TMEM16A/ANO1 Channels Contribute to the Myogenic Response in Cerebral Arteries


Rationale: Pressure-induced arterial depolarization and constriction (the myogenic response) is a smooth muscle cell (myocyte)-specific mechanism that controls regional organ blood flow and systemic blood pressure. Several different nonselective cation channels contribute to pressure-induced depolarization, but signaling mechanisms involved are unclear. Similarly uncertain is the contribution of anion channels to the myogenic response and physiological functions and mechanisms of regulation of recently discovered transmembrane 16A (TMEM16A), also termed Anoctamin 1, chloride (Cl\(^-\)) channels in arterial myocytes.

Objective: To investigate the hypothesis that myocyte TMEM16A channels control membrane potential and contractility and contribute to the myogenic response in cerebral arteries.

Methods and Results: Cell swelling induced by hyposmotic bath solution stimulated Cl\(^-\) currents in arterial myocytes that were blocked by TMEM16A channel inhibitory antibodies, RNAi-mediated selective TMEM16A channel knockdown, removal of extracellular calcium (Ca\(^{2+}\)), replacement of intracellular EGTA with BAPTA, a fast Ca\(^{2+}\) chelator, and Gd\(^{3+}\) and SKF-96365, nonselective cation channel blockers. In contrast, nimodipine, a voltage-dependent Ca\(^{2+}\) channel inhibitor, or thapsigargin, which depletes intracellular Ca\(^{2+}\) stores, did not alter swelling-activated TMEM16A currents. Pressure-induced (~40 mmHg) membrane stretch activated ion channels in arterial myocyte cell–attached patches that were inhibited by TMEM16A antibodies and were of similar amplitude to recombinant TMEM16A channels. TMEM16A knockdown reduced intravascular pressure-induced depolarization and vasoconstriction but did not alter depolarization-induced (60 mmol/L K\(^+\)) vasoconstriction.

Conclusions: Membrane stretch activates arterial myocyte TMEM16A channels, leading to membrane depolarization and vasoconstriction. Data also provide a mechanism by which a local Ca\(^{2+}\) signal generated by nonselective cation channels stimulates TMEM16A channels to induce myogenic constriction. (Circ Res. 2012; 111:1027-1036.)

Key Words: arterial smooth muscle ■ Cl\(_{Ca}\) channel ■ TMEM16A ■ ANO1 ■ myogenic tone ■ contractility ■ smooth muscle cells ■ membrane potential

Resistance-size cerebral arteries control brain regional blood flow and maintain perfusion during changes in arterial pressure. One important functional stimulus that controls cerebral artery contractility is intravascular pressure. An elevation in intravascular pressure stimulates depolarization, leading to the activation of smooth muscle cell voltage-dependent calcium (Ca\(^{2+}\)) channels, an intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) elevation, and vasoconstriction.\(^1\) This “myogenic response” regulates regional brain blood flow, maintains perfusion over a range of intravascular pressures, and provides a baseline diameter from which other stimuli can either dilate or constrict. Several pathologies, including hypertension, are associated with altered myogenic responsiveness.\(^2\) Therefore, defining mechanisms that control the myogenic response is critical to a better understanding of vascular diseases.

Arterial smooth muscle cell cation channels, including Ca\(_{1.2}\), several K\(^+\) and nonselective transient receptor potential (TRP) channels, control vascular contractility.\(^1,2\) Multiple TRP channels also contribute to pressure-induced depolarization, leading to vasoconstriction, although mechanisms involved are unclear.\(^2\) In contrast, vascular contractility
regulation by arterial smooth muscle cell anion channels is poorly understood. Chloride (Cl\(^-\)) is the most abundant intracellular anion in vascular smooth muscle cells, with intracellular [Cl\(^-\)] = 50 mmol/L.\(^3\) The estimated reversal potential (E\(_{\text{rev}}\)) for Cl\(^-\) in smooth muscle cells is between −30 and −20 mV.\(^4\) The entire working range of rat cerebral arteries, from fully dilated to fully constricted, occurs between membrane potentials of −60 and −20 mV, which elevates global arterial wall [Ca\(^{2+}\)] from 100 to 350 mmol/L.\(^5\) With physiological ionic gradients, Cl\(^-\) channel activation would result in Cl\(^-\) efflux and arterial myocyte depolarization and vasoconstriction.\(^1\) This is in contrast to some other cell types, including adult neurons, where Cl\(^-\) \(E_{\text{rev}}\) is −75 mV, a voltage near resting potential.\(^6\) The concept that Cl\(^-\) channels contribute to myogenic constriction has previously been suggested from experiments that used highly nonspecific pharmacological Cl\(^-\) channel modulators.\(^1,3,7,8\) Indeed, poor selectivity of pharmacological Cl\(^-\) channel modulators and uncertain molecular identity of the protein(s) involved has hindered progress in defining functions of Cl\(^-\) channels in contractile arterial smooth muscle cell and their involvement in the regulation of vascular contractility.

Transmembrane protein 16A (TMEM16A) channels, also termed Anoctamin 1, are recently discovered Ca\(^{2+}\)-activated Cl\(^-\) (Cl\(_{Ca}\)) channels.\(^9,10\) Our group and others recently demonstrated that TMEM16A channels are expressed in arterial smooth muscle cells and generate Cl\(_{Ca}\) currents.\(^12-15\) TMEM16A has recently been described as a negative regulator of arterial smooth muscle cell proliferation.\(^15\) However, regulation of contractility by arterial smooth muscle cell TMEM16A channels is unclear. In the present study, we demonstrate that cell swelling and pressure-induced membrane stretch stimulate TMEM16A channels in arterial smooth muscle cells, leading to depolarization and vasoconstriction. Data also suggest that membrane distention activates nonselective cation channels that stimulate TMEM16A channels through local Ca\(^{2+}\) signaling. Thus, we show that arterial smooth muscle cell TMEM16A channels are one component of a mechanosensitive mechanism that contributes to the myogenic response.

### 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 (age, 6–8 weeks) were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed and placed into physiological saline solution of composition (in mmol/L): 112 NaCl, 4.8 KCl, 24 NaHCO\(_3\), 1.8 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), and 10 glucose, gassed with 21% O\(_2\)-5% CO\(_2\)-74% N\(_2\) to pH 7.4. Resistance-size (≈200-μm diameter) posterior cerebral, cerebellar, and middle cerebral arteries were dissected from the brain, pooled, and used for experimentation, unless specified.

#### Patch-Clamp Electrophysiology

Smooth muscle cells were isolated from cerebral arteries as previously described.\(^16\) Patch-clamp electrophysiology was performed using isolated cerebral artery smooth muscle cells or human embryonic kidney 293 (HEK293) cells expressing recombinant TMEM16A channels. For whole-cell current measurements, the pipette solution contained (in mmol/L): 126 CsCl, 10 HEPES, 10 D-glucose, 1 EGTA or 1 BAPTA, 1 Mg ATP, 0.2 GTP-Na, and 40 sucrose with pH adjusted to 7.2 with CsOH. Total MgCl\(_2\) was adjusted to give final Mg\(^2+\) of 1 mmol/L. For whole-cell current experiments on arterial smooth muscle cells, pipette free Ca\(^{2+}\) was 200 nmol/L. For whole-cell current measurements in HEK293 cells expressing recombinant TMEM16A channels, pipette-free Ca\(^{2+}\) was 1 μmol/L. 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 for whole-cell recordings are described in Online Table I. For cell-attached patch measurements, the pipette solution contained (in mmol/L): 134 NaCl, 6 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, 1 TEA\(^+\) and 5-4-amino-pyridine (pH 7.4, NaOH). For cell-attached patch measurements, the bath solution contained (in mmol/L): 140 KCl, 10 glucose, 10 HEPES, 2 CaCl\(_2\), 1 MgCl\(_2\) (pH 7.4, KOH). To study anion permeability, Cl\(^-\) was replaced with equimolar 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).

Whole-cell Cl\(^-\) currents were measured by applying 1.5-second voltage steps between −80 mV and +80 mV in 20 mV increments, using an interpulse holding potential of −40 mV. Currents were normalized to membrane capacitance. Cell-attached currents were measured at a steady membrane potential of −80 mV. Pharmacological agents and rabbit monoclonal anti-TMEM16A antibody (Abcam) were introduced directly into the experimental chamber. Boiled (15 minutes at 98°C), denatured TMEM16A antibody served as a control for active antibody. Pressure-induced stretch was applied to the plasma membrane contained within the patch pipette, using an ez-gSEAL 100B controller (Neo Biosystems). Membrane currents were recorded using an Axopatch 200B amplifier equipped with a CV 203B headstage, Digidata 1332A, and Clampex 8 or 9 (Molecular Devices).

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 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\(_{\text{rev}}\)) and the constant field equation:

\[
P_{X/Cl^-} = \left( \frac{[(Cl^-)_{o}]}{[X]_{o}} \right) \left( e^{(\Delta \Delta \text{E}_{\text{rev}})} \right)
\]

where X is I\(^-\) or Asp and z\(_F\)/RT is −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 6000 g for 10 minutes 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 (1:5000 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, were inserted intracellularly into cerebral arteries using either reverse permeabilization, as described, 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 after reverse permeabilization or 3 days after electroporation at 37°C in a sterile incubator (1% O₂, 5% CO₂). 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 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard tissue culture conditions (21% O₂–5% CO₂; 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). Cells were used between 36 and 72 hours after transfection.

**Pressurized Artery Membrane Potential and Diameter Measurement**

Experiments were performed using endothelium-denuded middle cerebral arteries. Arteries were maintained in physiological saline solution containing (in mmol/L): 112 NaCl, 4.8 KCl, 26 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 10 glucose, gassed with 100% N₂, 5% CO₂, (pH 7.4). Artery segments 1 to 2 mm in length were cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation; Burlington, VT). Intravascular pressure was altered by means of 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 100×(1-active diameter/passive diameter). Endothelial denudation was confirmed using methods previously described.

Membrane potential measurements were obtained in arteries at 10 or 60 mmHg that had developed steady-state myogenic tone. This was done by maintaining arteries at steady pressure for at least 90 min before increasing pressure to 100 mmHg to allow time to develop myogenic tone and then returning to pressure of interest.
2 hours and confirmed using edge detection. Membrane potential was measured by inserting glass microelectrodes filled with 3 mol/L 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 on insertion; (2) stable voltage for at least 1 minute after entry; (3) a sharp positive voltage deflection on exit from the recorded cell; and (4) a <10% change in tip resistance after the impalement.

Statistical Analysis
OriginLab and GraphPad InStat software were used for statistical analyses. Values are expressed as mean±SEM. Student t test was used for comparing paired and unpaired data from 2 populations, and ANOVA with Student-Newman-Keuls post hoc test was used for multiple group comparisons. P<0.05 was considered significant. Power analysis was performed on all data in which P>0.05 to verify that sample size was sufficient to give a power value of >0.8. All-points histograms were fit with a multipeak gaussian function, using Microcal Origin.

Expanded materials and methods are provided in the Online Data Supplement.

Results
Cell Swelling Activates TMEM16A Currents in Arterial Smooth Muscle Cells
To study the mechanosensitivity of Cl− currents in arterial smooth muscle cells, we measured responses to hyposmotic bath solution, a commonly used method to induce cell swelling.8,22–24 Experiments were performed using solutions that abolish Na+ and K+ currents (Online Table I). In isosmotic solution (300 mOsm/L), smooth muscle cells generated outwardly rectifying currents (Figure 1A and 1B). Switching to hyposmotic bath solution (250 mOsm/L) increased currents from 4.0±0.5 to 11.3±2.0 pA/pF or 2.8-fold (at +80 mV) and linearized the current-voltage (I-V) relationship, reducing the rectification index (I80/I−80) from ≈3.1 to 2.3 (Figure 1A and 1B). The swelling-activated current peaked in ≈90 seconds (Online Figure I). Equimolar replacement of 126 mmol/L external Cl− with I− caused a −26.1±1.7-mV hyperpolarizing shift in Erev, indicating a PI/PCl of 2.9 (Figure 1B). In contrast, equimolar replacement of 126 mmol/L external Cl− with aspartate shifted Erev by +13.2±4.2 mV, indicating a PASp/PCl of 0.6 (Figure 1B). These data indicate that cell swelling activates a Cl− current (I Cl) in arterial smooth muscle cells.

Next, we examined the molecular identity of channels that generate swelling-activated Cl− currents in arterial smooth muscle cells. A TMEM16A antibody that inhibits currents generated by recombinant TMEM16A channels12 reduced mean swelling-activated Cl− currents in smooth muscle cells from 12.5±1.9 to 4.0±0.9 pA/pF, or by ≈71% (at +80 mV; Figure 1C and 1D). In contrast, boiled TMEM16A antibody did not alter swelling-activated Cl− currents (Figure 1D). RNAi was used as a complementary approach. Western blotting indicated that TMEM16A siRNA reduced arterial TMEM16A protein by 43±3% when compared with control siRNA (Figure 2A). In contrast, TMEM16A siRNA did not alter TRPC6, TRPM4, or TRPP2 expression (Figure 2B and Online Figure II). Hyposmotic (250 mOsm/L) bath solution increased Cl− currents from 4.2±0.8 to 14.1±2.7 pA/pF (at +80 mV), or ≈3-fold in control siRNA-treated smooth cells. A Representative Western blot illustrating that TMEM16A siRNA reduced TMEM16A expression in cerebral arteries. B, Mean data of the effect of TMEM16A siRNA on TMEM16A (n=9), TRPC6 (n=4), TRPM4 (n=5), and TRPP2 (n=9) protein. *P<0.05 when compared with TMEM16A siRNA. C, Exemplary recordings illustrating that TMEM16A knockdown attenuates swelling-activated Cl− currents in smooth muscle cells. D, Mean data: control siRNA: 300 mOsm/L, n=8; 250 mOsm/L, n=8; TMEM16A siRNA: 300 mOsm/L, n=9; 250 mOsm/L, n=10. *P<0.05 when comparing 300 mOsm/L control siRNA with 250 mOsm/L control siRNA.
muscle cells (Figure 2C and 2D). In contrast, hyposmotic bath solution increased Cl− current density only 1.9-fold (at +80 mV) in smooth muscle cells in which TMEM16A was knocked down. In hyposmotic bath solution, mean Cl− current density in TMEM siRNA-treated cells was ≈30% of that in control siRNA-treated cells (Figure 2D). These results indicate that cell swelling activates TMEM16A currents in arterial smooth muscle cells.

Nonselective Cation Channels Stimulate TMEM16A Currents Through Local Intracellular Ca2+ Signaling

Mechanisms by which cell swelling activates TMEM16A currents in arterial smooth muscle cells were investigated. Extracellular Ca2+ removal abolished swelling-activated TMEM16A currents, whereas nifedipine, a voltage-dependent Ca2+ channel inhibitor, and thapsigargin, a SR Ca2+-ATPase inhibitor that depletes SR Ca2+ load, did not alter swelling-activated TMEM16A currents (Figure 3A and 3B). These data suggest that swelling activates TMEM16A currents by inducing Ca2+ influx through a pathway that is independent of voltage-dependent Ca2+ channels. Therefore, we tested the hypothesis that swelling activates 1 or more nonselective cation channels, leading to Ca2+ influx that activates TMEM16A currents. SKF-96365 and Gd3+, nonselective cation channel blockers, inhibited swelling-activated TMEM16A currents in smooth muscle cells. In contrast, SKF-96365 and Gd3+ did not alter currents generated by recombinant TMEM16A channels expressed in HEK293 cells (Online Figure III). Next, we studied whether Ca2+ influx activates TMEM16A channels via a local signaling mechanism. This hypothesis has merit because the Ca2+-sensitivity of recombinant TMEM16A channels is lower than the physiological global [Ca2+] range in arterial smooth muscle cells.\(^5,10\) Consistent with our hypothesis, equimolar replacement of pipette solution EGTA for BAPTA, a fast Ca2+ chelator, abolished swelling-activated TMEM16A currents. These data indicate that swelling activates nonselective cation channels, which generate a local intracellular Ca2+ signal that stimulates TMEM16A currents, in arterial smooth muscle cells.

Pressure-Induced Membrane Stretch Stimulates TMEM16A Channels in Arterial Smooth Muscle Cells

As an alternative approach to investigate ICl activation by osmolarity, negative pressure was applied to cell-attached patches to induce membrane stretch. These experiments were performed using a pipette solution containing physiological 140 mmol/L NaCl and 2 mmol/L Ca2+. Experiments were performed at −80 mV with the pipette solution containing 1 mmol/L TEA+ and 5 mmol/L 4-aminopyridine to block large-conductance Ca2+-activated K+ (BKCa) and voltage-dependent K+ (Kv) channels, respectively. The bath solution contained 140 mmol/L KCl to depolarize smooth muscle cells and permit efficient voltage-clamp of the plasma membrane patch within the pipette. Application of −40 mm Hg pipette pressure reversibly activated channels in membrane patches (Figure 4A through 4E). In 29 of 37 patches, −40 mm Hg pressure stimulation multiple simultaneously gating ion channels (Figure 4A). Averaging these recordings indicated that pressure stimulated a mean current of ≈2.0±0.3 pA (Figure 4A). To determine the contribution of TMEM16A channels to these currents, experiments were repeated in the presence of the TMEM16A inhibitory antibody. The TMEM16A antibody reduced pressure-induced membrane currents by ≈50% (P<0.05, Figure 4A). In 8 of 37 patches, −40 mm Hg pressure activated single channels that when analyzed using all-points histograms had a mean amplitude of 0.36±0.01 pA (Figure 4B and 4C). Inclusion of the TMEM16A antibody in the pipette solution inhibited these single channels (Figure 4B and 4C). Next, we compared the properties of these stretch-activated single channels to recombinant TMEM16A channels expressed in HEK293 cells. At −80 mV, the amplitude of single recombinant TMEM16A channels was 0.37±0.02 pA (Figure 4D and 4E). This amplitude is almost identical to that of the stretch-activated, TMEM16A antibody–inhibited channels in smooth muscle cells (Figure 4B and 4C). The TMEM16A antibody also blocked recombinant TMEM16A channels (Figure 4D and 4E). These data indicate that pressure-induced membrane stretch activates TMEM16A channels in arterial smooth muscle cells.

TMEM16A Channels Contribute to Pressure-Induced Arterial Depolarization and Constriction

To examine physiological functions of smooth muscle cell TMEM16A channels, membrane potential regulation by intravascular pressure was measured in endothelium-denuded arteries. At 10 mm Hg, TMEM16A knockdown did not alter mean arterial smooth muscle cell membrane potential (Figure 5A and 5B). An intravascular pressure elevation from 10 to 60 mm Hg depolarized control siRNA-treated arteries from −66.8±2.3 to −35.5±1.5 mV, or by ≈31.3 mV (Figure 5A and 5B). In contrast, the same pressure...
elevation depolarized TMEM16A siRNA-treated arteries by ≈15.6 mV, or by ≈50% of that in control arteries (Figure 5A and 5B).

Myogenic tone was measured at intravascular pressures between 20 and 100 mm Hg in endothelium-denuded arteries. Elevating intravascular pressure induced a graded elevation in myogenic tone in control siRNA-treated arteries (Figure 6A and 6B). TMEM16A knockdown reduced myogenic tone at pressures between 40 and 100 mm Hg by between 43% and 49% (Figure 6A and 6B). In contrast, TMEM16A knockdown did not alter membrane depolarization-induced (60 mmol/L K+) vasoconstriction (Figure 6C). TMEM16A knockdown also did not alter passive arterial diameter (control siRNA, 243.1±11.6 μm, n=7; TMEM16A siRNA, 238.4±11.3 μm, n=7; P>0.05). These data indicate that intravascular pressure–induced smooth muscle cell TMEM16A channel

Figure 4. Pressure-induced membrane stretch activates TMEM16A channels in arterial smooth muscle cells. A, Reduction in pipette pressure from 0 to −40 mm Hg stimulates inward currents that are partially inhibited by the TMEM16A-inhibitory antibody. Traces represent the average of current recordings of 29 control and 18 TMEM16A antibody-exposed patches at −80 mV. B, Exemplary recordings of single channels activated by −40 mm Hg pressure and inhibited by the TMEM16A-inhibitory antibody. C, All-points histograms of arterial smooth muscle cell patches fit a gaussian function for −40 mm Hg pressure in the absence and presence of the TMEM16A antibody. D, Original cell-attached recordings of recombinant TMEM16A channels expressed in HEK293 cells in the absence and presence of the TMEM16A inhibitory antibody. No patch pressure was applied during experiments on HEK293 cells. E, All-points histograms of recombinant TMEM16A channels fit with a gaussian function in the absence and presence of the TMEM16A antibody.

Figure 5. TMEM16A channels contribute to pressure-induced arterial smooth muscle cell depolarization. A, Original membrane potential recordings at 10 and 60 mm Hg in arteries treated with control siRNA and TMEM16A siRNA. Traces show intracellular microelectrode impalement followed by removal. B, Mean data (10 mm Hg: control siRNA, n=5, TMEM16A siRNA, n=5; 60 mm Hg: control siRNA, n=6; TMEM16A siRNA, n=6). *P<0.05 when comparing 10 mm Hg with 60 mm Hg for both control siRNA and TMEM16A siRNA. #P<0.05 compared with control at 60 mm Hg.
activation contributes to arterial depolarization and thus, vasoconstriction.

Discussion

The regulation of vascular contractility by anion channels is poorly understood. Similarly unclear are physiological functions and mechanisms of regulation of TMEM16A channels in contractile arterial smooth muscle cells. In the present study, we show that TMEM16A channels control smooth muscle cell membrane potential and contractility and contribute to the myogenic response in cerebral arteries. We show that cell swelling activates TMEM16A currents and pressure-induced membrane stretch activates single TMEM16A channels in arterial smooth muscle cells. Our data also indicate that nonselective cation channels generate a local intracellular Ca\textsuperscript{2+} signal that activates TMEM16A currents. These data provide a mechanism by which pressure-induced activation of arterial myocyte nonselective cation channels stimulates TMEM16A currents, leading to arterial smooth muscle cell depolarization and vasoconstriction.

Two distinct types of Cl\textsubscript{Ca} currents are present in vascular myocytes: “classic” Cl\textsubscript{Ca} and eGMP-dependent Cl\textsubscript{Ca} currents.\textsuperscript{25,26} Cl\textsubscript{Ca} currents have been characterized in myocytes of several vascular beds as outwardly rectifying Cl\textsuperscript{−} currents that are activated by [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{3,27} Outward rectification of classic Cl\textsubscript{Ca} currents at nanomolar [Ca\textsuperscript{2+}]\textsubscript{i} is linearized by an elevation in [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{27} Cell swelling activates Cl\textsuperscript{−} currents in cerebral, pulmonary, and renal artery and portal vein smooth muscle cells.\textsuperscript{3,23,24} A reduction in extracellular Cl\textsuperscript{−} elevated myogenic tone and nonselective Cl\textsuperscript{−} channel blockers hyperpolarized and dilated pressurized cerebral arteries.\textsuperscript{7} Cl\textsuperscript{−} efflux, measured using self-referencing ion-selective electrodes, also correlated with the myogenic response.\textsuperscript{28}

Our data obtained using extracellular ionic replacement, inhibitory antibodies, RNAi, and comparison of single channel properties to recombinant TMEM16A channels indicate that cell swelling, pressure-induced membrane stretch, and intravascular pressure activate TMEM16A channels in arterial smooth muscle cells. We show that linearization of the I-V relationship by cell swelling and the relative permeability of swelling-activated currents to I\textsuperscript{−} and Cl\textsuperscript{−} is also similar to that of recombinant TMEM16A channels.\textsuperscript{9} We also demonstrate that selective TMEM16A knockdown attenuates intravascular pressure–induced arterial depolarization and vasoconstriction. These data indicate that smooth muscle cell TMEM16A channels contribute to myogenic constriction in cerebral arteries. Recent studies demonstrated that TMEM16A channels are expressed in smooth muscle cells of rat small cerebral and mouse conduit arteries, cultured rat pulmonary artery smooth muscle cells, and interstitial cells of Cajal.\textsuperscript{12–15,20} Another recent study demonstrated that T16Ainh-A01, a TMEM16A current inhibitor with unclear selectivity, reduced a chronic hypoxia-induced elevation in serotonin contraction in rat pulmonary arteries.\textsuperscript{30} These findings suggest that smooth muscle cell TMEM16A channels may regulate contractility not only in cerebral arteries but in anatomically diverse vasculature and other smooth muscle cell types.

We show that removal of extracellular Ca\textsuperscript{2+} and replacement of intracellular EGTA with BAPTA abolished swelling-activated TMEM16A currents. These data are similar to those from a previous study that measured Cl\textsuperscript{−} current regulation by cell swelling in smooth muscle cells of the basilar artery, a large cerebral vessel.\textsuperscript{21} In contrast, swelling-induced TMEM16A currents were not altered by thapsigargin or ni-modipine, arguing against the functional involvement of SR Ca\textsuperscript{2+} release and voltage-dependent Ca\textsuperscript{2+} channels. A previous report described that swelling activated nonselective cation currents but did not stimulate Cl\textsuperscript{−} currents in cerebral artery smooth muscle cells.\textsuperscript{22} In this earlier study, intracellular and extracellular solutions were Ca\textsuperscript{2+}-free. Therefore, these data are consistent with ours that swelling-induced Ca\textsuperscript{2+} influx activates TMEM16A currents. To determine the mechanism by which cell swelling activates TMEM16A channels, we tested the hypothesis that nonselective cation channels, which have been previously implicated in mediating myogenic constriction, were involved. This approach also permitted us to test the associated hypothesis that TMEM16A channels may be mechanosensitive. Our data show that Gd\textsuperscript{3+} and SKF96365 blocked swelling-activated TMEM16A currents but did not alter currents generated by recombinant TMEM16A channels in HEK293 cells. These data indicate that swelling activates nonselective cation channels, leading to Ca\textsuperscript{2+} influx that stimulates TMEM16A channels. Consistent with our data, Gd\textsuperscript{3+} blocked both swelling- and pressure-induced depolarization in cerebral artery smooth muscle cells.\textsuperscript{22} In contrast, swelling-activated Cl\textsuperscript{−} currents dissimilar to classic Cl\textsubscript{Ca} were not inhibited by BAPTA in portal vein smooth muscle cells.\textsuperscript{23} Depolarization-induced Cl\textsuperscript{−} currents attributed to TMEM16A have been described in interstitial cells of Cajal.\textsuperscript{29} Based in part on their activation latency after stimulation, these Cl\textsuperscript{−} currents have been suggested to be activated by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release.\textsuperscript{29} Collectively, these studies suggest that diverse mechanisms of Cl\textsubscript{Ca} current activation may exist in smooth muscle cells of different tissues. Future studies will determine if these different activation mechanisms also apply to regulation of TMEM16A channels. Our data indicate that a mechanosensitive mechanism stimulates nonselective cation channels that activate TMEM16A via local Ca\textsuperscript{2+} signaling in cerebral artery smooth muscle cells. These data also suggest that arterial smooth muscle cell TMEM16A currents are not directly activated by cell swelling.

The molecular identity of nonselective cation channels that stimulate TMEM16A currents was not determined in the present study. We show that membrane stretch induced by negative pipette pressure stimulates single TMEM16A channels in arterial smooth muscle cells. In a majority (>80%) of membrane patches, pressure activated currents to which multiple simultaneously gating channels contributed. The TMEM16A-inhibitory antibody reduced stretch-activated currents by >50%, indicating that TMEM16A channels contribute almost half of the current. In a minority of patches (>20%), single ion channels identical to recombinant TMEM16A channels were activated. These data suggest that TMEM16A channels may cluster in the plasma membrane with other stretch-activated channels, consistent with other
Evidence in this study that closely localized nonselective cation channels activate TMEM16A after membrane stretch. Several nonselective cation channels expressed in arterial smooth muscle cells are Ca$^{2+}$-permeant, including multiple TRPC and TRPM subfamily members, TRPP1/2, TRPV2, and TRPV4. Conceivably, 1 or more of these channels, including TRP heteromultimers, may control TMEM16A channel activity. Previous studies have indicated that TRPC6, TRPM4, and TRPP1/2 activation contributes to the myogenic response. Under physiological conditions, TRPC6 and TRPP1/2 activation would lead to both Na$^{+}$ and Ca$^{2+}$ influx. In contrast, TRPM4 channels are primarily Na$^{+}$-permeant. Our data support the concept that both local Ca$^{2+}$ signaling and Na$^{+}$ influx mediated by nonselective cation channels contribute to pressure-induced depolarization and vasoconstriction. Given the large number of potential candidates, that currently unidentified channels may be involved, more than 1 channel may be engaged, and heteromultimeric proteins may be involved, it was beyond the scope of this study to determine the molecular identity of nonselective cation channels that activate TMEM16A channels. Future studies should be designed to identify Ca$^{2+}$-permeant ion channels that control TMEM16A channels in arterial smooth muscle cells.

The contribution of TMEM16A channels to myogenic vasoconstriction was studied between 20 and 100 mm Hg, a range that encompasses physiological intravascular pressures in the cerebral circulation. Physiological cerebral artery membrane potential over this range of pressures is ≈−65 to −36 mV. The predicted E$_{rev}$ for Cl$^{-}$ is ≈−30 mV, indicating that TMEM16A channel–mediated Cl$^{-}$ efflux would contribute to membrane depolarization and myogenic constriction over the range of pressures studied. Elevating pressure above 100 mm Hg does not further depolarize cerebral arteries with a plateau at ≈−30 mV, a potential similar to the predicted Cl$^{-}$ E$_{rev}$. Data in the present study indicate that the RNi-mediated reduction in TMEM16A protein (≈43%) and myogenic response (43% to 49%) was similar over the entire pressure range. These data suggest that TMEM16A channels contribute equally to myogenic constriction over this pressure range. These observations could be interpreted as indicating that TMEM16A channels are the major contributor to pressure-induced depolarization and that the Cl$^{-}$ reversal potential determines maximal depolarization. However, multiple mechanisms can contribute to the pressure-induced depolarization plateau. At voltages more positive than ≈−30 mV, Cl$^{-}$ efflux will switch polarity to

Figure 6. TMEM16A channels contribute to pressure-induced vasoconstriction. A, Exemplary traces illustrating diameter responses to increasing pressure steps and 60 mmol/L K$^{+}$ at 60 mm Hg in arteries treated with control siRNA and TMEM16A siRNA. B, Mean data: control siRNA, n=7; TMEM16A siRNA, n=7. *P<0.05 when comparing control siRNA with TMEM16A siRNA. C, TMEM16A knockdown did not alter 60 mmol/L K$^{+}$-induced constriction; control siRNA, n=7; TMEM16A siRNA, n=7.
influx that will oppose depolarization mediated by nonselective cation current. Pressure-induced depolarization is also opposed through the activation of K+ channels, including Ks and BKCa.1 Although the RNAi-mediated reduction in TMEM16A protein and myogenic response were similar, the reduction in swelling-activated TMEM16A currents was larger. Explanations for this result include that a threshold level of TMEM16A protein may be required for the formation of functional ion channels in arterial smooth muscle cells. In addition, multiple processes contribute to pressure-induced depolarization and myogenic constriction, with some of these mechanisms interacting, as we show in the present study.2 Therefore, the partial loss of 1 signaling component may lead to amplification of functional effects. Future studies should therefore examine the relative contribution of nonselective cation and TMEM16A channels to myogenic vasoconstriction both in vitro and in vivo. This determination would require the molecular identification of the nonselective cation channels that communicate with TMEM16A.

In summary, data indicate that membrane stretch activates TMEM16A channels in arterial smooth muscle cells. TMEM16A channels regulate arterial smooth muscle cell membrane potential and contractility and contribute to the myogenic response. Data also suggest that nonselective cation channels activate TMEM16A channels through local Ca2+ signaling, leading to pressure-induced depolarization and vasconstriction.

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Disclosures

None.

References


### Novelty and Significance

The myogenic response is a physiological smooth muscle-specific mechanism that controls systemic blood pressure and regional organ blood flow. Cardiovascular diseases, including hypertension, are associated with an augmented myogenic response, which elevates blood pressure and can induce end-organ damage. Intravascular pressure has been proposed to activate several different nonselective cation channels, including members of the TRP family, to induce myogenic vasoconstriction. The concept that Cl\textsuperscript{−} channels regulate vascular contractility has been suggested from experiments that used Cl\textsuperscript{−} channel modulators with low or uncertain specificity. However, the molecular identity of Cl\textsuperscript{−} channels that control vascular contractility and contribute to the myogenic response was unclear. In the present study, we used a combination of molecular, electrophysiological, and functional approaches to show that membrane stretch activates TMEM16A channels in cerebral artery smooth muscle cells. Our data suggest that a stretch-induced local intracellular Ca\textsuperscript{2+} signal generated by nonselective cation channels stimulates TMEM16A channels. Intravascular pressure–induced TMEM16A channel activation contributes to membrane depolarization and vasoconstriction. These data indicate that TMEM16A channels are one component of a mechanosensitive mechanism that contributes to the myogenic response. These results also identify a new approach to modulate the myogenic response through the manipulation of TMEM16A channel activity.
TMEM16A/ANO1 Channels Contribute to the Myogenic Response in Cerebral Arteries
Simon Bulley, Zachary P. Neeb, Sarah K. Burris, John P. Bannister, Candice M.
Thomas-Gatewood, Wanchana Jangsangthong and Jonathan H. Jaggar

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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 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 10 glucose, which was gassed with 21% O₂-5% CO₂-74% N₂ 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.

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 MgCl₂ was adjusted to give final free Mg²⁺ of 1 mmol/L. For experiments on arterial myocytes, pipette free Ca²⁺ was 200 nM. For experiments on HEK293 cells expressing recombinant TMEM16A channels, pipette free Ca²⁺ was 1 µM. Free Mg²⁺ and Ca²⁺ were calculated using WebmaxC Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm) and confirmed using a Ca²⁺-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⁻ (Pᵢ/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:

\[
P_X/P_{Cl} = [\{Cl\}]_o e^{(\Delta E_{rev} zF/RT)}/[X]_o,\]

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, were inserted intracellularly into cerebral arteries using either reverse permeabilization, as described or a Bex CUY21 Vivo-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% O₂, 5% CO₂). 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% O₂-5% CO₂; 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 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 10 glucose, gassed with 74% N₂, 21% O₂, 5% CO₂ (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.

**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


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**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).