SUPPLEMENTAL MATERIAL

Novel Smooth Muscle Ca^{2+}-Signaling Nanodomains in Blood Pressure Regulation

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SUPPLEMENTAL METHODS

Animal protocols. All animal protocols were approved by the University of Virginia Animal Care and Use Committee (protocols 4,100 and 4,120). Because the smooth muscle myosin heavy chain (SMMHC) promoter-driven *Myh11*-ERT2 construct used to generate transgenic mice is inserted into the Y chromosome, only male mice were used in this study. C57BL6/J were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Before harvesting intestinal tissue, mice were euthanized with pentobarbital (90 mg/kg, i.p.; Diamondback Drugs, Scottsdale, AZ, USA) followed by cranial dislocation. Third-order mesenteric arteries (MAs; ~100 μm) were isolated into cold HEPES-buffered physiological salt solution (HEPES-PSS; 10 mM HEPES, 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$ hexahydrate, 2 mM $CaCl_2$ dihydrate, and 7 mM dextrose, pH adjusted to 7.4 using 1 M NaOH).[9]

For *in vivo* experiments, an independent team member performed random assignment of animals to groups and did not have knowledge of treatment assignment groups. All the *in vivo* experiments were blinded; information about the groups or treatments was withheld from the experimenter or from the team member who analyzed the data. All the experiments were performed in at least two independent batches.

Generation of $TRPV4_{SMC}^{-/-}$ Mice. $TRPV4^{fl/fl}$ mice[9] were crossed with *Myh11*-CreERT2 mice[43]. SMC-specific knockout of TRPV4 was induced by injecting 6-week-old $TRPV4^{fl/fl}$ Cre^{+} mice with tamoxifen (40 mg/kg/d for 10 days; i.p.). Tamoxifen-injected $TRPV4^{fl/fl}$ Cre^{-} mice were used as controls. Mice were used for experiments after a 2-week tamoxifen-washout period.[9] SMC-specific knockout of TRPV4 was confirmed by comparing TRPV4

immunostaining and mRNA levels in endothelial cell (EC)-denuded MAs (described below).

Genotyping. Genomic DNA was extracted by treating samples of ear tissue with HotSHOT lysis buffer (25 mM NaOH, 0.2 mM EDTA) and then neutralizing with an equal volume of 40 mM Tris-HCL. PCR was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 1 unit of Bioline MangoTaq polymerase and buffer (London, England), 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM 5' and 3' primers, and approximately 100–250 ng genomic DNA. Reaction products were then run on a 1% agarose gel containing 0.2 μg/μL ethidium bromide in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 90V using a Bio-Rad PowerPac HC High-Current Power Supply. Gels were visualized by exposing to 302 nm UV light, and the sizes of PCR products were calibrated using a 100-bp DNA Ladder (New England BioLabs, Ipswich, MA, USA).[9] Mice were genotyped using the following primer pairs: SMMHC Cre, F 5'-TGACCCCATCTCTTCACTCC-3', R 5'-AGTCCCTCACATCCTCAGGTT-3'; $TRPV4^{loxP}$, F 5'-CATGAAATCTGACCTCTTGTCCCC-3', R 5'-CGGACCACACGTCTGTCATGTGTT -3'.[9] All primers were obtained from Eurofins Genomics (Louisville, KY, USA).

Isolation of human paraspinal muscle tissue from non-hypertensive and hypertensive individuals. Paraspinal muscle tissue was obtained from non-hypertensive and hypertensive subjects during spinal surgeries, as approved by the University of Virginia Institutional Review Board (Protocol #18699). Informed consent was obtained from each subject as per the protocol. Hypertension was determined based on whether a patient was documented to be on anti-hypertensive medication pre-operatively. Review was performed to ensure the medication was not started for other indications (for

example, in one patient, a low-dose ACE inhibitor was started for diabetic nephropathy). In addition, patients in the “non-hypertensive” group were confirmed not to be overtly hypertensive based on pre-operative blood pressure measurements. For this group, borderline measurements with systolic blood pressures in the 140 mm Hg range were still accepted as “non-hypertensive.” Individuals treated with α1 adrenergic receptor antagonists as antihypertensive medication were excluded from the study. The detailed clinical information, including anti-hypertensive medications, reason for surgery, and other medication are listed in Table S1. Small arteries (~ 100 μm) were dissected out from the muscle tissue, placed in cold HEPES-PSS, and then used for pressure myography, *in situ* proximity ligation assay (PLA), or enzymatic isolation of SMCs for whole-cell patch-clamp.

Immunostaining. Immunostaining was performed on *en face* preparations of arteries.[9] Briefly, arteries were pinned down on SYLGARD blocks with the endothelium facing up and fixed with 4% paraformaldehyde at room temperature for 15 minutes. Fixed arteries were washed three times (5 minutes each) with phosphate-buffered saline (PBS), then permeabilized by incubating with 0.2% Triton-X PBS for 60 minutes at room temperature on a rocker. After blocking with 5% normal donkey serum (Abcam plc, Cambridge, MA, USA) for 1 hour at room temperature, arteries were incubated with a primary antibody against TRPV4 (1:200, LS_C94498; LifeSpan BioScience Inc., WA, USA or 1:100, NBP2-41262; Novus Biologicals, Littleton, CO, USA), anti-α_{1D}-AR (1:100, ab84402; abcam, Cambridge, UK), or anti-MaxiKα antibody (B-1) (1:100, sc-374142; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. After washing three times with PBS, arteries were incubated with Alexa Fluor 568-conjugated donkey anti-rabbit secondary

antibody (1:500; Life Technologies, Carlsbad, CA, USA) at room temperature for 1 hour in the dark, followed by addition of a fluorescein isothiocyanate (FITC)-conjugated anti-smooth muscle α-actin (α-SMA) antibody (1:500, F3777; MilliporeSigma, St. Louis, MO, USA)[19] or FITC-conjugated anti-CD31 antibody[19] (1:500, RM5201; Invitrogen, Carlsbad, CA, USA). Arteries were then washed three times with PBS and incubated with 0.3 μM 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 minutes at room temperature in the dark to stain nuclei. Images were acquired using a previously described Andor imaging system (Andor Technology, Belfast, UK).[9,19] Consecutive images were taken along the z-axis at a slice thickness of 0.1 μm from the top of ECs to the bottom of SMCs. DAPI staining was imaged by exciting at 409 nm and collecting the emitted fluorescence with a 447/60-nm band-pass filter. α-actin-FITC and CD31-FITC were imaged by exciting at 488 nm solid-state laser and emitted fluorescence was captured using a 525/36 nm band-pass filter.

Isolated SMCs were fixed by incubating with 4% paraformaldehyde at room temperature for 15 minutes and then permeabilized by treating with PBS containing 0.2% Triton-X for 60 minutes. Cells were then treated with 5% normal donkey serum for 1 hour and subsequently incubated with FITC-conjugated anti-α-actin antibody (1:500, F3777; MilliporeSigma)[19] for 1 hour at room temperature. After washing SMCs three times with PBS, nuclei were stained by incubating with 0.3 μM DAPI (Invitrogen) for 10 minutes at room temperature in the dark.

Isolation of SMCs from arteries. SMCs were freshly isolated from third-order MAs (~ 100 μm) from mice or human paraspinal muscle arteries. Briefly, artery segments were transferred to a 12 x 75 mm borosilicate glass culture tube containing 1 mL dissociation

solution (145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.05 mM $CaCl_2$ 10 mM glucose) and 0.5 mg/mL bovine serum albumin (BSA) and then incubated for 10 minutes at room temperature (~24°C). This solution was then replaced with 1 mL dissociation solution containing 1 mg/mL papain (MilliporeSigma) and 0.5 mg/mL dithiothreitol (MilliporeSigma) at 37°C for 20 minutes. Thereafter, 0.5 mL of the papain solution was carefully removed without displacing artery segments and replaced with 0.5 mL dissociation solution containing 2 mg/mL collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ, USA), 0.5 mg/ml elastase (MilliporeSigma), and 1 mg/ml soybean trypsin inhibitor (MilliporeSigma) and incubated at 37°C for 5 minutes. The enzyme solution was then removed and replaced with cold dissociation solution containing BSA. The tube containing digested arteries was placed on ice, and the solution was gently triturated every 15 minutes for 1 hour to yield a single-cell suspension. **Quantitative polymerase chain reaction (qPCR).** The endothelium was denuded by passing an air bubble through the artery lumen for 60 seconds followed by perfusion with PSS. MAs were then placed in 500 μL RLT buffer (Qiagen RNeasy Mini Kit, Hilden, Germany) containing 5 μL β-mercaptoethanol and homogenized using a Standard Microhomogenizer (PRO Scientific Inc., Oxford, CT, USA). RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen), treated with a DNA-free DNA Removal Kit (Invitrogen, Waltham, MA), and converted to cDNA using a iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed on a Bio-Rad CFX96 qPCR Detection System using a reaction mix containing Bio-Rad 2x SYBR Green Master Mix, 200 nM 5' and 3' primers, and 20 nM cDNA. qPCR primers for TRPV4 and GAPDH (internal control) were obtained from GeneCoepia Inc. (Rockville, MD, USA).[9] Results were analyzed using the ΔΔCt method.

Radiotelemetric blood pressure measurement. Continuous blood pressure measurements were performed using Ponemah 6.42 software (Data Sciences International, St. Paul, MN), as described previously.[9,19] Mice were anesthetized with isoflurane (1.5%), and a radiotelemetry catheter (HD-X10; Data Sciences International, St. Paul, MN, USA) was inserted in the left carotid artery.[9,19] The radiotransmitter was placed in a subcutaneous pouch along the flank. Before initiating arterial pressure measurements, mice were allowed to recover for 7 days after surgery to regain normal circadian rhythms. Following the recovery period, baseline systolic pressure, diastolic, and mean arterial pressure, and heart rate were recorded continuously over 72 hours at 1-minute intervals. Baseline daytime and nighttime recordings were obtained by averaging values over 3 days (6 AM to 6 PM) and 3 nights (6 PM to 6 AM), respectively. $TRPV4^{fl/fl}$ and $TRPV4_{SMC}^{-/-}$ mice were given a single bolus intraperitoneal injection of the α1AR antagonist prazosin (1 mg/kg), α1AR agonist phenylephrine (PE; 10 mg/kg), or BK channel inhibitor paxilline (8 mg/kg). Sterile saline solution (0.9%) or DMSO was used as a vehicle, and mice injected with vehicle only were used as a control group for statistical comparisons. Blood pressure and heart rate were recorded for 1 hour at 5-minute intervals. Blood pressures obtained 15 minutes after injection were compared between groups. Experiments were performed in a blinded manner.

Cardiac magnetic resonance imaging (MRI). MRI studies were conducted under protocols that complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, Revised 1996). Anesthetized mice (1.25% isoflurane) were positioned in the scanner and their body temperature was maintained at 37°C using thermostatic circulating water. A 30-mm diameter, cylindrical birdcage RF coil (Bruker,

Ettlingen, Germany) with an active length of 70 mm was used, and heart rate, respiration, and temperature were monitored during imaging using a fiber optic, MR-compatible system (Small Animal Imaging Inc., Stony Brook, NY, USA). MRI was performed on a 7 Tesla (T) Clinscan system (Bruker) equipped with actively shielded gradients with a full strength of 650 mT/m and a slew rate of 6666 mT/m/ms. Six short-axis slices were acquired from base to apex, with a slice thickness of 1 mm, in-plane spatial resolution of 0.2 × 0.2 mm^2, and temporal resolution of 8–12 ms. Baseline ejection fraction (EF), end-diastolic volume (EDV), end-systolic volume (ESV), myocardial mass, wall thickness, stroke volume, and cardiac output were assessed from cine images using the freely available software, Segment version 2.0 R5292 (http://segment.heiberg.se).

Pressure myography. Third-order MAs (~100 μm) were cannulated onto two glass micropipettes on a custom-made pressure myography chamber (Instrumentation and Model Facility, University of Vermont, Burlington, VT, USA), pressurized to 80 mm Hg with a pressure servo controller peristaltic pump (Living Systems Instrumentation, St Albans, VT, USA), and superfused at 37°C with PSS (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$ 160 hexahydrate, 2.5 mM $CaCl_2$ dihydrate, 7 mM dextrose, and 24 mM $NaHCO_3$, maintained at pH 7.4 by bubbling the solution with 21% O_2 and 5% CO_2). MAs were allowed to develop spontaneous pressure-induced constriction (myogenic tone) at 80 mm Hg before initiating experimental treatments.[9] Dilation to NS309 (1 μM), a direct opener of intermediate/small-conductance Ca^{2+}-sensitive K^+ (IK/SK) channels, was used to functionally assess the health of endothelial cells.[9] Diameter changes in response to prazosin (1 μM) and to cumulative administration of PE (10 nM to 10 μM), U46619 (1, 3, 10, 30, 100, 300 nM) (Cayman Chemicals, Ann Arbor,

MI, USA), Ang II (1 pM to 10 nM) (Bachem, Torrance, CA, USA) or paxilline (1 μM) were recorded. MAs were exposed to each concentration of agonist until a stable diameter was attained (5–10 minutes). The contribution of α1AR and TRPV4 channels to PE-induced vasoconstriction was evaluated using prazosin (1 μM) and the selective TRPV4 channel antagonist GSK2193874 (GSK219; 100 nM), respectively. At the end of each experiment, maximum passive diameter was evaluated by incubating MAs with Ca^{2+}-free PSS (119 mM NaCl, 4.7 mM KCl,1.2 mM KH_2PO_4,1.2 mM $MgCl_2$, 7 mM glucose, 24 mM $NaHCO_3$, 5 mM EGTA (pH 7.4). Internal diameter was recorded at 10 frames/s using a CCD camera and analyzed using IonOptix edge-detection software (IonOptix LLC, Westwood, MA, USA). Diameter changes in response to agonists and antagonists were normalized to the resting diameter and expressed as a percentage.

Myogenic constriction at a perfusion pressure of 80 mm Hg was calculated as:

$$[(Diameter_{basal} - Diameter_{constricted})/Diameter_{basal}] \times 100$$

Myogenic constriction at perfusion pressures of 20, 40, 60, 80, 100, and 120 mm Hg[44,45] was calculated as:[9]

$$[(Diameter_{Ca\ free} - Diameter_{constricted})/Diameter_{Ca\ free}] \times 100,$$

where $Diameter_{constricted}$ is the diameter of the artery after drug-induced constriction or pressure-induced myogenic constriction, and $Diameter_{Ca\ free}$ is the maximum passive diameter at the relative perfusion pressure.

Percent dilation was calculated as:[9]

$$[(Diameter_{dilated} - Diameter_{basal})/(Diameter_{Ca\ free} - Diameter_{basal})] \times 100,$$

where $Diameter_{basal}$ is the diameter before treatment, $Diameter_{dilated}$ is the diameter after treatment, and $Diameter_{Ca\ free}$ is the maximum passive diameter at a perfusion pressure of 80 mm Hg.

Ca^{2+} imaging. Ca^{2+}-imaging studies were performed as described previously.[9,46] Briefly, third-order MAs from mice or human paraspinal muscle arteries (~100 μm) were incubated with Fluo-4 AM (10 μM)[9] and pluronic acid (0.04%) at 37°C for 1 hour, after which MAs were cannulated and pressurized to 80 mm Hg. Ca^{2+} images were acquired at 30 frames per second using an Andor Revolution WD (with Borealis) spinning-disk confocal imaging system (Andor Technology) comprising an upright Nikon microscope with a 40X water-dipping objective (numerical aperture, 0.8) and an electron-multiplying CCD camera. MAs were superfused with PSS (pH 7.4), and all experiments were performed at 37°C. Fluo-4 was excited using a 488 nm solid-state laser, and emitted fluorescence was captured using a 525/36 nm band-pass filter. MAs were treated with the sarco-endoplasmic reticulum (SR/ER) Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA; 20 μM) and L-type Ca^{2+} channel inhibitor nifedipine (1 μM) for 10 minutes at 37°C prior to imaging to eliminate intracellular Ca^{2+}-release signals and extracellular Ca^{2+}-influx signals, respectively. We previously showed that CPA *per se* does not alter the activity of TRPV4 sparklets in endothelial cells.[46] SMC TRPV4 sparklet activity was determined before and 5 minutes after the administration of the specific TRPV4 agonist GSK1016790A (GSK101; 30 nM). The influence of pharmacological agents (GSK101, PE, U73122) on TRPV4 sparklet activity was further evaluated. SMC TRPV4 sparklet inhibition was confirmed 10 minutes after incubation with GSK219 (100 nM). Ryanodine (RyR inhibitor; 1 μM) and xestospongin C (IP_3R inhibitor, 8 μM; Cayman Chemicals, Ann

Arbor, MI, USA) were used for studies of RyR Ca^{2+} sparks and IP_3R Ca^{2+} pulsars, respectively. Ca^{2+} images were analyzed using custom-designed SparkAn software developed by Dr. Adrian Bonev (University of Vermont). Fractional fluorescence traces (F/F_0) were obtained by placing a 1.7 μm^2 (5 × 5 pixels) region of interest (ROI) at the peak event amplitude. Representative F/F_0 traces were filtered using a Gaussian filter and a cutoff corner frequency of 4 Hz.[9] SMC TRPV4 sparklet measurements in endothelium-denuded MAs showed that the absence of endothelium does not alter the activity of SMC TRPV4 sparklets.

Analysis of the activity of SMC TRPV4 sparklets. Ca^{2+} signals were assessed as increased fluorescence relative to baseline fluorescence obtained by averaging 10 quiescent images prior to stimulation. TRPV4 Ca^{2+} sparklets were assessed based on previously established methods.[9,16] Average TRPV4 sparklet activity is defined as NP_O, where N is the number of TRPV4 channels per site and P_O is the open state probability of the channel. NP_O was calculated using the Single Channel Search module of Clampfit, quantal amplitudes derived from all-points histograms (0.3 $\Delta F/F_0$ for Fluo-4-loaded MAs; Figure S3B),[9,18] and the following equation:

$$\mathbf{NP_O} = (T_{level1} + 2T_{level2} + 3T_{level3} + 4T_{level4})/T_{total},$$

where T represents the dwell time at each quantal level and T_{total} is the total recording duration. Average NP_O per site was obtained by averaging the NP_O for all sites in a field of view. NP_O per site from all fields in an artery were averaged to obtain NP_O per site for that artery. The total number of sites per cell indicates all sparklet sites in a field divided

by the number of cells in that field. Sparklet sites per cell were averaged for all fields in an artery to obtain sparklet sites per cell for that artery.

Construction of all-points histograms. All-points histograms were constructed as described previously.[18] Briefly, images were filtered using a Kalman filter adapted from an ImageJ plug-in that utilizes an acquisition noise variance estimate of 0.05 and a filter gain of 0.8 (Christopher Philip Mauer, Northwestern University, Chicago, IL). The inclusion criteria were a stable baseline containing at least five steady points and a steady peak containing at least five peak points. Sparklet traces were exported to ClampFit10.3 for construction of an all-points histogram, which was fit with the multiple Gaussian function,

$$f(F/F_0) = \sum_{i=1}^{N} \frac{a_i}{\sqrt{2\pi\sigma_i}} \exp\left[\frac{-(\frac{F}{F_0} - \mu_i)^2}{2\sigma_i^2}\right]$$

where F/F_0 represents fractional fluorescence, *a* represents the area, μ represents the mean value, and σ^2 represents the variance of the Gaussian distribution. While detected sparklets can have multiple amplitudes corresponding to quantal level 1, 2, 3 or 4, the baseline (level 0) was the same for all detected sparklets regardless of their amplitude. Therefore, higher counts were obtained for baselines compared with all other events.

Analysis of RyR Ca^{2+} sparks and IP_3R Ca^{2+} signals. RyR and IP_3R signals in Fluo-4AM–loaded, pressurized MAs were automatically detected with SparkAn using a threshold of 1.25 F/F_0 and a scan box of 7 × 7 pixels. To account for a changing baseline (movement of the artery or change in focus), we created an average image and obtained F_{min} every 10 images. The number of IP_3R events was determined by subtracting the

number of events in the presence of the IP_3R inhibitor xestospongin C (8 μM) from the number before xestospongin C. RyR events were determined by subtracting the number of events in the presence of the RyR inhibitor ryanodine (1 μM) from the number before ryanodine. The numbers in different fields of view from an artery were averaged for each artery.

Whole-cell patch-clamp electrophysiology. Patch electrodes were pulled with a Narishige PC-100 puller (Narishige International USA, Inc, Amityville, NY) and polished using a MicroForge MF-830 polisher (Narishige International USA). The pipette resistance was (3–5 ΩM). Data were acquired using a Multiclamp 700B amplifier connected to a Digidata 1550B system and analyzed using Clampfit 11.1 software (Molecular Devices, San Jose, CA, USA) and MATLAB R2018a (MathWorks, Natick, MA, USA). TRPV4 channel currents were recorded from freshly isolated SMCs as described previously.[9,18] GSK101 (30 nM)-induced outward currents through TRPV4 channels were assessed using the whole-cell patch-clamp configuration in the presence of ruthenium red (RuR), included to prevent Ca^{2+} influx through TRPV4 channels and activation of BK channels. The intracellular solution consisted of 10 mM HEPES, 30 mM KCl, 10 mM NaCl, 110 mM K-aspartate and 1 mM $MgCl_2$ (adjusted to pH 7.2 with NaOH). HEPES-PSS was used as the bath solution. TRPV4 currents before and after PE (1 μM) were measured using a voltage-clamp protocol in which voltage-ramp pulses (-100 mV to +100 mV) were applied over 200 ms from a holding potential of -50 mV. Currents were measured before and 5 minutes after treatment with GSK219 (100 nmol/L),[9] with GSK219-sensitive currents corresponding TRPV4 channel-mediated currents. TRPV4 channel- or α1AR-induced activation of BK channels was evaluated by applying GSK101 (30 nM) or PE (1

μM), respectively. BK currents were measured using a voltage ramp from -140 mV to +60 mV over 250 ms. Currents were measured before and 5 minutes after treatment with paxilline (1 μM), with paxilline-sensitive currents corresponding to BK currents. The BK channel activator GoSlo SR 5-69 (1 μM) was also used to assess direct activation of BK channels.

***In situ* proximity ligation assay (PLA)**. Mouse third-order MAs and human paraspinal muscle arteries were isolated and pinned down *en face* on a SYLGARD block.[47] Arteries were fixed in paraformaldehyde (4%) for 15 minutes, washed three times with PBS, and then permeabilized by incubating in a solution containing 0.2% Triton X for 30 minutes at room temperature. After blocking by incubating with 5% normal donkey serum (Abcam plc) and 300 mM glycine at room temperature for 1 hour, arteries were washed three times with PBS and incubated overnight at 4°C with anti-TRPV4 (1:200, LS_C94498; LifeSpan BioScience Inc.), anti-α_{1D}-AR (1:100, sc-390884; Santa Cruz, Dallas, TX, USA), anti-Slo1/BKα channel (1:100, 75-408; NeuroMab, Davis, CA, USA), PKCα (1:100, sc-17769; Santa Cruz, Dallas, TX, USA or 1:100, 59754S; Cell Signaling Technology, Danvers, MA, USA) or anti-KCNMA1 (1:100, APC-107; alomone labs, Jerusalem, Israel) primary antibodies. The following day, PLA protocol from Duolink PLA Technology kit (MilliporeSigma) was followed. At the end of the protocol, nuclei were stained by treating arteries with 0.3 μM DAPI (Invitrogen). Images were acquired using an Andor Revolution spinning-disk confocal imaging system and analyzed using a Duolink Image Tool (MilliporeSigma). Images were obtained along the z-axis at a slice thickness of 0.1 μm from the top of the endothelial cells to the bottom of the smooth muscle cells. Image analysis was performed using IMARIS version 9.3. Images were analyzed by normalizing

the number of positive puncta by the number of nuclei in a field of view. Knockout mice and secondary antibody alone were used as negative controls for PLA experiments.[9,19]

Superresolution microscopy. A dSTORM imaging system (Abbelight, Paris, France) built around an inverted microscope (DMi8; Leica, Wetzlar, Germany) was used for acquisition of superresolution images. The microscope was configurated with a 63X HC Plan-Apochromat (numerical aperture, 1.47; Leica) oil-immersion objective and an electron multiplying CCD camera (Sona 4.2B-6; Andor Technology). Cells were seeded in a dish containing poly-d-lysine–coated microwells (MatTek, Ashland, MA, USA), fixed with 4% paraformaldehyde for 20 minutes, and then treated with 0.2% Triton-X PBS for 60 minutes at room temperature. Thereafter, cells were treated with 5% normal donkey serum (Abcam plc) for 1 hour at room temperature and subsequently incubated with anti-TRPV4 (1:200, LS_C94498, LifeSpan BioScience Inc.) or anti-α_{1D}-AR (1:100, sc-390884, Santa Cruz Biotechnology) primary antibody overnight at 4°C. Cells were than washed three times with PBS and incubated with Alexa Fluor 555-conjugated goat anti-mouse (1:500; Life Technologies) or Atto 488-conjugated goat anti-rabbit (1:500; MilliporeSigma) secondary antibody, as appropriate, at room temperature for 1 hour in the dark. Cells treated with secondary antibody-only and SMCs from MAs of $TRPV4_{SMC}^{-/-}$ mice were used as negative controls (Figure S19A, 19B). After three PBS washes, cells were incubated with Alexa Fluor 647-conjugated anti-MaxiKα antibody (B-1) (1:100, sc-374142 AF647; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 1 hour in the dark. For imaging, cells were embedded in STORM mounting buffer (Abbelight). Fluorophores were excited using 488 nm (50 mW), 560 nm (200 mW), and 640 nm (500 mW) lasers.

Optimal image results were achieved through a combination of corrections and background removal using NEO analysis software (Abbelight).

Co-localization of BK channels or α_1AR within 100 nm of TRPV4 channels (TRPV4:α_1AR and TRPV4:BK) in dSTORM images was assessed using a built-in Matlab R2019b co-localization feature in IMARIS (version 9.3; Bitplane AG, Zurich, Switzerland). The percentage of TRPV4 channels that colocalized with α_1AR or BK channels or both (triple co-localization) was determined from automated counts of the total number of TRPV4 channel puncta in each field of view.

Chronic angiotensin II (Ang II) infusion in mice. Mice were infused with Ang II (1 μg/kg/min) or 0.9% saline for 14 days.[2] Briefly, mice were anesthetized with isoflurane (1.5%) and implanted with an osmotic micropump (Alzet Model 1004; DURECT Corporation, Cupertino, CA, USA) containing Ang II or saline. Blood pressure was monitored in conscious mice using radiotelemetric blood pressure measurements as described above.

SMC Ca^{2+} concentration analysis. SMC Ca^{2+} concentrations were estimated using the maximal fluorescence method.[48] A maximum fluorescence intensity (F_{max}) was obtained by adding the Ca^{2+} ionophore, ionomycin (10 μM), and 20 mM external Ca^{2+}. An estimate of $[Ca^{2+}]_i$ is obtained for a fluorescence intensity (F) from a ROI placed on each SMC using the equation:

$$\mathrm{Ca}^{2+} = K_d \frac{F \,/\, F_{max} - 1 \,/\, R_f}{1 - F \,/\, F_{\max}}$$

where K_d is the Fluo-4 AM dissociation constant (340 nM) and R_f is the ratio of Fluo-4 AM fluorescence in vitro at saturation relative to 0 [Ca^{2+}] (=100).

Drugs and chemical compounds. Drugs were obtained from Tocris Bioscience (Minneapolis, MN, USA), except as stated otherwise. PE and Ang II were dissolved in water; nifedipine was dissolved in ethanol; and CPA, GSK101, GSK219, U73122, prazosin hydrochloride, paxilline, and GoSlo SR 5-69 were dissolved in DMSO. Subsequent dilutions of drugs were made in PSS.

Statistical analysis. Results are presented as means ± SEM. N=1 is defined as one artery in imaging experiments (Ca^{2+} imaging and immunofluorescence), one cell for patch-clamp experiments, one mouse for blood pressure measurements, one artery for pressure myography experiments, and one mouse for qPCR experiments. Data were obtained from at least two independent experiments performed using at least three mice each. Data were graphically presented using CorelDraw Graphics Suite X7 (Ottawa, ON, Canada) and statistically analyzed using GraphPad Prism 8.3.0 (San Diego, CA, USA). Group sizes and study power (>0.8) were determined by power analysis using GLIMMPSE software ($\alpha = 0.05$; >20% change). Normality was determined using a Shapiro-Wilk test, which showed that all data were normally distributed; therefore, parametric statistics were performed. Data were analyzed using two-tailed, paired or independent t-test, one-way analysis of variance (ANOVA) or two-way ANOVA. Tukey's correction was performed for multiple comparisons with one-way ANOVA, and a Bonferroni correction was performed for multiple comparisons with two-way ANOVA. P-

values less than 0.05 were considered statistically significant. Individual P-values are indicated in figure legends, ns indicates not significant (P greater than or equal to 0.05).

SUPPLEMENTAL DATA

Table S1. Clinical parameters of human subjects, HTN: hypertensive

Patient	Gender	Anti-HTN medicines	Other medicines	Blood pressure (mm Hg)	Reason for surgery
Non-HTN					
1	F	None	None	133/82	Lumbar fusion
2	M	None	None	134/87	Thoracolumbar fusion
3	M	None	None	123/72	Lumbar decompression
4	M	None	None	146/79	Lumbar decompression
5	M	None	Ibuprofen	133/79	Lumbar decompression
6	F	None	Aspirin	139/79	Thoracolumbar fusion
7	F	None	Diclofenac	110/68	Lumbar fusion
8	M	None	None	121/72	Lumbar fusion
9	M	None	None	128/68	Cervical fusion
10	M	None	Diclofenac	146/68	Lumbar decompression
HTN					
1	M	Losartan	Ibuprofen	153/94	Lumbar decompression
2	M	Lisinopril	None	157/87	Lumbar fusion
3	F	Lisinopril, Metoprolol	None	137/71	Lumbar decompression
4	M	Lisinopril	None	148/70	Lumbar fusion
5	F	Furosemide, Metoprolol	None	136/98	Lumbar fusion
6	M	Losartan, Metoprolol	Prednisone	130/77	Cervical decompression
7	M	Losartan	Aspirin	149/73	Lumbar fusion
8	M	Carvedilol, Lisinopril	None	101/51	Thoracolumbar epidural phlegmon/abscess causing stenosis
9	M	Amlodipine, Trandolapril	Aspirin	176/87	Cervical decompression
10	M	Amlodipine, Losartan, Triamterene-HCTZ	None	146/81	Cervical decompression
11	M	Atenolol, lisinopril	None	147/103	Cervical decompression

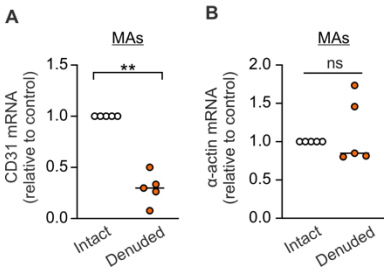

Figure S1. (A) CD31 mRNA levels and (B) smooth muscle α-actin mRNA levels in intact MAs (n = 4) and endothelium-denuded MAs (n = 5) of control C57BL6 mice (**P < 0.01; ns, not significant; unpaired t-test).

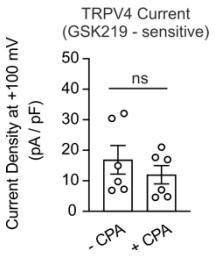

Figure S2**.** Averaged outward currents in SMCs isolated from MAs of Control mice (n = 6) at +100 mV in the absence or presence of CPA (20 μM) and GSK219 (100 nM) (ns, not significant; paired t-test).

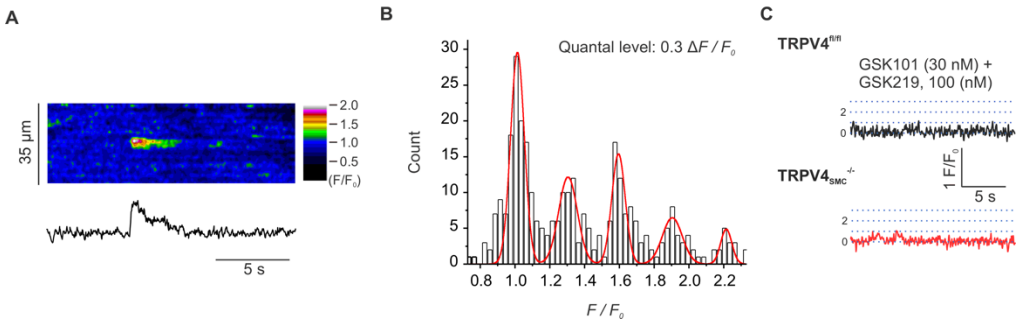

Figure S3. Local Ca^{2+}-influx events through TRPV4 channels (TRPV4 sparklets), recorded in pressurized MAs loaded with Fluo-4AM (10 µM). (A) Pseudo–line-scan image and associated trace recorded from a single site over the same interval. (B) All-points amplitude histogram of sparklet traces in pressurized MAs from $TRPV4^{fl/fl}$ mice. (C) Traces showing inhibition of sparklet activity in the presence of CPA (20 µM; used to eliminate interference from Ca^{2+} release from the sarcoplasmic reticulum), nifedipine (1 µM), GSK101 (30 nM), and the TRPV4 inhibitor GSK219 (100 nM) in $TRPV4^{fl/fl}$ and $TRPV4_{SMC}^{-/-}$ mice. Dotted blue lines represent quantal levels (single-channel amplitudes) determined from all-points histograms.

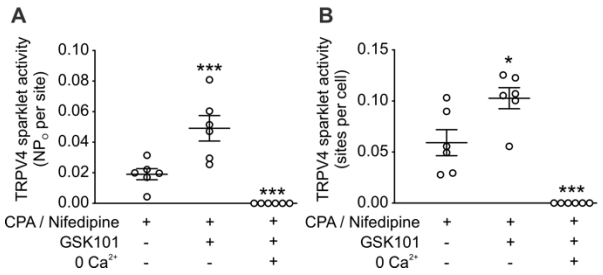

Figure S4**.** TRPV4 sparklet activity in intact SMCs from pressurized MAs. Averaged TRPV4 sparklet activity (NP_o) (A) and TRPV4 sparklet sites per cell (B) under basal conditions (CPA/nifedipine, 1 μM) in the presence of GSK101 (30 nM) and GSK101 + Ca^{2+}-free PSS (0 Ca^{2+}) in MAs from $TRPV4^{fl/fl}$ mice (n = 6) (*P < 0.05, ***P < 0.001 vs. CPA/nifedipine; one-way ANOVA).

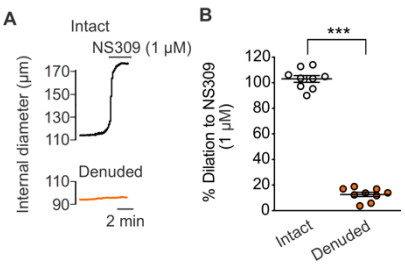

Figure S5. (A) Representative diameter traces for NS309 (1 μM)-induced dilation in intact MAs and endothelium-denuded MAs of control C57BL6 mice. (B) Percent dilation of intact MAs (n = 9) and endothelium-denuded MAs (n = 9) from C57BL6 mice in response to NS309 (***P < 0.001 vs. Intact; unpaired t-test).

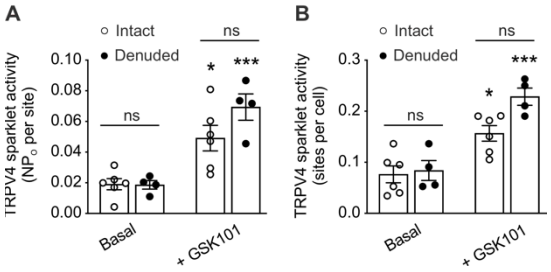

Figure S6**.** TRPV4 sparklet activity in SMCs from pressurized intact and endothelium-denuded MAs. Averaged TRPV4 sparklet activity (NP_o) (A) and TRPV4 sparklet sites per cell (B) under basal conditions (CPA/nifedipine, 1 μM) in the presence of GSK101 (30 nM) in MAs from $TRPV4^{fl/fl}$ mice (Intact, n = 6; Denuded, n = 4; ***P < 0.001, *P < 0.05 vs. Basal; ns, not significant; two-way ANOVA).

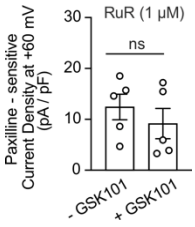

Figure S7**.** Averaged paxilline-sensitive currents in isolated SMCs at +60 mV in the presence of RuR (1 μM) with (n = 5) or without (n = 5) GSK101 (30 nM) (ns, not significant; unpaired t-test).

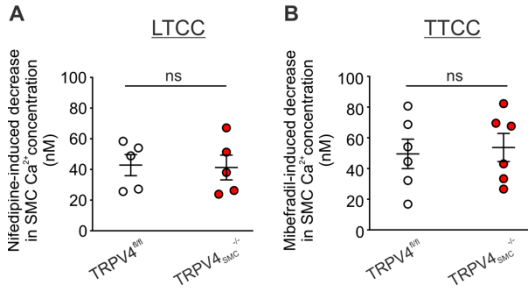

Figure S8. (A) Data points representing nifedipine (LTCC inhibitor, 1 μM)–sensitive whole cell Ca^{2+} levels in SMCs from pressurized MAs of $TRPV4^{fl/fl}$ (n = 5) and $TRPV4_{SMC}^{-/-}$ (n = 5) mice. (B) Data points representing mibefradil (TTCC inhibitor, 1 μM)–sensitive whole cell Ca^{2+} levels in SMCs from pressurized MAs of $TRPV4^{fl/fl}$ (n = 6) and $TRPV4_{SMC}^{-/-}$ (n = 6) mice (ns, not significant; unpaired t-test).

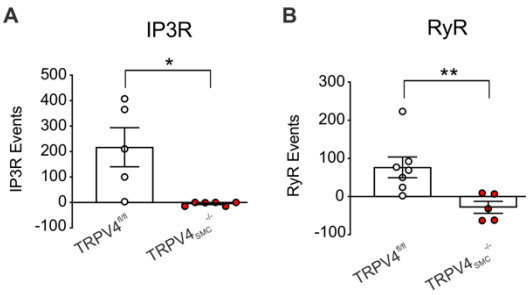

Figure S9**.** (A) Number of IP3R events per field of view in 30 seconds, indicating Xestospongin C (IP3R inhibitor, 8 μM)-sensitive Ca^{2+} events in MAs from $TRPV4^{fl/fl}$ (n = 5) and $TRPV4_{SMC}^{-/-}$ (n = 6) mice. *P < 0.05 vs. $TRPV4^{fl/fl}$, unpaired t-test. (B) Number of RyR events per field of view in 30 seconds, indicating ryanodine (RyR inhibitor, 1 μM) – sensitive Ca^{2+} events in MAs from $TRPV4^{fl/fl}$ (n = 7) and $TRPV4_{SMC}^{-/-}$ mice (n = 5). **P < 0.01 vs. $TRPV4^{fl/fl}$; unpaired t-test.

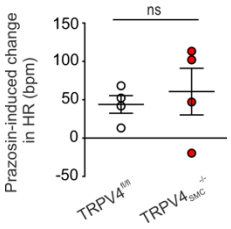

Figure S10**.** Change in heart rate (HR) after a single bolus injection of prazosin (1 mg/kg; i.p., n = 4) (ns, not significant; unpaired t-test).

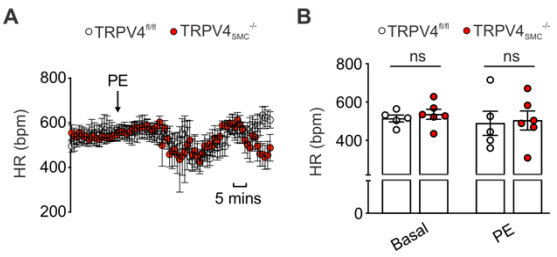

Figure S11. (A) Heart rate (HR; beats per minute [bpm]) in $TRPV4^{fl/fl}$ (n = 5) and $TRPV4_{SMC}^{-/-}$ (n = 6) mice, averaged before and after the administration of PE. (B) HR after a single bolus injection of PE over 30 minutes (ns, not significant; two-way ANOVA).

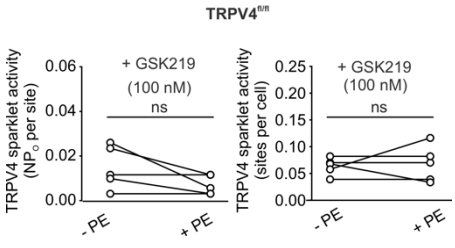

Figure S12. TRPV4$_{SMC}$ sparklets in MAs from TRPV4$^{fl/fl}$ mice, expressed as activity per site (*left*) and sites per cell (*right)*. Fluo-4AM-loaded, pressurized MAs from TRPV4$^{fl/fl}$ (n = 9) mice were pretreated with CPA (20 μM), nifedipine (1 μM), and GSK101 (30 nM). Experiments were performed before and after adding PE, with and without GSK219 (100 nM) pretreatment (ns, not significant; paired t-test).

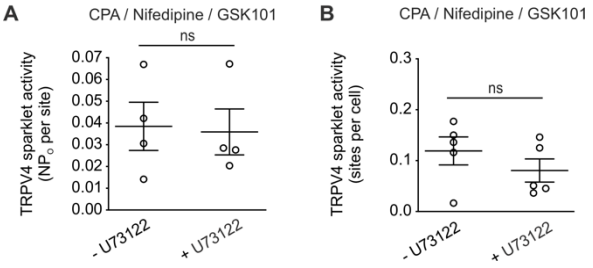

Figure S13**.** TRPV4 sparklet activity in intact SMCs from pressurized MAs. Effects of U73122 (3 μM) on GSK101 (30 nM)-induced (A) TRPV4 sparklet activity (NP_O) and (B) TRPV4 sparklet sites per cell in MAs from control mice (n = 5; ns, not significant; unpaired t-test).

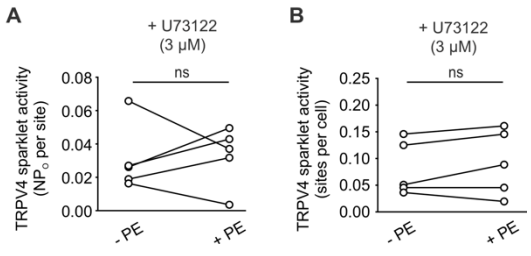

Figure S14**.** Effects of the PLC inhibitor U73122 (3 μM; n = 5) on PE-induced increase in $TRPV4_{SMC}$ sparklet activity per site (A) and sites per cell (B) in MAs from $TRPV4^{fl/fl}$ mice (ns, not significant; paired t-test).

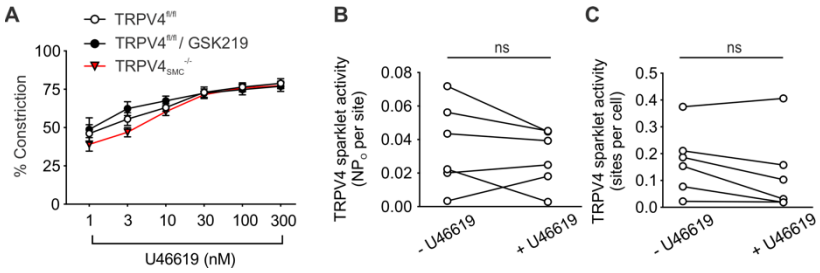

Figure S15. (A) U46619 (TXA_2 receptor agonist)-induced constriction of MAs from $TRPV4^{fl/fl}$ and $TRPV4_{SMC}^{-/-}$ mice in the absence or presence of the TRPV4 inhibitor GSK219 ($TRPV4^{fl/fl}$, n = 5; $TRPV4_{SMC}^{-/-}$, n = 6). Effect of U46619 on $TRPV4_{SMC}$ sparklet activity (B) and sparklet sites per cell (C) in MAs from $TRPV4^{fl/fl}$ mice (n = 5; ns, not significant; paired t-test).

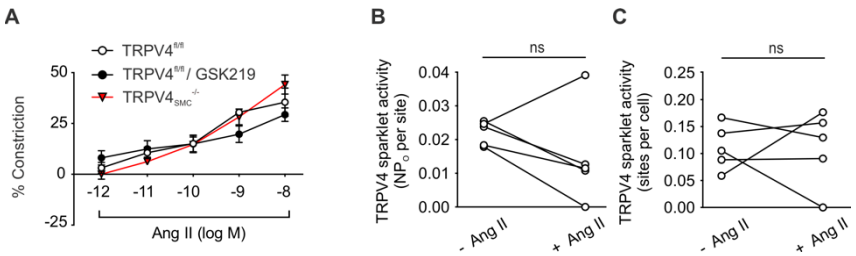

Figure S16. (A) Effects of the TRPV4 inhibitor GSK219 (100 nM) on Ang II-induced constriction of MAs from $TRPV4^{fl/fl}$ (n = 5) and $TRPV4_{SMC}^{-/-}$ (n = 5) mice. Effects of Ang II (1 nM) on TRPV4 sparklet activity (B) and sparklet sites per cell (C) in MAs from $TRPV4^{fl/fl}$ mice (n = 5; ns, not significant; paired t-test).

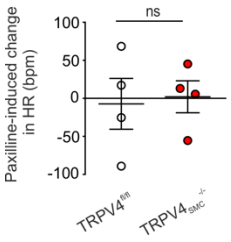

Figure S17**.** Change in heart rate (HR) after a single bolus injection of paxilline (8 mg/kg, i.p.; $TRPV4^{fl/fl}$, n = 4; $TRPV4_{SMC}^{-/-}$, n = 4), compared 15 minutes after paxilline administration (ns, not significant; unpaired t-test).

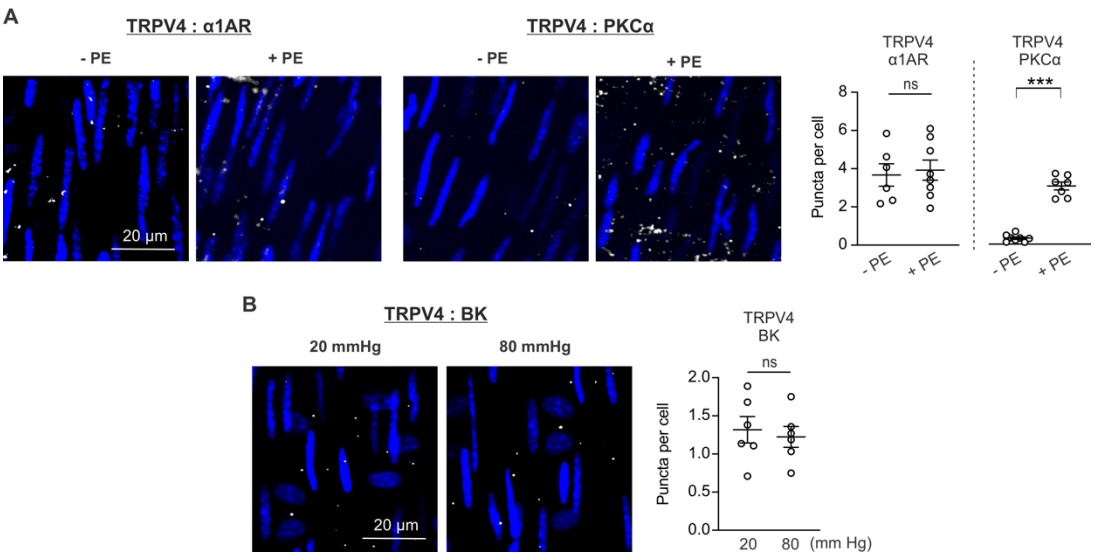

Figure S18. (A) Representative *in situ* PLA images showing SMC nuclei (blue), TRPV4$_{SMC}$:α1AR or TRPV4$_{SMC}$:PKCα co-localization (white puncta) in *en face* preparations of control (- PE, TRPV4$_{SMC}$:α1AR, n = 6; TRPV4$_{SMC}$:PKCα, n = 8) and phenylephrine (PE)–treated (+ PE, TRPV4$_{SMC}$:α1AR, n = 8; TRPV4$_{SMC}$:PKCα, n = 7) MAs from wild-type C57BL6 mice. ***Right***, quantification of TRPV4$_{SMC}$:α1AR and TRPV4$_{SMC}$:PKCα co-localization in control and PE–treated MAs from wild-type C57BL6 mice (***P < 0.001 vs. - PE; ns, not significant; unpaired t-test). (B) ***Left***, representative *in situ* PLA images showing SMC nuclei (blue) and TRPV4$_{SMC}$:BK co-localization (white puncta) in *en face* preparations of pressurized MAs from wild-type C57BL6 mice. ***Right***, quantification of TRPV4$_{SMC}$:BK co-localization in pressurized MAs (n = 6; not significant; unpaired t-test).

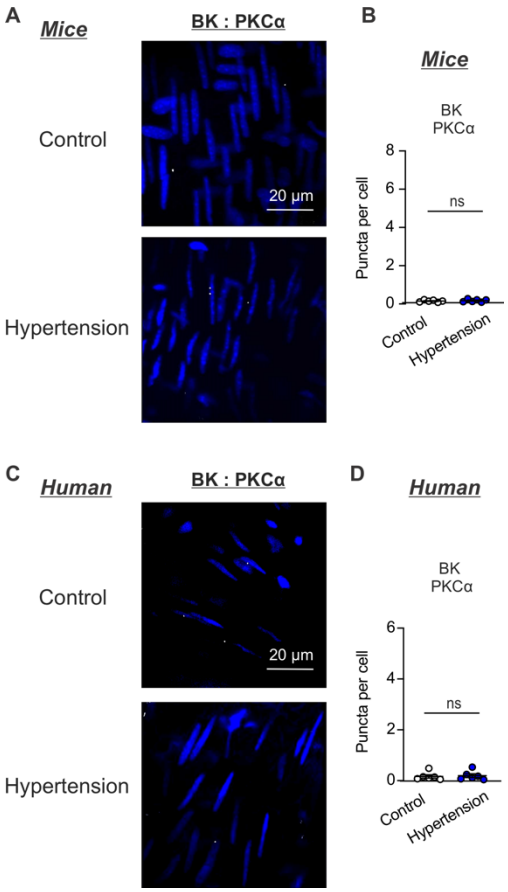

Figure S19. (A) Representative *in situ* PLA images showing SMC nuclei (blue) and BK:PKCα co-localization (white puncta) in *en face* preparations of MAs from Ang II-induced hypertensive mice and control mice. (B) Quantification of BK:PKCα (Control, n = 6; Hypertension, n = 6) co-localization in MAs from Ang II-induced hypertensive mice and control mice (ns, not significant; unpaired t-test). (C) Representative *in situ* PLA images showing SMC nuclei (blue) and BK:PKCα co-localization (white puncta) in *en face* preparations of paraspinal muscle arteries from non-hypertensive (control) and hypertensive individuals. (D) Quantification of BK:PKCα (Control, n = 6; Hypertension, n = 6) colocalization in paraspinal muscle arteries from non-hypertensive and hypertensive individuals (ns, not significant; unpaired t-test).

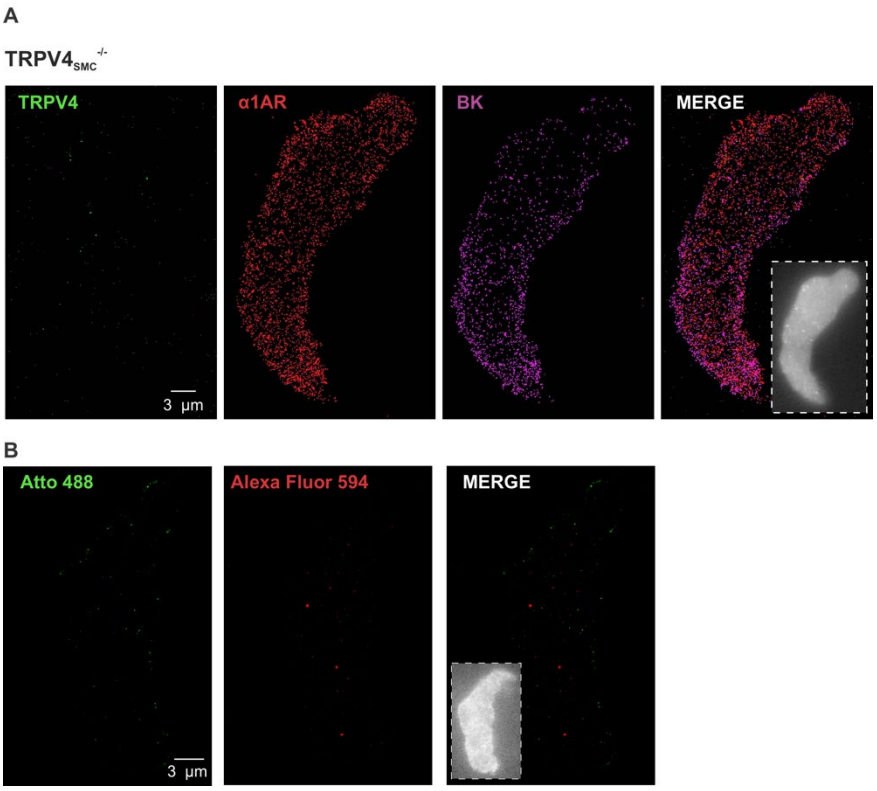

Figure S20**.** Verification of TRPV4 channel, α1AR and BK channel primary antibodies. (A) dSTORM images of TRPV4 channel, α1AR and BK channel primary antibodies with corresponding secondary antibodies in a mesenteric artery SMC from a $TRPV4_{SMC}^{-/-}$ mouse. Inset (dotted rectangle) shows a snapshot of an entire SMC. (B) Images of a mesenteric artery SMC from a control mouse exposed to secondary antibody only (Atto 488 or Alexa Fluor 594). Inset (dotted rectangle) shows a snapshot of an entire SMC.

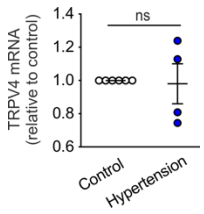

Figure S21. TRPV4 mRNA levels in endothelium-denuded MAs of control mice (n = 6) and Ang II-induced hypertensive mice (n = 4; ns, not significant; unpaired t-test).

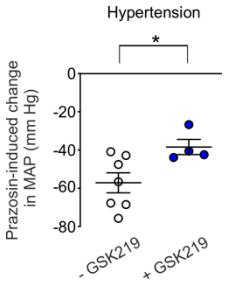

Figure S22. Decrease in mean arterial pressure (MAP) of Ang II-induced hypertensive mice after a single bolus injection of prazosin (1 mg/kg, i.p.) in the absence (n = 7) or presence (n = 4) of GSK219 (1 mg/kg, i.p., *P < 0.05 vs. – GSK219; unpaired t-test).

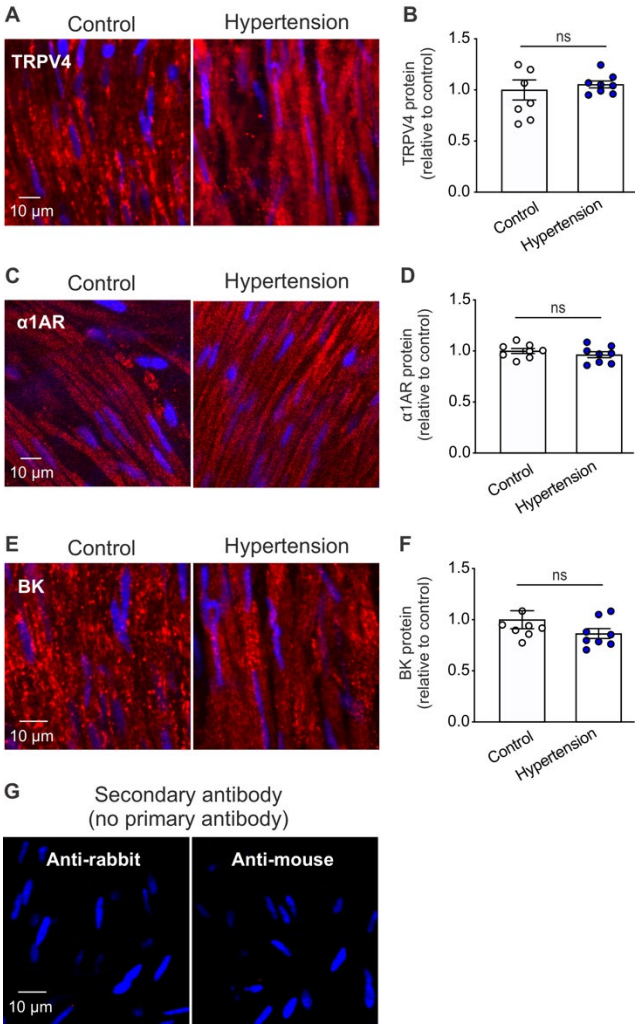

Figure S23. Representative immunostaining images showing SMC nuclei (blue), $TRPV4_{SMC}$ (red) (A), α1AR (red) (C), or BK (red) (E) immunofluorescence in *en face* preparations of vessels from non-hypertensive (control) subjects and hypertensive patients. Quantification of $TRPV4_{SMC}$ (B), α1AR (D) and BK (F) immunofluorescence intensity in *en face* preparations of vessels from non-hypertensive (control; n = 8) and hypertensive individuals (n = 8) (ns, not significant; unpaired t-test). (G) Images of *en face* preparations of paraspinal muscle arteries from non-hypertensive (control) individual exposed to secondary antibody only (anti-rabbit or anti-mouse).

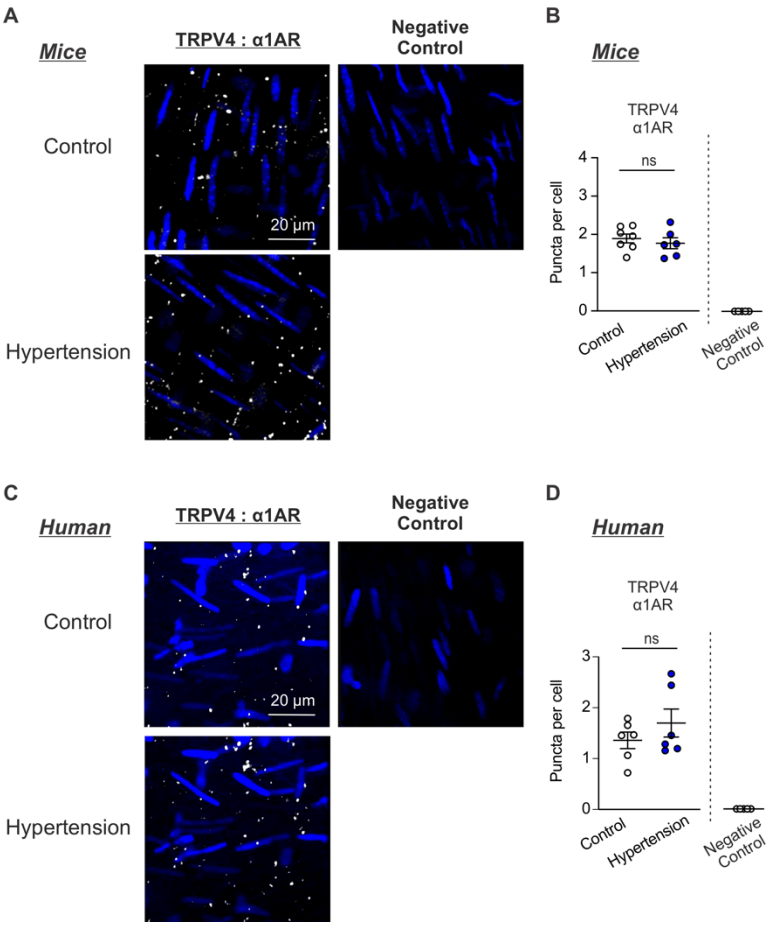

Figure S24. (A) Representative *in situ* PLA images showing SMC nuclei (blue) and TRPV4$_{SMC}$:α1AR co-localization (white puncta) in *en face* preparations of MAs from Ang II-induced hypertensive mice and control mice. (B) Quantification of TRPV4$_{SMC}$:α1AR (Control, n = 7; Hypertension, n = 6) and negative control (no primary antibody) co-localization in MAs from Ang II-induced hypertensive mice and control mice (ns, not significant; unpaired t-test). (C) Representative *in situ* PLA images showing SMC nuclei (blue) and TRPV4$_{SMC}$:α1AR co-localization (white puncta) in *en face* preparations of paraspinal muscle arteries from non-hypertensive (control) and hypertensive individuals. (D) Quantification of TRPV4$_{SMC}$:α1AR (Control, n = 6; Hypertension, n = 6), and negative

control (no primary antibody) co-localization in paraspinal muscle arteries from non-hypertensive (control) and hypertensive individuals (ns, not significant; unpaired t-test).