Title: Store-operated STIM1 translocation and interaction with TRPC1 at the plasma membrane stimulates PLC activity to induce channel gating in vascular smooth muscle cells

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Running title: STIM1-evoked PLC activity induces TRPC1 channel stimulation

Key words: STIM1, TRPC1, PLC, vascular smooth muscle

Key point summary

- Depletion of Ca^{2+} stores activates store-operated channels (SOCs), which mediate Ca^{2+} entry pathways that regulate cellular processes as contraction, proliferation, and gene expression.

- In vascular smooth muscle cells (VSMCs), stimulation of SOCs composed of canonical transient receptor potential 1 (TRPC1) proteins requires Gαq/PLCβ1/PKC activity. We studied the role of stromal interaction molecule 1 (STIM1) in coupling store depletion to this activation pathway using patch clamp recording, GFP-PLCδ1-PH imaging, and co-localisation techniques.

- Store-operated TRPC1 channel and PLCβ1 activities were inhibited by STIM1 shRNA and absent in $TRPC1^{-/-}$ cells, and store-operated PKC phosphorylation of TRPC1 was inhibited by STIM1 shRNA. Store depletion induced interactions between STIM1 and TRPC1, Gαq, and PLCβ1, which required STIM1 and TRPC1. Similar effects were produced with noradrenaline.

- These findings identify a new activation mechanism of TRPC1-based SOCs in VSMCs, and a novel role for STIM1, where store-operated STIM1-TRPC1 interactions stimulate Gαq/PLCβ1/PKC activity to induce channel gating.

Abbreviations: CPA, cyclopiazonic acid; CRACs, calcium release activated channels; ER/SR, endoplasmic/sarcoplasmic reticulum; Gαq, G protein alpha q subunit; IP, immunoprecipitation; MARCKS, myristoylated alanine-rich C kinase substrate; NP_o, open probability; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA, proximity ligation assay; PLC, phospholipase C; PM, plasma membrane; shRNA, short hairpin RNA; SOCs, store-operated channels; STIM1, stromal interaction molecule 1; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamineed; TRPC, canonical transient receptor potential; WB, Western blotting; VSMCs, vascular smooth muscle cells; WT, wild-type

Abstract

In vascular smooth muscle cells (VSMCs), stimulation of TRPC1-based SOCs mediate Ca^{2+} entry pathways which regulate contractility, proliferation and migration. It is therefore important to understand how these channels are activated. Studies have shown that stimulation of TRPC1-based SOCs requires Gαq/PLCβ1 activities and PKC phosphorylation, but it is unclear how store depletion stimulates this gating pathway. The present work examines this issue by focusing on the role of STIM1, an endo/sarcoplasmic reticulum Ca^{2+} sensor. Store-operated TRPC1 channel activity was inhibited by TRPC1 and STIM1 antibodies and STIM1 shRNA in wild-type VSMCs, and was absent in $TRPC1^{-/-}$ VSMCs. Store-operated PKC phosphorylation of TRPC1 was reduced by knockdown of STIM1. Moreover, store-operated PLCβ1 activity measured with the fluorescent $PIP_2/InsP_3$ biosensor GFP-PLCδ1-PH was reduced by STIM1 shRNA and absent in $TRPC1^{-/-}$ cells. Immunocytochemistry, co-immunoprecipitation, and proximity ligation assays revealed that store depletion activated STIM1 translocation from within the cell to the plasma membrane (PM) where it formed STIM1-TRPC1 complexes, which then associated with Gαq and PLCβ1. Noradrenaline also evoked TRPC1 channel activity and associations between TRPC1, STIM1, Gαq and PLCβ1, which were inhibited by STIM1 knockdown. Effects of N-terminal and C-terminal STIM1 antibodies on TRPC1-based SOCs and STIM1 staining suggest that channel activation may involve insertion of STIM1 into the PM. Our findings identify a new activation mechanism of TRPC1-based SOCs in VSMCs, and a novel role for STIM1, in which store-operated STIM1-TRPC1 interactions stimulate PLCβ1 activity to induce PKC phosphorylation of TRPC1 and channel gating.

Introduction

Ca^{2+}-permeable store-operated channels (SOCs) are physiologically activated by stimulation of the classical phosphoinositol signalling pathway involving Gαq-coupled receptors, PLC activation, PIP_2 hydrolysis, $InsP_3$ generation, and $InsP_3$-mediated depletion of endo/sarcoplasmic reticulum (ER/SR) Ca^{2+} stores. In vascular smooth muscle cells (VSMCs), SOCs mediate Ca^{2+} entry pathways which regulate cellular functions such as contractility, proliferation and migration, and are potential therapeutic targets for cardiovascular diseases such as hypertension and atherosclerosis (Abramowitz & Birnbaumer, 2009; Beech, 2013; Earley & Brayden, 2015). Identifying molecules involved in composing and activating SOCs are important objectives in vascular biology.

There is increasing evidence that cells express two distinct types of SOCs (Cheng *et al*, 2013). Prototypical SOCs, termed calcium release-activated channels (CRACs) responsible for the calcium release activated current (I_{crac}), have several characteristic properties such as high Ca^{2+} permeability, pronounced inward rectification, unitary conductances in the fS range, and are composed of pore-forming Orai1 proteins (Prakriya & Lewis, 2015). Orai1-based CRACs are gated by store depletion through stromal interaction molecule 1 (STIM1), a Ca^{2+} sensor within ER/SR stores which following store depletion undergoes oligomerisation and approaches the cytosolic surface of the plasma membrane (PM) where it interacts directly with Orai1 to induce channel opening (Prakriya & Lewis, 2015; Soboloff *et al*, 2012). It is also apparent that many cell types express SOCs which have very different properties to those of Orai1-based CRACs such as much lower Ca^{2+} permeability, relatively linear rectification, considerably larger unitary conductances, and are proposed to be composed by the canonical transient receptor potential family of Ca^{2+}-permeable non-selective cation channel proteins (TRPC1-C7) (Cheng *et al*, 2013). It is relatively well-accepted that TRPC1 are regulated by store depletion, but there is less evidence for the other channel subtypes. Although TRPC-based SOCs are likely to form a diverse family of structures as TRPC subunits form heteromeric channels (Alfonso *et al*, 2008; Saleh *et al*, 2008; Shi *et al*, 2012; Cheng *et al*, 2013). Whether TRPC proteins form a distinct family of SOCs remains controversial as it is unclear on how store depletion couples to channel activation. However, there is growing support for STIM1 also being involved in activation of TRPC-based SOCs through both Orai1-dependent and -independent mechanisms (Ambudkar *et al*, 2007; Worley *et al*, 2007; Yuan *et al*, 2009; Cheng *et al*, 2011; Cheng *et al*, 2013; Liao *et al*, 2014).

It is proposed that VSMCs differentially express Orai1-based CRACs and/or TRPC-based SOCs according to their phenotype. In cells expressing a contractile phenotype, such as acutely isolated VSMCs and primary cultured VSMCs maintained in low serum conditions, SOCs have relatively linear rectification, unitary conductances of about 2pS, and are composed of a heteromeric TRPC1/C5 molecular template that may also contain other TRPC subunits (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012). As TRPC1 is essential for conferring gating by store depletion these channels are termed TRPC1-based SOCs (Shi *et al*, 2012). Importantly, SOCs with properties similar to Orai1-based CRACs have not been described in contractile VSMCs (Shi *et al*, 2012), and Orai proteins are poorly expressed in these cells (Berra-Romani *et al*, 2008; Trebak, 2012). In contrast, long-term cultured VSMCs maintained in high serum conditions, which display a non-contractile, synthetic phenotype associated with cell proliferation and migration express both TRPC1-based SOCs (Li *et al*, 2008; Ng *et al*, 2009, 2010) and Orai1-based CRACs (Potier *et al*, 2009; Li *et al*, 2011; Beech, 2012; Trebak, 2012). In the present work we examined the activation mechanisms of native TRPC1-based SOCs in contractile VSMCs, which are likely to mediate Ca^{2+} entry pathways involved in regulating vascular tone and switching of VSMCs from contractile to synthetic phenotypes (Matchkov *et al*, 2013).

Our previous findings have revealed that PKC activity is pivotal for stimulation of TRPC1-based SOCs in contractile VSMCs (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012). It is proposed that store-operated PKC phosphorylation of TRPC1 is necessary for PIP_2 binding to occur, which acts as the gating ligand (Saleh *et al*, 2009; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012, 2014). This process is controlled by the PIP_2-binding protein myristoylated alanine-rich C kinase substrate (MARCKS) which behaves as a reversible PIP_2 buffer to control local PIP_2 levels required for channel activation (Shi *et al*, 2014). In a recent study, we investigated how depletion of stores induces this activation pathway and revealed that store depletion stimulates Gαq-evoked PLCβ1 activity to induce PKC phosphorylation of TRPC1 proteins (Shi *et al*, 2015). However, these studies did not elucidate how store depletion is coupled to Gαq/PLCβ1 activity. The present study investigates this question, and identifies a new activation mechanism of TRPC1-based SOCs in VSMCs, and a novel role for STIM1, in which store depletion induces formation of STIM1-TRPC1 complexes that stimulate PLC activity.

Methods

Cell isolation

New Zealand White rabbits (2-3kg) were killed using i.v. sodium pentobarbitone (120mg/kg) and WT and TRPC1$^{-/-}$ mice were killed using cervical dislocation according to UK Animals Scientific Procedures Act of 1986 and as revised by European Directive 2010/63/EU. All experiments were carried out according to guidelines laid down by St. George's, University of London animal welfare committee, and conform to the principles and regulations described by Grundy (2015). Portal veins from rabbit or 2nd order mesenteric arteries from mice were dissected free, and cleaned of fat, connective tissue and endothelium in physiological salt solution containing (mM): 126 NaCl, 6 KCl, 10 glucose, 11 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$, pH adjusted to 7.2 using 10M NaOH. Vessels were enzymatically dispersed into single VSMCs as described previously (Shi *et al*, 2016).

Primary cell culture

VSMCs were seeded into culture plates; maintained using DMEM/F-12 media containing 1% serum, and incubated at 37°C in 95%O_2:5%CO_2 at 100% humidity for up to 7 days. In 1% serum, VSMCs maintained their contractile phenotype and had similar properties to TRPC1 channel currents in freshly dispersed VSMCs (Shi *et al*, 2016), which suggest that compensatory changes to channel properties were unlikely in these cell culture conditions.

Electrophysiology

Whole-cell and single-channel cation currents were made with an AXOpatch 200B amplifier (Axon Instruments, Union City, CA, USA) at room temperature (20–23°C) as described previously (Shi et al, 2016). Whole-cell currents were filtered at 1kHz (−3dB, low-pass 8-pole Bessel filter, Frequency Devices model LP02, Scensys, Aylesbury, UK) and sampled at 5kHz (Digidata 1322A and pCLAMP 9.0 software, Molecular Devices, Sunnydale, CA, USA). Whole-cell current/voltage (I/V) relationships were obtained by applying 750ms duration voltage ramps from +100 to −150mV every 30s from a holding potential of 0mV. Single channel currents were filtered between 0.1-0.5kHz and acquired at 1-5kHz. Single channel I/V relationships were evaluated by manually altering the holding potential of −80mV between −120 and +120mV. Single channel current amplitudes were calculated from idealised traces of ≥60s in duration using the 50% threshold method and analysed using pCLAMP 9.0 software. Events lasting for <6.664ms (2× rise time for a 100Hz (−3dB) low-pass filter) were excluded from analysis to maximize the number of channel openings reaching their full current amplitude. Open probability (NP_O) was used as a measure of

channel activity and was calculated automatically by pCLAMP 9. Single channel current amplitude histograms were plotted from the event data of the idealised traces with a 0.01pA bin width. Amplitude histograms were fitted using Gaussian curves with peak values corresponding to channel open levels. Mean channel amplitudes at different membrane potentials were plotted, and I/V relationships were fitted by linear regression with the gradient determining conductance values. Figures were prepared using MicroCal Origin 6.0 software (MicroCal Software, Northampton, MA, USA), in which inward single channel openings are shown as downward deflections.

Whole-cell recording bath solution contained (mM): 126 NaCl, 1.5 $CaCl_2$, 10 HEPES, 11 glucose, 0.1 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS), 0.1 niflumic acid, and 0.005 nicardipine, pH to 7.2 with NaOH. Under these conditions, voltage-dependent Ca^{2+} channels, and Ca^{2+}-activated and swell-activated Cl^- conductances are blocked allowing cation conductances to be recorded in isolation. Whole-cell patch pipette and inside-out patch bathing solutions contained (mM): 18 CsCl, 108 cesium aspartate, 1.2 $MgCl_2$, 10 HEPES, 11 glucose, 1 Na_2ATP, and 0.2 NaGTP, pH adjusted to 7.2 with Tris. Free $[Ca^{2+}]_i$ was set at 100nM by adding 4.8mM $CaCl_2$ plus 10mM 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid(acetoxymethyl ester) (BAPTA) or 0.48mM $CaCl_2$ plus 1mM BAPTA for whole-cell and inside-out recordings respectively using EqCal software (Biosoft, Cambridge, UK). In cell-attached patch experiments the membrane potential was set to 0mV by perfusing cells in a KCl external solution containing (mM): 126 KCl, 1.5 $CaCl_2$, 10 HEPES and 11 glucose, pH adjusted to 7.2 with 10M KOH. 5μM Nicardipine was included to prevent smooth muscle cell contraction by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels. The patch pipette solution used for both cell-attached and inside-out patch recording (extracellular solution) was K^+ free and contained (mM): 126 NaCl, 1.5 $CaCl_2$, 10 HEPES, 11 glucose, 10 TEA, 5 4-AP, 0.0002 iberiotoxin , 0.1 DIDS, 0.1 niflumic acid and 0.005 nicardipine, pH adjusted to 7.2 with NaOH.

Knockdown of STIM1 and PLCβ1

We used lentiviral-mediated delivery of pLKO.1-puro based shRNA expression plasmids purchased from Sigma-Aldrich to knockdown STIM1 and PLCβ1 (Gillingham, UK). Infected VSMCs were selected with 2.5 μg/ml puromycin (Invitrogen, San Diego, US) for 2 days prior to the experiments, and STIM1 and PLCβ1 levels were determined by Western blotting. Two STIM1 shRNA were used to knockdown proteins in rabbits (Sequence 1: 5'-CACCTTCCATGGTGAGGATAA-3' and Sequence 2: 5'-GGCTGCTGGTTTGCCTATATC-

3') and two different sequences were used to knockdown STIM1 in mice (Sequence 1: 5'-CACCTTCCATGGTGAGGATAA-3' and Sequence 2: 5'-CCCTTCCTTTCTTTGCAATAT-3'). PLCβ1 shRNA1 (5'-GCAGATAAACATGGGCATGTA-3') and shRNA2 (5'-GCTGTCTTTGTCTACATAGAA-3') were used to knockdown PLCβ1 in both rabbits and mice (Shi *et al*, 2016). Scrambled shRNA sequences from STIM1 shRNA1/2 and PLCβ1 shRNA1/2 were used as controls.

Imaging of GFP-PLCδ-PH-mediated signals

VSMCs were transfected with GFP-PLCδ-PH (Addgene (plasmid ID:21179, Addgene) using Nucleofector™ according to manufacturer's instructions (Amaxa Biosystems, Gaithersburg, MD). 0.2–0.4μg plasmid DNA was added to $1x10^5$ cells re-suspended in 20 μl Nucleofector™ solution, and cells were kept in primary cell culture conditions for up to 3 days. Transfected cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope and associated software (Carl Zeiss, Jena, Germany). Excitation was produced by 488/405 nm lasers and delivered via a Zeiss Apochromat 63 oil-immersion objective (numerical aperture, 1.4). Two-dimensional images cut horizontally through approximately the middle of the cells were captured (1024x1024 pixels). Final images were produced using PowerPoint (Microsoft XP;Microsoft, Richmond, WA, USA).

Immunoprecipitation and Western blot

Freshly isolated vessel segments or primary cultured cells were lysed by sonication for 3h in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then transferred to a microcentrifuge tube and cleared by centrifuging at 1000g for 10 min at 4^{o}C. Total cell lysate protein was immunoprecipitated using antibodies raised against targeted proteins with a Millipore Catch and Release Kit (Millipore, Billerica, MA, USA) followed by 1-dimensional protein gel electrophoresis (15–20μg of total protein/lane). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, and then membranes were incubated with 5% (weight/volume) non-fat milk in PBS containing 0.1% Tween 20 (PBST) to block non-specific binding, and then primary antibodies were added and the membrane left overnight at 4°C. Visualization was performed with a horseradish peroxidase-conjugated secondary antibody (80ng/ml) and ECL chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1min and exposure to photographic films. Band intensities were calculated using Image Studio software (Li-Cor Biosciences Ltd.,

Cambridge, UK) and then were normalized to control bands. Data shown represent findings from ≥3 different animals.

Immunocytochemistry

Freshly isolated VSMCs were fixed with 4% paraformaldehyde (Sigma-Aldrich, Gillingham, UK) for 10min, washed with phosphate-buffered saline (PBS), and permeabilised with PBS containing 0.1% Triton X-100 for 20min at room temperature. Cells were incubated with PBS containing 1% bovine serum albumin (BSA) for 1h at room temperature and then were incubated with primary antibodies in PBS containing 1% BSA overnight at 4°C. In control experiments, cells were incubated without the primary antibody. The cells were washed and incubated with secondary antibodies conjugated to a fluorescent probe. Unbound secondary antibodies were removed by washing with PBS, and nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Sigma-Aldrich). Cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The excitation beam was produced by an argon (488nm) or helium/neon laser (543nm and 633nm), and delivered to the specimen via a Zeiss Apochromat X63 oil immersion objective (numerical aperture, 1.4). Emitted fluorescence was captured using LSM 510 software (release 3.2; Carl Zeiss). Two-dimensional images cut horizontally through approximately the middle of the cells were captured (1024x1024 pixels). Raw confocal imaging data were processed and analysed using Zeiss LSM 510 software. Final images were produced using PowerPoint (Microsoft XP;Microsoft, Richmond, WA, USA).

Proximity Ligation Assay

Freshly isolated VSMCs were studied using the Duolink in situ PLA detection kit 563 (Olink, Uppsala, Sweden) (Soderberg *et al*, 2008). Cells were adhered to coverslips, fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15min, and permeabilised in PBS containing 0.1% Triton X-100 for 15min. Cells were blocked for 1hr at 37°C in blocking solution, and incubated overnight at 4°C with anti-STIM1 and anti-TRPC1 antibodies (both at 1:200) in antibody diluent solution. Cells were labelled with combinations of either anti-goat PLUS/anti-rabbit MINUS or anti-goat PLUS/anti-mouse MINUS depending on animal species used for 1hr at 37°C. Hybridised oligonucleotides were ligated for 30min at 37°C prior to amplification for 100min at 37°C. Red fluorescently-labelled oligonucleotides were then hybridised to rolling circle amplification products, and visualised using a Confocal LSM 510 (Zeiss, Germany).

Reagents

Rabbit anti-TRPC1 antibody was generated by GenScript (Piscataway, NJ, USA) using peptide sequences from a previously characterized putative extracellular region (Xu & Beech, 2001). Goat anti-TRPC1 (sc-15055), mouse anti-STIM1 (sc-393705), goat anti-STIM1 (sc-79106), goat anti-TRPC6 (sc-19196), mouse anti-Gαq (sc-136181), goat anti-PLCβ1 (sc-31755), mouse anti-PLCβ1 (sc-5291) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). All secondary antibodies were obtained from Santa Cruz Biotechnology. Fluor 488-conjugated donkey anti-rabbit antibodies and Fluor 546-conjugated donkey anti-mouse antibodies were from Alexa. Mouse anti-β-actin antibody (A1978) was obtained from Sigma-Aldrich (Gillingham, UK). Rabbit anti-STIM1 antibody against the N-terminal EF hand (11565-1-AP) was obtained from Proteintech (Chicago, IL, USA), mouse anti-GOK/STIM1 (610954) against the N-terminal EF hand was obtained from BD Biosciences (Oxford, UK), and mouse anti-STIM1 antibody against the C-terminal region (SC-66173) was obtained from Santa Cruz. All other drugs were purchased from Sigma-Aldrich, or Tocris (Abingdon, UK). Agents were dissolved in distilled H_2O or 0.1% dimethyl sulfoxide (DMSO). DMSO alone had no effect on whole-cell currents or single channel activity.

Statistical analysis

This was performed using paired (comparing the effects of agents on the same cell) or unpaired (comparing the effects of agents between cells) Student's *t* tests with the level of significance set at a value of $P<0.05$.

Results

TRPC1 compose SOCs in contractile VSMCs

In our initial experiments, we confirmed that native TRPC1-based SOCs are functionally expressed in contractile VSMCs using freshly isolated mesenteric artery VSMCs from wild-type (WT) and TRPC1$^{-/-}$ mice, and antibodies raised against TRPC1 as channel blockers (Xu & Beech, 2001; Xu et al, 2005; Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert, 2011; Shi *et al*, 2012; Shi *et al*, 2016). In WT VSMCs, passive depletion of internal Ca^{2+} stores following cell dialysis with a patch pipette solution containing high concentrations of BAPTA and no added Ca^{2+} evoked whole-cell cation currents with a relatively linear current-voltage (I/V) relationship and an E_{rev} of about +20mV, which were inhibited by bath application of TIE3, a TRPC1 antibody raised against a putative extracellular channel pore site (Xu & Beech, 2001; Xu *et al*, 2005), by over 80% at all membrane potentials tested (Figure 1A). In WT VSMCs, bath application of the cell permeable Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA-AM), also activated single channel activity in cell-attached patches with a unitary conductance of about 2pS, which was inhibited by bath application of an TRPC1 antibody raised against an putative intracellular site by about 85% at -80mV following patch excision into the inside-out configuration (Figure 1B). These whole-cell and single channel current properties are similar to those of native TRPC1-based SOCs previously described in VSMCs from various vascular beds and different animal species (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert, 2011; Shi *et al*, 2012; Shi *et al*, 2016). In further agreement with earlier findings, store-operated whole-cell conductances and single channel activities were absent in TRPC1$^{-/-}$ VSMCs (Figures 1A & B, Shi *et al*, 2012). These results clearly indicate that native TRPC1-based SOCs are functionally expressed in contractile VSMCs.

STIM1 is obligatory for activation of TRPC1 SOCs

We next examined if STIM1 is involved in activating TRPC1-based SOCs in contractile VSMCs using a knockdown strategy, and an antibody raised against the N-terminal EF hand of STIM1 (Spassova *et al*, 2006; Hewavitharana *et al*, 2008). Western blot studies showed that STIM1 protein is expressed in primary cultured rabbit portal vein VSMCs, maintained in low concentrations of fetal calf serum to retain their contractile phenotype (Shi *et al*, 2016), and that two different STIM1 shRNA sequences reduced STIM1 expression by about 80% compared to scrambled shRNA sequences (Figure. 2A). In control experiments, STIM1 shRNA1 did not alter TRPC1, Gαq, PLCβ1, and β-actin expression levels (data not shown).

In VSMCs expressing scrambled shRNA, development of store-operated whole-cell TRPC1 currents remained unaffected but peak currents were inhibited by over 80% at all membrane potentials tested following bath application of the N-terminal STIM1 antibody (Figure 2B). Treatment of cells with STIM1 shRNAs reduced development of store-operated whole-cell TRPC1 currents by over 60% at all membrane potentials tested (Figure 2B), and inhibited BAPTA-AM-evoked single TRPC1 channel activities by over 80% at -80mV (Figure 2C). In addition, the SR Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA) and the cell-permeable low affinity Ca^{2+} chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamineed (TPEN) also activated TRPC1 channel activities which were reduced by STIM1 shRNA1 and 2 (data not shown). These results clearly indicate that STIM1 is essential for activation of native TRPC1-based SOCs in contractile VSMCs.

Store-operated PKC phosphorylation of TRPC1 requires STIM1

We have previously shown that PKC phosphorylation of TRPC1 is a pivotal event in the activation of TRPC1-based SOCs in contractile VSMCs (Shi *et al*, 2016), and therefore we investigated if STIM1 is involved in this gating pathway. Immunoprecipitation of isolated vessel lysates with a mixture of anti-phosphorylated serine and threonine antibodies followed by Western blotting with an anti-TRPC1 antibody identified that TRPC1 proteins expressed low basal phosphorylation, which was greatly increased following pre-treatment with BAPTA-AM (Figures 3A & B). Expression of STIM1 shRNA1 and shRNA2 sequences and co-application of the PKC inhibitor GF09203X greatly reduced BAPTA-AM-induced TRPC1 phosphorylation by over 80% (Figures 3A & B). These findings clearly indicate that STIM1 is required for PKC phosphorylation of TRPC1 proteins by store depletion.

Store depletion induces PLCβ1 activity mediated by STIM1 and TRPC1

In a recent study we reported that store depletion stimulates Gαq-evoked PLCβ1 activity, which drives PKC activity and gating of native TRPC1-based SOCs in contractile VSMCs (Shi *et al*, 2016). However, it remains unclear how store depletion stimulates this pathway. The above findings reveal that STIM1 is central for store-operated PKC phosphorylation of TRPC1 and channel activation, and therefore we investigated if this ER/SR Ca^{2+} sensor couples store depletion to stimulation of PLCβ1 activity. To monitor store-operated PLCβ1 activity, we transfected primary cultured VSMCs with GFP-PLCδ1-PH, a fluorescent biosensor with a high affinity for PIP_2 and $InsP_3$ (Balla & Vamai, 2009; Szentpetery *et al*, 2009), and measured signal changes (measured as relative fluorescent units), at the PM (Fm) and within the cytosol (Fc) as previously described (Shi *et al*, 2016).

In un-stimulated VSMCs, containing scrambled or STIM1 shRNAs, GFP-PLCδ1-PH signals had a mean Fm/Fc ratio of about 7, reflecting a predominant location of PIP_2 at the PM (Figure 4A). In scrambled shRNA VSMCs, bath application of BAPTA-AM produced a significant reduction in the mean Fm/Fc ratio. This GFP-PLCδ1-PH signal change represent PLCβ1 activity stimulated by store depletion causing PIP_2 hydrolysis at the PM and subsequent generation of cytosolic $InsP_3$ as previously described (Balla & Vamai, 2009; Szentpetery *et al*, 2009; Shi *et al*, 2016). In further agreement with our earlier study (Shi *et al*, 2016), BAPTA-AM-induced GFP-PLCδ1-PH signal changes were inhibited by co-application the PLC inhibitor U73122 (Figure 4A). Similar effects on mean Fm/Fc ratio were also produced with CPA and TPEN (data not shown).

Knockdown of STIM1 with shRNA1 and shRNA2 sequences greatly reduced translocation of GFP-PLCδ1-PH signals induced by BAPTA-AM (Figures 4B & C), and CPA and TPEN (data not shown) indicating that STIM1 is the likely mediator that stimulates PLC activity when stores are depleted by BAPTA-AM, CPA or TPEN.

Stimulation of endogenously expressed α1 Gαq-coupled adrenoreceptors by bath application of noradrenaline induced translocation of GFP-PLCδ1-PH signals from the PM to the cytosol in the presence of STIM1 shRNA1 and 2 (Figure 4B & C). These results indicate that STIM1-independent pathways have a dominant role in stimulating PLC activity by this concentration of noradrenaline, and that knockout of STIM1 *per se* does not prevent activation of PLC. These findings do not exclude the possibility that noradrenaline-stimulated STIM1 activity produces a small contribution to overall PLC activity, which is sufficient to activate TRPC1-based SOCs (see Figure 11).

As our findings indicate that STIM1 is obligatory for activation of TRPC1-based SOCs and store-operated PLC activity but it is not required for PLC activity *per se*, we questioned whether TRPC1 is also essential for coupling store depletion to stimulation of PLC activity. In VSMCs from WT mice, BAPTA-AM evoked similar translocation of GFP-PLCδ1-PH signals from the PM to the cytosol to those in rabbit VSMCs (see Figure 4A), and these signal changes were also reduced by co-application of U73122 (Figure 5A). In contrast, BAPTA-AM failed to alter the cellular distribution of GFP-PLCδ1-PH signals in $TRPC1^{-/-}$ VSMCs, although noradrenaline was capable of evoking substantial translocation of GFP-PLCδ1-PH signals (Figure 5B). Similar effects between WT and $TRPC1^{-/-}$ mice were produced with CPA and TPEN (data not shown). These data suggest that TRPC1, similar

to STIM1, is an important determinant for store-operated PLC activity but it is not an absolute requirement for Gαq-coupled receptor-stimulated PLC activity.

Store depletion induces interactions between STIM1, TRPC1, Gαq, and PLCβ1

The current work proposes that both STIM1 and TRPC1 are required for store depletion to stimulate PLCβ1 activity, and therefore we hypothesised that store depletion is likely to induce associations between STIM1 and TRPC1 at, or near, the PM.

In freshly isolated un-stimulated VSMCs, immunocytochemical studies showed that STIM1 staining (red) was mainly located within the cytosol, TRPC1 staining (green) was predominantly found at the PM, and there were sparse apparent regions of co-localisation (Figure 6A). Pre-treatment with BAPTA-AM caused translocation of STIM1 signals from the cytosol to the PM, and also stimulated co-localisations between TRPC1 and STIM1 (yellow) at puncta-like sites (Figure 6B). Moreover, proximity ligation assays (PLA) showed no apparent signals between STIM1 and TRPC1 in un-stimulated VSMCs whereas robust fluorescent signals (red) were found at the PM following pre-treatment with BAPTA-AM (Figures 6C & D). These findings clearly indicate that store depletion stimulates formation of STIM1-TRPC1 complexes at the PM. Interestingly, in TRPC1$^{-/-}$ VSMCs, BAPTA-AM caused the translocation of STIM1 signals to the PM which had a relatively even distribution without obvious discrete puncta-like formation (Figure 6E).

Since Gαq/PLCβ1 activity is obligatory for TRPC1 channel activation in VSMCs (Shi *et al*, 2016), we next proposed that store-operated STIM1-TRPC1 complexes are also likely to encompass Gαq and PLCβ1. In un-stimulated primary cultured VSMCs expressing scrambled shRNA, co-immunoprecipitation studies showed that TRPC1 did not associate with STIM1, Gαq, or PLCβ1 (Figure 7A). However, pre-treatment with BAPTA-AM induced significant interactions between these molecules (Figure 7A). Similar interactions between TRPC1 and STIM1 were also obtained with CPA (data not shown). Knockdown of STIM1 with STIM1 shRNA1 significantly decreased BAPTA-AM-stimulated associations between TRPC1 and STIM1, Gαq, and PLCβ1 (Figures 7A & B). Correspondingly, BAPTA-AM-activated interactions between STIM1 and TRPC1, Gαq, and PLCβ1 measured using co-immunoprecipitation (Figure 7D) and PLA (Figure 8) were present in WT but absent in TRPC1$^{-/-}$ cell lysates.

In control experiments, BAPTA-AM and CPA did not alter expression levels of TRPC1, STIM1, Gαq, and PLCβ1 (Shi *et al*, 2016), TRPC1 expression was not altered in the presence of shRNA STIM1, and STIM1 expression was not changed in WT and TRPC1$^{-/-}$ vessel lysates (data not shown).

TRPC6 subunits form receptor-operated Ca^{2+}-permeable cation channels in VSMCs which are not activated by store depletion (Abramowitz & Birnbaumer, 2009; Large *et al*, 2009; Albert, 2011; Earley & Brayden, 2015). In accordance with these findings, we found that TRPC6 proteins were not associated with STIM1 at rest or following pre-treatment with BAPTA-AM in vessel lysates (Figure 7C).

Taken together, these results suggest that store-operated formation of STIM1-TRPC1 complexes is required before interactions with Gαq and PLCβ1 occur. To further explore this idea, we studied the effect of decreasing expression of PLCβ1 on store-operated interactions between TRPC1 and STIM1. In VSMCs expressing scrambled shRNA, PLA studies showed that BAPTA-AM induced interactions between TRPC1 and PLCβ1, and STIM1 (Figures 9A & C). However, in the presence of PLCβ1 shRNA1 and 2, BAPTA-AM-evoked interactions between TRPC1 and PLCβ1 were greatly reduced whereas association between TRPC1 and STIM1 remained unaffected (Figures 9B & C). Similar results following expression of PLCβ1 shRNA1 and 2 were obtained for BAPTA-AM-induced interactions between TRPC1 and PLCβ1, and STIM1 using co-immunoprecipitation (Figures 9D & E). In control experiments, PLCβ1 shRNA1 and 2 did not alter expression of TRPC1, Gαq, and STIM1 (data not shown).

STIM1 acts as a cell surface transmembrane protein to activate TRPC1-based SOCs

In the present study, we have shown that application of the N-terminal EF hand STIM1 antibody to the extracellular surface of VSMCs inhibited store-operated TRPC1-based SOCs (Figure 2B). This raises the possibility that store depletion may induce translocation of STIM1 from the intracellular compartment to the PM where it acts a transmembrane protein to interact with TRPC1 and stimulate PLCβ1 activity. To further investigate this idea, we compared the effect of the N-terminal STIM1 antibody with an antibody raised against a C-terminal region of STIM1 (Prakriya & Lewis, 2015), on activation of TRPC1-based SOCs and on store-operated translocation of STIM1 from the cytoplasm to the PM.

In freshly isolated WT VSMCs, bath application of the N-terminal STIM1 antibody greatly reduced the mean amplitude of store-operated whole-cell currents from 4.6 ± 0.8pA/pF to 1.6 ± 0.4pA/pF (n=6) at -80mV (also see Figure 2B), but had little effect on BAPTA-AM-evoked single channel activity when bath applied to the cytosolic surface of inside-out patches (Figures 10A & B). In contrast, bath application of the C-terminal STIM1 antibody had little effect on store-operated whole-cell currents but produced pronounced inhibition of the mean NP_o value of BAPTA-AM-evoked single channel activity from 0.63 ± 0.05 to 0.13 ± 0.02 (n=6) at -80mV when applied to the cytosolic surface of inside-out patches (Figures 10A & B). Immunocytochemical studies also revealed differential effects of N-terminal and C-terminal STIM1 antibodies on STIM1 staining according to whether cells were permeabilised with triton. In un-stimulated VSMCs, N-terminal and C-terminal antibodies only produced staining for STIM1 when cells were treated with triton (Figures 10C & D). However, BAPTA-AM-evoked translocation of STIM1 signals to the PM were recorded using the N-terminal STIM1 antibody in both triton and non-triton treated VSMCs, whereas BAPTA-AM-evoked STIM1 translocation to the PM was only recorded with the C-terminal STIM1 antibody in cells pre-treated with triton (Figures 10C & D).

These findings further indicate that in resting cells STIM1 is likely to be found within the cell not the PM. Furthermore, activation of TRPC1-based SOCs by STIM1 may involve store-operated STIM1 translocation to the PM where it acts as a transmembrane protein with its N-terminal region exposed to the extracellular environment and its C-terminal remaining within the cytosol. It is perhaps in this configuration that TRPC1-STIM1 interactions stimulate PLC activity.

Noradrenaline-evoked TRPC1 activation requires STIM1

Our results clearly demonstrate that STIM1 is critical for stimulation of native TRPC1 channels by agents that deplete internal Ca^{2+} stores. In our final series of experiments we investigated if STIM1 is involved in activation of TRPC1 channels by the vasoconstrictor noradrenaline. In WT VSMCs expressing scrambled shRNA, bath application of noradrenaline activated 2pS TRPC1 channel activity in cell-attached patches held at -80 mV in a concentration-dependent manner as previously described (Shi *et al*, 2016). In the presence of STIM1 shRNAs, stimulation of TRPC1 channel activities by concentrations of noradrenaline above 1μM were reduced by over 80% (Figures 11A & B). In PLA experiments, noradrenaline induced interactions between STIM1 and TRPC1, Gαq, and

PLCβ1 (Figure 11C). These results strongly suggest that STIM1 is required for activation of native TRPC1 channel activity by noradrenaline.

Discussion

The present work reveals that in contractile VSMCs depletion of intracellular Ca^{2+} stores causes STIM1 to translocate from within the cell to the PM where it interacts with TRPC1 to induce channel opening through stimulation of Gαq-evoked PLCβ1 activity. In other cell types, STIM1 has been proposed to combine directly with TRPC-based SOCs and Orai1-based CRACs to cause channel opening (Worley *et al*, 2007; Yuan *et al*, 2009; Lee *et al*, 2014; Asannov *et al*, 2015; Prakriya & Lewis, 2015), and therefore these findings represent a novel activation pathway of TRPC1-based SOCs and a previously unrecognised role for STIM1.

TRPC1-based SOCs require STIM1 for activation in contractile VSMCs

We show that several well-established store depletion protocols activate whole-cell conductances with a relatively linear I/V relationship and an E_{rev} of about +20 mV and single channel currents with a unitary conductance of about 2pS, which are inhibited by TRPC1 antibodies and absent in $TRPC1^{-/-}$ VSMCs. Knockdown of STIM1 using two STIM1 shRNA sequences, and two antibodies raised against N- and C-terminal regions of ER/SR Ca^{2+} store sensor STIM1 also produced pronounced inhibition of these store-operated whole-cell and single channel currents. It is likely that these antibodies have a selective action as they identify a protein band with a molecular weight corresponding to STIM1, which has a reduced density in the presence of shRNA STIM1. In addition, the N-terminal STIM1 antibody has been shown to reduce STIM1-evoked I_{crac} (Spassova *et al*, 2006). Knockdown of STIM1 also reduced store-operated PKC phosphorylation of TRPC1, which is a known pivotal event for activation of TRPC1-based SOCs in contractile VSMCs (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012). These findings confirm earlier reports that native TRPC1-based SOCs are expressed in contractile VSMCs (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012), and highlight for the first time that STIM1 as an obligatory molecule in the PKC-mediated gating pathway of these channels.

Store-operated STIM-TRPC1 interactions stimulate PLCβ1 activity

There is substantial evidence that PKC activity is essential for stimulation of TRPC1-based SOCs in contractile VSMCs (Saleh *et al*, 2008; Albert *et al*, 2009; Large *et al*, 2009; Albert 2011; Shi *et al*, 2012), and in a recent study we reported that store depletion stimulates Gαq-evoked PLCβ1 activity to drive PKC activity and channel opening (Shi *et al*, 2016). We

656 therefore hypothesised that as STIM1 is an ER/SR Ca^{2+} sensor, its role in activating TRPC1-
657 based SOCs may be through coupling store depletion to stimulation of Gαq/PLCβ1 activity.
658
659 Store-operated PLC activity recorded using the $PIP_2/InsP_3$ biosensor GFP-PLCδ1-PH (Balla
660 & Vamai, 2009; Szentpetery *et al*, 2009; Shi *et al*, 2016) was inhibited by knockdown of
661 STIM1, and was absent in $TRPC1^{-/-}$ VSMCs, which indicates that both STIM1 and TRPC1
662 are required to stimulate PLC activity. Immunocytochemical and PLA studies identified that
663 store depletion stimulated the translocation of STIM1 from within the cell to the PM, where
664 it formed STIM1-TRPC1 complexes with puncta-like distributions. The discrete pattern of
665 STIM1-TRPC1 complex distribution seemed to be dependent on TRPC1 as relatively even,
666 non-puncta-like STIM1 staining was produced by store depletion in $TRPC1^{-/-}$ cells. In further
667 agreement with STIM1-TRPC1 complexes stimulating Gαq/PLCβ1 activity, co-
668 immunoprecipitation studies revealed that store depletion activate associations between
669 TRPC1 and Gαq, and PLCβ1 and also between STIM1 and Gαq, and PLCβ1, with these
670 interactions requiring both STIM1 and TRPC1. Knockdown of PLCβ1 did not affect formation
671 of store-operated STIM1-TRPC1 interactions, which further implies that STIM1-TRPC1
672 interactions occur before Gαq/PLCβ1 binding. It is not yet known if store-operated STIM1-
673 TRPC1 interactions require Gαq subunits, and if association and stimulation of Gαq and
674 PLCβ1 activities occur with STIM1-TRPC1 interactions in a sequential pathway.
675
676 Store depletion failed to induce interactions between STIM1 and TRPC6, which forms non-
677 TRPC1 containing receptor-operated Ca^{2+}-permeable channels in VSMCs that are not
678 activated by store depletion (Large et al, 2009; Albert, 2011). This agrees with over-
679 expression studies showing that store depletion activates interactions between STIM1 and
680 TRPC1-, TRPC4-, and TRPC5-based SOCs, but does not stimulate interactions between
681 STIM1 and TRPC3, TRPC6, and TRPC7 that are not activated by store depletion (Worley
682 *et al*, 2007). Thus interactions with STIM1 are likely to be key in determining whether TRPC
683 channels are activated by store depletion.
684
685 STIM1 has diverse cellular partners including ion channels such as Orai1 channels (Prakriya
686 & Lewis, 2015), TRPC channels (Cheng *et al*, 2013), voltage-gated Ca^{2+} channels (Park *et*
687 *al*, 2010; Wang *et al*, 2010), SR and PM Ca^{2+}-ATPases (Jousset *et al*, 2007; Ritchie *et al*,
688 2012), and adenylyl cyclases (Lefkimmiatis *et al*, 2009). The present study makes an
689 important addition to this list; store-operated formation of STIM1-TRPC1 complexes which
690 stimulate Gαq/PLCβ1 activity.

STIM1 acts at the PM to activate TRPC1 channels

It is not clear how store-operated STIM1-TRPC1 interactions induce Gαq/PLCβ1 activity. Over-expression studies generally indicate that activation of Orai1-based CRACs and TRPC-based SOCs by STIM1 involves movement of the ER membrane containing activated STIM1 towards the PM, where junction-like complexes are formed and STIM1 binds to intracellular domains of channel proteins to assemble Orai1-based CRACs by protein-protein interactions (Prakriya & Lewis, 2015), and directly gate TRPC1-based SOCs using electrostatic and protein-protein interactions (Worley *et al*, 2007; Yuan *et al*, 2009; Lee *et al*, 2014; Asannov *et al*, 2015). Whereas electrostatic gating is unique to TRPC1, protein-protein interactions between STIM1 and CRAC and STIM1 and TRPC1 channels involve the same 100 amino acid stretch of STIM1 referred to as SOAR or CAD (Asannov et al, 2015). STIM1 has also been suggested to act at, or within, the PM to activate Orai1-based CRACs (Spassova *et al*, 2006; Hewavitharana *et al*, 2008), and constitutively active STIM1 present in the PM is proposed to be required for activation of arachidonic acid-regulated Ca^{2+} (ARC) channels composed of Orai1 and Orai3 subunits (Thomson & Shuttleworth, 2013).

Our findings of differential inhibitory actions of N- and C-terminal STIM1 antibodies on activation of TRPC1-based SOCs suggest that in contractile VSMCs store-operated STIM1 is translocated from the SR into the PM. Here it is likely to continue to act as a transmembrane protein through its proposed transmembrane domain located between amino acids 214-234 (Soboloff *et al*, 2012), with its N-terminal region presented to the extracellular environment and its C-terminal region remaining within the cell. It is in this configuration that STIM1 is likely to form complexes with TRPC1 which allow subsequent binding and stimulation of Gαq/PLCβ1 activity. These conclusions indicate that STIM1 involved in interacting with TRPC1 and stimulating Gαq/PLCβ1 activity is derived from within the cell, and is not part of STIM1 pools constitutively present in the PM. Future experiments are needed to examinine if previously identified interactions sites between STIM1 and TRPC1 (Worley *et al*, 2007; Yuan *et al*, 2009; Lee *et al*, 2014; Asannov *et al*, 2015) are involved in association with Gαq and PLCβ1. Understanding associations between STIM1-TRPC1 and Gαq subunits are particularly important as these are likely to be the trigger for initiating PLCβ1 activity that drives channel gating.

Are Orai proteins involved in the composition and activation of TRPC1-based SOCs in contractile VSMCs?

There is debate on whether functional TRPC-based SOCs require involvement of Orai proteins, which may act as pore-forming subunits or obligatory accessory proteins, or may mediate Ca^{2+} signals that regulate TRPC expression at the PM (Cheng et al, 2011; Cheng et al, 2013; Liao *et al,* 2014; Prakriya & Lewis, 2015). In synthetic, non-contractile VSMCs, store depletion stimulates Ca^{2+} entry involving both TRPC1 and Orai1, and also activates a conductance with I_{crac}-like properties (Li *et al*, 2008; Ng *et al*, 2009, 2010; Li *et al*, 2011; Beech, 2012; Trebak, 2012). In contractile VSMCs, the current study failed to detect store-operated conductances with characteristics of Orai1-, Orai2-, and Orai3-based SOCs such as strong inward rectification and a E_{rev} >+50mV (Lis et al, 2007; Prakriya & Lewis, 2015) in $TRPC1^{-/-}$ VSMCs, and in WT cells when TRPC1-based SOCs were inhibited with a TRPC1 antibody. This indicates that Orai proteins are unlikely to contribute to activation of TRPC1-based SOCs in contractile VSMCs, which is in agreement with a low level of Orai protein expression in these cells (Berra-Romani *et al*, 2008; Trebak, 2012). In a wider context, this present work provides further evidence that cells express multiple SOCs composed of TRPC-based SOCs and Orai-based CRACs, which can exist independently of one another.

It is possible that Orai-based CRACs are present in contractile VSMCs but that opening of these channels produces small irresolvable conductances using electrophysiological techniques. An alternative approach may be to investigate whether Orai-based Ca^{2+} sparklets are present using total internal reflection fluorescence microscopy, which can detect Ca^{2+} entry through opening of Ca^{2+}-permeable channels at localised regions of the PM (Sonkusare *et al*, 2014). It is clear that a detailed comparison on the role of Orai proteins in activation of native TRPC1-based SOCs in contractile and synthetic VSMCs is needed.

Physiological importance of STIM1-activated TRPC1 channels

TRPC1 channel activity induced by the endogenous α1 adrenergic Gαq-coupled receptor agonist and vasoconstrictor noradrenaline was prevented by knockdown of STIM1. Noradrenaline also evoked interactions between TRPC1 and STIM1, STIM1 and Gαq, and STIM1 and PLCβ1 at the PM. These results indicate that STIM1 is important for activation of TRPC1 channels by a physiologically agonist, and suggests that this may involve a similar activation pathway induced by agents that deplete Ca^{2+} stores. This is further highlighted by previous data showing that noradrenaline-evoked TRPC1 channel activity is also inhibited by knockdown of PLCβ1 (Shi *et al*, 2016).

Physiological vasoconstrictors acting through Gαq-coupled receptors stimulate PLC, leading to $InsP_3$-mediated depletion of Ca^{2+} stores and DAG-mediated PKC activation. It therefore might be expected that the store-operated STIM1/PLCβ1/PKC pathway described in the present study represents a feed forward mechanism to induce TRPC1 channel opening. However, our data indicate that STIM1 and PLCβ1 contribute little to overall PLC activation by noradrenaline (see Figures 4B & C, and Figure 5B, and Shi *et al*, 2016). This may suggest that the STIM1/PLCβ1/PKC pathway involved in activating TRPC channels is uniquely stimulated following store depletion. To confirm these ideas it will be important to determine which PLC isoform is coupled to stimulation of α1 Gαq-coupled adrenoreceptors, and to also identify if Gαq receptor/PLC-coupled DAG generation activates a different PKC isoform from that induced by store-operated PLCβ1 activity. Moreover, these ideas should be tested using different vasoconstrictor agents such as angiotensin II and endothelin-1 which are known to stimulate TRPC1-based SOCs (Albert *et al*, 2009; Large *et al*, 2009).

Conclusion

This study describes a novel activation pathway of TRPC1-based SOCs in native contractile VSMCs which is depicted in Figure 12. Store depletion stimulates translocation of STIM1 from the SR to the PM where it is inserted as a transmembrane protein and forms STIM1-TRPC1 complexes, which subsequently bind and stimulation of Gαq/PLCβ1 activities. This increase in PLCβ1 activity stimulates channel opening through DAG-evoked PKC phosphorylation of TRPC1.

Additional Interests

Competing interests

None declared

Author Contributions

All authors approved final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. APA, WAL, LB, JS, FM contributed to the conception or design of the work, analysis of data or interpretation of data for the work, and were involved in drafting the work or revising it critically for important intellectual content. JS was involved in acquisition of data.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BB/J007226/1 and BB/M018350/1 to APA) and was also supported in part by the NIH Intramural Research Program (Project Z01-ES-101684 to L.B).

Acknowledgements

APA was funded by BBSRC and BHF. LB was funded by NIH.

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Zhang SL, Yu Y, Roos J, Kozack A, Deerinck TJ, Ellisman MH, Stauderman KA & Cahalan MD (2005). STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* **437**, 902-905.

Figure Legends

Figure 1.

TRPC1 compose SOCs in contractile VSMCs. A, Development of a store-operated whole-cell current in a mesenteric artery VSMC from a WT mouse following break-in into the whole-cell configuration (w.c.) was inhibited by bath application of T1E3. Vertical deflections represent currents evoked by voltage ramps from +100mV to -150mV (750 ms duration) every 30s from a holding potential of 0 mV. Development of a store-operated whole-cell TRPC1 current was absent in a $TRPC1^{-/-}$ VMSC. Mean I/V relationships of store-operated whole-cell currents demonstrate that T1E3 reduced store-operated whole-cell currents at all membrane potentials tested in WT VSMCs and that store-operated currents were absent in $TRPC1^{-/-}$ VSMCs (each point is at least n=6). B, BAPTA-AM evoked single channel activity in a cell-attached patch (c/a) held at -80mV from a WT VSMC was maintained following patch excision into the inside-out configuration (i/o). Bath application of an intracellular acting anti-TRPC1 antibody to the cytosolic surface of the inside-out patch inhibited BAPTA-evoked channel activity. BAPTA-AM failed to activate channel activity in a cell-attached held at -80 mV from a $TRPC1^{-/-}$ VSMC. Mean data of the inhibitory effect of the anti-TRPC1 antibody on BAPTA-evoked channel activity. Note that channel activities were maintained on changing from cell-attached to inside-out patch configurations (n=7, ***P<0.001).

Figure 2.

TRPC1-based SOCs are dependent on STIM1. A, Western blots and mean data confirm that 2 different STIM1 shRNA sequences (shRNA1 and shRNA2) reduced STIM1 expression (n=3 primary rabbit portal vein VSMC culture preparations, **P<0.01). B, Representative traces and mean I/V relationships showing that peak amplitude of store-operated whole-cell TRPC1-based currents were greatly reduced at all membrane potentials tested following transduction of rabbit portal vein VSMCs with shRNA sequences compared to scrambled shRNA sequences. In the presence of scrambled sequences, store-operated TRPC1-based currents were inhibited by an anti-STIM1 antibody (n=6). C, Representative recordings and mean data showing that BAPTA-AM-evoked TRPC1-based SOCs were reduced by shRNA sequences targeting STIM1 compared to scrambled shRNA in VSMCs (n=6, ***P<0.001).

Figure 3.

Store-operated phosphorylation of TRPC1 proteins requires STIM1. A, Co-immunoprecipitation of rabbit portal vein tissue lysates with anti-phosphorylated serine (pSer) and threonine (pThr) antibodies followed by Western blotting (WB) with an anti-TRPC1 antibody shows that basal TRPC1 phosphorylation is increased by pre-treatment with BAPTA-AM for 10min, and that this increase was reduced by STIM1 shRNA and co-application of GF109203X. B, Mean relative band densities normalised to BAPTA-AM bands (n=3 different tissue lysate preparations, *P<0.05, ** P<0.01).

Figure 4.

Store-operated PLC activity is mediated by STIM1. A, Representative image from a single rabbit portal vein VSMC showing that in control conditions the location of GFP-PLCδ1-PH-mediated signals (measured as relative fluorescence units (RFU)) was predominantly expressed at the PM (black). In the same cell, pre-treatment with BAPTA-AM for 10min induced translocation of signals to the cytosol (blue), and co-application of U73122 for 5min reversed these cytosolic signals back to the PM (orange). Graphs of RFU of line scans for the region denoted by white dotted lines show GFP-PLCδ1-PH signals across the cell width. Mean Fm/Fc ratios of GFP-PLCδ1-PH-mediated signals represent n=20 cells from 3 different experiments (***P<0.001). B & C, Representative images and mean data show that transduction of rabbit portal vein VSMCs with either STIM1 shRNA1 or shRNA2 sequences prevented BAPTA-AM inducing translocation of GFP-PLCδ1-PH signals to the cytosol. In both these conditions, application of noradrenaline for 5min (red, applied in the presence of 1μM wortmannin to prevent cell contraction) was still able to induce translocation of GFP-PLCδ1-PH signals from the PM to the cytosol (n=20 cells for each STIM1 shRNA sequence from 3 different experiments, ***P<0.01).

Figure 5.

Store-operated PLC activity requires TRPC1. A, In WT mesenteric artery VSMCs, application of BAPTA-AM for 10min induced translocation of GFP-PLCδ1-PH-mediated signals from the PM to the cytosol which was prevented by co-application of U73122 for 5min (n=20 cells from 3 different experiments, ***P<0.001). B, In TRPC1$^{-/-}$ VSMCs, BAPTA-AM did not alter the cellular distribution of GFP-PLCδ1-PH signals whereas application of noradrenaline for 5min induced translocation of GFP-PLCδ1-PH signals from the PM to the cytosol (n=20 cells from 3 different experiments, ***P<0.001).

Figure 6.

Store-depletion induces co-localisations between STIM1 and TRPC1 at the PM. A & B, Representative images from two different rabbit portal vein VSMCs showing TRPC1 (green) and STIM1 (red) staining. Changes in the relative fluorescence of TRPC1 and STIM1 staining across the cell width were determined from the line scan (dotted white line). In a resting cell (A), TRPC1 staining was predominantly present at the PM whereas labelling for STIM1 was located within the cytosol. In a cell treated with BAPTA-AM for 10min (B), both TRPC1 and STIM1 were located at the PM in discrete puncta. The inset image shows co-localisation between TRPC1 and STIM1 staining (yellow) at the PM. C & D, Representative PLA images showing BAPTA-AM induced fluorescent signals between STIM1 and TRPC1 which were predominantly at the PM in rabbit portal vein and mice mesenteric artery VSMCs. E, Representative immunocytochemical image showing that in TRPC1$^{-/-}$ mesenteric artery VSMCs, BAPTA-AM induced translocation of STIM1 from the cytosol to the PM where it produced uniform, non-puncta-like staining.

Figure 7.

Store-depletion evokes associations between TRPC1, STIM1, Gαq, and PLCβ1. A, Representative Western blots showing that pre-treatment with BAPTA-AM for 10min induced associations between TRPC1 and STIM1, Gαq, and PLCβ1 which were reduced by STIM1 shRNA1. Primary cultured rabbit portal vein VSMC lysates were initially immunoprecipitated (IP) with anti-TRPC1 antibodies and were then Western blotted (WB) with anti-STIM1, anti-Gαq or anti-PLCβ antibodies. B, Mean data for relative band intensities of BAPTA-AM-evoked interactions with TRPC1 shown in A (n=3 different cell lysates, **P*<0.05). C, Application of BAPTA-AM for 10min did not alter interactions between TRPC6 and STIM1 (left panel) or change expression levels of TRPC6 (right panel) in rabbit portal vein tissue lysates. D, In primary cultured WT mesenteric artery VSMCs, BAPTA-AM evoked interactions between STIM1 and TRPC1, Gαq, and PLCβ1, which were absent in cell lysates from TRPC1$^{-/-}$ VSMCs.

Figure 8.

Store-operated induced interactions between STIM1, Gαq and PLCβ1 require TRPC1. A, PLA images from WT mesenteric artery VSMCs showing that BAPTA-AM induced interactions between STIM and Gαq, and also between STIM1 and PLCβ1. B, BAPTA-AM-

evoked interactions between STIM and Gαq, and STIM1 and PLCβ1 were absent in TRPC1$^{-/-}$ VSMCs.

Figure 9.

Store-operated interactions between TRPC1 and STIM1 do not require PLCβ1. A, B & C, Application of BAPTA-AM for 10min induced interactions between TRPC1 and PLCβ1 in rabbit portal vein VSMCs measured using PLA which were reduced by expression of PLCβ1 shRNA1 and shRNA2 sequences, whereas associations between TRPC1 and STIM1 were unaffected (n=3 different preparations, **P*<0.05). D & E, BAPTA-AM-induced interactions between TRPC1 and PLCβ1 measured using co-immunoprecipitation were reduced by expression of PLCβ1 shRNA1 and shRNA2 sequences, whereas associations between TRPC1 and STIM1 were unaffected (n=3 different rabbit portal vein cell lysates, **P*<0.05).

Figure 10.

Differential actions of antibodies raised against N-terminal and C-terminal regions of STIM1 on activation of TRPC1-based SOCs.

A, Original trace showing that a store-operated whole-cell current from a WT mesenteric artery VSMC was inhibited by bath application of a N-terminal but not a C-terminal STIM1 antibody. B, Representative recording showing that BAPTA-AM-evoked single channel activity in an inside-out patch from a WT VSMC held at -80 mV was inhibited by bath application of a C-terminal but not a N-terminal STIM1 antibody. C, Representative images from two different VSMCs treated with triton, in which both N-terminal and C-terminal STIM1 antibodies WT identified translocation of STIM1 signalling (red) from the cytosol to the PM following treatment with BAPTA-AM for 10min. D, Representative images from two different WT VSMCs not treated with triton, in which N-terminal nor C-terminal STIM1 antibodies identified STIM1 staining in un-stimulated cells, and only the N-terminal antibody revealed STIM1 staining at the PM following treatment with BAPTA-AM.

Figure 11.

Noradrenaline-evoked TRPC1 channel activity is mediated by STIM1. A, Traces showing that bath application of noradrenaline evoked TRPC1 channel activity in a concentration-dependent manner in cell-attached patches from WT mesenteric artery VSMCs held at -80 mV, which were reduced in VSMCs expressing STIM1 shRNA1 and shRNA2 sequences compared to scrambled shRNA. B, Mean data show the inhibitory

actions of STIM1 shRNA1 and shRNA2 on noradrenaline-evoked TRPC1 channel activity (n=at least 6 patches in which every concentration of noradrenaline tested was cumulatively applied, **P<0.01, ***P<0.001). C, Representative PLA images from single WT VSMCs showing that application of noradrenaline for 5min induced fluorescent signals (red), which indicated interactions between TRPC1 and STIM1, STIM1 and Gαq, and STIM1 and PLCβ1 predominantly at the PM.

Figure 12.

Proposed activation model of TRPC1-based SOCs in contractile VSMCs. A, In resting VSMCs, SR stores are filled with Ca^{2+}, TRPC1-based SOCs do not interact with Gαq, PLCβ1 or STIM1, and the channels are in a closed state. B, Following store depletion of the SR, STIM1 proteins (red) are activated and translocated from the SR into the PM, where they interact with TRPC1. Note that following translocation, the N-terminal EF hand of STIM1, which acts as a Ca^{2+} sensor within the SR, is exposed on the external surface of the cell, whilst the C-terminal region is maintained within the cytosol. C, Formation of store-operated STIM1-TRPC1 interactions are able to bind Gαq and PLCβ1 which stimulate PLC activity, leading to PIP_2 hydrolysis, formation of DAG, PKC stimulation, phosphorylation of TRPC1 subunits, and channel opening.

Figure 1

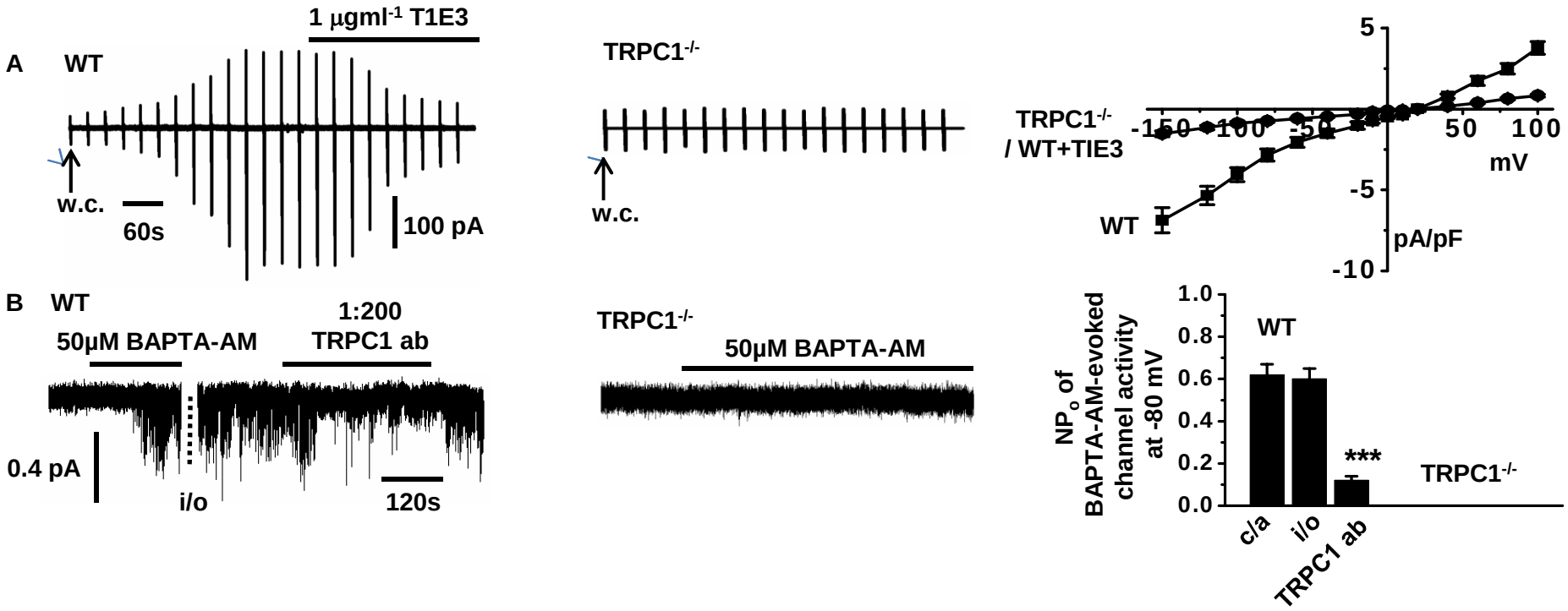

A
WT
1 μgml-1 T1E3
w.c.
60s
100 pA
B
WT
50μM BAPTA-AM
1:200
TRPC1 ab
0.4 pA
i/o
120s
TRPC1-/-
w.c.
TRPC1-/-
50μM BAPTA-AM
TRPC1-/-
/ WT+TIE3
WT
5
-5
-10
-150
-100
-50
50
100
mV
pA/pF
NPo of
BAPTA-AM-evoked
channel activity
at -80 mV
1.0
0.8
0.6
0.4
0.2
0.0
WT
***
TRPC1-/-
c/a
i/o
TRPC1 ab

Figure 2

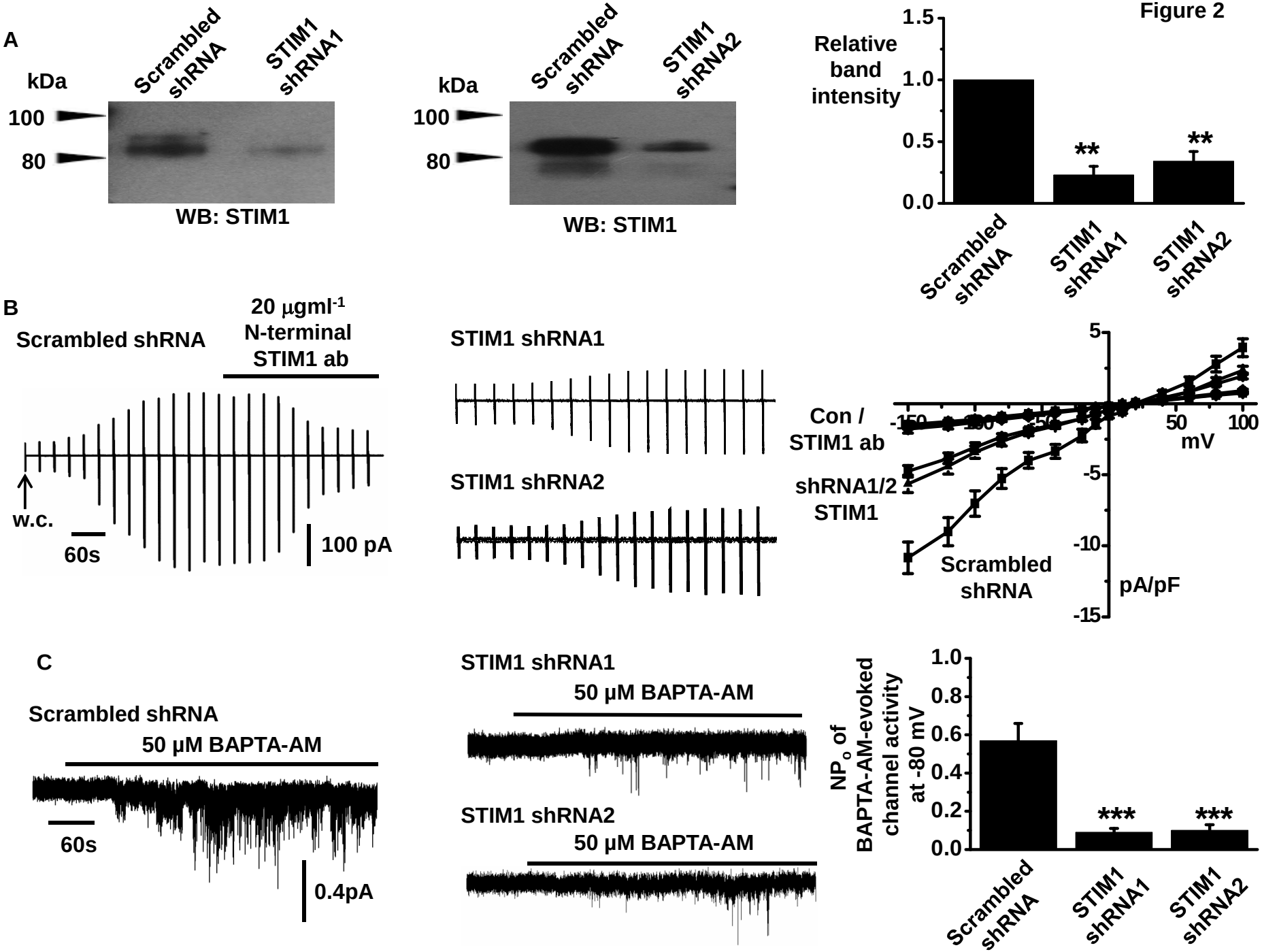
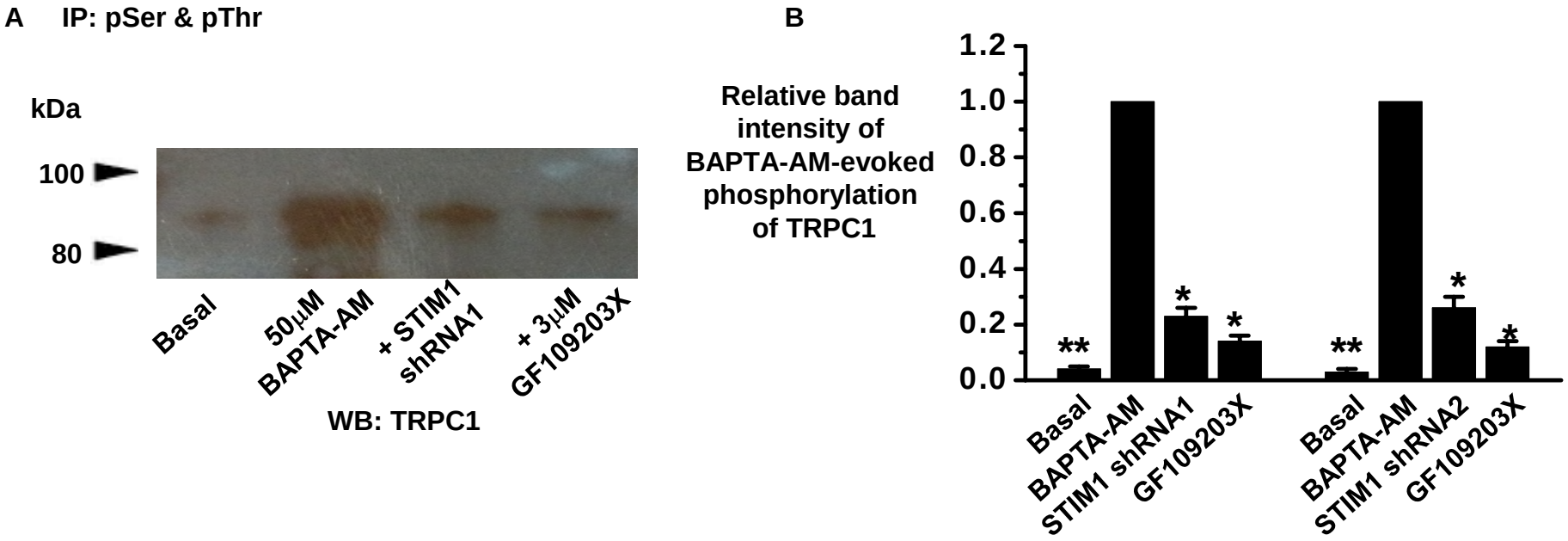

A
kDa
Scrambled shRNA
STIM1 shRNA1
100
80
WB: STIM1
kDa
Scrambled shRNA
STIM1 shRNA2
100
80
WB: STIM1
Relative band intensity
1.5
1.0
0.5
0.0
**
**
Scrambled shRNA
STIM1 shRNA1
STIM1 shRNA2
B
Scrambled shRNA
20 μgml-1 N-terminal STIM1 ab
w.c.
60s
100 pA
STIM1 shRNA1
STIM1 shRNA2
Con / STIM1 ab
shRNA1/2 STIM1
5
-5
-10
-15
50
100
mV
Scrambled shRNA
pA/pF
C
Scrambled shRNA
50 μM BAPTA-AM
60s
0.4pA
STIM1 shRNA1
50 μM BAPTA-AM
STIM1 shRNA2
50 μM BAPTA-AM
NPo of BAPTA-AM-evoked channel activity at -80 mV
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Scrambled shRNA
STIM1 shRNA1
STIM1 shRNA2

Figure 3


A
IP: pSer & pThr
kDa
100
80
Basal
50μM BAPTA-AM
+ STIM1 shRNA1
+ 3μM GF109203X
WB: TRPC1
B
Relative band intensity of BAPTA-AM-evoked phosphorylation of TRPC1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
**
*
*
Basal
BAPTA-AM
STIM1 shRNA1
GF109203X
Basal
BAPTA-AM
STIM1 shRNA2
GF109203X

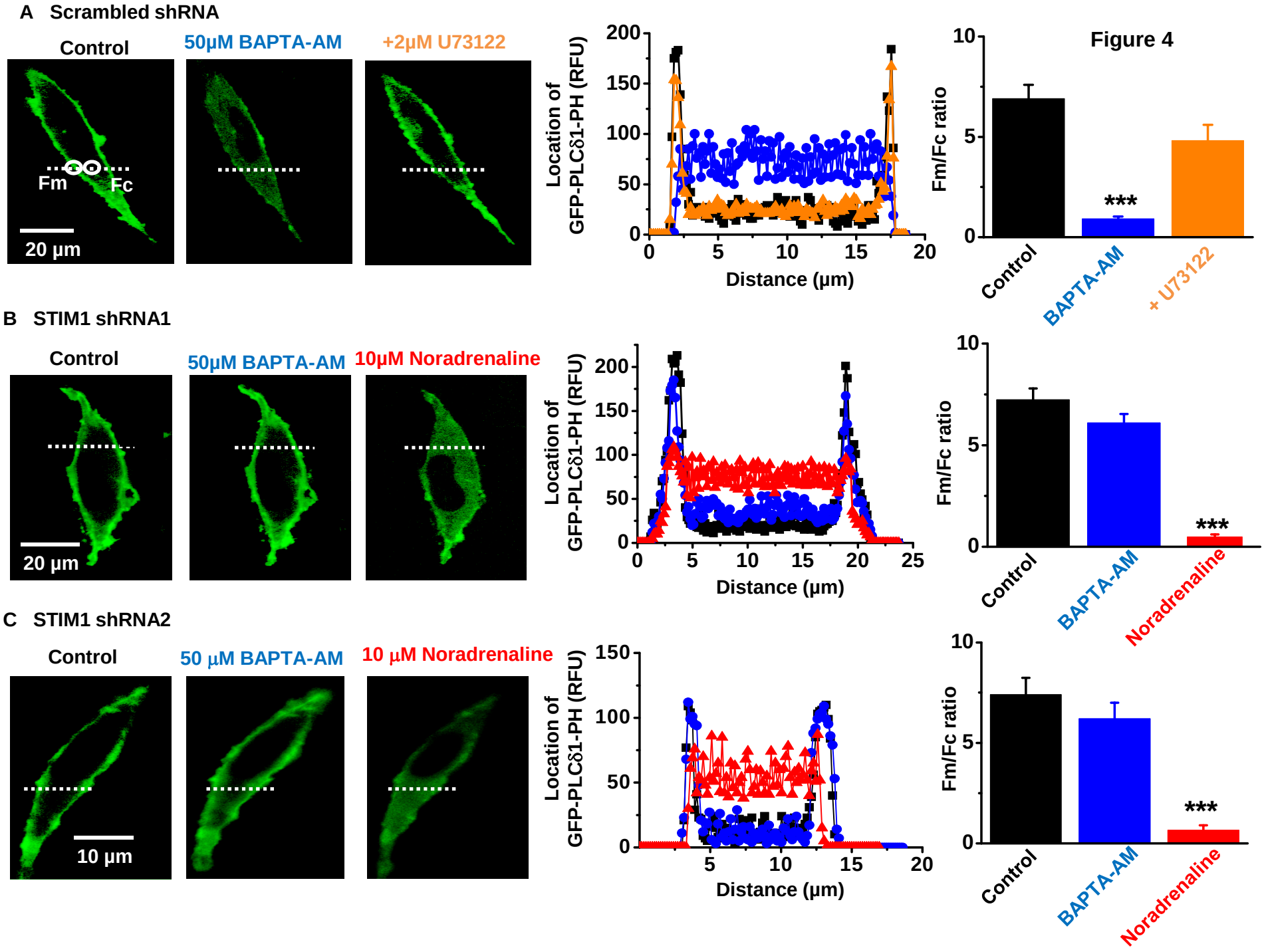
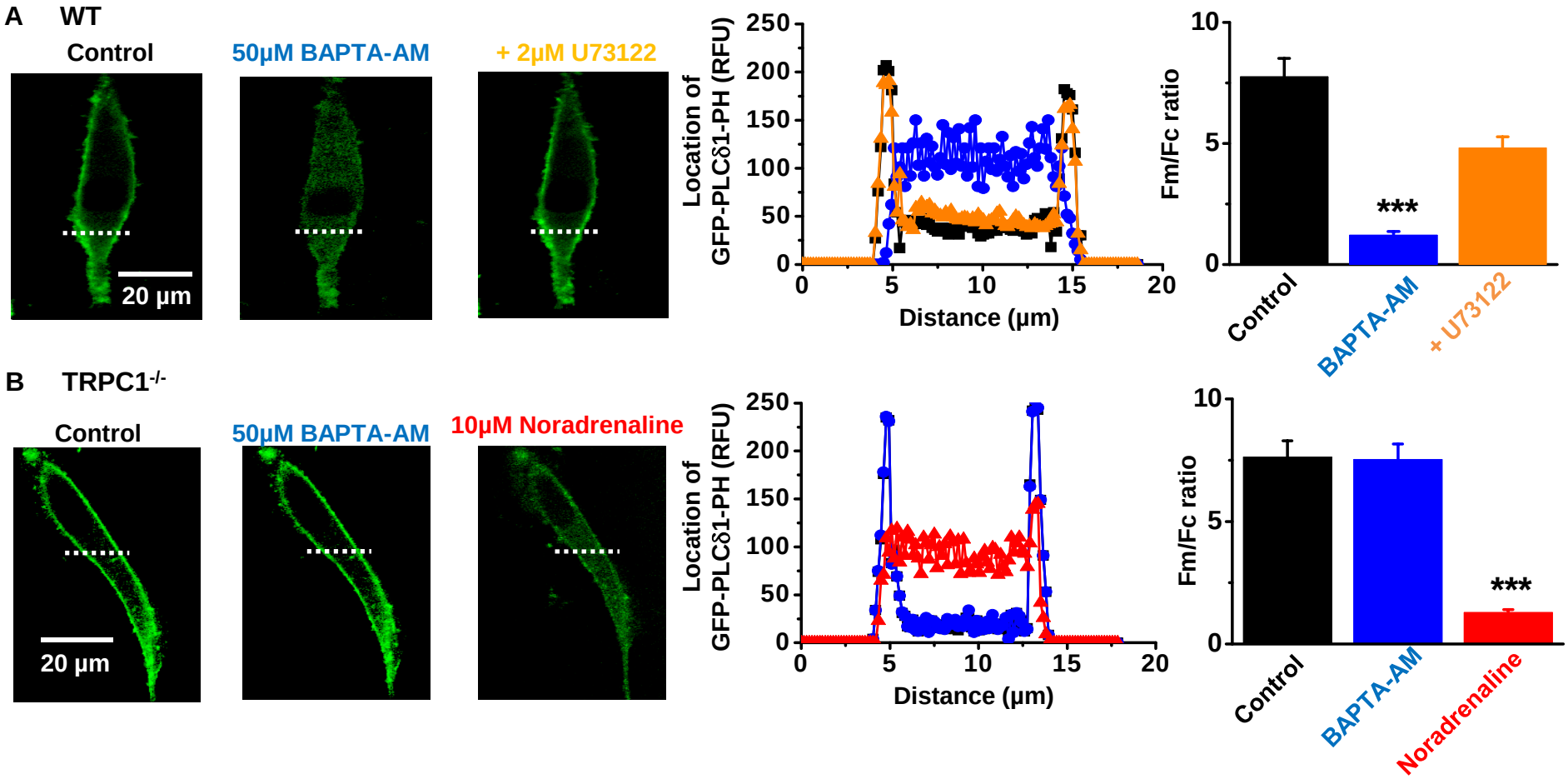

A Scrambled shRNA
Control
50μM BAPTA-AM
+2μM U73122
Fm
Fc
20 μm
Location of GFP-PLCδ1-PH (RFU)
Distance (μm)
Fm/Fc ratio
***
Control
BAPTA-AM
+ U73122
Figure 4
B STIM1 shRNA1
Control
50μM BAPTA-AM
10μM Noradrenaline
20 μm
Noradrenaline
C STIM1 shRNA2
Control
50 μM BAPTA-AM
10 μM Noradrenaline
10 μm

Figure 5


A WT
Control
50μM BAPTA-AM
+ 2μM U73122
20 μm
Location of GFP-PLCδ1-PH (RFU)
Distance (μm)
Fm/Fc ratio
***
Control
BAPTA-AM
+ U73122
B TRPC1-/-
Control
50μM BAPTA-AM
10μM Noradrenaline
20 μm
Location of GFP-PLCδ1-PH (RFU)
Distance (μm)
Fm/Fc ratio
***
Control
BAPTA-AM
Noradrenaline

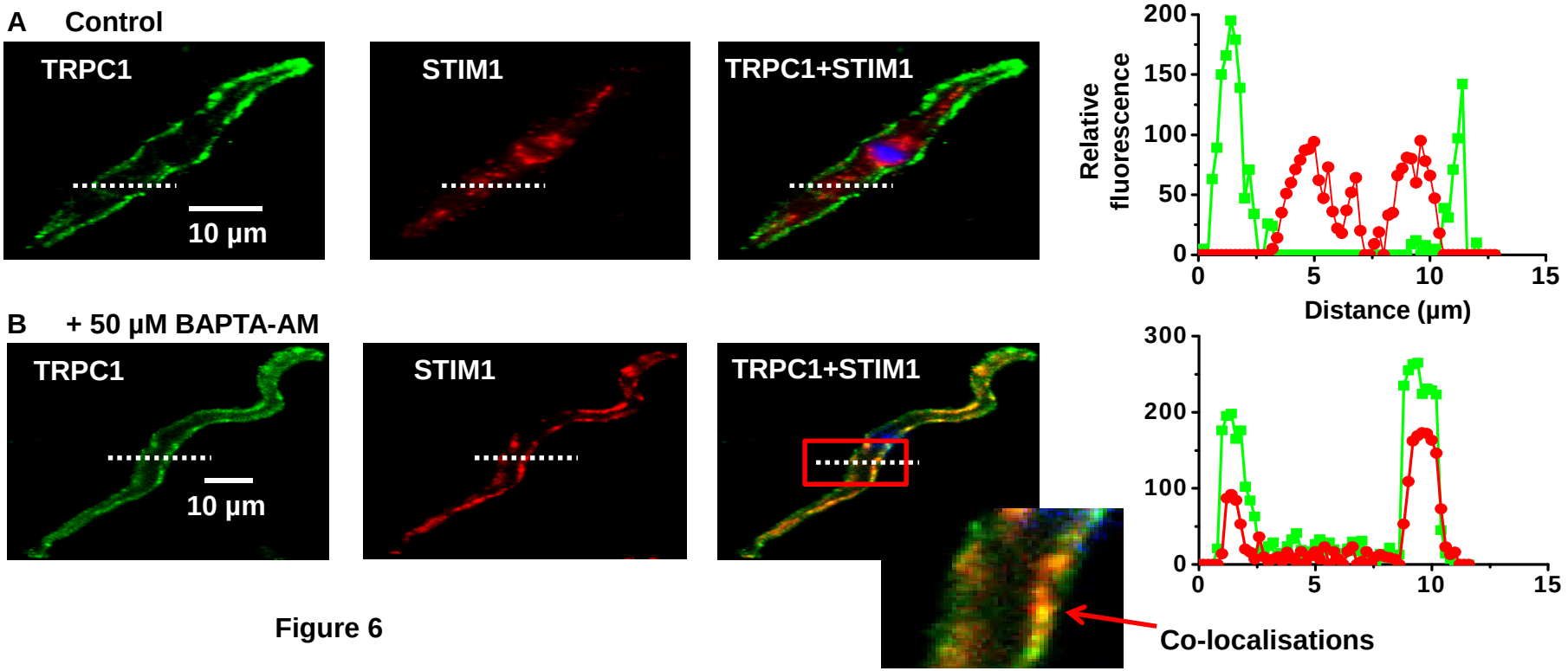
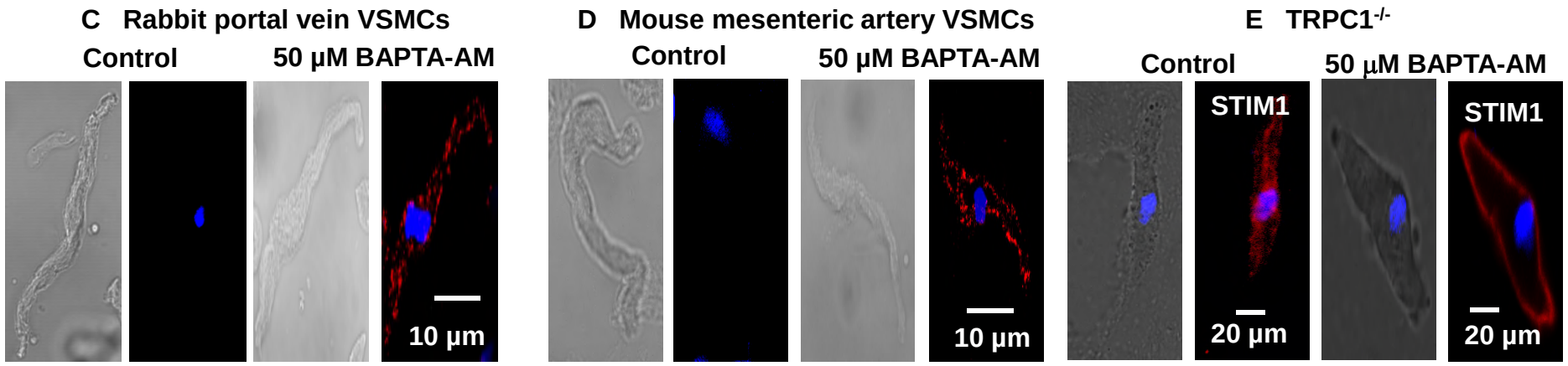

A Control
TRPC1
STIM1
TRPC1+STIM1
10 µm
Relative fluorescence
200
150
100
50
0
0
5
10
15
Distance (µm)
B + 50 µM BAPTA-AM
TRPC1
STIM1
TRPC1+STIM1
10 µm
300
200
100
0
0
5
10
15
Co-localisations

Figure 6


C Rabbit portal vein VSMCs
Control
50 µM BAPTA-AM
10 µm
D Mouse mesenteric artery VSMCs
Control
50 µM BAPTA-AM
10 µm
E TRPC1-/-
Control
50 µM BAPTA-AM
STIM1
20 µm
STIM1
20 µm

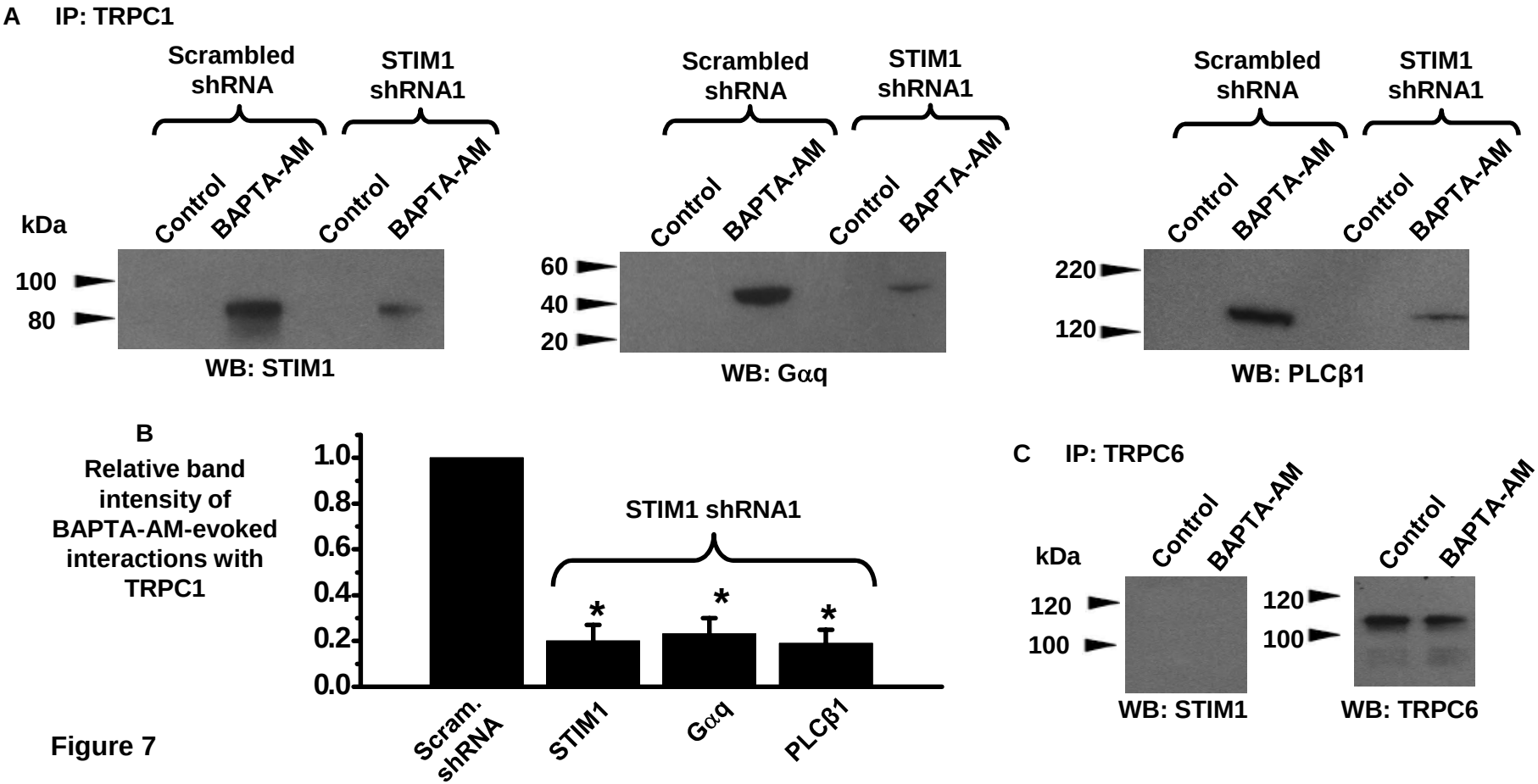
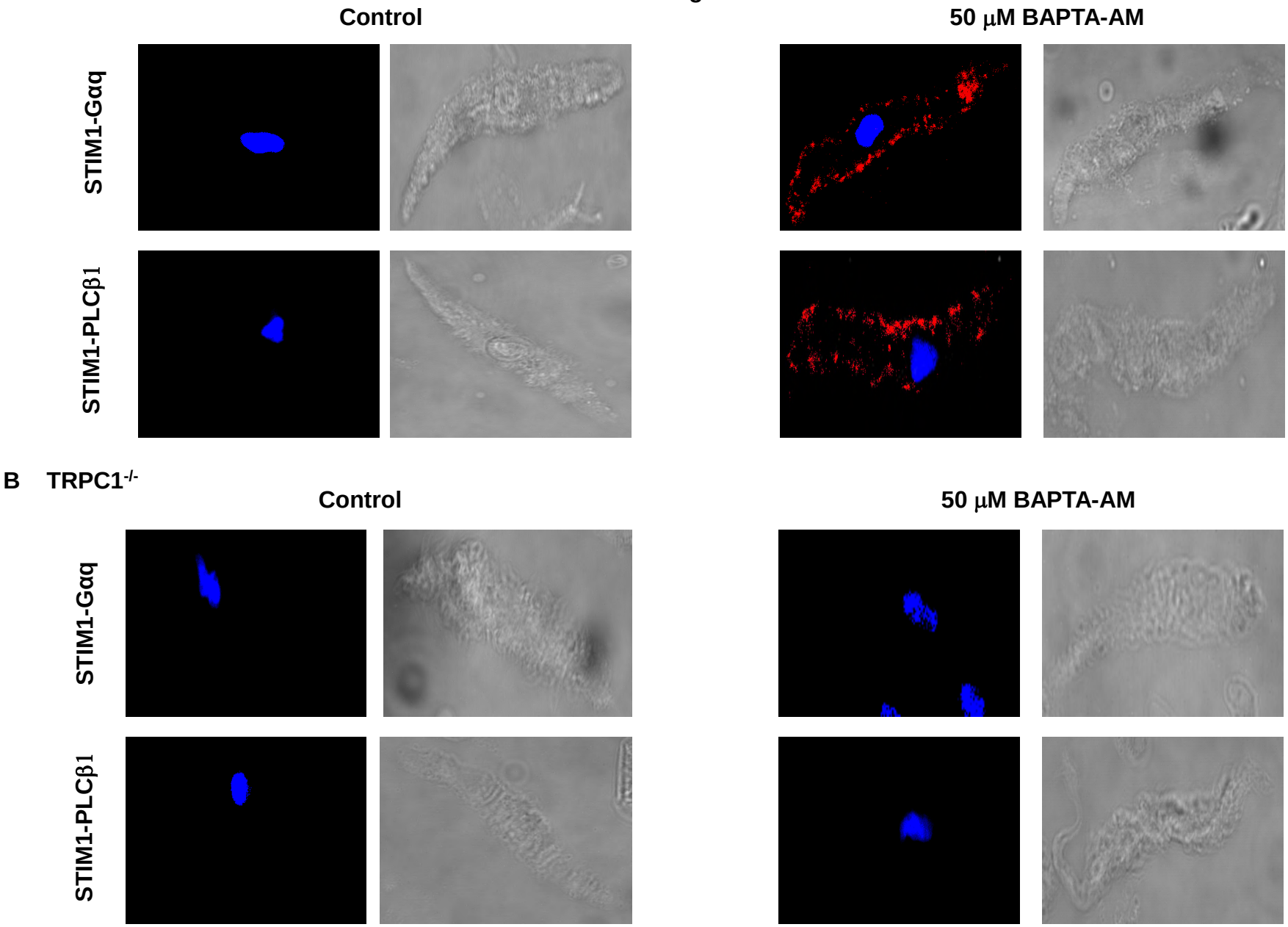

A
IP: TRPC1
Scrambled shRNA
STIM1 shRNA1
Control
BAPTA-AM
kDa
100
80
WB: STIM1
60
40
20
WB: Gαq
220
120
WB: PLCβ1
B
Relative band intensity of BAPTA-AM-evoked interactions with TRPC1
1.0
0.8
0.6
0.4
0.2
0.0
STIM1 shRNA1
*
Scram. shRNA
STIM1
Gαq
PLCβ1
C
IP: TRPC6
WB: STIM1
WB: TRPC6
D
IP: STIM1
WT
TRPC1-/-
WB: TRPC1
WB: Gαq
WB: PLCβ1

Figure 7

Figure 8


A TRPC1 WT
Control
50 μM BAPTA-AM
STIM1-Gαq
STIM1-PLCβ1
B TRPC1-/-
Control
50 μM BAPTA-AM
STIM1-Gαq
STIM1-PLCβ1

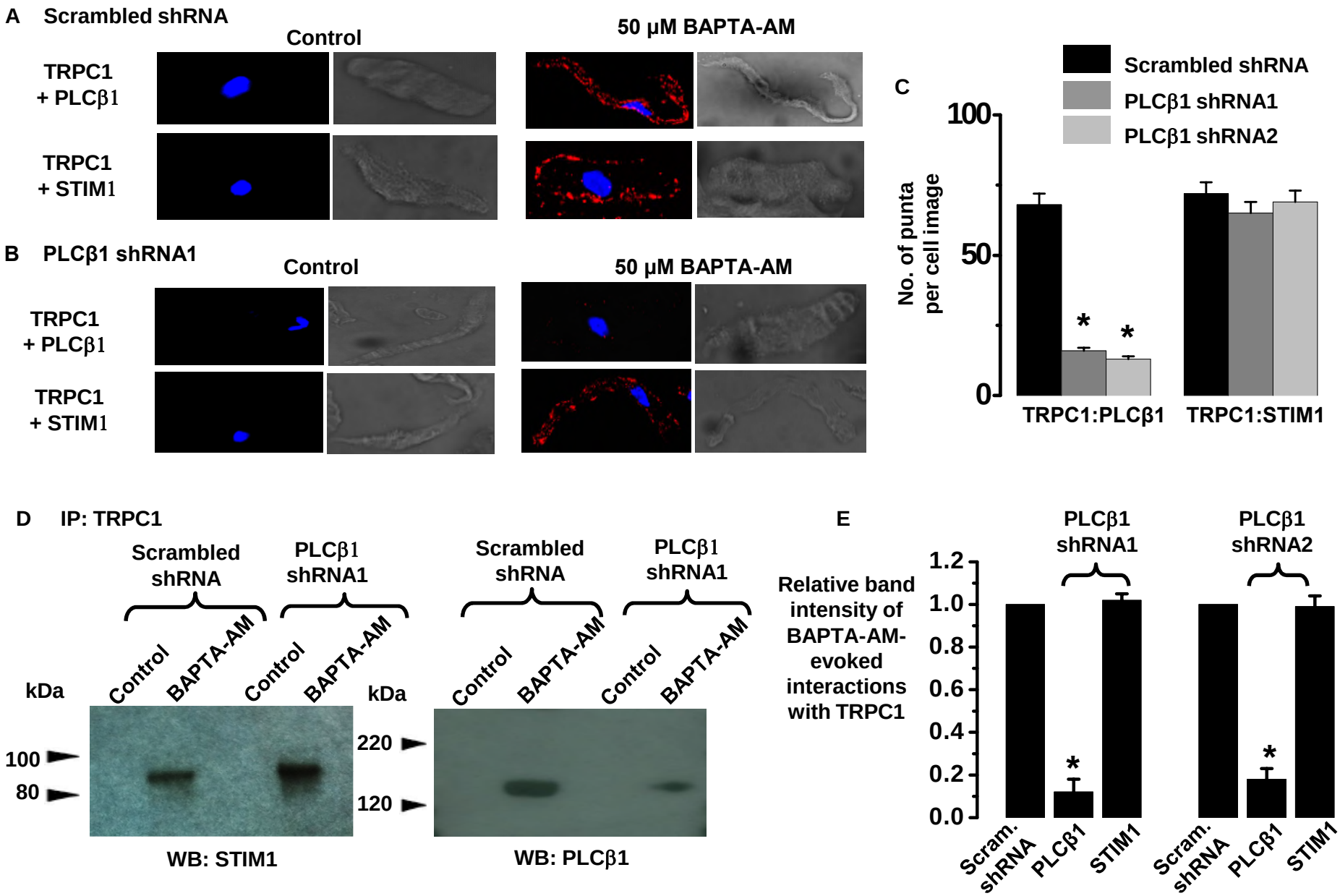

A Scrambled shRNA
Control
50 μM BAPTA-AM
TRPC1 + PLCβ1
TRPC1 + STIM1
B PLCβ1 shRNA1
Control
50 μM BAPTA-AM
TRPC1 + PLCβ1
TRPC1 + STIM1
C
Scrambled shRNA
PLCβ1 shRNA1
PLCβ1 shRNA2
No. of punta per cell image
100
50
0
*
*
TRPC1:PLCβ1
TRPC1:STIM1
D IP: TRPC1
Scrambled shRNA
PLCβ1 shRNA1
Scrambled shRNA
PLCβ1 shRNA1
Control
BAPTA-AM
Control
BAPTA-AM
Control
BAPTA-AM
Control
BAPTA-AM
kDa
100
80
WB: STIM1
kDa
220
120
WB: PLCβ1
E
Relative band intensity of BAPTA-AM-evoked interactions with TRPC1
PLCβ1 shRNA1
PLCβ1 shRNA2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Scram. shRNA
PLCβ1
STIM1
Scram. shRNA
PLCβ1
STIM1

Figure 9

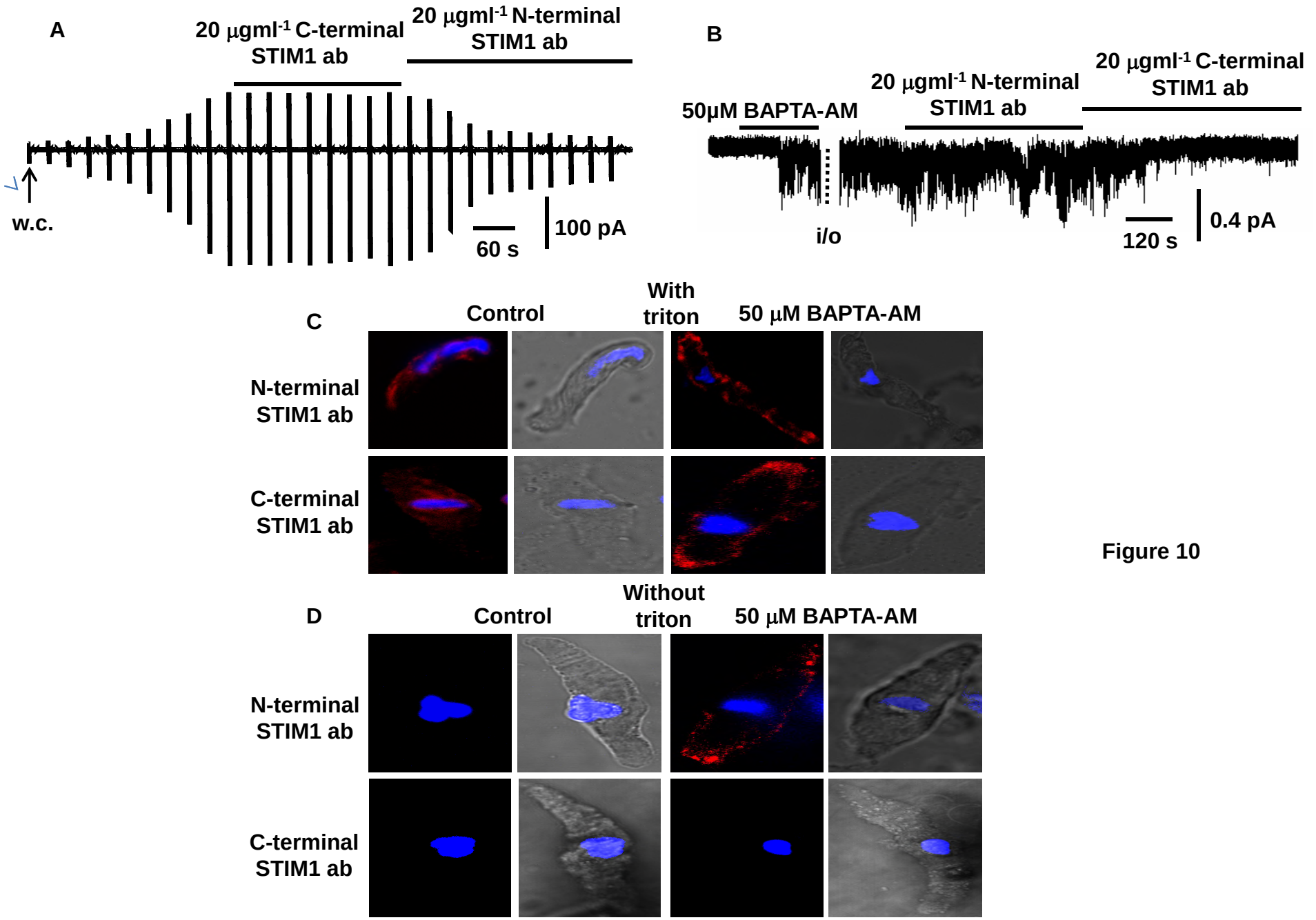
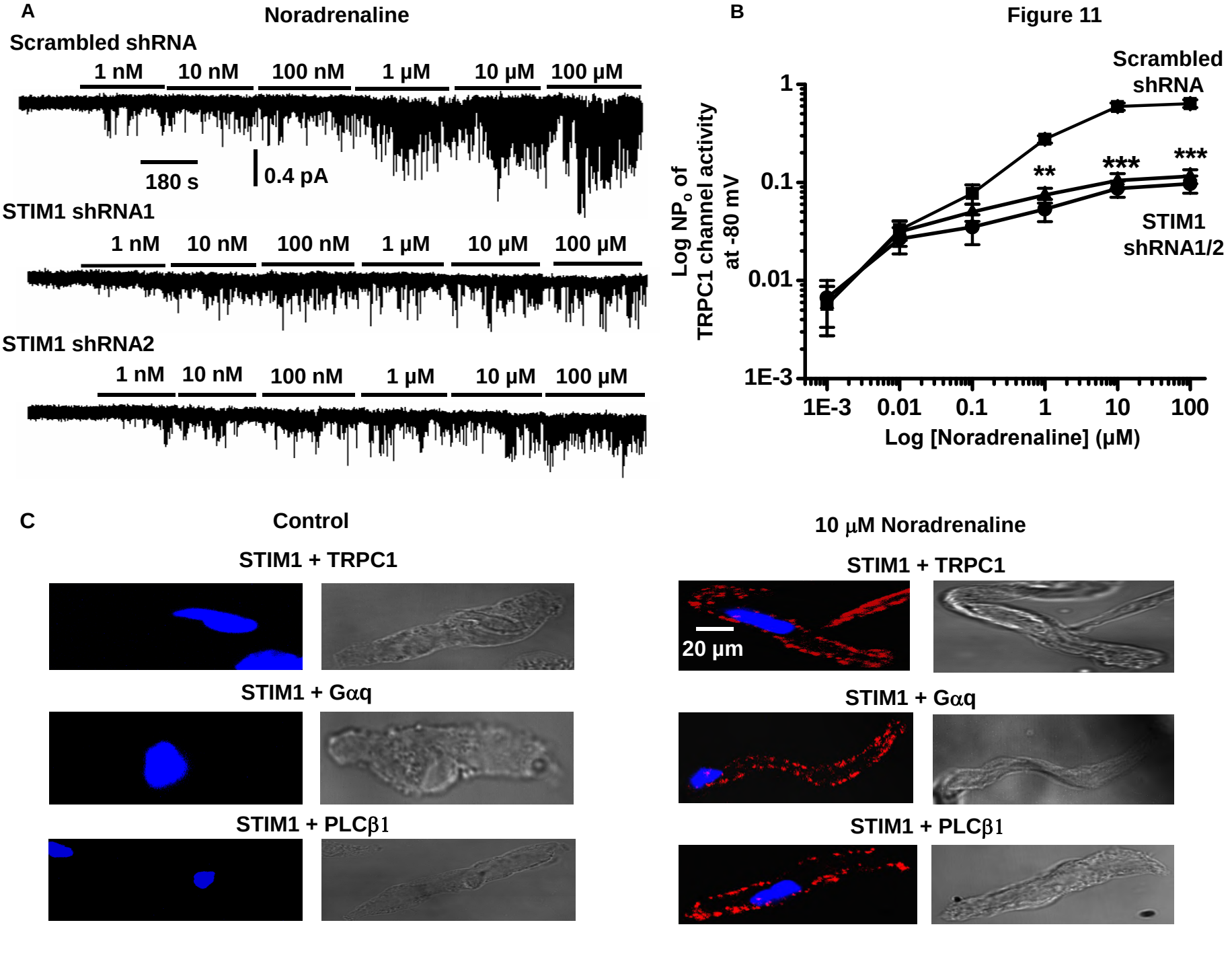

A
20 μgml-1 C-terminal STIM1 ab
20 μgml-1 N-terminal STIM1 ab
w.c.
60 s
100 pA
B
50μM BAPTA-AM
20 μgml-1 N-terminal STIM1 ab
20 μgml-1 C-terminal STIM1 ab
i/o
120 s
0.4 pA
C
Control
With triton
50 μM BAPTA-AM
N-terminal STIM1 ab
C-terminal STIM1 ab
D
Control
Without triton
50 μM BAPTA-AM
N-terminal STIM1 ab
C-terminal STIM1 ab

Figure 10

Figure 11


A
Noradrenaline
Scrambled shRNA
1 nM
10 nM
100 nM
1 μM
10 μM
100 μM
180 s
0.4 pA
STIM1 shRNA1
STIM1 shRNA2
B
Scrambled shRNA
STIM1 shRNA1/2
Log NPo of TRPC1 channel activity at -80 mV
Log [Noradrenaline] (μM)
1
0.1
0.01
1E-3
**
***
***
C
Control
10 μM Noradrenaline
STIM1 + TRPC1
STIM1 + Gαq
STIM1 + PLCβ1
20 μm

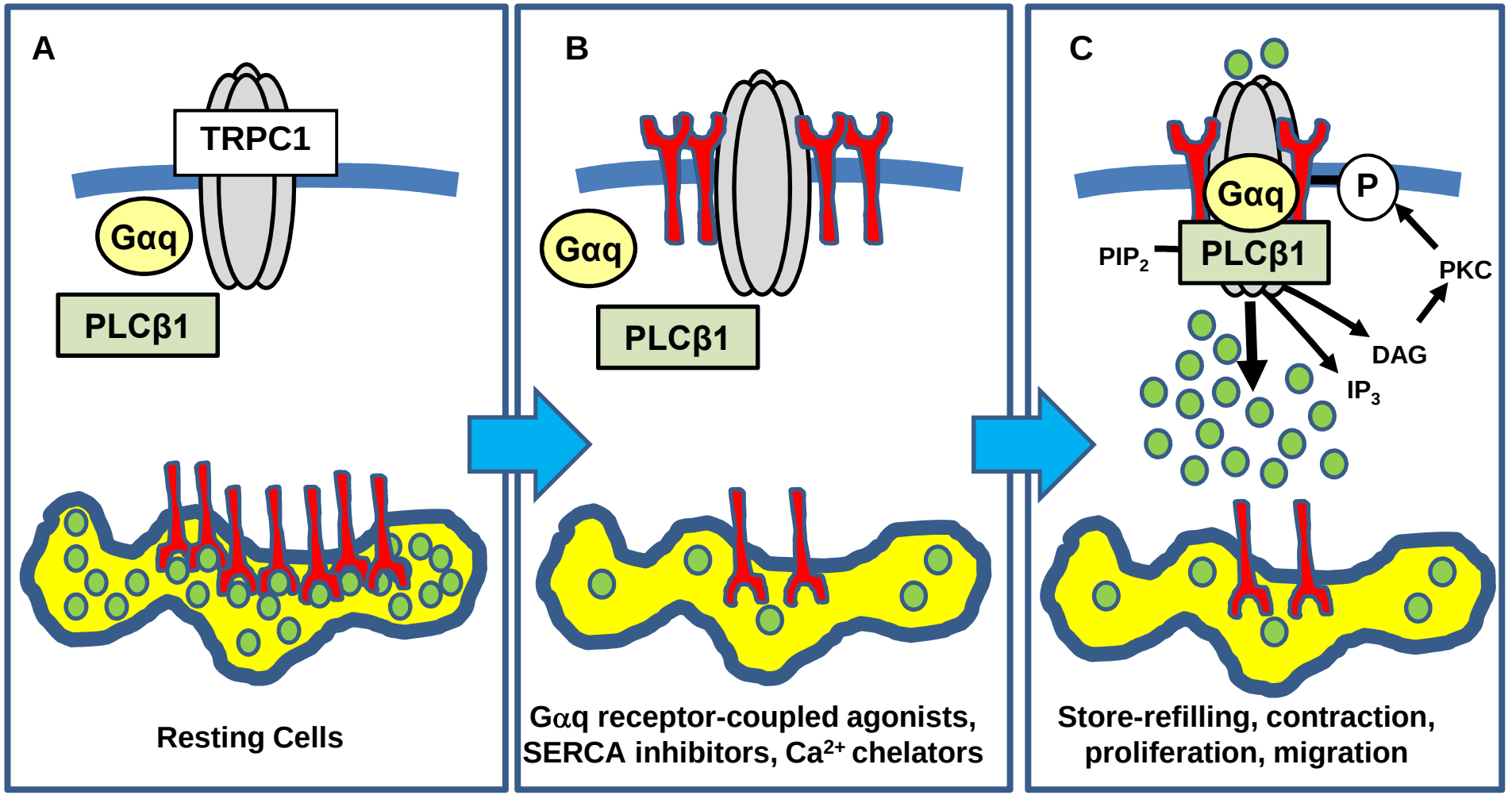

A
TRPC1
Gαq
PLCβ1
Resting Cells
B
Gαq
PLCβ1
Gαq receptor-coupled agonists,
SERCA inhibitors, Ca2+ chelators
C
Gαq
P
PIP2
PLCβ1
PKC
DAG
IP3
Store-refilling, contraction,
proliferation, migration

Figure 12