bringing it close to TRPC1, and that this translocation is dependent on the Ser172 and Thr313 phosphorylation sites of TRPC1.

Inhibitory effect of the PKG activator 8-Br-cGMP on 4 α PDD-stimulated [Ca²⁺]_i transient and cation current

4 α PDD is a synthetic phorbol ester that activates TRPV4-C1 heteromeric channels^[18,19]. We used 4 α PDD to stimulate Ca²⁺ influx into primary MAECs (Figure 3). Pretreatment with the PKG activator 8-Br-cGMP (2 mmol/L) for 10 min markedly reduced the magnitude of the 4 α PDD-induced [Ca²⁺]_i transient (Figure 3A–3C) and cation current (Figure 3E–3F). The 4 α PDD-induced [Ca²⁺]_i transient was inhibited by Ruthenium red (RuR) (a blocker of TRPV4-C1 channels), as shown in Figure 3B and 3C^[7]. The 4 α PDD-induced cation current was inhibited by T1E3 (a TRPC1-specific blocking antibody, diluted by 1:100) (Figure 3G)^[7]. On the other hand, KT5823 (2 μ mol/L) and DT3 (1 μ mol/L), which are potent and highly-specific

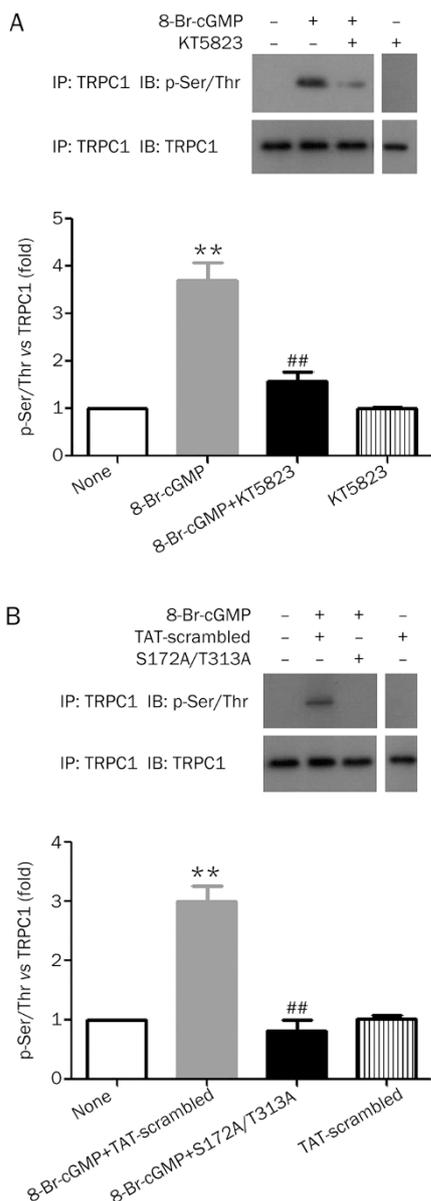


Figure 2. Protein kinase G (PKG)-mediated phosphorylation at Ser172 and Thr313 of TRPC1. Representative images are shown for the PKG dependent phosphorylation of native TRPC1 proteins in MAECs (upper panel), and a data summary is shown (lower panel). (A) MAECs were treated with KT5823 (2 $\mu\text{mol/L}$) for 20 min before the addition of 8-Br-cGMP (2 mmol/L) for 30 min. (B) MAECs were treated with TAT-scrambled (600 nmol/L) or S172A/T313A (TAT-TRPC1^{S172A} plus TAT-TRPC1^{T313A}, 600 nmol/L). IP and IB are abbreviations designating immunoprecipitation and immunoblotting, respectively. Mean \pm SEM. $n=4-6$. ** $P<0.01$ vs no 8-Br-cGMP or TAT. ## $P<0.01$ vs 8-Br-cGMP but no KT5823 or S172A/T313A.

PKG inhibitors, abolished the inhibitory action of 8-Br-cGMP (Figure 3C and 3F). 11,12-EET (3 $\mu\text{mol/L}$) is a major type of physiological epoxyeicosatrienoic acid (EET) that activates TRPV4-C1 heteromeric channels^[20-22]. 8-Br-cGMP also inhibited 11,12-EET-induced $[\text{Ca}^{2+}]_i$ transients, and the inhibitory

effects of 8-Br-cGMP on the action of 11,12-EET were reversed by KT5823 (Figure 3D). Endothelial $[\text{Ca}^{2+}]_i$ was also measured *in situ* in freshly isolated mouse thoracic aortas. The inhibitory effects of 8-Br-cGMP on the action of 4 α PDD or 11,12-EET were also reversed by KT5823 (2 $\mu\text{mol/L}$) or DT3 (1 $\mu\text{mol/L}$) (Figure 3H-3K). These data demonstrate that the activation of PKG is necessary to inhibit the 4 α PDD- or 11,12-EET-induced $[\text{Ca}^{2+}]_i$ transients and cation current in endothelial cells.

TRPC1 phosphorylation sites Ser172 and Thr313 are required for the inhibitory effect of 8-Br-cGMP on the 4 α PDD-stimulated cation current

We showed that the treatment of MAECs with both fusion peptides, S172A/T313A, abolished the inhibitory effect of 8-Br-cGMP on the 4 α PDD-induced $[\text{Ca}^{2+}]_i$ transients (Figure 4A, 4B) and cation current (Figure 4C, 4D) in primary MAECs. In addition, S172A/T313A also reversed the inhibitory effect of 8-Br-cGMP on the 4 α PDD-induced $[\text{Ca}^{2+}]_i$ transients *in situ* (Figure 4E, 4F). These data strongly suggest that cGMP/PKG phosphorylates TRPC1, thereby suppressing the 4 α PDD-stimulated endothelial Ca^{2+} influx.

8-Br-cGMP inhibits vasodilation by phosphorylating Ser172 and Thr313 of TRPC1 in intact arterial segments

In a wire myography study, the segments were pre-treated with phenylephrine (Phe) (3-10 $\mu\text{mol/L}$). 4 α PDD induced vascular relaxation in a concentration-dependent manner in small rat mesenteric artery segments (Figure 5A). However, 4 α PDD failed to induce relaxation in arteries that were endothelium denuded, indicating that 4 α PDD-induced relaxation is endothelium dependent (Figure 5B). In artery segments with intact endothelium, 8-Br-cGMP (2 mmol/L) markedly reduced 4 α PDD-induced relaxation. The application of TAT-TRPC1^{S172} plus TAT-TRPC1^{T313} abolished the inhibitory effect of 8-Br-cGMP (Figure 5B). Together, these data also suggest that PKG phosphorylates TRPC1 and thereby inhibits 4 α PDD-induced vascular relaxation.

Discussion

In the present study, we found that the cGMP/PKG pathway inhibits endothelial cell Ca^{2+} entry and vasodilation induced by 4 α PDD through the PKG-targeted residues Ser172 and Thr313 of TRPC1 in TRPV4-C1 channels. PKG-mediated phosphorylation of TRPC1 was found in native endothelial cells. More interestingly, we demonstrated that the PKG-mediated phosphorylation of TRPV4-C1 channels was due to translocation-induced spatial proximity between PKG1 α and TRPC1.

Endothelial cell Ca^{2+} entry is known to stimulate the production of NO, which subsequently activates guanylate cyclase, leading to the elevation of cellular cGMP^[5]. The elevated cGMP may, in turn, inhibit Ca^{2+} entry via a PKG-dependent pathway, thereby providing a negative feedback mechanism through which Ca^{2+} -influx is finely regulated. However, the molecular identity of such PKG-inhibitable Ca^{2+} -entry channels is unknown. In the present study, we demonstrated that,

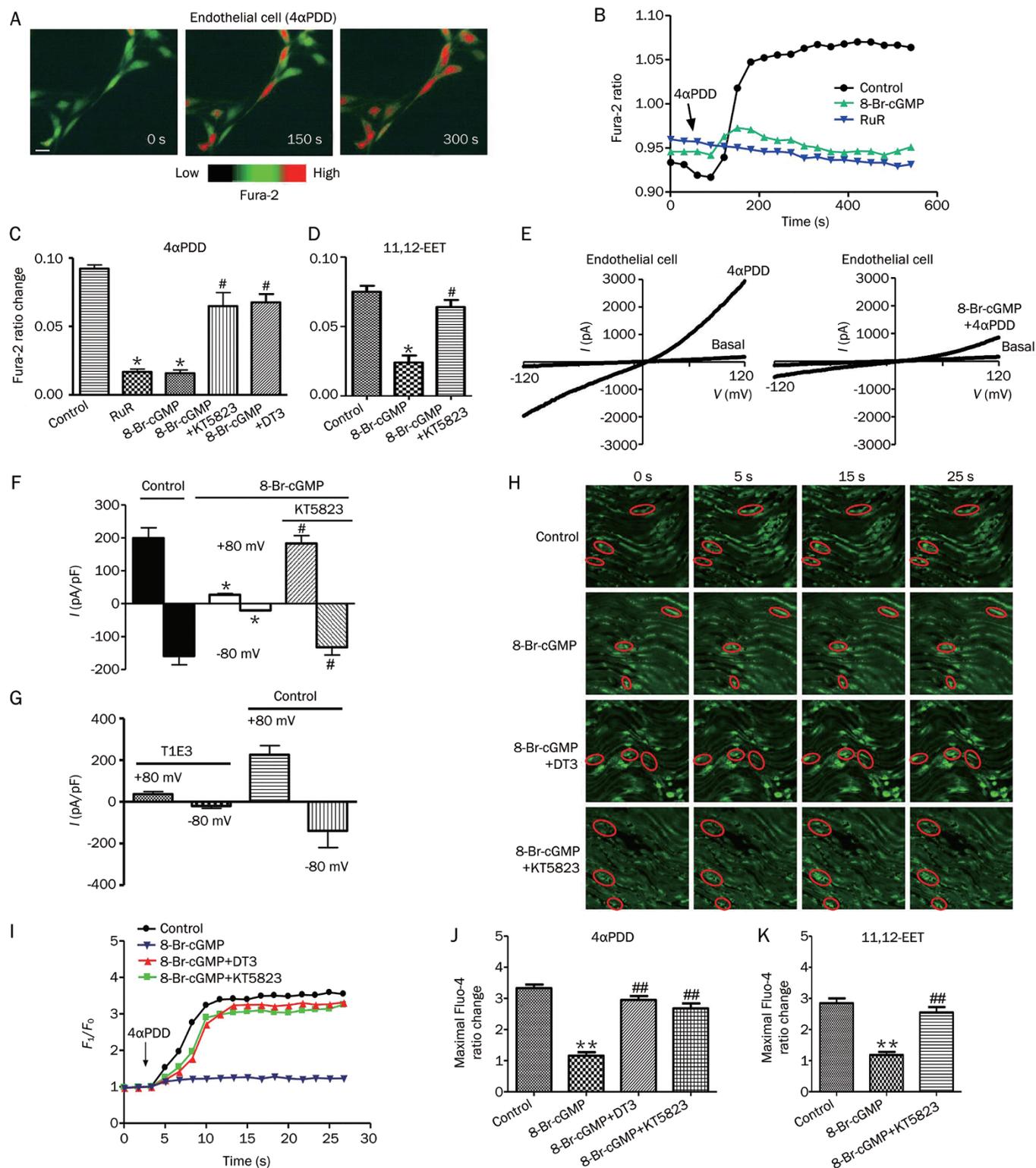


Figure 3. Inhibitory effect of 8-Br-cGMP on 4 α PDD-stimulated $[Ca^{2+}]_i$ transients and cation current. (A and B) Representative images and time-course of 4 α PDD-stimulated Ca^{2+} entry into primary cultured endothelial cells. (C) Summary for the maximal Ca^{2+} entry in B ($n=5$ to 6 experiments; 8 to 20 cells per experiment; from 3–4 animals). (D) Summary for the maximal Ca^{2+} entry induced by 11,12-EET. $n=5$ to 6 experiments; 8 to 20 cells per experiment; from 3–4 animals. (E) Representative $I-V$ curves for 4 α PDD-stimulated cation current. (F and G) Summary data. 8-Br-cGMP (2 mmol/L) with KT5823 (2 μ mol/L), DT3 (1 μ mol/L) or T1E3 was introduced 10 min before 4 α PDD ($n=4$ experiments, from 3 animals). (H and I) Representative images and time-course of 4 α PDD-stimulated Ca^{2+} entry into endothelial cells *in situ*. (J) Summary for the maximal Ca^{2+} entry in (H). $n=4$ experiments, from 3–4 animals. (K) Summary for the maximal Ca^{2+} entry induced by 11,12-EET. $n=4$ experiments, from 3–4 animals. Note that 4 α PDD and 11,12-EET are TRPV4-C1 activators, 8-Br-cGMP is a PKG activator, and KT5823 is PKG inhibitor. RuR and T1E3 are TRPV4-C1 blockers. Mean \pm SEM. * $P<0.05$, ** $P<0.01$ vs control. # $P<0.05$, ## $P<0.01$ vs 8-Br-cGMP. Scale bar, 10 μ m.

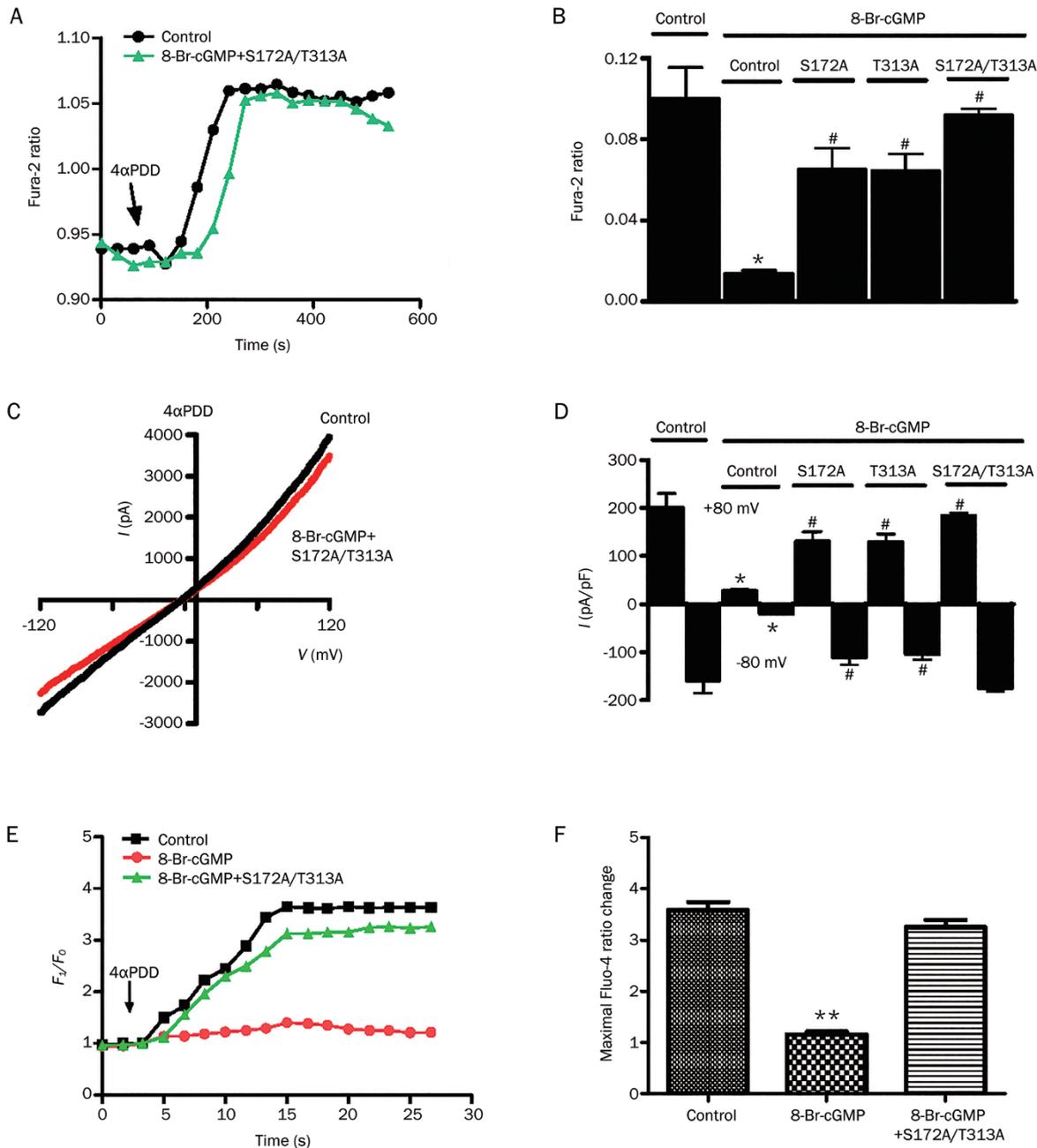


Figure 4. TRPC1 phosphorylation sites Ser172 and Thr313 are required for the inhibitory effect of 8-Br-cGMP on the 4 α PDD-stimulated $[Ca^{2+}]_i$ transients in endothelial cells. (A and B) Traces (A) and summary (B) for 4 α PDD-stimulated Ca^{2+} entry as the maximal change in $[Ca^{2+}]_i$. $n=4$ experiments, 8 to 20 cells per experiment, from 3–4 animals. (C and D) Representative $I-V$ curves and summary for 4 α PDD-stimulated cation current. $n=4$ experiments, from 3–4 animals. (C) Representative $I-V$ curves pretreated with 8-Br-cGMP (2 mmol/L) plus S172A/T313A (TAT-TRPC1^{S172A} plus TAT-TRPC1^{T313A}). (D) Summary for the currents at ± 80 mV. 8-Br-cGMP (2 mmol/L) with control or S172A/T313A was introduced 15 min before the application of 4 α PDD. (E) Time-course of 4 α PDD-stimulated Ca^{2+} entry into endothelial cells *in situ*. (F) Summary for the maximal Ca^{2+} entry *in situ* in (E). $n=4$ experiments, from 3–4 animals. Values are mean \pm SEM. * $P<0.05$ vs without 8-Br-cGMP. # $P<0.05$ vs cells treated with 8-Br-cGMP.

in primary endothelial cells, 8-Br-cGMP increased the plasma membrane distribution of PKG1 α and enhanced the FRET efficiency of TRPC1 and PKG1 α . The action of 8-Br-cGMP on the spatial proximity of PKG1 α and TRPC1 was abrogated by blocking TRPC1-PKG phosphorylation sites with an excessive supply of the exogenous PKG substrates TAT-TRPC1^{S172} and

TAT-TRPC1^{T313}. Furthermore, phosphorylation assays demonstrated that PKG phosphorylated TRPC1 at Ser172 and Thr313 in native endothelial cells. Our data support the speculation that the phosphorylation of TRPC1 recruits PKG to the plasma membrane. However, the mechanism by which PKG translocation regulates other downstream targets requires further

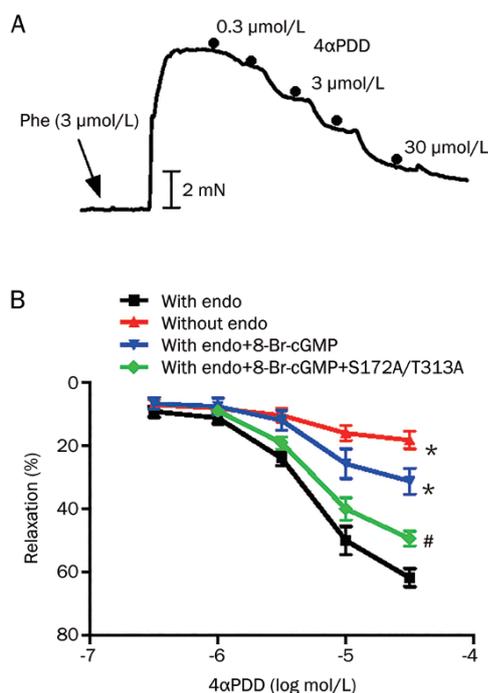


Figure 5. Role of TRPC1 phosphorylation sites, Ser172 and Thr313 in vascular relaxation. (A and B) Traces and summary data for dose-dependent relaxation in response to 4αPDD (0.3–30 μmol/L) and effect of 8-Br-cGMP pretreated with S172A/T313A (TAT-TRPC1^{S172A} plus TAT-TRPC1^{T313A}). 8-Br-cGMP (2 mmol/L) with control or S172A/T313A was introduced 15 min before the application of 4αPDD. The segments were pre-treated with phenylephrine (Phe) (3–10 μmol/L). Values are mean±SEM. *n*=4 experiments, from 4 animals. **P*<0.05 vs control. #*P*<0.05 vs 8-Br-cGMP.

study. The TRPV4-C1 heteromeric channel is important for Ca²⁺ influx and flow-induced vasodilation^[6–8]. We next determined the action of the cGMP/PKG pathway on the function of the TRPV4-C1 heteromeric channel. 8-Br-cGMP prevented 4αPDD-induced endothelial cell Ca²⁺ influx and cation current. This inhibition was reversed by KT5823, thereby suggesting the involvement of PKG. Furthermore, another publication by our group has demonstrated that EET targets the TRPV4-C1 heteromeric channel^[22]. In this study, we found that 8-Br-cGMP inhibits 11,12-EET-induced Ca²⁺ influx through PKG, an effect that is consistent with that on 4αPDD. The inhibitory action of 8-Br-cGMP on 4αPDD-induced Ca²⁺ influx and vascular relaxation were abrogated by shielding TRPC1-PKG phosphorylation sites with an excessive supply of the exogenous PKG substrates TAT-TRPC1^{S172} and TAT-TRPC1^{T313}. As a result, we established that the cGMP/PKG pathway enables the phosphorylation of TRPC1 in TRPV4-C1 channels, thereby causing the inhibition of 4αPDD-induced Ca²⁺ influx and vascular relaxation. It has already been well established that the TRPV4-C1 heteromeric channel in endothelial cells prolongs Ca²⁺ influx, thus producing more NO, cGMP and active PKG. Our study provides novel evidence that cGMP/PKG can act on TRPC1 in the TRPV4-C1 channel and thereby counter the function of the TRPV4-C1 channel, perhaps further inhibit-

ing the cGMP/PKG pathway. The presence of two separate control mechanisms, one that positively stimulates Ca²⁺ entry via Ca²⁺ influx channels and another that negatively attenuates Ca²⁺ entry through PKG, allows intracellular Ca²⁺ concentrations to be finely regulated.

Conclusion

In the present study, we demonstrated that the plasma membrane distribution of PKG1α is dependent on Ser172 and Thr313 of TRPC1. Closer spatial proximity between PKG1α and TRPC1 is also dependent on Ser172 and Thr313 of TRPC1, and PKG inhibits endothelial Ca²⁺-entry and vasodilation by phosphorylating Ser172 and Thr313 of TRPC1, indicating an important role for TRPC1 in the regulation of vascular tone and blood pressure.

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Author contribution

Xin MA, Xiao-qiang YAO, and Jian JIN conceived the project and designed the experiments; Peng ZHANG, Ai-qin MAO, and Chun-yuan SUN performed the experiments with help from Xiao-dong ZHANG, Qiong-xi PAN, Dan-tong YANG, Chun-lei TANG, Zhen-yu YANG, and Xiao-jie LU; Peng ZHANG, Ai-qin MAO, and Chun-yuan SUN analyzed the data; Xin MA and Peng ZHANG wrote the manuscript.

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