Online Supplement File

Materials and Methods

Tissue Preparation and Smooth Muscle Cell Isolation

Posterior cerebral, cerebellar, and middle cerebral arteries (100–200 μm) were harvested from rat brain maintained in ice-cold (4°C) oxygenated (21% O_2-5% CO_2-74% N_2) physiological saline solution (PSS) containing: (in mmol/L) 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, and 11 glucose (pH 7.4). Individual smooth muscle cells were dissociated from the cerebral arteries using a HEPES-buffered solution containing: (in mmol/L) 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.3), as previously described.[1] Briefly, cerebral arteries were placed into isolation solution containing 0.7 mg/ml papain, 1 mg/ml dithioerythreitol and 1 mg/ml bovine serum albumin (BSA) for 12 minutes at 37 ^oC. Arteries were then immediately transferred to isolation solution containing 1 mg/ml collagenase F and H (2:1), 100 μmol/L $CaCl_2$ and 1 mg/ml BSA for 10 minutes at 37 ^oC. Arteries were subsequently washed in isolation solution and triturated using a fire-polished glass pipette to yield single smooth muscle cells. Cells were maintained at 4°C, and used for experimentation within 8 hours.

Immunofluorescence resonance energy transfer (immuno-FRET)

Freshly isolated cerebral artery smooth muscle cells were allowed to adhere to poly-L-lysine coated coverslips. Cells were fixed with 3.7% paraformaldehyde in Phosphate-Buffered Saline (PBS) for 15 min. Paraformaldehyde-fixed cells were then washed three times with PBS and permeabilized with 0.1% triton X-100 for 1 min at room temperature. Following a 1 h incubation in PBS containing 5% bovine serum albumin (BSA), smooth muscle cells were treated for 1 h at 37ºC with primary antibodies: mouse monoclonal anti-IP_3R1 (NeuroMab) and either rabbit polyclonal anti-TRPC3 (Alomone), rabbit polyclonal anti-TRPC6 (Alomone), or rabbit polyclonal anti-TRPM4 (Affinity BioReagents, Golden CO), each at a dilution of 1:100 in PBS containing 5% BSA. After a wash and block with PBS containing 5% BSA, smooth muscle cells were incubated for 1 h at 37ºC with secondary antibodies: Cy3-conjugated Donkey Anti-Mouse for IP_3R1 (Jackson ImmunoResearch) and Cy2-conjugated Goat Anti-Rabbit (Jackson ImmunoResearch) for TRPC3, TRPM4, or TRPC6. Following wash and mount, fluorescence images were acquired using a Zeiss LSM Pascal laser-scanning confocal microscope. Cy2 and Cy3 were excited at 488 and 543 nm and emission collected at 505-530 and >560 nm, respectively. Negative controls were prepared by omitting primary antibodies. Images were background-subtracted and N-FRET was calculated on a pixel-by-pixel basis for the entire image and in regions of interest (within the boundaries of the cell) using the Xia method [2] and Zeiss LSM FRET Macro tool version 2.5.

Reverse transcription polymerase chain reaction (RT-PCR)

Briefly, total RNA was prepared from isolated cerebral artery smooth muscle cells using Absolutely RNA nanoprep kit (Stratagene, La Jolla, CA). cDNA was synthesized from DNase-treated RNA samples using AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene). cDNA products were amplified by nested-PCR using gene-specific oligonucleotide primer pairs. The oligonucleotide primer sequences used for RT-PCR were as follows: TRPC3 channel CIRB domain, first-round PCR: 5′-ATCATGAGGATCACTAACTTTTCCA-3′ (forward) and 5′-TATCGTGTTGGCTGATTGAGA-3′ (reverse); nested-PCR: 5′-GGAACTGGGCATGGGTAACTC-3′ (forward) and 5′-TGGCTGATTGAGAATGCTGTTA-3′ (reverse). TRPC6 channel CIRB domain, first-round PCR: 5′-TGACAGAAATCAGCTGGCAC-3′ (forward) and 5′-TGGGCCTGCAGTACGTATCT-3′ (reverse); nested-PCR: 5′-GAAATCAGCTGGCACACAACAAA-3′ (forward) and 5′-CATGATTTTCTGATACTGTCTTGGA-3′ (reverse). GeneBank accession numbers used to design TRPC3 and TRPC6 CIRB domains were NM_021771.1 and NM_053559.1, respectively. PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY) with the

following reaction conditions: an initial denaturation at 94 ^{o}C for 2 min, followed by 40 cycles (denaturation at 94 ^{o}C for 30 s, annealing at 50 ^{o}C for 30 s, and extension at 72 ^{o}C for 45 s), with a final extension at 72 ^{o}C for 10 min. PCR products were separated by agarose gel (1.5%) electrophoresis and sequenced.

Short Hairpin RNA Silencing Vector Construction
Using pRNA-U6.1/Neo as a template, silencing vectors were constructed to express short hairpin (sh)RNA (GenScript Corp, Piscataway, NJ) as previously described.[3] Expressed DNA sequences were as follows: for TRPC3shV, GTTCATACTTTACTCCTACTA; and for TRPC6shV, AGCTCAGAAGATTTCCATTTA

Reverse Permeabilization
Silencing vectors were inserted intracellularly into cerebral artery segments using a reverse permeabilization procedure, as previously described.[4] Arteries were placed into serum-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin and incubated at 37°C (95%O2, 5% CO2) for 4 days.

Western Immunoblotting
Western Immunoblotting was performed as previously described.[3, 5] Briefly, rat cerebral artery lysate protein concentrations were determined spectrophotometrically with amido black solution. Proteins were separated by 4-15% gradient SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes using a Mini Trans Blot Cell (Bio-Rad, Hercules, CA). Membranes were then incubated in respective antibodies and developed using enhanced chemiluminescence (Pierce, Rockford IL).

Co-immunoprecipitation
For each experiment, lysate was harvested from cerebral arteries pooled from ~20 rats using ice-cold lysis buffer, giving ~1 mg total protein. The composition of lysis buffer was (in mmol/L): 140 NaCl, 8 sodium phosphate, 2 potassium phosphate, 10 KCl, and 0.2% Triton X-100, plus 1% protease inhibitor cocktail (Sigma). Arterial lysate was incubated with control mouse IgG or IP_3R1 mouse monoclonal antibody (5 μg) at 4 $^{\circ}$C overnight. Samples were then incubated with 50 μL protein A Sepharose beads (Pierce, Rockford, IL) for 2 h at room temperature. Beads were subsequently collected by centrifugation at 5000 g for 5 minutes at 4 $^{\circ}$C and washed three times with lysis buffer. Laemmli sample buffer (2x, 25 μL) was added to protein beads and incubated for ~ 10 min at room temperature, followed by heating in boiling water for 5 min. Protein samples were then analyzed by Western blotting using mouse monoclonal anti-IP_3R1 (NeuroMab), mouse polyclonal anti-TRPC3 (Abnova), rabbit polyclonal anti-TRPC6 (Alomone), and horseradish peroxidase-conjugated secondary antibodies, as previously described.[3, 5]

Surface Biotinylation
For each experiment, intact cerebral arteries from ~ 2 rats were incubated for 1 h at room temperature in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Pierce) in PBS pH 7.4. Unbound biotin was removed by quenching with PBS supplemented with 100 mmol/L glycine and excess removed by washing with PBS. Arteries were then homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. The total protein concentration of the supernatant was determined and used to calculate the amount of lysate to be used for Avidin (Monomeric Avidin, Pierce) pull-down of biotinylated surface proteins. This ensured that the same amount of total protein was present in all samples. Following pull-down, biotinylated proteins remained bound to the Avidin beads, whereas the supernatant contained non-biotinylated cytosolic proteins. Biotinylated proteins were eluted from Avidin beads by boiling in 1x SDS-buffer containing 2% 2-mercaptoethanol. Western blotting was used to quantify surface and cytosolic TRPC3 protein using rabbit polyclonal anti-TRPC3 (Abnova) and horseradish peroxidase-conjugated secondary antibody, as previously described.[3, 5] Mouse monoclonal anti-Hsp90 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Band intensity was determined using Quantity One software (BioRad). The percentage of total protein at the surface was calculated as 100 × [biotinylated/(biotinylated+non-biotinylated)].

Cell culture and Transfection

Human Embryonic Kidney 293 (HEK293) cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard tissue culture conditions (21% O_2-5% CO_2; 37°C). HEK293 cells were transfected with vectors encoding GFP (control), GFP-tagged TRPC3, or GFP-tagged TRPC6 (2 μg of each). Transfection was done using the Ca^{2+} phosphate method. TRPC3-GFP and TRPC6-GFP constructs were kindly provided by Dr. James Putney (NIEHS, Research Triangle Park) and Dr. Jochen Reiser (University of Miami Miller School of Medicine), respectively. The vectors used for transfection were confirmed by PCR analysis (Online Figure IV). Transfected cells grown on sterile glass coverslips were used for electrophysiological experiments. Transfected cells grown on 35 mm culture dishes were used for Western blotting experiments. Cells were used between 36 and 72 h after transfection.

Patch-Clamp Electrophysiology

Patch-clamp electrophysiology was performed on isolated arterial smooth muscle cells or HEK293 cells. Isolated smooth muscle cells were allowed to attach to a glass coverslip in the bottom of a chamber for 15 min prior to experimentation. Transfected HEK293 cells expressing GFP, GFP-tagged TRPC3, or GFP-tagged TRPC6 channels were grown on glass coverslips, which were then placed in the bottom of a patch-clamp chamber. GFP-positive HEK293 cells were visualized using a Nikon TS100 microscope using an epi-fluorescence attachment. Fluorescent HEK cells that were not attached to neighboring cells were used to obtain whole cell patch-clamp recordings. Membrane currents were measured using the patch-clamp technique (Axopatch 200B, Clampex 8.2). I_{Cat} was measured using the conventional whole cell patch-clamp configuration, as we have done previously.[3, 5] Whole cell currents were measured by applying 940-ms voltage ramps between -120 and + 20 mV with a holding potential of -40 mV. Bath solution for conventional whole-cell experiments contained (in mmol/L): 140 NaCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes, and 10 glucose (pH 7.4). The pipette solution contained (in mmol/L): 140 CsCl, 10 HEPES, 10 glucose, 5 Mg-ATP, and 5 EGTA (with pH adjusted to 7.2 with CsOH), and 100 nmol/L free Ca^{2+}. Where applicable, anti-TRPC3 and -TRPC6 antibodies were included in the pipette solution. Antibodies were denatured by incubation at 95 ^{o}C for 20 minutes. Cells were dialyzed with antibodies or peptides for 10 minutes prior to current recordings. Effects of Gd^{3+} were measured in paired experiments. Current amplitude at -120 mV was analyzed offline using pClamp 9 (Axon Instruments).

Pressurized Artery Diameter Measurement

An arterial segment 1–2 mm in length was cannulated at each end in a perfusion chamber (Living Systems Instrumentation; Burlington, VT). The chamber was continuously perfused with PSS equilibrated with a mixture of 21 % O_2-5 % CO_2-74 % N_2, and maintained at 37°C. Intravascular pressure was altered using an attached reservoir and monitored using a pressure transducer. Pressurized arteries were observed with a charge-coupled device camera attached to an inverted microscope (Nikon TE 200). Arterial diameter was measured at 1 Hz using the automatic edge-detection function of IonWizard software (Ionoptix; Milton, MA). Pharmacological compounds were applied via chamber perfusion.

Chemicals

Unless otherwise stated, all reagents were purchased from Sigma Chemical (St. Louis, MO). Papain was purchased from Worthington Biochemical (Lakewood, NJ) and 2,3,6-Tri-O-Butyryl-myo-Inositol 1,4,5-Trisphosphate-Hexakis(propionoxymethyl) Ester (Bt-IP_3) was purchased from SiChem (Bremen, Germany). CIRB and IP3RntP peptides were synthesized by Sigma-Genosys (Woodlands, TX) while CIRBPscrm, CIRBP-TAT, and CIRBPscrm-TAT were synthesized by Molecular Resource Center, UTHSC (Memphis, TN).

Statistical Analysis

GraphPad Instat software (GraphPad Prism, San Diego, CA) was used for statistical analysis.

References

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4. Lesh RE, Somlyo AP, Owens GK, Somlyo AV. Reversible permeabilization. A novel technique for the intracellular introduction of antisense oligodeoxynucleotides into intact smooth muscle. *Circ Res.* 1995;77:220-230.
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Online Figure Legends

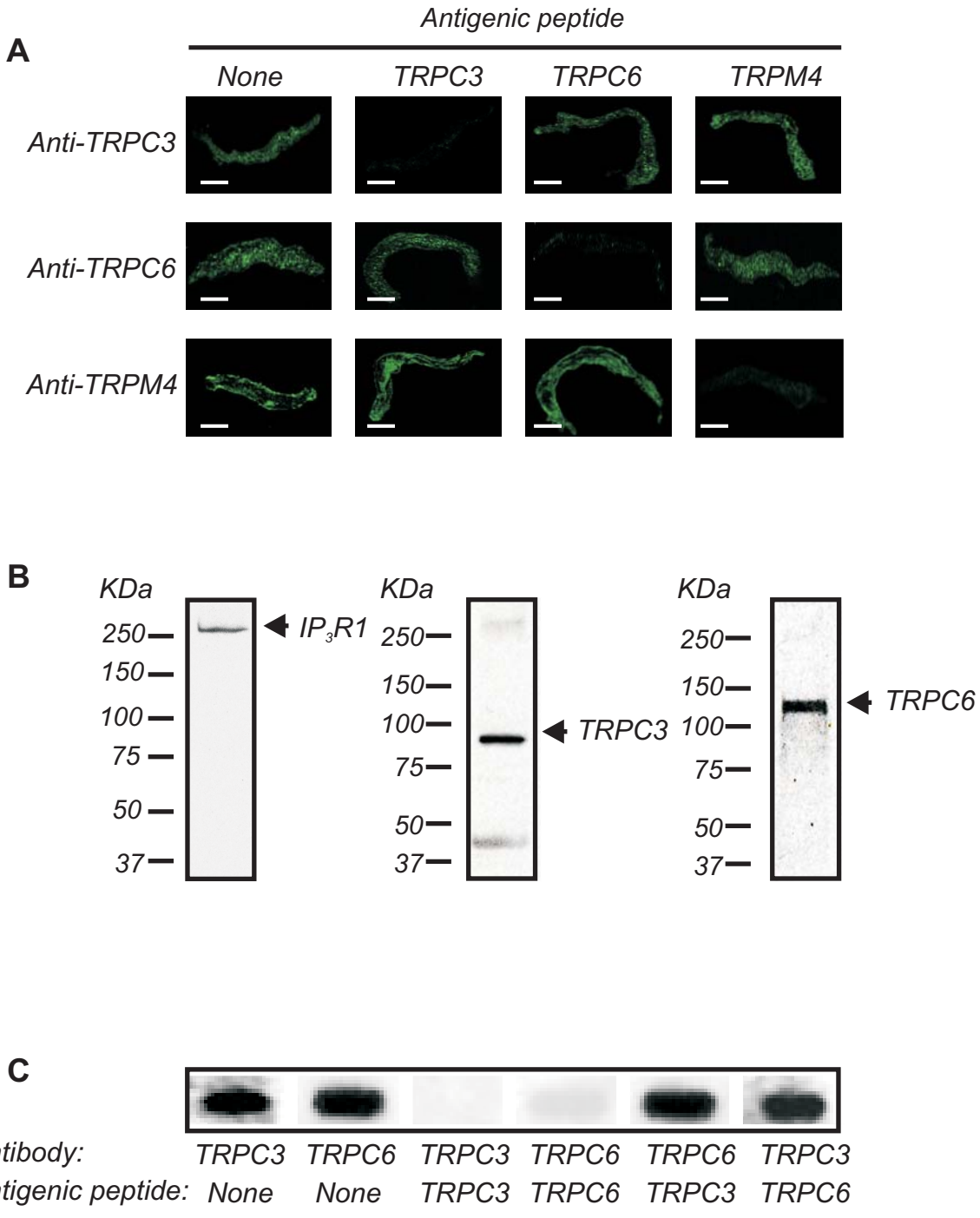
Online Figure I. **A**, Antigenic peptides for each TRP channel primary antibody specifically block immunofluorescence produced by that antibody, but do not alter immunofluorescence detection by the other two primary antibodies. **B**, Whole western blot illustrating detection of IP_3R1 (~270 kDa), TRPC3 (~90 kDa), and TRPC6 (~110 kDa) proteins by their respective antibodies in cerebral arteries. **C**, TRPC3 antigenic peptide blocked TRPC3 detection, but had no effect on TRPC6 recognition. Similarly, a TRPC6 antigenic peptide blocked TRPC6 detection, but had no effect on TRPC3 recognition.

Online Figure II. A,TRPC3 (left panel) and TRPC6 (right panel) antibodies selectively detect overexpressed recombinant TRPC3 and TRPC6 in HEK293 cells. Each lane represents corresponding construct transfected into the cells. Faint TRPC3 and TRPC6 bands indicate endogenous TRPC3 and TRPC6 in HEK293 cells, as previously demonstrated.[6] **B**, Exemplary recordings of I_{Cat} measured in HEK293 cells transfected with vectors encoding GFP (control), GFP-TRPC3, or GFP-TRPC6 and effects of TRPC3 and TRPC6 antibodies and heat-denatured antibodies (2 μg/mL each).

Online Figure III. Surface labeling of myocytes using Texas red-conjugated streptavidin in non-biotinylated (left) and biotinylated (right) intact cerebral arteries. Scale bar = 20 μm.

Online Figure IV. First-round PCR analysis of TRPC3 and TRPC6 vector constructs using specific primers.

Online Figure V. Proposed mechanisms by which IP_3R coupling to TRPC3 channels regulate IP_3-induced arterial smooth muscle cell contractility.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Antigenic peptide
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TRPC3
TRPC6
TRPM4
Anti-TRPC3
Anti-TRPC6
Anti-TRPM4
B
KDa
250
150
100
75
50
37
IP3R1
TRPC3
TRPC6
C
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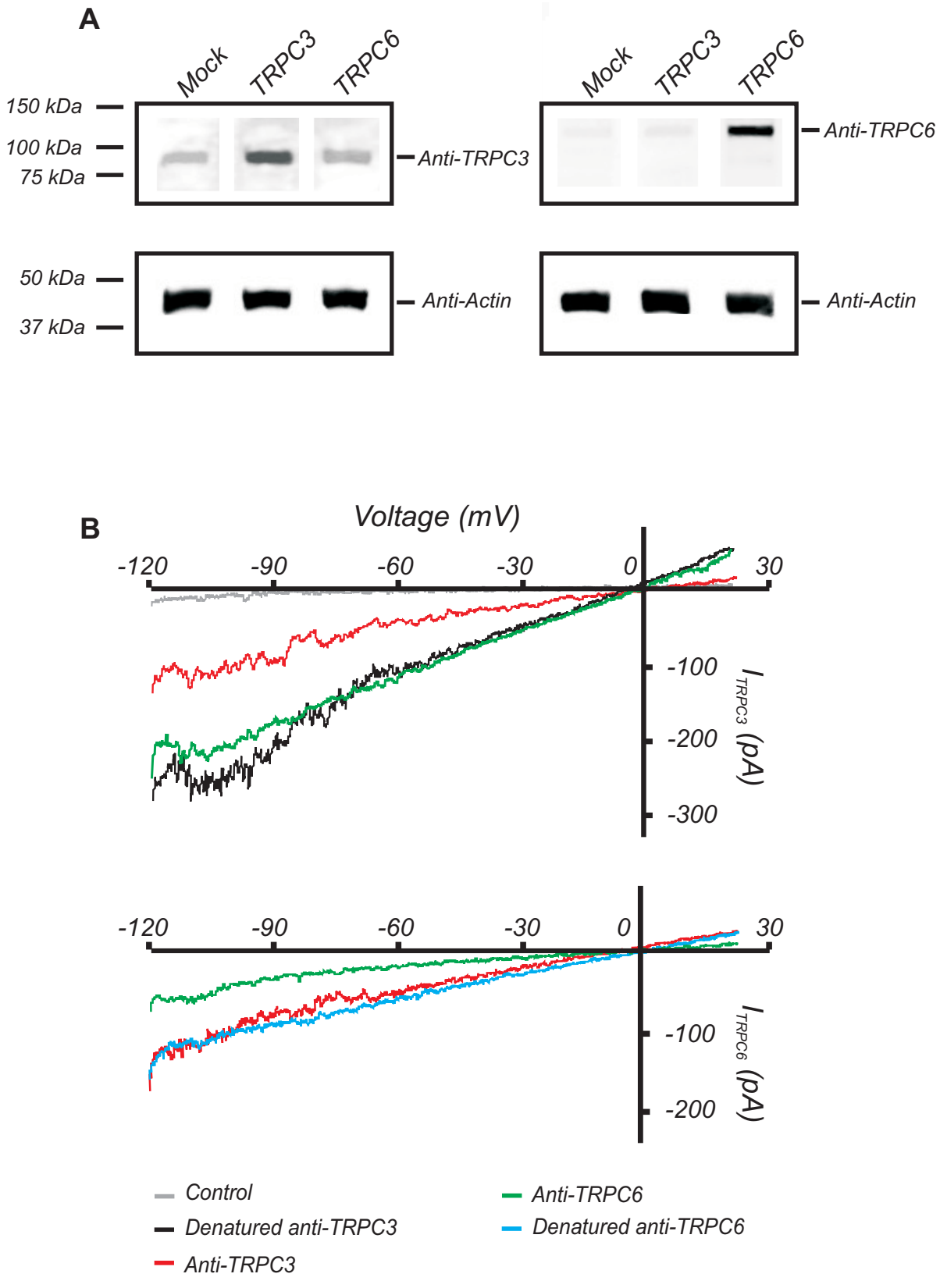
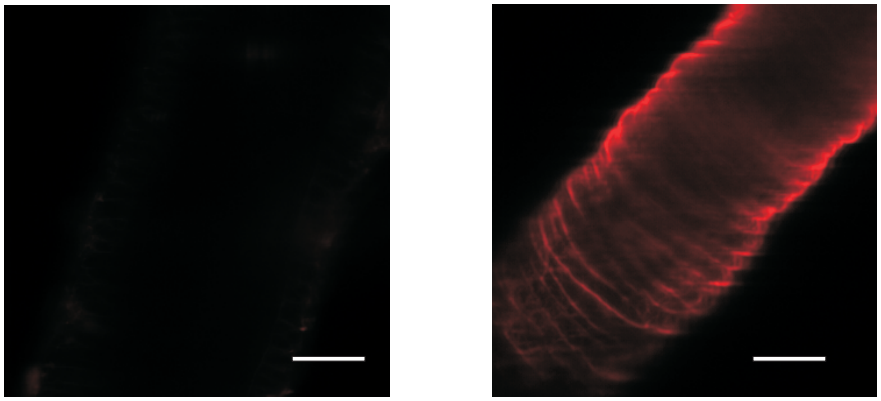
Online Fig. I



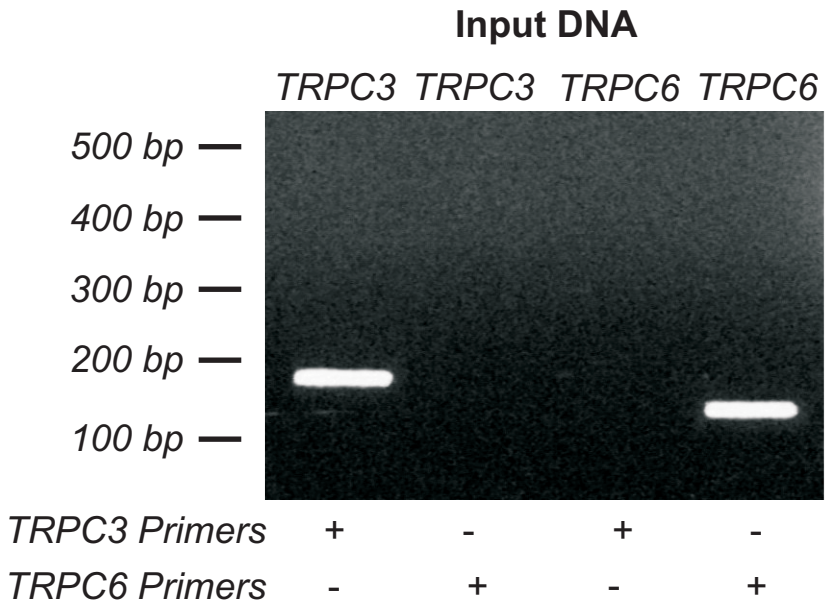
Online Fig. II

Control

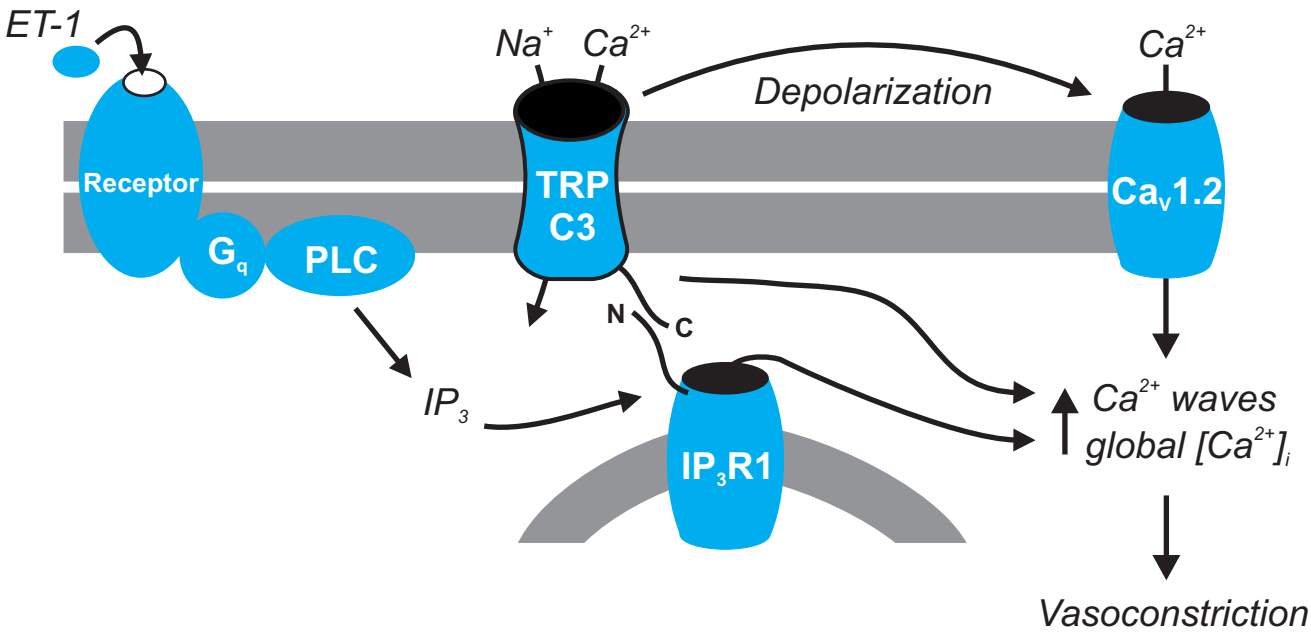
Biotin



Online Fig. III



Online Fig. IV


ET-1
Receptor
G_q
PLC
IP_3
Na^+ Ca^{2+}
TRP C3
N
C
IP_3R1
Depolarization
Ca^{2+}
$Ca_V1.2$
Ca^{2+} waves
global $[Ca^{2+}]_i$
Vasoconstriction

Online Fig. V