Supplemental Material

Expanded Methods

Tissue and Cell Preparation

Animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Male Sprague-Dawley rats (6-8 weeks) were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Only male rats were used to avoid potential confounding effects of the estrus cycle on ion channel expression and regulation. The brain was removed and placed into physiological saline solution (PSS) of composition: (in mmol/L) 112 NaCl, 4.8 KCl, 24 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 10 glucose, which was gassed with 21% O_2-5% CO_2-74% N_2 to pH 7.4. Resistance-size (~200 μm diameter) cerebral (posterior cerebral, cerebellar, middle cerebral) arteries were dissected from the brain and used for experimentation. Smooth muscle cells were isolated from cerebral arteries as previously described [1].

Patch Clamp Electrophysiology

Patch-clamp electrophysiology was performed using isolated cerebral artery myocytes or human embryonic kidney 293 (HEK293) cells expressing recombinant TMEM16A channels. Membrane currents were recorded using an Axopatch 200B amplifier equipped with a CV 203BU headstage, Digidata 1332A, and Clampex 8 or 9 (Molecular Devices). Pipettes were pulled from borosilicate glass, heat polished to 1-3 MΩ, and waxed to reduce capacitance. Whole cell currents were filtered at 1 kHz using a low pass Bessel filter and digitized at 4 kHz. Single channel currents were filtered at 2 kHz and digitized at 8 kHz. The pipette solution contained (in mmol/L): 126 CsCl, 10 HEPES, 10 D-Glucose, 1 EGTA or 1 BAPTA, 1 MgATP, 0.2 GTP·Na, and 40 sucrose and pH adjusted to 7.2 with CsOH. Total $MgCl_2$ was adjusted to give final free Mg^{2+} of 1 mmol/L. For experiments on arterial myocytes, pipette free Ca^{2+} was 200 nM. For experiments on HEK293 cells expressing recombinant TMEM16A channels, pipette free Ca^{2+} was 1 μM. Free Mg^{2+} and Ca^{2+} were calculated using WebmaxC Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm) and confirmed using a Ca^{2+}-sensitive and reference electrode (Corning; Acton, MA). Bath solutions used are described in Online Table I. To study anion permeability, Cl^- was replaced with either aspartate or I^-. The osmolarity of solutions was measured using a Wescor 5500 Vapor Pressure Osmometer (Logan, UT). To minimize junction potential, the reference Ag/AgCl electrode was immersed in a solution of 3 mmol/L KCl continuous with an agar bridge (4% agar in 3 mmol/L KCl). Junction potentials (provided in Online Table I) were accounted for in voltage step protocols. Cl^- currents were measured by applying 1 s voltage steps to between -80 mV and +100 mV in 20 mV increments using an interpulse holding potential of -40 mV. Currents were normalized to membrane capacitance. Pharmacological agents and rabbit monoclonal anti-TMEM16A antibody (Abcam) were introduced directly into the experimental chamber. Boiled (15 min at 98 °C) denatured TMEM16A antibody served as a control for active antibody. The relative anion permeability ratio of I^- to Cl^- (P_I/P_{Cl}) or aspartate (Asp) to Cl^- (P_{Asp}/P_{Cl})was calculated using the shift in reversal potential (E_{rev}) and the constant field equation:

$P_X/P_{Cl} = [[Cl^-]_o(e^{(\Delta Erev(zF/RT)}]/[X]_o$, where X was I^- or Asp and zF/RT was -0.039 at 25°C.

Western Blotting
Cerebral arteries were homogenized using Laemmli sample buffer (2.5% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol in 100 mmol/L Tris·HCl, pH 6.8) and centrifuged at 6,000x g for 10 min to remove cellular debris. Proteins (40 μg/lane) were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Blots were physically cut at 75 kDa to permit probing for TMEM16A, TRPC6, TRPM4 or TRPP2 at the higher molecular weight and for actin at the lower molecular weight. Membranes were incubated with rabbit monoclonal anti-TMEM16A (1:100, Abcam), rabbit anti-TRPC6 (1:250, Sigma), rabbit anti-TRPM4 (1:500, Thermo Scientific), rabbit anti-TRPP2 (1:1000, Johns Hopkins Polycystic Kidney Disease Research and Clinical Core Center or 1:100, Santa Cruz) and mouse monoclonal anti-actin (1:5,000 dilution, Chemicon International) primary antibodies overnight at 4°C in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk. Proteins were visualized using horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Pierce) and a chemiluminescent detection kit (Pierce). Band intensity was quantified by digital densitometry using Quantity One software (Bio-Rad). Protein band intensity was normalized to actin.

TMEM16A Channel Knockdown
Three small interference RNA (siRNA) sequences targeting TMEM16A or negative control siRNA (Invitrogen), as used previously [2], were inserted intracellularly into cerebral arteries using either reverse permeabilization, as described [3-7] or a Bex CUY21Vivo-SQ electroporator. Arteries were then maintained in serum-free DMEM F12 media supplemented with 1% penicillin-streptomycin (Sigma) for 4 days following reverse permeabilization or 3 days after electroporation at 37°C in a sterile incubator (21% O_2, 5% CO_2). Western blotting was used to compare the effect of TMEM16A siRNA with control siRNA on protein expression. Band intensity of proteins from arteries treated with either TMEM16A siRNA or control siRNA were compared on the same membranes. Reverse permeabilization and electroporation similarly reduced TMEM16A protein (reverse permeabilization, 62±5% of control siRNA, n=7; electroporator, 56±1% of control siRNA, n=3) in arteries ($P>0.05$).

Cell culture and Transfection
HEK293 (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 transiently transfected with pcDNA3 encoding full-length recombinant TMEM16A (2 μg), a kind gift from Dr. Luis Galietta, Istituto Giannina Gaslini, Italy. Transfection was done using Effectene (Qiagen), according to the manufacturer's instructions. Transfected cells grown on sterile glass coverslips were used for electrophysiological experiments. Whole-cell currents were recorded from cells 36 to 72 h post-transfection.

Pressurized Artery Membrane Potential Measurements
Endothelium-denuded arteries were maintained at either 10 or 60 mmHg for 2 hours to ensure steady-state myogenic tone had occurred, as confirmed using edge-detection myography Membrane potential was measured by inserting glass microelectrodes filled with 3 M KCl (50–90 mΩ) into the adventitial side of pressurized arteries. Membrane potential was recorded using a WPI FD223 amplifier and digitized using pClamp 9.2 software (Axon Instruments) and a

personal computer. Criteria for successful intracellular impalements were 1) a sharp negative change in potential upon insertion; 2) stable voltage for at least 1 min after entry; 3) a sharp positive voltage deflection upon exit from the recorded cell; and 4) a <10% change in tip resistance after the impalement.

Pressurized Artery Diameter Measurements

Experiments were performed using PSS containing (in mmol/L): 112 NaCl, 4.8 KCl, 26 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 MgSO4, 1.2 KH_2PO4, and 10 glucose, gassed with 74% N_2, 21% O_2, 5% CO_2 (pH 7.4). Endothelium-denuded artery segments 1–2 mm in length were cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation; Burlington, VT). Intravascular pressure was altered using a reservoir and monitored using a pressure transducer. Arterial wall diameter was measured at 1 Hz using a CCD camera attached to a Nikon TS100-F microscope and the automatic edge-detection function of IonWizard software (Ionoptix, Milton, MA). Luminal flow was absent during experiments. Myogenic tone (%) was calculated as 100x(1-active diameter/passive diameter). Endothelial denudation was confirmed using methods previously described [4].

Statistical Analysis

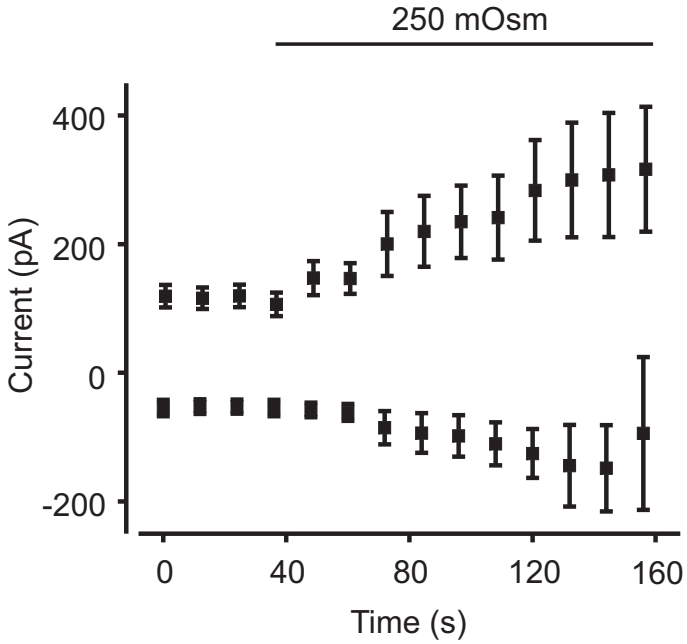
OriginLab and GraphPad InStat software were used for statistical analyses. Values are expressed as mean±SEM. Student's *t*-test was used for comparing paired and unpaired data from two populations, and ANOVA with Student–Newman–Keuls post-hoc test used for multiple group comparisons. $P<0.05$ was considered significant. Power analysis was performed on all data where $P>0.05$ to verify that sample size was sufficient to give a power value of >0.8.

Supplemental References

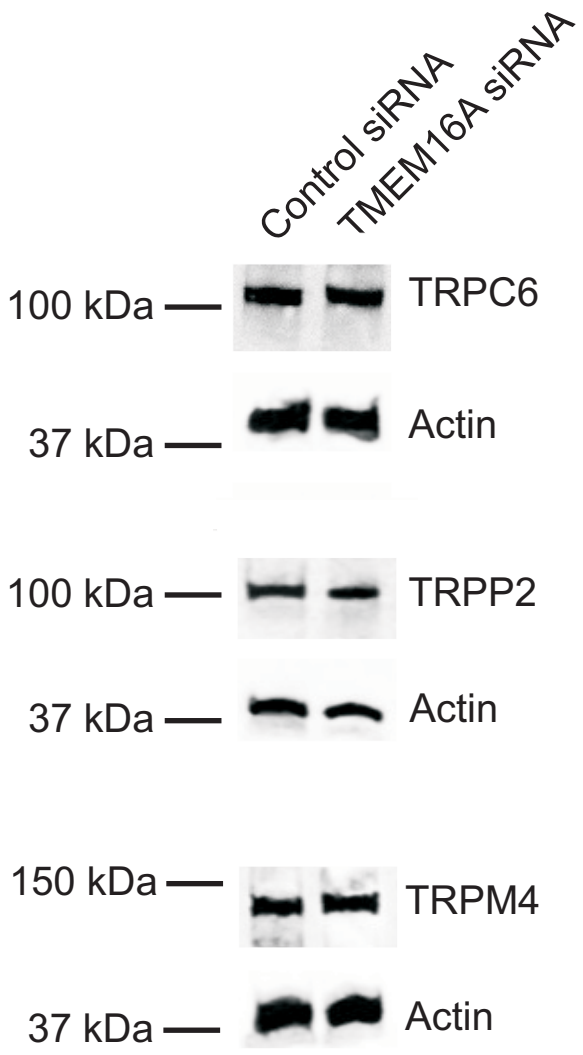
1. Jaggar JH. Intravascular pressure regulates local and global Ca^{2+} signaling in cerebral artery smooth muscle cells. *Am J Physiol.* 2001;281:C439-C448.
2. Thomas-Gatewood C, Neeb ZP, Bulley S, Adebiyi A, Bannister JP, Leo MD, Jaggar JH. TMEM16A channels generate Ca^{2+}-activated Cl^- currents in cerebral artery smooth muscle cells. *Am J Physiol Heart Circ Physiol.* 2011;301:1819-27.
3. Adebiyi A, Zhao G, Narayanan D, Thomas CM, Bannister JP, Jaggar JH. Isoform-selective physical coupling of TRPC3 channels to IP_3 receptors in smooth muscle cells regulates arterial contractility. *Circ Res.* 2010;106:1603-12.
4. Bannister JP, Adebiyi A, Zhao G, Narayanan D, Thomas CM, Feng JY, Jaggar JH. Smooth muscle cell $\alpha_2\delta$-1 subunits are essential for vasoregulation by $Ca_V1.2$ channels. *Circ Res.* 2009;105:948-55.
5. Bannister JP, Thomas-Gatewood CM, Neeb ZP, Adebiyi A, Cheng X, Jaggar JH. $Ca_V1.2$ channel N-terminal splice variants modulate functional surface expression in resistance size artery smooth muscle cells. *J Biol Chem.* 2011;286:15058-66.
6. 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;77:220-30.
7. Zhao G, Adebiyi A, Blaskova E, Xi Q, Jaggar JH. Type 1 inositol 1,4,5-trisphosphate receptors mediate UTP-induced cation currents, Ca^{2+} signals, and vasoconstriction in cerebral arteries. *Am J Physiol.* 2008;295:C1376-C1384.

	NMDG-Cl	NMDG-I	NMDG-Asp	HEPES	EGTA	Sucrose	$MgCl_2$	$CaCl_2$	Glucose	pH	Junction Potential
Normosmotic	126	–	–	10	–	40	1	2	10	7.4	9.9
Hyposmotic	126	–	–	10	–	–	1	2	10	7.4	9.9
Aspartate/low Cl^- hyposmotic	–	–	126	10	–	–	1	2	10	7.4	-0.3
Iodide/low Cl^- hyposmotic	–	126	–	10	–	–	1	2	10	7.4	10
Ca^{2+}-free normosmotic	126	–	–	10	1	40	1	–	10	7.4	10.2
Ca^{2+}-free hyposmotic	126	–	–	10	1	–	1	–	10	7.4	10.2

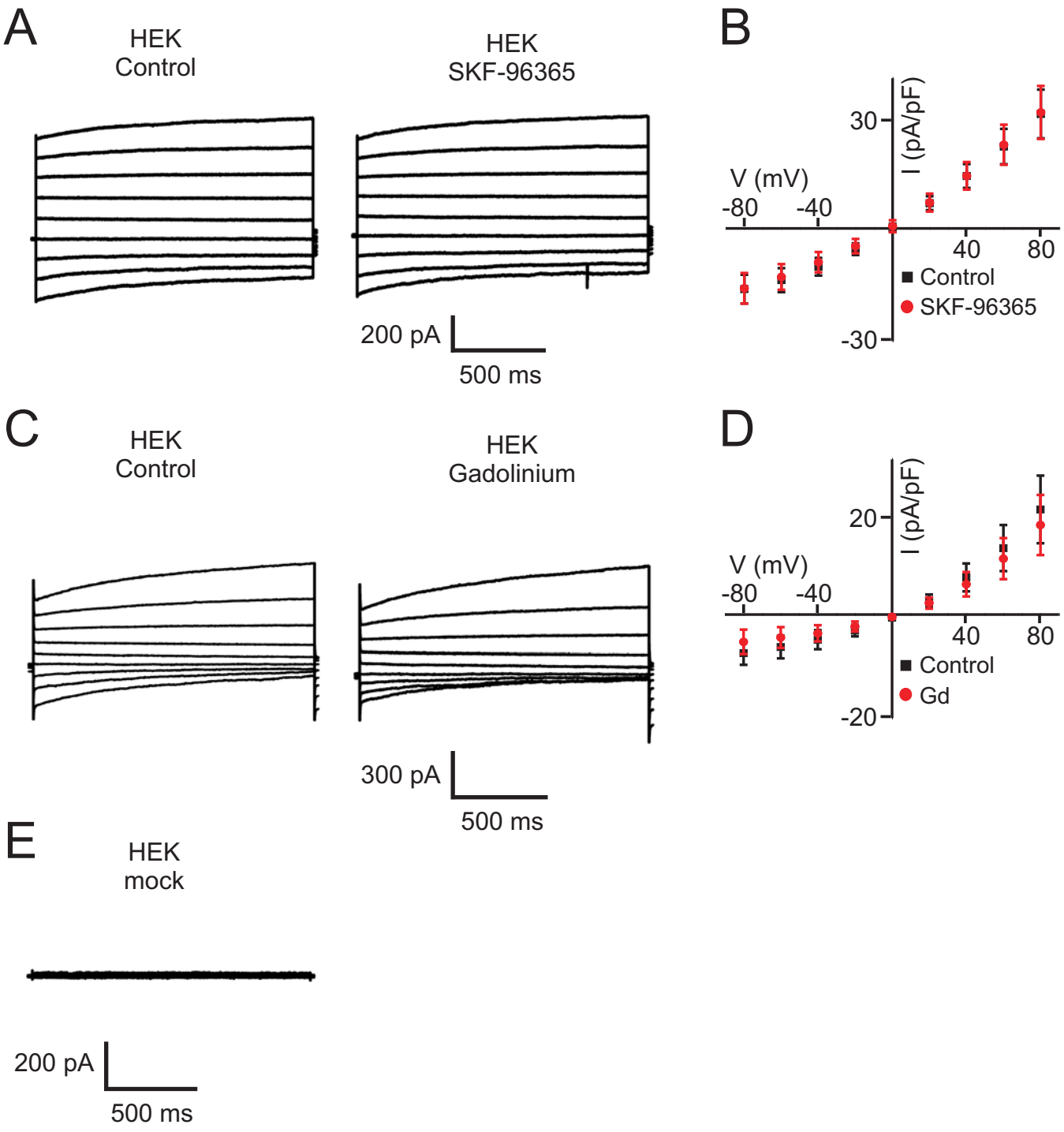
Online Table I. Composition of bath solutions used for patch-clamp electrophysiology experiments. Concentrations given are mmol/L



Online Figure I. Hyposmotic bath solution activates TMEM16A currents in cerebral artery smooth muscle cells. A, Time course of swelling-activated TMEM16A currents in hypotonic 250 mOsm solution at +80 mV and -80 mV (n=5).



Online Figure II. TMEM16A siRNA did not reduce TRPC6, TRPM4 or TRPP2 expression in cerebral arteries. Representative Western blots. Blots were physically cut at 75 kDa to permit simultaneous probing for TMEM16A/TRPC6/TRPM4/TRPP2 and actin, respectively.



Online Figure III. SKF96365 and Gd^{3+} do not inhibit currents generated by recombinant TMEM16A channels in HEK293 cells. A, Exemplary recordings from the same HEK293 cell transfected with vectors encoding TMEM16A channels in control and SKF96365 (10 µmol/L). 1 µmol/L free Ca^{2+} was present in the pipette solution. **B,** Mean data: control and SKF96365 (10 µmol/L), n=4 for each. P>0.05 at all voltages. **C,** Original recordings from the same HEK293 cell transfected with vectors encoding TMEM16A channels in control and Gd^{3+} (10 µmol/L). 1 µmol/L free Ca^{2+} was present in the pipette solution. **D,** Mean data: control and Gd^{3+} (10 µmol/L), n=5 for each. P>0.05 at all voltages. **E,** Original recording from a HEK293 cell that underwent the transfection procedure without inclusion of the vector encoding TMEM16A channels (representative of 3 experiments).