**Title page.**

# Adenylate cyclase-cAMP-protein kinase A signaling pathway inhibits endothelin type A receptor-operated $Ca^{2+}$ entry mediated via TRPC6 channels.

Takahiro Horinouchi, Tsunaki Higa, Hiroyuki Aoyagi, Tadashi Nishiya,

Koji Terada, and Soichi Miwa*

Department of Cellular Pharmacology, Hokkaido University Graduate School of Medicine,

Hokkaido 060-8638, Japan.

**Running title page.**

**Running title:** PKA-mediated inhibition of $ET_AR$-induced $Ca^{2+}$ entry via TRPC6.

**Corresponding author:** Soichi Miwa, M.D., Ph.D., Professor.

Department of Cellular Pharmacology,

Hokkaido University Graduate School of Medicine,

North 15, West 7, Sapporo-City, Hokkaido 060-8638, Japan.

Tel: +81-11-706-6921, Fax: +81-11-706-7824

E-mail: smiwa@med.hokudai.ac.jp

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**Abbreviations:** AC, adenylate cyclase; 8-Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphothioate; $[Ca^{2+}]_i$, intracellular free $Ca^{2+}$ concentration; DAG, diacylglycerol; EPAC, exchange protein activated by cAMP; ET-1, endothelin-1; $ET_AR$, endothelin type A receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; $G_q$PCR, $G_q$ protein-coupled receptor; HEK293, human embryonic kidney 293; $IP_3$, inositol 1,4,5-trisphosphate; 8-pCPT-2'-O-Me-cAMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate sodium salt; PDE, phosphodiesterase; PKA, protein kinase A; PKG, protein kinase G; PLC, phospholipase C; ROCC, receptor-operated $Ca^{2+}$ channel; ROCE, receptor-operated $Ca^{2+}$ entry; Rp-8-Br-cAMP, Rp-8-bromoadenosine 3',5'-cyclic monophosphothioate sodium salt; SOCC, store-operated $Ca^{2+}$ channel; SOCE, store-operated $Ca^{2+}$ entry; SQ-22,536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; TG, thapsigargin; TRPC, transient receptor potential canonical.

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**Abstract**

Receptor-operated $Ca^{2+}$ entry (ROCE) via TRPC6 (transient receptor potential canonical channel 6) is an important machinery for an increase in intracellular $Ca^{2+}$ concentration triggered by activation of $G_q$ protein-coupled receptors. TRPC6 is phosphorylated by various protein kinases including protein kinase A (PKA). However, the regulation of TRPC6 activity by PKA is still controversial. The purpose of this study is to elucidate the role of adenylate cyclase/cAMP/PKA signaling pathway in regulation of $G_q$ protein-coupled endothelin type A receptor ($ET_AR$)-mediated ROCE via TRPC6. For this purpose, HEK293 cells stably co-expressing human $ET_AR$ and TRPC6 (wild-type) or its mutants possessing a single point mutation of putative phosphorylation sites for PKA were used to analyze ROCE and amino acids responsible for PKA-mediated phosphorylation of TRPC6. $Ca^{2+}$ measurements with thapsigargin-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration protocol to estimate ROCE showed that stimulation of $ET_AR$ induced marked ROCE in HEK293 cells expressing TRPC6 compared with control cells. The ROCE was inhibited by forskolin and papaverine to activate cAMP/PKA pathway, while it was potentiated by Rp-8-Br-cAMP, a PKA inhibitor. Inhibitory effects of forskolin and papaverine were partially cancelled by replacing $Ser^{28}$ ($TRPC6^{S28A}$) but not $Thr^{69}$ ($TRPC6^{T69A}$) of TRPC6 with alanine. In vitro kinase assay with Phos-tag$^{TM}$ biotin to determine phosphorylation level of TRPC6 revealed that wild-type and mutant ($TRPC6^{S28A}$ and $TRPC6^{T69A}$) TRPC6 proteins were phosphorylated by PKA, but the phosphorylation level of these mutants was lower (about 50%) than that of wild-type. These results suggest that TRPC6 is negatively regulated by PKA-mediated phosphorylation of $Ser^{28}$ but not of $Thr^{69}$.

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## Introduction

$Ca^{2+}$ signaling regulates various important physiological and pathophysiological events, including cell constriction, cell proliferation, cell differentiation, and activation of immune cells. Increased $Ca^{2+}$ influx via TRPC6 (transient receptor potential canonical channel 6), a voltage-independent, $Ca^{2+}$-permeable non-selective cation channel, is particularly a major stimulus for the development of cardiovascular diseases such as idiopathic pulmonary arterial hypertension (IPAH) associated with the continued stimulation of endothelin type A receptor ($ET_AR$) resulting from excessive production of endothelin-1 (ET-1) (Abramowitz and Birnbaumer, 2009). TRPC6 has been identified as a potential candidate for a receptor-operated $Ca^{2+}$ channel (ROCC) rather than a store-operated $Ca^{2+}$ channel (SOCC), and it is operated by phospholipase C (PLC)-mediated diacylglycerol (DAG) production following stimulation of $G_q$ protein-coupled receptors ($G_qPCR$) such as $\alpha_1$-adrenergic and angiotensin type I receptors (Watanabe *et al.*, 2008). However, it remains unclear whether activation of $G_q$ protein-coupled $ET_AR$ with its agonist, ET-1, triggers ROCE through TRPC6.

The activity of TRPC6 is positively and negatively regulated by various protein kinases: the channel is activated by $Ca^{2+}$/calmodulin-dependent protein kinase II and the Src tyrosine kinase family (Hisatsune *et al.*, 2004; Shi *et al.*, 2004), while it is inactivated by protein kinase C and protein kinase G (PKG) (Kim and Saffen, 2005; Kinoshita *et al*., 2010; Nishida *et al*., 2010). TRPC6 is also phosphorylated by protein kinase A (PKA), which is a downstream target of cAMP, whereas PKA-mediated phosphorylation of TRPC6 is reported not to affect the channel function (Hassock *et al.*, 2002). However, Nishioka *et al.* (2011) have shown that PKA-mediated phosphorylation of TRPC6 at threonine 69 ($Thr^{69}$) is essential for the vasorelaxant effects of phosphodiesterase type 3 (PDE3) inhibition against the angiotensin II-induced constriction of vascular smooth muscle cells. In contrast to the negative regulation of TRPC6 activity by PKA-mediated phosphorylation, cAMP is reported to activate TRPC6 via the PI3K (phosphoinositide 3-kinase)/PKB (protein kinase B)/MEK (mitogen-activated protein kinase kinase)/ERK1/2 (extracellular-signal-regulated kinase 1/2) signaling pathway (Shen *et al.*, 2011). Thus, post-translational modification of TRPC6 by protein kinases plays a critical role in the regulation of channel activity.

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Drug therapy designed to elevate intracellular contents of cAMP and cGMP with $G_s$ protein-coupled prostaglandin $I_2$ receptor agonists (e.g., beraprost) and cGMP-specific PDE5 inhibitors (e.g., sildenafil), respectively, is highly effective against IPAH, which is closely correlated with either the prolonged activation of $ET_AR$ or the augmentation of $Ca^{2+}$ influx through up-regulated TRPC6 (Kunichika *et al.*, 2004; Yu *et al.*, 2004). Many lines of evidence indicate that the PKA- and PKG-dependent phosphorylation of TRPC6 at $Thr^{69}$ inhibits the channel activity, leading to the vasorelaxant and anti-hypertrophic effects, respectively (Kinoshita *et al.*, 2010; Nishida *et al.*, 2010; Nishioka *et al.*, 2011). In terms of the substrate specificity of PKA, a study with an oriented peptide library has demonstrated that PKA is an arginine-directed serine/threonine protein kinase and arginine residue is preferred at positions -4 to -1 amino-terminal to the phosphorylation site (Songyang *et al.*, 1994). In addition, the greatest selectivity was observed at residues -3 and -2, that is, $R_2$-X-S/T, and the lysine residue at the -2 position was the second preferred amino acid (R-L-X-S/T) (Songyang *et al.*, 1994). Searching for the primary sequence of TRPC6, we have found that in addition to R-R-Q-T sequence surrounding $Thr^{69}$, potential sequences for PKA-mediated phosphorylation are present within TRPC6 sequence, namely R-R-G-G-S at serine 14 ($Ser^{14}$), R-R-N-E-S at $Ser^{28}$, and R-K-L-S at $Ser^{321}$. Unlike $Thr^{69}$ responsible for PKA-mediated negative regulation of TRPC6 activity (Nishioka *et al.*, 2011), there is no conclusive evidence for the functional role of these serine residues in the regulation of TRPC6 activity by PKA.

In the present study, we tried to clarify whether TRPC6 functions as $ET_AR$-operated $Ca^{2+}$ channels by using $Ca^{2+}$ measurements with thapsigargin (TG)-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration protocol. In addition, we made and used TRPC6 mutants possessing a single point mutation of putative phosphorylation sites for PKA in order to identify key amino acid(s) responsible for the regulation of TRPC6 activity by PKA-mediated phosphorylation. For this purpose, we improved in vitro kinase assay by using Phos-tag™ biotin (a phosphate-specific ligand with biotin tag) to detect specifically phosphorylated proteins (Kinoshita *et al.*, 2006). We here show that although PKA can phosphorylate TRPC6 on $Ser^{28}$ and $Thr^{69}$, $ET_AR$-operated $Ca^{2+}$ entry through TRPC6 is negatively regulated

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by the activation of AC/cAMP/PKA signaling pathway, via phosphorylation of TRPC6 on $Ser^{28}$ but not on $Thr^{69}$ of the N-terminal.

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## Materials and methods

*Materials*

The following drugs and reagents were used in the present study: synthetic human ET-1 (Peptide Institute, Osaka, Japan); fura-2/acetoxymethyl ester (fura-2/AM), Pluronic F-127 (Dojindo Laboratories, Kumamoto, Japan); gadolinium (III) chloride, G418, thapsigargin (TG), probenecid, aprotinin, leupeptin, pepstatin, sodium deoxycholate, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), $Na_3VO_4$, NaF, puromycin dihydrochloride, forskolin, 1,9-dideoxyforskolin, 8-Br-cAMP (8-bromoadenosine 3',5'-cyclic monophosphothioate), papaverine hydrochloride, adenosine triphosphate (ATP), bovine serum albumin (BSA) (Sigma-Aldrich Co., St. Louis, MO, U.S.A.); Rp-8-Br-cAMP (Rp-8-bromoadenosine 3',5'-cyclic monophosphothioate sodium salt), 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate sodium salt (8-pCPT-2'-O-Me-cAMP) (Enzo Life Sciences International Inc., PA, U.S.A.); SQ-22,536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) (Calbiochem, San Diego, CA, U.S.A.), cAMP-dependent protein kinase catalytic subunit (Promega Corp., Madison, WI, U.S.A.); rAPid alkaline phosphatase (Roche Applied Science, Mannheim, Germany). All cell culture media and supplements except fetal calf serum (FCS; Invitrogen Corp., Grand Island, NY, U.S.A.) were obtained from Sigma-Aldrich. Antibodies for FLAG peptide, a green fluorescent protein (GFP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), horseradish peroxidase (HRP) conjugated FLAG peptide (HRP-FLAG) and HRP conjugated streptavidine (HRP-SA) were obtained from Sigma-Aldrich, Clontech Laboratories Inc. (Mountain View, CA, U.S.A.), Santa Cruz Biotechnology Inc. (Delaware Avenue Santa Cruz, CA, U.S.A.), Medical and Biological Laboratories Co., LTD. (Aichi, Japan) and Thermo Fisher Scientific Inc. (Rockford, IL, U.S.A.), respectively. Phos-tag$^{TM}$ biotin was obtained from NARD Institute, Ltd. (Hyogo, Japan). The other reagents used were of the highest grade in purity.

*Construction of retrovirus vectors*

The pCI-neo mammalian expression vector encoding TRPC6 (pCI-neo-TRPC6) was

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generously provided by Dr. Yasuo Mori (Kyoto University). The insert cDNA of wild-type TRPC6 was generated from the pCI-neo-TRPC6 as a template by a PCR reaction with specific primers containing the restriction enzyme sites, which are BamHI at the 5'-end and AgeI at the 3'-end, for subcloning into the pCR$^{TM}$-Blunt II-TOPO$^{TM}$ vector (Invitrogen Corp.). The resulting pCR$^{TM}$-Blunt II-TOPO$^{TM}$ vector and the pEGFP-N1 vector encoding a red-shifted variant of GFP (Clontech Laboratories Inc.) were digested with two restriction enzymes (BamHI/AgeI, AgeI/NotI or BamHI/NotI) simultaneously. The cDNA fragments were ligated into the BamHI/NotI-treated pMXrmv5 retrovirus vector to yield the pMXrmv5 vectors encoding GFP and TRPC6 fused with GFP at the C terminus (TRPC6-GFP). All of the constructs were verified by DNA sequencing.

To identify the target site(s) of TRPC6 for phosphorylation by PKA, serine residues at positions 14, 28, and 321, and threonine at 69 were replaced with alanine by using KOD -Plus- Mutagenesis Kit (TOYOBO Co., LTD., Osaka, Japan). The sequences of the resulting mutants tagged with GFP or FLAG peptide at the C terminus were confirmed by DNA sequencing.

*Cell culture*

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v $v^{-1}$) FCS, penicillin (100 units $ml^{-1}$), and streptomycin (100 μg $ml^{-1}$) at 37°C in humidified air with 5% $CO_2$.

*Stable expression of human $ET_AR$ in HEK293 cells*

The pDisplay mammalian expression vector containing cDNA of human $ET_AR$ fused with an influenza hemagglutinin (HA) epitope tag at the N terminus (HA-$ET_AR$) was transfected into HEK293 cells using TransIT$^{TM}$-293 transfection kit (Mirus Bio Corporation, Madison, WI, U.S.A) according to the manufacture's instructions. Stable transformants were selected in medium containing 800 μg $ml^{-1}$ G418 for 3 weeks. Clonal cell lines were obtained by limiting dilution. Clones were expanded and screened for expression levels by whole cell radioligand binding assay and Western blot analysis. The resulting suitable clone

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(HA-$ET_AR$/HEK293 cells) was grown up for further experiments.

*Stable expression of TRPC6 and its mutants*

To generate HA-$ET_AR$-positive HEK293 cells stably expressing GFP, TRPC6-GFP, TRPC6-FLAG, or their mutants, these genes were introduced into HA-$ET_AR$/HEK293 cells by retroviral gene transfer. Briefly, retroviruses were produced by triple transfection of HEK293T cells with retroviral constructs along with gag-pol and vesicular stomatitis virus G glycoprotein expression constructs (Yee *et al.*, 1994). The supernatants containing virus were collected 24 h after transfection and added to HA-$ET_AR$/HEK293 cells. The HA-$ET_AR$/HEK293 cells were then centrifuged at 900 × g for 45 min at 25°C followed by incubation for 6 h at 37°C in 5% $CO_2$-95% air. Then, fresh culture media were added to dilute supernatants containing virus. GFP- or TRPC6-positive HA-$ET_AR$/HEK293 cells were selected for growth in medium containing 5 μg $ml^{-1}$ puromycin for a week.

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

Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was monitored by using a fluorescent $Ca^{2+}$ indicator, fura-2/AM, as described previously (Horinouchi *et al.*, 2009; Higa *et al.*, 2010). Briefly, HEK293 cells grown in 3.5-cm dish were incubated with 4 μM fura-2/AM admixed with 2.5 mM probenecid and 0.04% Pluronic F-127 at 37°C for 45 min under reduced light. After collecting and washing cells, the cells were suspended in $Ca^{2+}$-free Krebs-HEPES solution (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM D-(+)-glucose, 10 mM HEPES; adjusted to pH 7.3 with NaOH) at $4 \times 10^5$ cells $ml^{-1}$. $CaCl_2$ was added to 0.5-ml aliquot of the cell suspension at the final concentration of 2 mM, when necessary. Changes of $[Ca^{2+}]_i$ in cells were measured at 30°C using CAF-110 spectrophotometer (JASCO, Tokyo, Japan) with the excitation wavelengths of 340 and 380 nm, and emission wavelength of 500 nm.

*Confocal microscopy*

Confocal microscopy was carried out using FluoView™ FV300 (Olympus Corporation,

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Tokyo, Japan) with a 63 × oil-immersion lens.

*In vitro kinase assay*

Wild-type and mutant TRPC6-FLAG protein-expressed HA-$ET_AR$/HEK293 cells grown in 10-cm dish were washed twice with ice-cold PBS, and lysed in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 6.8), 1% NP-40, 0.5 % sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM $Na_3VO_4$, 20 mM NaF, 10 μg $ml^{-1}$ leupeptin, 10 μg $ml^{-1}$ aprotinin, and 10 μg $ml^{-1}$ pepstatin) supplemented with EDTA-free, protease inhibitor cocktail (PIERCE Biotechnology Inc., Rockford, IL, U.S.A.). The cell lysates were sonicated for 10 s on setting 10 of a handy sonicator (UR-20P, TOMY SEIKO Co., LTD., Tokyo, Japan) and centrifuged at 20,000 × g for 20 min at 4ºC. Protein content of supernatant was measured according to the method of Bradford (1976) using BSA as standard. Immunoprecipitation was carried out using Immunoprecipitation Kit (Dynabeads™ Protein G, Invitrogen Corp.). Briefly, the Dynabeads were incubated with a primary antibody (anti-FLAG, 1:100 dilution) for 1 h at room temperature. The Dynabeads-antibody complex were washed twice with washing buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 6.8), 1% NP-40, 0.5 % sodium deoxycholate, 0.1% SDS) by gentle pipetting, and then incubated with lysates containing equal protein amounts for 1 h at room temperature. Western blot analysis showed that the expression of TRPC6 proteins relative to total protein was quantitatively similar between wild-type and mutant proteins (supplemental Figure S1). The resulting Dynabeads were washed three times with the washing buffer. The Dynabeads-binding TRPC6 proteins were then incubated with alkaline phosphatase for 1 h at 37°C to reduce the basal phosphorylation levels. After rinsing with the washing buffer three times, the dephosphorylated TRPC6 proteins were incubated in phosphorylation buffer (20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM ATP and cAMP-dependent protein kinase catalytic subunit (40 units per reaction)) at 37°C for 30 min. This amount of cAMP-dependent protein kinase catalytic subunit produced approximately 50% response of the maximum TRPC6 phosphorylation (supplemental Figure S2). The TRPC6 proteins bound to the Dynabeads were eluted in the phosphorylation buffer by adding SDS sample buffer (62.5 mM

Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol (2-ME), 2.5% SDS, 0.1% bromphenol blue) followed by incubation at 37°C for 30min. The phosphorylation levels of TRPC6 protein were analyzed by Western blotting.

*Western blot analysis*

The proteins in immunoprecipitated samples and whole cell lysates were separated on a 5-20% polyacrylamide gel (SuperSep™, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and electrotransferred to a polyvinylidene fluoride membrane (Immobilon™-P, pore size 0.45 μm, Millipore Corp., Bedford, MA, U.S.A.) with a semidry electroblotter. After transfer, the membranes were washed three times for 5 min with Tris-buffered saline-Tween 20 (TBS-T; 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.1% Tween-20) followed by blocking (2% BSA in TBS-T) of nonspecific binding for 1 h at room temperature. The membranes were incubated with anti-FLAG-HRP antibody or Phos-tag™ biotin-bound HRP-SA complex (which was prepared according to the manufacturer's instruction) at room temperature for 6 h or with a monoclonal antibody for GFP or GAPDH as a primary antibody overnight at 4°C. The anti-FLAG-HRP antibody and Phos-tag™ biotin-bound HRP-SA complex were detected with ECL Western blotting Analysis System (GE Healthcare Limited, Little Chalfont, Buckinghamshire, U.K.). The primary antibody was detected with a secondary horseradish peroxidase-conjugated anti-mouse IgG antibody and enhanced chemiluminescence (ECL; GE Healthcare Limited). The blots were exposed to Amersham Hyperfilm™ ECL (GE Healthcare Limited). Phosphorylation levels of wild-type and mutant TRPC6 proteins were analyzed with National Institutes of Health Image J1.37 software.

*Data Analysis*

Data regarding change in $[Ca^{2+}]_i$ were collected and analyzed by using a MacLab/8s with Chart (v. 3.5) software (ADInstruments Japan, Tokyo, Japan). All data are presented as means ± S.E.M. where *n* refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISM™ (version 3.00, GraphPad Software Inc., San Diego, CA, U.S.A.) by Student's paired or unpaired *t*-test. A *P*

value less than 0.05 was considered to indicate significant differences.

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## Results

*Characterization of $ET_AR$-operated $Ca^{2+}$ entry through TRPC6*

To determine whether the stimulation of $ET_AR$ with its agonist, ET-1, induces receptor-operated $Ca^{2+}$ entry (ROCE) via TRPC6, we employed the TG-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration protocol to measure store-operated $Ca^{2+}$ entry (SOCE) followed by $ET_AR$ stimulation to measure ROCE (Boulay, 2002). 10 μM $Gd^{3+}$ in the extracellular medium was used throughout the experiments to inhibit the endogenous SOCE (capacitative $Ca^{2+}$ entry) that masks the ROCE via TRPC3 and TRPC7 in HEK293 cells (Okada *et al.*, 1999). In GFP- and TRPC6-GFP-expressing HA-$ET_AR$/HEK293 cells, SOCE induced by TG-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration was significantly inhibited by addition of 10 μM $Gd^{3+}$ (210.2 ± 11.5 nM to 46.0 ± 2.1 nM for GFP and 177.2 ± 4.8 nM to 41.2 ± 1.9 nM for TRPC6-GFP, $n$ = 6 for each). Figures 1A and 1B show that in nominally $Ca^{2+}$-free solution containing 10 μM $Gd^{3+}$, 2 μM TG evoked $Ca^{2+}$ release from the endoplasmic reticulum (ER), causing a transient increase in $[Ca^{2+}]_i$ that promptly returned to near baseline. In the TG-treated HA-$ET_AR$/HEK293 cells expressing GFP as a control, stimulation with 10 nM ET-1 after restoration of extracellular $Ca^{2+}$ to 2 mM did not produce further increase in $[Ca^{2+}]_i$ (Figure 1A), indicating that under this condition, $ET_AR$ stimulation cannot elicit either $Ca^{2+}$ release from $Ca^{2+}$ store, SOCE or ROCE. On the other hand, the stimulation of $ET_AR$ with 10 nM ET-1 elicited $Gd^{3+}$-insensitive transient increase in $[Ca^{2+}]_i$ resulting from ROCE through TRPC6 in the TG-treated HA-$ET_AR$/HEK293 cells expressing TRPC6-GFP (Figure 1B and Table 1). These results clearly suggest that the activation of $ET_AR$ is able to elicit ROCE mediated through TRPC6-GFP.

*Negative regulation of $ET_AR$-operated, TRPC6-mediated $Ca^{2+}$ entry by activation of adenylate cyclase (AC)/cAMP/protein kinase A (PKA) signaling pathway*

Next, we examined the effects of cAMP and PKA on TRPC6-mediated ROCE in response to $ET_AR$ stimulation. The ROCE was markedly suppressed by either 10 μM forskolin, an AC activator (Figure 2B), or 10 μM papaverine, a non-selective phosphodiesterase (PDE) inhibitor (Figure 2D), both of which increase the intracellular cAMP

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level, thereby activating PKA. The inhibitory effect of forskolin on TRPC6-mediated ROCE was cancelled by treatment with 1 mM SQ-22,536, a membrane-permeable AC inhibitor, that also weakly but significantly augmented the ROCE (supplemental Figure S3). Since it is well-known that forskolin can exhibit pleiotropic effects in an AC-independent manner (Laurenza *et al.*, 1989), we examined the effect of 1,9-dideoxyforskolin, an inactive analogue of forskolin as a negative control (Pinto *et al*., 2008; 2009), on the $ET_AR$-operated $Ca^{2+}$ influx via TRPC6. Surprisingly, 10 μM 1,9-dideoxyforskolin as well as 10 μM forskolin inhibited the TRPC6-mediated $Ca^{2+}$ entry (supplemental Figure S3). The inhibitory effect of 10 μM 1,9-dideoxyforskolin was cancelled by 1 mM SQ-22,536, indicating the possibility that 1,9-dideoxyforskolin was able to directly activate AC. 100 μM Rp-8-Br-cAMP, a membrane-permeable PKA inhibitor, enhanced $ET_AR$-operated $Ca^{2+}$ influx (Figure 2C), while 500 μM 8-Br-cAMP, a membrane-permeable direct PKA activator, suppressed the $Ca^{2+}$ response to ET-1 (supplemental Figure S3). The effects of these drugs on $ET_AR$-induced ROCE via TRPC6 were summarized in Figure 2E and supplemental Figure S3. Our findings indicate the possibility that the activity of TRPC6 is negatively regulated by its phosphorylation by PKA, resulting in the inhibition of $ET_AR$-operated $Ca^{2+}$ entry via TRPC6.

*Effect of activation of cAMP/PKA signaling pathway on subcellular localization of TRP*C6

Wild-type TRPC6 protein expressed in HEK293 cells is reported to be mainly present on the plasma membrane (Lussier *et al*., 2008; Graham *et al*., 2010). We confirmed the subcellular localization of TRPC6-GFP expressed in HA-$ET_AR$/HEK293 cells under basal condition using a confocal laser-scanning microscopic approach. Although GFP expressed in HA-$ET_AR$/HEK293 cells as a control was localized in the cytosol and nucleus (Figure 3A), wild-type TRPC6-GFP was predominantly targeted to the plasma membrane (Figure 3B). The intracellular distribution of GFP and TRPC6-GFP after treatment with 10 μM forskolin (Figures 3C and D), and 10 μM papaverine (Figures 3E and F) was similar to that in the control cells. Likewise, pharmacological inhibition of AC and PKA with 1 mM SQ-22,536 and 100 μM Rp-8-Br-cAMP, respectively, seemed to not affect the intracellular distribution of GFP and TRPC6-GFP (supplemental Figure S4).

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*Determination of the amino acid residue responsible for inhibition of TRPC6-mediated $Ca^{2+}$ influx by forskolin and papaverine*

To determine the critical amino acid residue(s) involved in the inhibition of ROCE via TRPC6 by forskolin and papaverine, we searched for the PKA phosphorylation candidate sites in the TRPC6 sequence. As a result, we found three serine residues at positions 14 (R-R-G-G-S), 28 (R-R-N-E-S), and 321 (R-K-L-S), and single threonine residue at 69 (R-R-Q-T): all of these sequences were present on the intracellular N-terminal region of TRPC6. To identify the target residue(s) of PKA phosphorylation, we made HA-$ET_A$R/HEK293 cells stably expressing GFP-tagged TRPC6 mutants carrying alanine substitution for these serine/threonine residues. Functional study with $Ca^{2+}$ measurement demonstrated that there was no significant difference in the magnitude of ROCE between wild-type and these mutants (Table 1). Replacement of $Ser^{28}$ (S28A) but not other residues ($Ser^{14}$, $Thr^{69}$, and $Ser^{321}$) by alanine (S14A, T69A, and S321A) attenuated the inhibitory effects of 10 μM forskolin (Figures 4A and 4B) and 10 μM papaverine (Figures 5A and 5B). The effects of these drugs on $ET_A$R-induced ROCE via wild-type and mutant TRPC6 proteins were summarized in Figures 4C and 5C.

*Identification of PKA phosphorylation sites on TRPC6 by using in vitro kinase assay with Phos-tag™ biotin*

To directly demonstrate that TRPC6 is a substrate for PKA, PKA-mediated phosphorylation of TRPC6 was estimated by using Phos-tag™ biotin that is a biotinylated phosphate-specific ligand and can specifically detect phosphorylated proteins (Kinoshita *et al.*, 2006). Figure 6A shows that immunoblotting with Phos-tag™ biotin-bound HRP-SA complex detected wild-type and mutant phosphorylated TRPC6 proteins under basal condition which were reduced by treatment of immunoprecipitated TRPC6 proteins with phosphatase, indicating the ability of Phos-tag™ biotin to specifically identify phosphorylated proteins. The basal phosphorylation level of wild-type TRPC6 was similar to that of mutant TRPC6 proteins (Figure 6A). To analyze a change in the levels of TRPC6 phosphorylation

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by PKA in vivo, the HA-$ET_AR$/HEK293 cells stably expressing wild-type or mutant TRPC6-FLAG protein were treated with a combination of 10 μM forskolin and 10 μM papaverine to enhance cAMP production and to inhibit cAMP breakdown by PDE, respectively. However, there was little or no change in the phosphorylation levels of wild-type and mutant TRPC6 following the activation of AC/cAMP/PKA signaling pathway (Figure 6B). We considered the possibility that the high basal phosphorylation levels masked the effects of cAMP-elevating agents. Therefore, we next attempted to perform in vitro kinase assay on immunoprecipitated TRPC6 proteins which were pretreated with a phosphatase to reduce their basal phosphorylation levels. Incubation with exogenous PKA catalytic subunit induced phosphorylation of wild-type TRPC6 protein dephosphorylated with a phosphatase pretreatment (Figure 6C, upper panel), demonstrating that the target site(s) for PKA-mediated phosphorylation is present within TRPC6 sequence. There was no significant difference in an increase in phosphorylation level of TRPC6 protein by PKA between wild-type and $TRPC6^{S14A}$ mutant. Notably, in vitro kinase assay using TRPC6 mutants with $Ser^{28}$ ($TRPC6^{S28A}$) and $Thr^{69}$ ($TRPC6^{T69A}$) replaced to alanine showed significant loss of phosphorylation, suggesting that PKA can phosphorylate TRPC6 on these sites and the $Ser^{28}$ and $Thr^{69}$ residues contributed to approximately 50% of the phosphorylation of TRPC6 by PKA.

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**Discussion**

Activation of $G_q$PCR and tyrosine kinase receptor induces formation of the second messengers such as inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) via PLC. Binding of $IP_3$ to its receptor on endoplasmic reticulum (ER) triggers $Ca^{2+}$ release from ER, resulting in store-operated (capacitative) $Ca^{2+}$ entry mediated through voltage-independent $Ca^{2+}$-permeable cation channels including TRPC and Orai proteins (Lee *et al.*, 2010). In addition, ROCE is activated and it is mediated via certain TRPC categorized as ROCCs that operate independently of store depletion. TRPC6 is reported to be involved in ROCE triggered by stimulation of $G_q$PCRs such as muscarinic (Bousquet *et al.*, 2010) and $\alpha_1$-adrenergic receptors (Suzuki *et al.*, 2007). In the present study, we showed that activation of $G_q$ protein-coupled $ET_AR$ induced TRPC6-mediated ROCE but not SOCE. That is, an increase in $[Ca^{2+}]_i$ induced by TG-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration in GFP-expressing cells was not significantly different from that in TRPC6-GFP-expressing cells, indicating that TRPC6 does not contribute to SOCE. Different from no significant $Ca^{2+}$ influx in response to ET-1 after TG-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration in the GFP-expressing cells, 10 nM ET-1 was capable of producing additional $Ca^{2+}$ influx in the TRPC6-expressing cells where SOCE had been maximally activated by TG-induced $Ca^{2+}$-depletion/$Ca^{2+}$-restoration. In addition, our fluorescent confocal microscopic observations were consistent with the previous report of Graham *et al.* (2010) that unlike diffuse distribution of GFP, TRPC6-GFP is dominantly present in the plasma membrane or sub-plasma membrane of the cells. These data, taken together, indicate that TRPC6 located in plasma membrane functions as $ET_AR$-activated ROCC but not SOCC.

More recent studies have indicated that the negative regulation of TRPC6 by PKA in addition to PKG is an important mechanism underlying the protective effect of cAMP-elevating agent against cardiovascular diseases such as hypertension and cardiac hypertrophy (Kinoshita *et al.*, 2010; Nishida *et al.*, 2010; Nishioka *et al.*, 2011). PKA is the major target of the intracellular second messenger cAMP which is synthesized from ATP via AC and inactivated by some members of PDE superfamily (Pearce *et al.*, 2010; Boswell-Smith *et al.*, 2006). To elucidate the functional role of AC/cAMP/PKA signaling

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pathway in the regulation of $ET_AR$-operated $Ca^{2+}$ influx via TRPC6, we used pharmacological agents targeting this pathway. The TRPC6-mediated ROCE in response to $ET_AR$ stimulation was markedly reduced in the presence of cAMP-elevating agents, forskolin and papaverine. In contrast, the response was significantly potentiated by pretreatment with a membrane-permeant PKA inhibitor, Rp-8-Br-cAMP, that competitively binds to cAMP-binding domain of the PKA regulatory subunit and inhibits dissociation of the catalytic subunit from the regulatory subunit (Schwede *et al.*, 2000). Furthermore, either forskolin or papaverine did not change the membrane localization of TRPC6 protein (Figure 3). cAMP can activate not only PKA but also EPAC (exchange protein activated by cAMP) that functions as guanine nucleotide exchange factors for both Rap1 and Rap2, members of the Ras family of small G proteins (Gloerich and Bos, 2010). However, the possibility that cAMP-dependent EPAC activation by forskolin or papaverine inactivates TRPC6 could be ruled out, since the selective EPAC agonist, 8-pCPT-2'-O-Me-cAMP (200 μM), had no or little effect on the ROCE via TRPC6 (data not shown). Taken together, these findings suggested the inactivation of $ET_AR$-activated TRPC6 by the AC/cAMP/PKA signaling pathway. This supports the observation by Nishioka *et al.* (2011) that cAMP-dependent PKA activation induced by cilostazol, a selective PDE3 inhibitor, results in the suppression of TRPC6-mediated $Ca^{2+}$ entry. However, conflicting results have been reported on the role of cAMP-dependent signaling pathways in the regulation of TRPC6 activity. As opposed to the findings described in the present and previous studies (Nishioka *et al.*, 2011), cAMP/PKA pathway did not affect SOCE-independent, nonselective cation entry (ROCE) via TRPC6 (Hassock *et al.*, 2002), while TRPC6-mediated $Ca^{2+}$ entry was triggered by cAMP-dependent activation of PI3K/PKB/MEK/ERK1/2 signaling pathway (Shen *et al.*, 2011). The reason for the discrepancy in these experimental results is not clear. However, the discrepancy may be attributed to the difference in basal phosphorylation state of TRPC6, since TRPC6 is phosphorylated by several types of protein kinases under basal conditions (Figure 6) (Bousquet *et al.*, 2011).

Interestingly, we have found that 1,9-dideoxyforskolin, an inactive analogue of forskolin (Pinto *et al*., 2008; 2009), also inhibited $ET_AR$-operated $Ca^{2+}$ influx via TRPC6 (supplemental

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Figure S3). The inhibition of the ROCE by 10 μM 1,9-dideoxyforskolin seems to be due to activation of AC, since the inhibitory effect is sensitive to 1 mM SQ-22,536, an AC inhibitor. More recent studies with purified catalytic AC subunits clearly have suggested that 1,9-dideoxyforskolin binds to AC, whereas the stimulatory activity in AC is not observed (Pinto *et al*., 2008; 2009). Activation of AC requires both the binding of diterpenes including forskolin and 1,9-dideoxyforskolin to the catalytic subunits of AC and an additional conformational switch via yet unidentified step (Pinto *et al*., 2009). In native systems such as intact cells, the binding of 1,9-dideoxyforskolin to AC may trigger a second conformational switch that results in activation of catalysis.

Does the activation of PKA via AC/cAMP signaling pathway induce phosphorylation of TRPC6? Hassock *et al*. (2002) have reported that pharmacological stimulation of cAMP/PKA signaling pathway actually phosphorylates an unidentified phosphorylation site(s) of TRPC6 endogenously expressed in human platelets and exogenously overexpressed in QBI-293A cells (a sub-clone of HEK 293 cells). Other studies have shown that $Thr^{69}$ residue within TRPC6 sequence is phosphorylated by PKA in addition to by PKG (Nishida *et al*., 2010; Nishioka *et al*., 2011). We analyzed the primary sequence of TRPC6 and found some serine/threonine residues other than $Thr^{69}$, i.e., $Ser^{14}$, $Ser^{28}$, and $Ser^{321}$, as potential sites for PKA phosphorylation. Both our site-directed mutagenesis and $[Ca^{2+}]_i$ measurement experiments have provided first functional evidence that $Ser^{28}$ but not $Thr^{69}$ is involved in the inhibition of TRPC6-mediated ROCE in response to $ET_AR$ stimulation by cAMP-elevating agents.

Finally, we have conducted immunoblotting analysis with Phos-tag™ biotin to clarify whether PKA can phosphorylate TRPC6 on $Ser^{28}$ residue. Phos-tag™ biotin is a phosphate-specific ligand with biotin tag and allows us to detect specifically phosphorylated proteins (Kinoshita *et al.*, 2006). As shown in Figure 6A, we have confirmed that Phos-tag™ biotin can discriminate between phosphorylated and unphosphorylated states of TRPC6 proteins. Bousquet *et al.* (2011) have reported that TRPC6 protein stably expressed in HEK293 cells is phosphorylated under basal conditions and the $Ser^{814}$ residue contributes to 50% of the basal phosphorylation state, although its functional significance is unknown.

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Our site-directed mutagenesis approach has revealed that the contribution of $Ser^{14}$, $Ser^{28}$, and $Thr^{69}$ residues to the basal phosphorylation of TRPC6 is minor (Figures 6A and 6B). In vivo treatment of wild-type and mutant cells with a combination of forskolin and papaverine to activate PKA via the AC/cAMP signaling pathway induced little or no significant change in the phosphorylation level, indicating that the high basal phosphorylation masks the relatively weak PKA-mediated phosphorylation of TRPC6. Therefore, we have carried out in vitro kinase assay with immunoprecipitated wild-type and mutant TRPC6 proteins which are dephosphorylated by phosphatase treatment. Surprisingly, in vitro kinase assay has revealed that PKA phosphorylates TRPC6 on not only $Ser^{28}$ but also $Thr^{69}$, although only $Ser^{28}$ is involved in the negative regulation of $ET_AR$-mediated ROCE via TRPC6 by the activation of AC/cAMP/PKA signaling pathway. This discrepancy between $[Ca^{2+}]_i$ measurement assay and in vitro kinase assay could result from that $Thr^{69}$ mutant of TRPC6 is functionally resistant to increased PKA activity, since $Thr^{69}$ but not $Ser^{28}$ is maximally phosphorylated under basal condition. Another possibility is that PKA catalytic subunit at 40 units used in the present study nonspecifically phosphorylates $Thr^{69}$ as a PKG phosphorylation site in addition to $Ser^{28}$ as a PKA phosphorylation site, since PKA and PKG are known to have very similar consensus sites.

In summary, we have identified a new phosphorylation site ($Ser^{28}$) on TRPC6 for PKA in addition to $Thr^{69}$. We have provided first evidence that activation of AC/cAMP/PKA signaling pathway inhibits $ET_AR$-mediated ROCE via TRPC6 by phosphorylation of $Ser^{28}$ but not $Thr^{69}$, although both sites could be phosphorylated by PKA in vitro. In the treatment of IPAH attributable to excessive $ET_AR$ signaling and/or $Ca^{2+}$ entry via TRPC6 (Kunichika *et al.*, 2004; Yu *et al.*, 2004), prostacyclin and its analogs have been used as cAMP-generating drugs to relax contracted pulmonary artery and to inhibit proliferation of PASMC (Clapp *et al.*, 2002). Taken together, our findings imply that the negative regulation of $ET_AR$-activated TRPC6 via PKA phosphorylation may be an important therapeutic target for treatment of PAH with cAMP-elevating agents.

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**Authorship Contributions.**

**Participated in research design:** Horinouchi, Nishiya, and Miwa.

**Conducted experiments:** Horinouchi, Higa, Aoyagi, and Tarada.

**Contributed new reagents or analytic tools:** Horinouchi.

**Performed data analysis:** Horinouchi, Higa, and Aoyagi.

**Wrote or contributed to the writing of the manuscript:** Horinouchi, Nishiya, and Miwa.

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## References

Abramowitz J and Birnbaumer L (2009) Physiology and pathophysiology of canonical transient receptor potential channels. *FASEB J.* **23**:297-328.

Boswell-Smith V, Spina D and Page CP ( 2006) Phosphodiesterase inhibitors. *Br J Pharmacol* **147**:S252-S257.

Boulay G (2002) $Ca^{2+}$-calmodulin regulates receptor-operated $Ca^{2+}$ entry activity of TRPC6 in HEK-293 cells. *Cell Calcium* **32**:201-207.

Bousquet SM, Monet M and Boulay G (2010) Protein kinase C-dependent phosphorylation of transient receptor potential canonical 6 (TRPC6) on serine 448 causes channel inhibition. *J Biol Chem* **285**:40534-40543.

Bousquet SM, Monet M and Boulay G (2011) The serine 814 of TRPC6 is phosphorylated under unstimulated conditions. *PLoS One* **6**:e18121.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.

Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ and Tinker A (2002) Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery. *Am J Respir Cell Mol Biol* **26**:194–201.

Gloerich M and Bos JL (2010) Epac: defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol* **50**:355-375.

Graham S, Ding M, Ding Y, Sours-Brothers S, Luchowski R, Gryczynski Z, Yorio T, Ma H and Ma R (2010) Canonical transient receptor potential 6 (TRPC6), a redox-regulated cation channel. *J Biol Chem* **285**:23466-23476.

Hassock SR, Zhu MX, Trost C, Flockerzi V and Authi KS (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood* **100**:2801-2811.

Higa T, Horinouchi T, Aoyagi H, Asano H, Nishiya T, Nishimoto A, Muramatsu I and Miwa S (2010) Endothelin type B receptor-induced sustained $Ca^{2+}$ influx involves $G_{q/11}$/phospholipase C-independent, p38 mitogen-activated protein kinase-dependent

activation of $Na^+/H^+$ exchanger. *J Pharmacol Sci* **113**:276-280.

Hisatsune C, Kuroda Y, Nakamura K, Inoue T, Nakamura T, Michikawa T, Mizutani A and Mikoshiba K (2004) Regulation of TRPC6 channel activity by tyrosine phosphorylation. *J Biol Chem*. **279**:18887-18894. .

Horinouchi T, Asano H, Higa T, Nishimoto A, Nishiya T, Muramatsu I and Miwa S (2009) Differential coupling of human endothelin type A receptor to $G_{q/11}$ and $G_{12}$ proteins: the functional significance of receptor expression level in generating multiple receptor signaling. *J Pharmacol Sci* **111**:338-351.

Kim JY and Saffen D (2005) Activation of M1 muscarinic acetylcholine receptors stimulates the formation of a multiprotein complex centered on TRPC6 channels. *J Biol Chem* **280**:32035-32047.

Kinoshita E, Kinoshita-Kikuta E, Takiyama K and Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* **5**:749-757.

Kinoshita H, Kuwahara K, Nishida M, Jian Z, Rong X, Kiyonaka S, Kuwabara Y, Kurose H, Inoue R, Mori Y, Li Y, Nakagawa Y, Usami S, Fujiwara M, Yamada Y, Minami T, Ueshima K and Nakao K (2010) Inhibition of TRPC6 channel activity contributes to the antihypertrophic effects of natriuretic peptides-guanylyl cyclase-A signaling in the heart. *Circ Res* **106**:1849-1860.

Kunichika N, Landsberg JW, Yu Y, Kunichika H, Thistlethwaite PA, Rubin LJ and Yuan JX (2004) Bosentan inhibits transient receptor potential channel expression in pulmonary vascular myocytes. *Am J Respir Crit Care Med* **170**:1101-1107.

Laurenza A, Sutkowski EM and Seamon KB (1989) Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol Sci* **10**:442-447.

Lee KP, Yuan JP, Hong JH, So I, Worley PF and Muallem S (2010) An endoplasmic reticulum/plasma membrane junction: STIM1/Orai1/TRPCs. *FEBS Lett* **584**:2022-2027.

Lussier MP, Lepage PK, Bousquet SM and Boulay G (2008) RNF24, a new TRPC interacting protein, causes the intracellular retention of TRPC. *Cell Calcium* **43**:432-443.

Nishida M, Watanabe K, Sato Y, Nakaya M, Kitajima N, Ide T, Inoue R and Kurose H (2010) Phosphorylation of TRPC6 channels at $Thr^{69}$ is required for anti-hypertrophic effects of phosphodiesterase 5 inhibition. *J Biol Chem* **285**:13244-13253.

Nishioka K, Nishida M, Ariyoshi M, Jian Z, Saiki S, Hirano M, Nakaya M, Sato Y, Kita S, Iwamoto T, Hirano K, Inoue R and Kurose H (2011) Cilostazol suppresses angiotensin II-Induced vasoconstriction via protein Kinase A-mediated phosphorylation of the transient receptor potential canonical 6 channel. *Arterioscler Thromb Vasc Biol* **31**:in press.

Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, Imoto K and Mori Y (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. $Ca^{2+}$-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. *J Biol Chem* **274**:27359-27370.

Pearce LR, Komander D and Alessi DR (2010) The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* **11**:9-22.

Pinto C, Hübner M, Gille A, Richter M, Mou TC, Sprang SR and Seifert R (2009) Differential interactions of the catalytic subunits of adenylyl cyclase with forskolin analogs. *Biochem Pharmacol* **78**:62-69.

Pinto C, Papa D, Hübner M, Mou TC, Lushington GH and Seifert R (2008) Activation and inhibition of adenylyl cyclase isoforms by forskolin analogs. *J Pharmacol Exp Ther* **325**:27-36.

Schwede F, Maronde E, Genieser H and Jastorff B (2000) Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacol Ther* **87**:199-226.

Shen B, Kwan HY, Ma X, Wong CO, Du J, Huang Y and Yao X (2011) cAMP activates TRPC6 channels via the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-mitogen-activated protein kinase kinase (MEK)-ERK1/2 signaling pathway. *J Biol Chem* **286**:19439-19445.

Shi J, Mori E, Mori Y, Mori M, Li J, Ito Y and Inoue R (2004) Multiple regulation by calcium of murine homologues of transient receptor potential proteins TRPC6 and TRPC7

expressed in HEK293 cells. *J Physiol* **561**:415-432.

Songyang Z, Blechner S, Hoagland N, Hoekstra MF, Piwnica-Worms H and Cantley LC (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr Biol* **4**:973-982.

Suzuki F, Morishima S, Tanaka T and Muramatsu I (2007) Snapin, a new regulator of receptor signaling, augments $\alpha_{1A}$-adrenoceptor-operated calcium influx through TRPC6. *J Biol Chem* **282**:29563-29573.

Watanabe H, Murakami M, Ohba T, Takahashi Y and Ito H (2008) TRP channel and cardiovascular disease. *Pharmacol Ther* **118**:337-351.

Yee JK, Friedmann T and Burns JC (1994) Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol* **43**:99-112.

Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigno DD, Thistlethwaite PA, Rubin LJ and Yuan JX (2004) Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A* **101**:13861-13866.

**Footnotes.**

**Footnote to the title:**

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**Reprint requests should be addressed to:**

Soichi Miwa, M.D., Ph.D., Professor.
Department of Cellular Pharmacology, Hokkaido University Graduate School of Medicine, North 15, West 7, Sapporo-City, Hokkaido 060-8638, Japan.
Tel: +81-11-706-6921; Fax: +81-11-706-7824; E-mail: smiwa@med.hokudai.ac.jp.

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**Legends for figures**

**Figure 1.**

Characterization of ROCE induced by activation of $ET_AR$ in HEK293 cells stably co-expressing human $ET_AR$ and TRPC6-GFP. Representative traces for thapsigargin (TG)-induced SOCE and $ET_AR$-activated ROCE after store depletion in the presence of 10 μM $Gd^{3+}$ in GFP- (C) and TRPC6-GFP-transfected (D) HEK293 cells stably expressing human $ET_AR$. 2 μM TG-induced $Ca^{2+}$ release from ER in nominally $Ca^{2+}$-free medium followed by SOCE upon restoration of 2 mM extracellular $Ca^{2+}$. ROCE was triggered by stimulation of $ET_AR$ with 10 nM endothelin-1 (ET-1) 12 min after the addition of extracellular $Ca^{2+}$.

**Figure 2.**

Effects of forskolin, Rp-8-Br-cAMP, and papaverine on ROCE via TRPC6-GFP induced by 10 nM ET-1. Data are presented as means ± S.E.M of the results obtained from 4 – 6 experiments. $^{**}P < 0.01$, versus its vehicle.

**Figure 3.**

Effects of forskolin and papaverine on the intracellular distribution of GFP and TRPC6-GFP expressed in $ET_AR$/HEK293 cells. The cells were treated with 10 μM forskolin and 10 μM papaverine for 30 min. The subcellular localization of GFP and TRPC6-GFP was visualized by fluorescence confocal microscopy.

**Figure 4.**

Effects of alanine substitutions for putative PKA phosphorylation sites on inhibition of ROCE via TRPC6-GFP by forskolin. (A and B) Representative traces for inhibitory effects of 10 μM forskolin on ROCE via TRPC6 induced by 10 nM ET-1 in TRPC6(S28A)-GFP (A) and TRPC6(T69A)-GFP (B). (C) Inhibitory effects of 10 μM forskolin on ROCE in response to 10 nM ET-1 in wild-type and mutants of TRPC6. Data are presented as means ± S.E.M of

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the results obtained from 4 – 6 experiments. $^{**}P < 0.01$, versus its wild-type.

**Figure 5.**

Effects of alanine substitutions for putative PKA phosphorylation sites on inhibition of ROCE via TRPC6-GFP by papaverine. (A and B) Representative traces for inhibitory effects of 10 μM papaverine on ROCE via TRPC6 induced by 10 nM ET-1 in TRPC6(S28A)-GFP (A) and TRPC6(T69A)-GFP (B). (C) Inhibitory effects of 10 μM papaverine on ROCE in response to 10 nM ET-1 in wild-type and mutants of TRPC6. Data are presented as means ± S.E.M of the results obtained from 4 – 6 experiments. $^{**}P < 0.01$, versus its wild-type.

**Figure 6.**

Phosphorylation of wild-type and mutant TRPC6 proteins stably expressed in HA-$ET_A$R/HEK293 cells. (A) Effects of phosphatase treatment on the basal phosphorylation of FLAG-tagged TRPC6 proteins immunoprecipitated with anti-FLAG antibody. Immunoprecipitated TRPC6 proteins were incubated in the dephosphorylation buffer for 1 h. (B) Effects of forskolin and papaverine on the basal phosphorylation of TRPC6-FLAG proteins in vivo. HA-$ET_A$R/HEK293 cells expressing wild-type and mutant TRPC6-FLAG were treated with a combination of 10 μM forskolin and 10 μM papaverine for 30 min. Subsequently, immunoprecipitation and immunoblotting of TRPC6 were performed to estimate changes in the phosphorylation level of TRPC6 proteins. (C) Phosphorylation of TRPC6 on $Ser^{28}$ and $Thr^{69}$ by PKA. Immunoprecipitated TRPC6 proteins were incubated in the PKA phosphorylation buffer for 30 min. The histogram represents the relative ratio of PKA-induced phosphorylation of mutant TRPC6 proteins to that of wild-type TRPC6. The PKA-induced phosphorylation of each sample was calculated as the ratio of the phosphorylation level of TRPC6 (phospho TRPC6) in the present of PKA catalytic subunit to that in the absence of PKA catalytic subunit. These phosphorylation levels were normalized by the quantity of total TRPC6 protein (total TRPC6). Data are presented as means ± S.E.M of the results obtained from 4 experiments. $^{**}P < 0.01$, versus its wild-type. (A-C) Upper panels are representative immunoblots with Phos-tag$^{TM}$ biotin-bound HRP-SA complex

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detecting phosphorylated TRPC6 proteins (indicated as phospho TRPC6). Lower panels are representative immunoblots with anti-FLAG-HRP antibody to determine the quantity of TRPC6 in the immunoprecipitate (indicated as total TRPC6).

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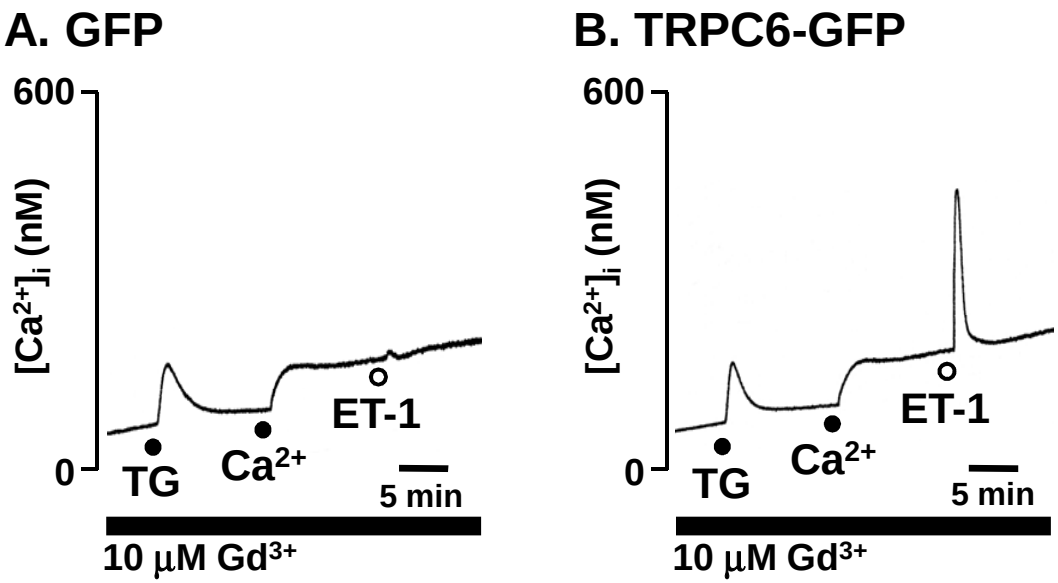
**Table 1** Comparison of $ET_AR$-operated $Ca^{2+}$ entry mediated through wild-type and mutant TRPC6 in HEK293 cells stably co-expressing human $ET_AR$ and TRPC6 proteins.

| Mutation | Sequences | *n* | $[Ca^{2+}]_{iMAX}$ (nM) |
|---|---|---|---|
| Wild-type | - | 8 | 266.4 ± 40.1 |
| S14A | R-R-G-G-$S^{14}$ | 6 | 243.8 ± 32.6 |
| S28A | R-R-N-E-$S^{28}$ | 7 | 247.4 ± 26.6 |
| T69A | R-R-Q-$T^{69}$ | 7 | 282.8 ± 27.6 |
| S321A | R-K-L-$S^{321}$ | 6 | 221.0 ± 38.8 |

Results are presented as means ± S.E.M. of *n* number of experiments. Sequences represent amino acids at amino-terminal to phosphorylation sites for PKA. $[Ca^{2+}]_{iMAX}$ (nM) is the peak of ROCE induced by 10 nM ET-1. Abbreviations for amino acid residues are: S, serine; A, alanine; T, threonine; G, glycine; N, asparagine; E, Glutamic acid; Q, glutamine; K, lysine; L, leucine.

# Figure 1.



# Figure 2.

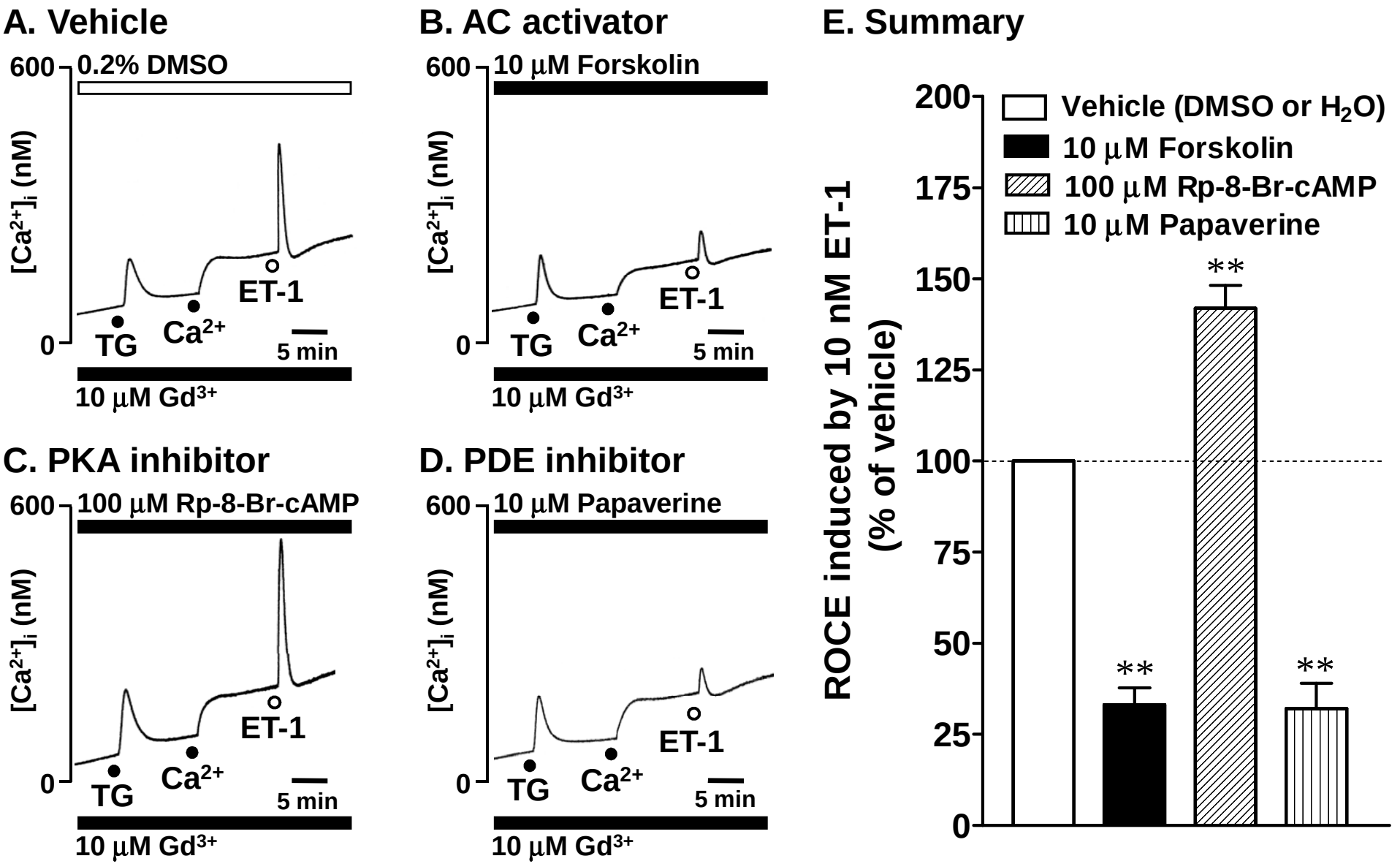
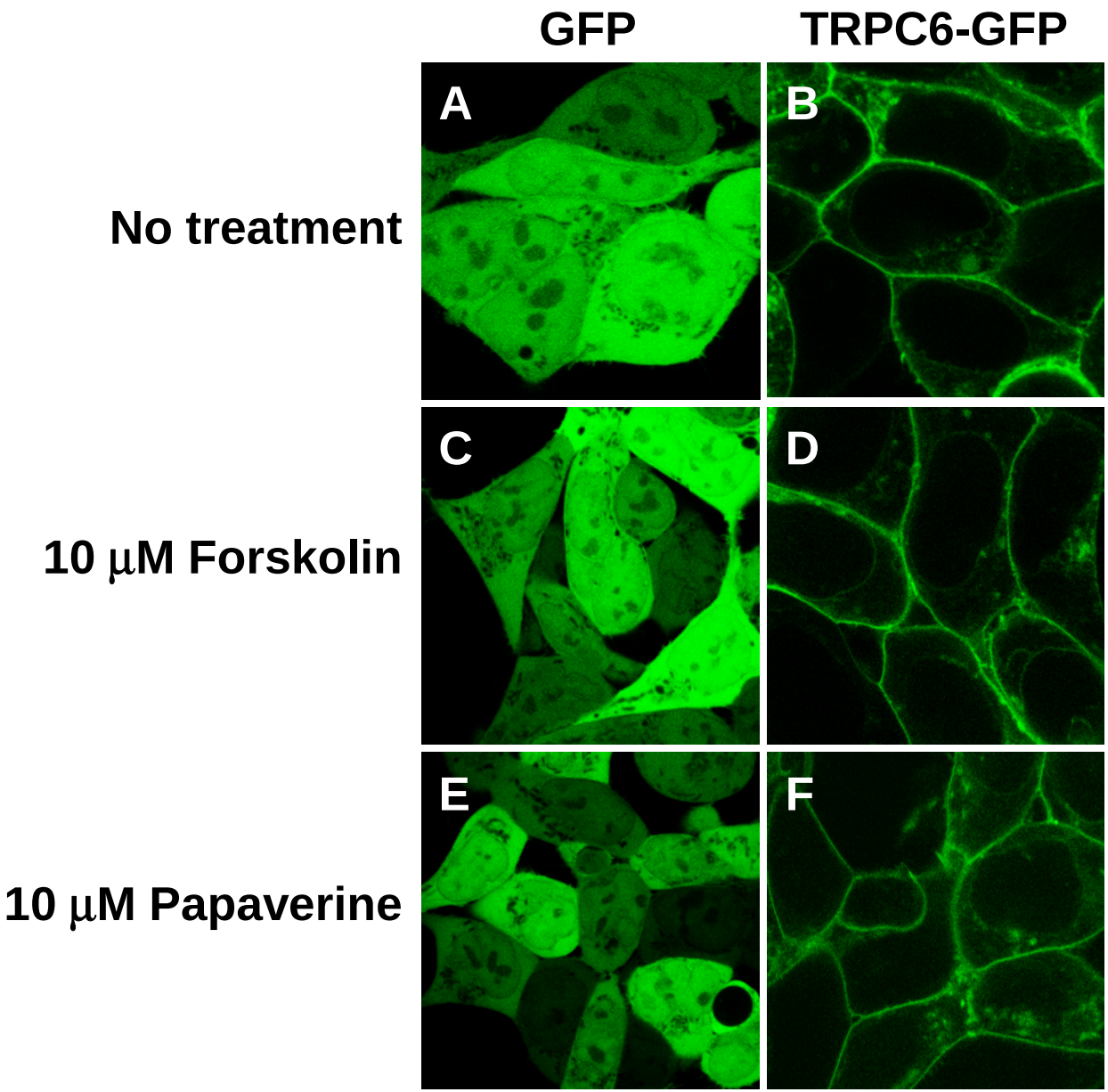


**Figure 3.**



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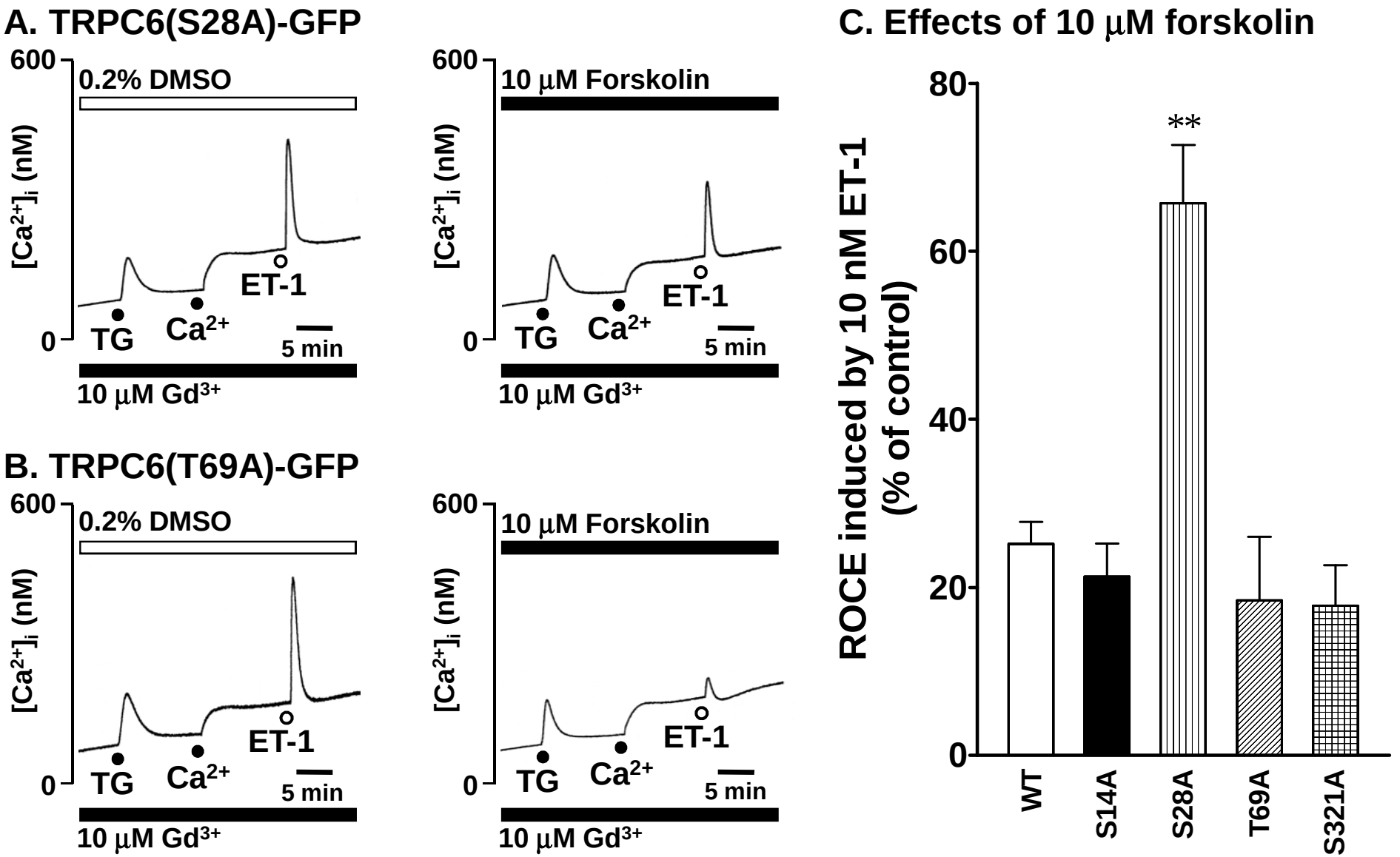

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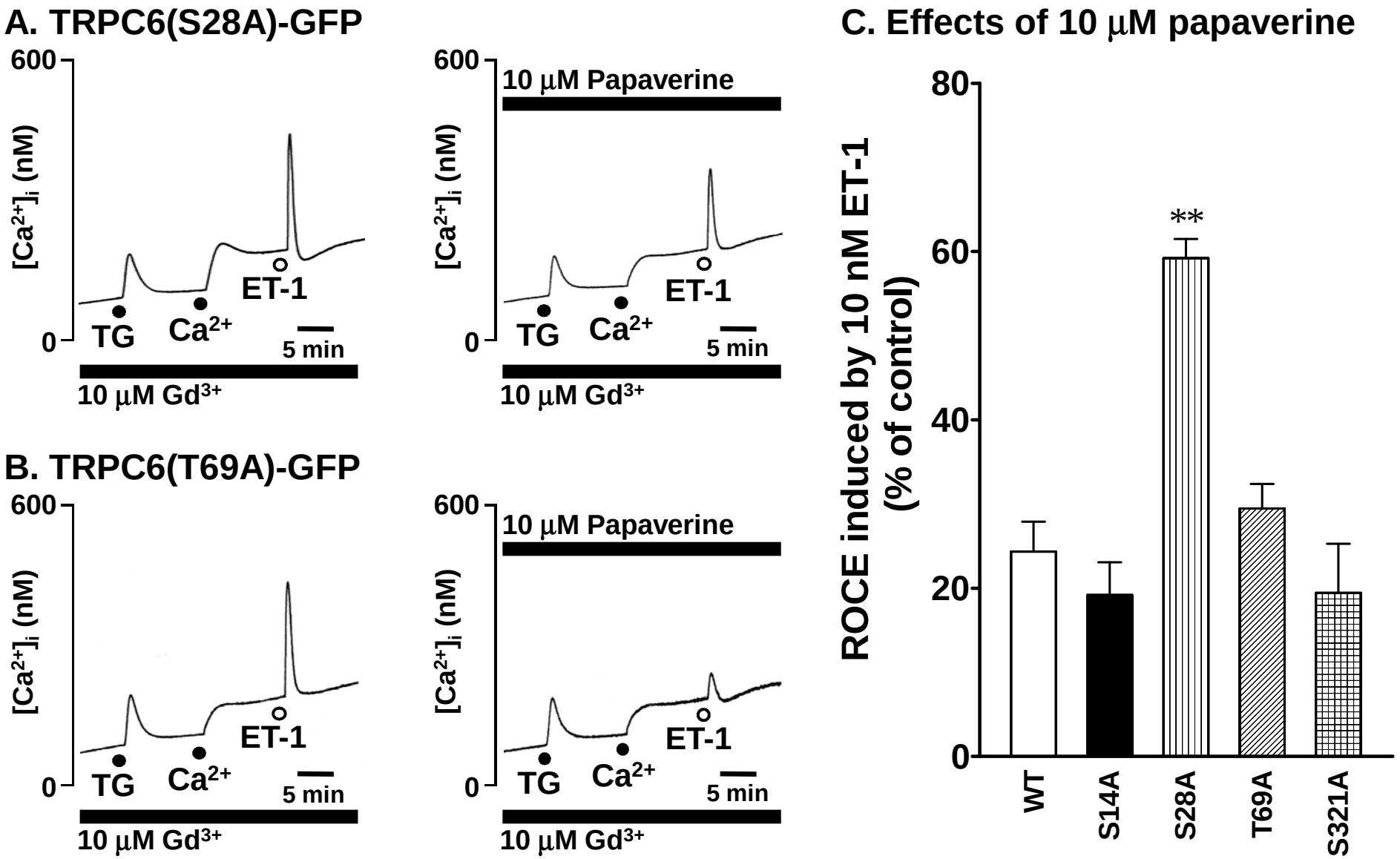
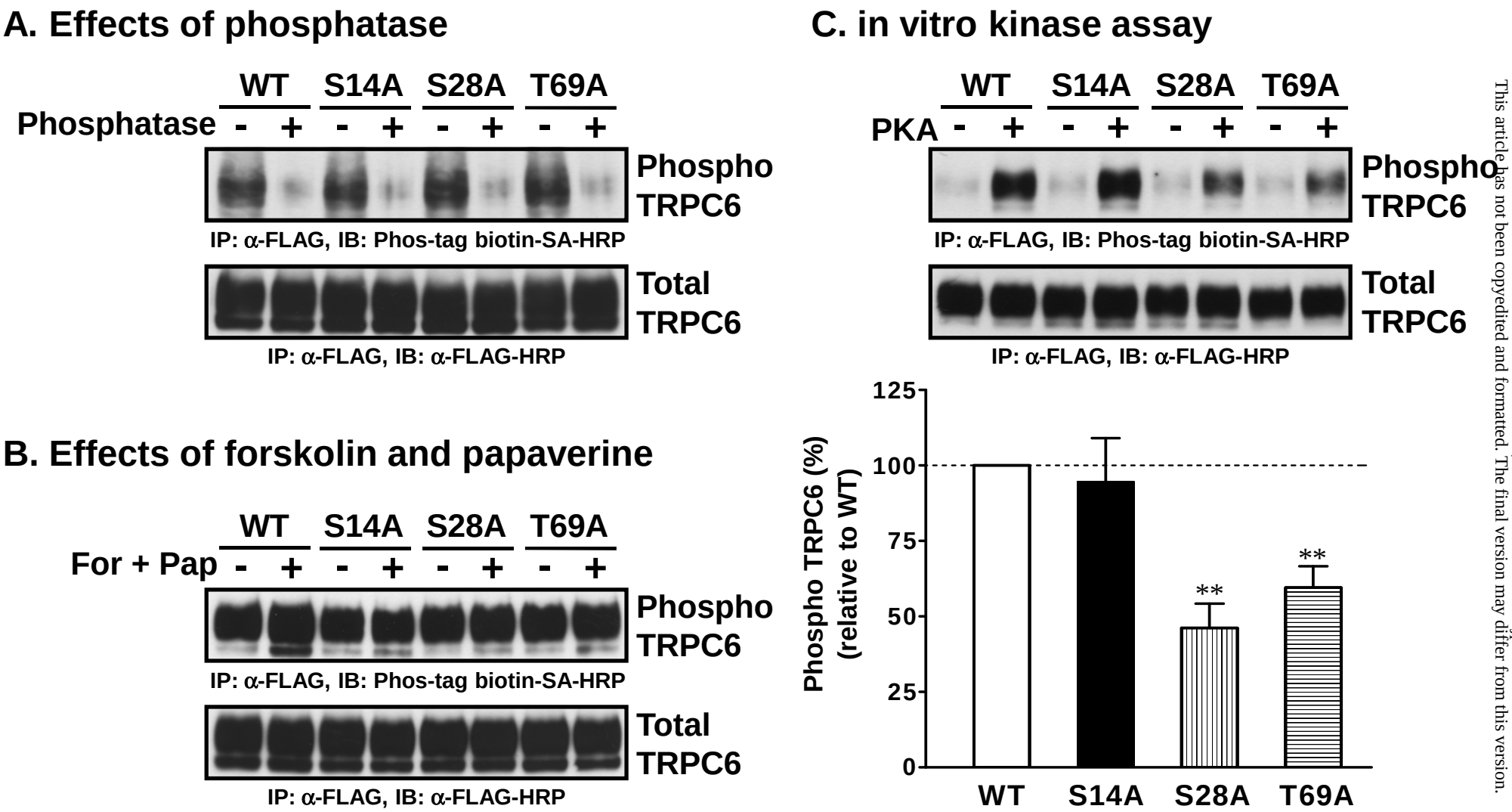


# Figure 6.



**Supplemental data**

**JPET MS# 187500**

# Adenylate cyclase-cAMP-protein kinase A signaling pathway inhibits endothelin type A receptor-operated $Ca^{2+}$ entry mediated via TRPC6 channels.

Takahiro Horinouchi, Tsunaki Higa, Hiroyuki Aoyagi, Tadashi Nishiya,
Koji Terada, and Soichi Miwa.

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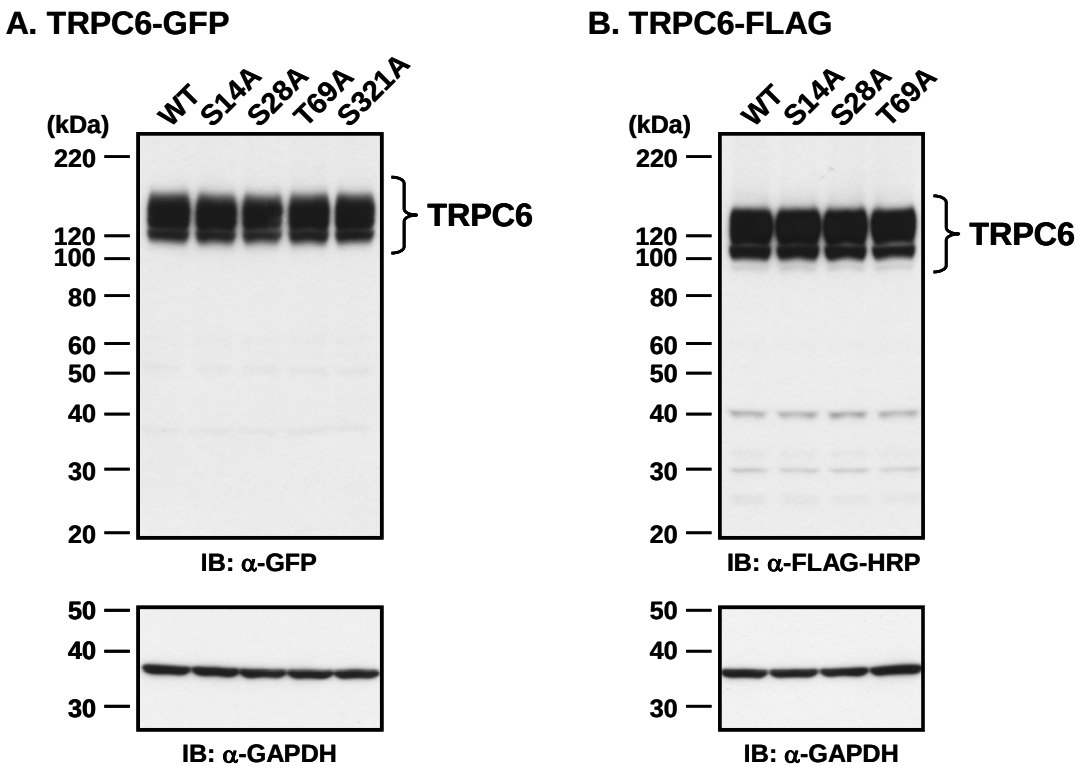


**Figure S1.**

Comparison of protein expression levels between wild-type (WT) and mutant (S14A, S28A, T69A, and S321A) TRPC6 fused with GFP (A) or FLAG tag peptide (B). The upper panels are representative immunoblots to detect wild-type and mutant TRPC6 proteins. The lower panels are representative immunoblots to detect GAPDH as a loading control. The locations of the bands for wild-type and mutant TRPC6-GFP or TRPC6-FLAG are indicated to the right. The positions of protein size markers are indicated to the left in kDa.

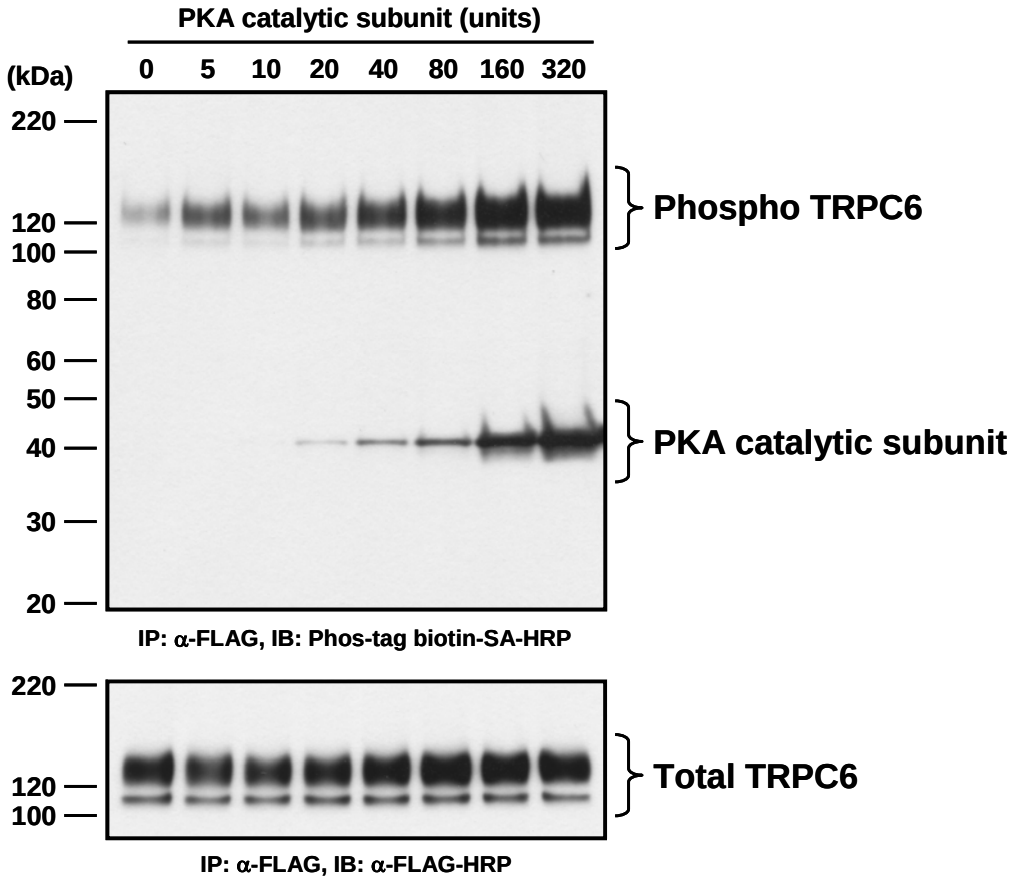


**Figure S2.**

Dose-response relationship for PKA-mediated phosphorylation of wild-type TRPC6 protein. Immunoprecipitated TRPC6 protein was incubated in the dephosphorylation buffer for 1 h and then was phosphorylated with PKA catalytic subunit at indicated doses for 30 min. The upper panel is a representative immunoblot with Phos-tag$^{TM}$ biotin-bound HRP-SA complex to detect phosphorylated TRPC6 protein (indicated as phospho TRPC6). Lower panel is representative immunoblots with anti-FLAG-HRP antibody to determine the quantity of TRPC6 in the immunoprecipitate (indicated as total TRPC6). The locations of the bands for TRPC6-FLAG and PKA catalytic subunit are indicated to the right. The positions of protein size markers are indicated to the left in kDa.

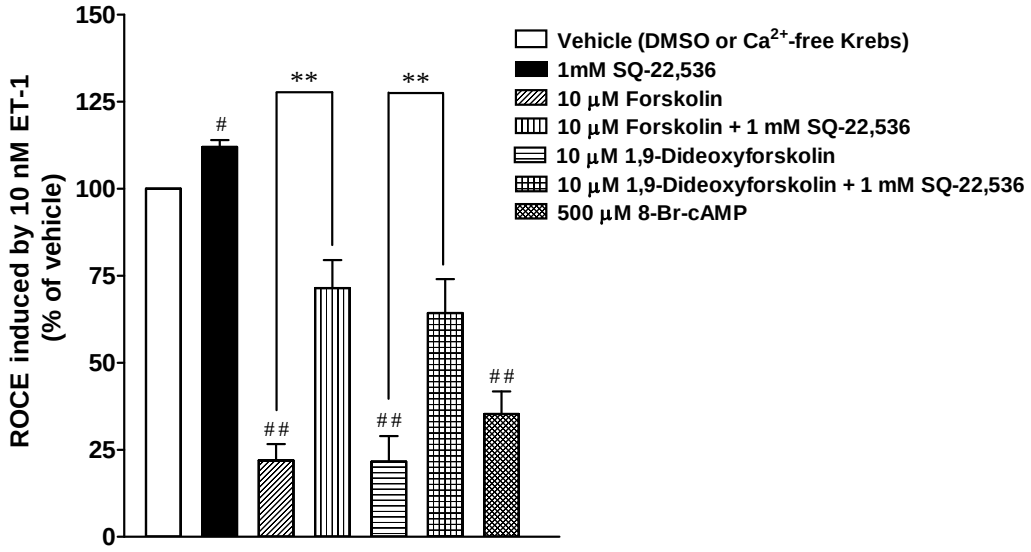


**Figure S3.**

Effects of SQ-22,536, forskolin, 1,9-dideoxyforskolin, 8-Br-cAMP, and a combination of SQ-22,536 and forskolin or 1,9-dideoxyforskolin on ROCE via TRPC6-GFP induced by 10 nM ET-1. Data are presented as means ± S.E.M of the results obtained from 4 experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus its vehicle. $^{**}P < 0.01$, between indicated groups.

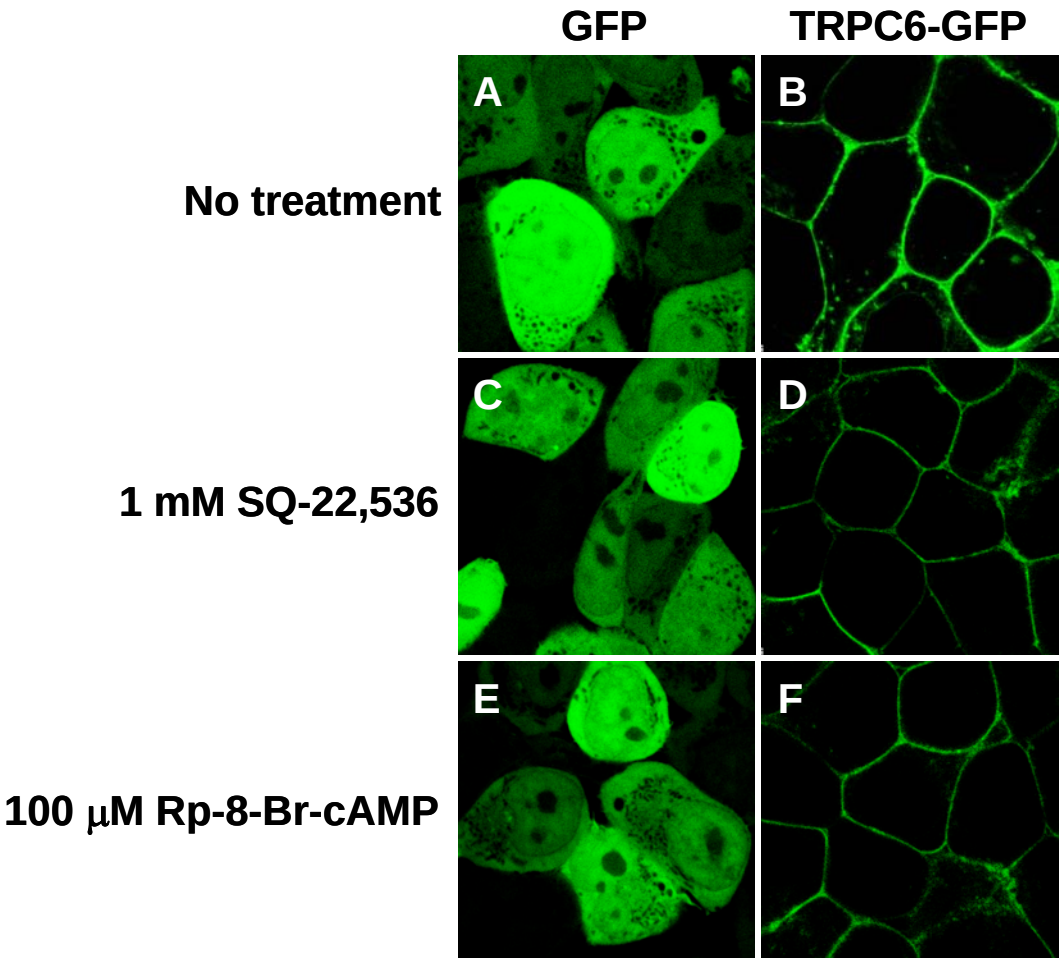


**Figure S4.**

Effects of SQ-22,536 and Rp-8-Br-cAMP on the intracellular distribution of GFP and TRPC6-GFP expressed in $ET_AR$/HEK293 cells. The cells were treated with 1 mM SQ-22,536 and 100 μM Rp-8-Br-cAMP for 30 min. The subcellular localization of GFP and TRPC6-GFP was visualized by fluorescence confocal microscopy.