Pharmacological inhibition of TRPM4 hyperpolarizes vascular smooth muscle

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Gonzales AL, Garcia ZI, Amberg GC, Earley S. Pharmacological inhibition of TRPM4 hyperpolarizes vascular smooth muscle. Am J Physiol Cell Physiol 299: C1195–C1202, 2010. First published September 8, 2010; doi:10.1152/ajpcell.00269.2010.—The contractile state of vascular smooth muscle cells is regulated by small changes in membrane potential that gate voltage-dependent calcium channels. The melastatin transient receptor potential (TRP) channel TRPM4 is a critical mediator of pressure-induced membrane depolarization and arterial constriction. A recent study shows that the tricyclic compound 9-phenanthrol inhibits TRPM4, but not the related channel TRPM5. The current study investigated the specificity of 9-phenanthrol and the effects of the compound on pressure-induced smooth muscle depolarization and arterial constriction. Patch-clamp electrophysiology revealed that 9-phenanthrol blocks native TRPM4 currents in freshly isolated smooth muscle cells in a concentration-dependent manner (IC50 = 10.6 µM). 9-Phenanthrol (30 µM) had no effect on maximum evoked currents in human embryonic kidney cells expressing recombinant TRPC3 or TRPC6 channels. Large-conductance Ca2+/activated K+, voltage-dependent K+, inwardly rectifying K+, and voltage-dependent Ca2+ channel activity in native cerebral artery myocytes was not altered by administration of 9-phenanthrol (30 µM). Using intracellular microelectrodes to record smooth muscle membrane potential in isolated cerebral arteries pressurized to 70 mmHg, we found that 9-phenanthrol (30 µM) reversibly hyperpolarized the membrane from −40 mV to −70 mV. In addition, we found that myogenic tone was reversibly abolished when vessels were exposed to 9-phenanthrol. These data demonstrate that 9-phenanthrol is useful for studying the functional significance of TRPM4 in vascular smooth muscle cells and that TRPM4 is an important regulator of smooth muscle cell membrane depolarization and arterial constriction in response to intraluminal pressure.

Several members of the transient receptor potential (TRP) superfamily of cation channels are present in vascular smooth muscle cells and contribute to the regulation of smooth muscle membrane potential and contractility (2–4, 6, 19, 24). Antisense-mediated gene silencing has been used to demonstrate that expression of TRPM4 (5, 6, 18) and TRPC6 (24) channels is required for pressure-induced smooth muscle cell depolarization and myogenic vasoconstriction of cerebral arteries. TRPC3 channels mediate receptor-dependent responses in these vessels (19). Further investigation into the regulation of TRP channels and how they influence vascular function has been hampered by the lack of selective pharmacological inhibitors. Recently, it was reported that the hydroxytricyclic compound 9-hydroxyphenanthrene (9-phenanthrol) blocks TRPM4 channels in a human embryonic kidney (HEK) cell expression system with a half-maximal inhibitory concentration (IC50) of 16.7 ± 4.5 µM under conventional whole cell patch-clamp conditions (10). The related channel TRPM5 is not inhibited by 9-phenanthrol at a concentration of 100 µM, suggesting that the compound has selectivity for TRPM4 (10). However, the effects of the compound on other TRP, K+, and Ca2+ channels involved in smooth muscle cell function have not been previously reported. Therefore, the goals of the current study were to further examine the selectivity of 9-phenanthrol and to determine the effects of the compound on cerebral artery smooth muscle cell membrane potential and contractility. We find that 9-phenanthrol blocks sustained TRPM4 currents in freshly isolated cerebral artery myocytes with an IC50 that is consistent with the value reported for recombinant TRPM4 channels expressed in HEK cells. In addition, we find that 9-phenanthrol is without effect on TRPC3 and TRPC6 currents in HEK expression systems. Our studies also demonstrate that 9-phenanthrol is without effect on K+ and VDCCs that are involved in the regulation of smooth muscle membrane potential and contractility, suggesting that the compound may be useful for investigation of the physiological function of TRPM4 in these cells. Block of TRPM4 currents with 9-phenanthrol in intact cerebral vessels at physiological intraluminal pressure results in substantial hyperpolarization of vascular smooth muscle cell membrane potential and dilation of arteries with myogenic tone. These findings show that TRPM4 currents play a major role in establishing the membrane potential of arterial myocytes and maintaining myogenic tone.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300–350 g; Harlan, Indianapolis, IN) were used for these studies. Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and euthanized by exsanguination according to a protocol approved by the Institutional Animal Care and Use Committee of Colorado State University. Brains were isolated in ice-cold MOPS-buffered saline containing (in mM) 3 MOPS (pH 7.4), 145 NaCl, 5 KCl, 1 MgSO4, 2.5 CaCl2, 1 KH2PO4, 0.02 EDTA, 2

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pyruvate, 5 glucose, and 1% bovine serum albumin. Cerebral and cerebellar arteries were dissected from the brain, cleaned of connective tissue, and stored in MOPS-buffered saline on ice before further manipulation.

Cerebral Artery Smooth Muscle Cell Preparation

To isolate smooth muscle cells, vessels were cut into 2-mm segments and placed in the following Mg²⁺-based physiological saline solution (Mg-PSS) (in mM): 140 NaCl, 5 KCl, 2 MgCl₂, 10 glucose, and 10 HEPES (pH 7.2). Arterial segments were initially incubated at room temperature in 0.6 mg/ml papain (Worthington) and 1 mg/ml dithiothreitol for 17 min, followed by a 15-min incubation at 37°C in 1 mg/ml type II collagenase (Worthington). Digested segments were washed three times in Mg-PSS and triturated to release smooth muscle cells. Cells were stored on ice in Mg-PSS for use the same day.

HEK Cell Culture

HEK 293 cells were cultured in Dulbecco’s 1× high glucose modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.5% penicillin-streptomycin (Gibco). Cells were incubated at 37°C with 6% CO₂, media were changed the same day. HEK 293 cells were cultured in Dulbecco’s 1× high glucose modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.5% penicillin-streptomycin (Gibco). Cells were incubated at 37°C with 6% CO₂, media were changed the same day.

General methods.

Currents were recorded using an AxoPatch 200B amplifier equipped with an Axon CV 203BU headstage (Molecular Devices). Recording electrodes (1–3 MΩ) were pulled, polished, and coated with wax to reduce capacitance. G1 seals were obtained in Mg-PSS. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. Clampex and Clampfit versions 10.2 (Molecular Devices) were used for data acquisition and analysis, respectively. All patch-clamp experiments were performed at room temperature (22–25°C).

TRPC3 and TRPC6 currents in HEK cells. The pipette solution used to record both TRPC3 and TRPC6 currents contained (in mM) 130 CsCl, 110 aspartic acid, 15 CsCl, 1 MgCl₂, 3.6 CaCl₂, 10 EGTA, and 10 HEPES (pH 7.4). The extracellular bath solution for TRPC3 solutions and 0.9 mV for the TRPC6 solution. Reversal potentials for monovalent anions are 1.6 mV for the TRPC3 solution and −1 mV for the TRPC6 solution.

Large-conductance Ca²⁺-activated K⁺ (KCa) channels following transient release of Ca²⁺ from ryanodine receptors located on the sarcoplasmic reticulum (SR) and represent BKCa activity under physiological conditions in smooth muscle cells (15). Transient inward cation currents (TICCs) are the result of activation of TRPM4 channels by SR Ca²⁺ released from inositol triphosphate (IP₃) receptors (7). For the current study, both STOCs and TICCs were recorded using the perforated patch-clamp configuration. Isolated smooth muscle cells were transferred to a recording chamber (Warner Instruments) and allowed to adhere to glass coverslips for 20 min at room temperature. Amphotericin B (40 μM) was included in the pipette solution to perforate the membrane. Acceptable perforation was determined by a series resistance of <50 MΩ. STOCs and TICCs were recorded in normal external bathing solution containing (in mM) 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose at pH of 7.4 (NaOH). The pipette solution contained 110 mM K-aspartate, 1 mM MgCl₂, 30 mM KCl, 10 mM NaCl, 10 mM HEPES, and 5 μM EGTA at pH 7.2 (NaOH). STOCs were recorded at a membrane potential of +20 mV, and TICCs were recorded at −70 mV. The calculated reversal membrane potential for the perforated patch solutions is −1.8 mV for total monovalent cations and −30.6 mV for monovalent anions (Cl⁻). STOCs were defined as a transient event >10 pA (more than one BKCa channel), and the frequency was calculated by dividing the number of events by the time between the first and last event. TICC activity at −70 mV was calculated as the sum of the open channel probability (NPₒ) of multiple open states of 1.75 pA. This value was based on the reported unitary conductance of TRPM4 (25 pS). Channel open probability (NPₒ) was calculated using the following equation:

\[
NP_{o} = \frac{\sum_{j=1}^{N} (T_{j})}{T}
\]

where \(j\) is time spent in seconds with \(j = 1, 2, \ldots, N\) channels open; \(N\) is maximum number of channels observed; and \(T\) is duration of measurement.

Voltage-dependent K⁺ (KV) currents in smooth muscle cells. The conventional whole cell patch-clamp configuration was used to examine the effects of 9-phenanthrol on KV currents in smooth muscle cells. The extracellular bath solution contained (in mM) 5 KCl, 140 NaCl, 2 MgCl₂, 10 glucose, and 10 HEPES. The pipette solution contained (in mM) 87 K⁺ aspartate, 20 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 EGTA, and 5 MgATP. Cells were initially voltage clamped at −80 mV, and voltage steps from −80 mV to +30 mV were applied for 400 ms in 10-mV increments, followed by a step to −40 mV for 200 ms. Recordings were performed in the presence of saxipiline (5 μM) to block BKCa activity. Recordings were obtained in the presence of the vehicle for 9-phenanthrol (DMSO) and when 9-phenanthrol (30 μM) was present in the superfusion bath. Average current density (in pA/pF) during the steady state for each voltage step was used to generate current-voltage relationships.

Inwardly rectifying K⁺ currents in smooth muscle cells. Conventional patch-clamp electrophysiology was used to measure whole cell inwardly rectifying K⁺ (KIR) currents in isolated smooth muscle cells using a previously described method (20). The pipette solution for these experiments contained (in mM) 5 NaCl, 35 KCl, 100 K⁺ gluconate, 1 CaCl₂, 10 HEPES, 10 EGTA, 2.5 Tris-ATP, and 0.2 GTP (pH 7.2). Cells were voltage clamped at −60 mV and equilibrated for 15 min in a bath solution containing (in mM) 135 NaCl, 5 KCl, 0.1 MgCl₂, 10 HEPES, 5 glucose, and 0.1 CaCl₂ (pH 7.4). Following
equilibration, bath [K+] was elevated from 5 to 60 mM by replacement of NaCl by KCl. Voltage ramps between −120 and 20 mV (0.047 mV/ms) were applied in the presence and absence of 30 μM BaCl2 and K1/2 (Ba2+-sensitive) currents were calculated by current subtraction. K1/2 currents recorded in the presence of 9-phenanthrol were compared with those recorded in the presence of vehicle (DMSO).

Voltage-dependent Ca2+ currents in smooth muscle cells. The conventional whole cell patch-clamp technique was used to record VDCCs in smooth muscle cells. Freshly prepared cerebral arterial myocyte suspensions were transferred to a recording chamber and the cells were allowed to adhere to glass coverslips for 20 min at room temperature. During experiments, cells were superfused with a solution containing (in mM) 120 NMDG+, 5 CsCl, 1 MgCl2, 10, glucose, 10 HEPES, and 20 CaCl2, adjusted to pH 7.4 with HCl. Pipettes were filled with a solution composed of (in mM) 87 Cs-aspartate, 20 CsCl, 1 MgCl2, 5 MgATP, 0.1 NaGTP, 1 NADPH, and 10 EGTA, adjusted to pH 7.2 with CsOH. Cells were initially voltage clamped at −70 mV, and VDCC currents were evoked by stepping the membrane potential to +30 mV. Currents recorded in the presence of vehicle (DMSO) were compared with those recorded in the presence of 9-phenanthrol (30 μM).

Isolated Vessel Experiments

Arteries were harvested and transferred to a vessel chamber (Living Systems). The proximal end of the vessel was cannulated with a glass micropipette and secured with monofilament thread. Blood was gently clamped until vessel length was constant. Arteries pressurized to 10 mmHg were exposed to isotonic solution containing (in mM): 119 NaCl, 4.7 KCl, 1.8 CaCl2, 1.2 MgSO4, 24 NaHCO3, 0.2 KH2PO4, 10.6 glucose, and 1.1 EDTA) and superfused (5 ml/min) with warmed solution of (in mM) 120 NMDG+, 5 CsCl, 1 MgCl2, 10, glucose, 10 HEPES, and 20 CaCl2, adjusted to pH 7.4 with HCl. Pipettes were filled with a solution composed of (in mM) 87 Cs-aspartate, 20 CsCl, 1 MgCl2, 5 MgATP, 0.1 NaGTP, 1 NADPH, 10 HEPES, and 10 EGTA, adjusted to pH 7.2 with CsOH. Cells were initially voltage clamped at −70 mV, and VDCC currents were evoked by stepping the membrane potential to +30 mV. Currents recorded in the presence of vehicle (DMSO) were compared with those recorded in the presence of 9-phenanthrol (30 μM).

Smooth Muscle Cell Membrane Potential

For measurement of smooth muscle cell membrane potential, cerebral arteries were isolated and pressurized to 70 mmHg, and smooth muscle cells were impaled through the adventitia with glass micropipettes (tip resistance 100–200 MΩ). A WPI Intra 767 amplifier was used for recording membrane potential (Em). Analog output from the amplifier was recorded using Ionwizard software (sample frequency 20 Hz). Criteria for acceptance of Em recordings were 1) an abrupt negative deflection of potential as the microelectrode was advanced into a cell; 2) stable membrane potential for at least 1 min; and 3) an abrupt change in potential to ~0 mV after the electrode was retracted from the cell.

Calculations and Statistics

All data are means ± SE. Values of n refer to number of arteries for isolated vessel experiments or number of cells for patch-clamp experiments. Statistical methods conform to the recommendations of the editors of Circulation Research (22). A level of P ≤ 0.05 was accepted as statistically significant for all experiments.

RESULTS

9-Phenanthrol Selectively Blocks Sustained TRPM4 Currents

A recent report from our laboratory demonstrates that TICCs, TRPM4 currents under near-physiological conditions, can be recorded from cerebral artery smooth muscle cells voltage clamped using the amphotericin B perforated patch configuration (7). TICC currents are diminished when TRPM4 expression is attenuated using small interfering RNA (siRNA), suggesting that TICCs represent TRPM4 activity under physiological conditions in cerebral artery smooth muscle cells (7). To characterize the effects of 9-phenanthrol on native TRPM4 currents, we examined the concentration-response relationship for TICC inhibition. We find that 9-phenanthrol inhibits TICC activity in a concentration-dependent manner with an IC50 of 10.6 μM, (Fig. 1, A and B) in agreement with the prior report [16.7 ± 4.5 μM for TRPM4 channel in an HEK expression system, whole cell conditions (10)]. These data indicate that 9-phenanthrol effectively blocks TRPM4 currents recorded under near-physiological conditions in native smooth muscle cells.

The effects of 9-phenanthrol on other ion channels involved in smooth muscle cell function have not been previously reported. We began our investigation into the specificity of the compound by examining its effects on TRPC3 and TRPC6 currents. These channels were selected because both are involved in the regulation of cerebral artery smooth muscle membrane potential and contractility (12, 19, 24). To study the effects of 9-phenanthrol on TRPC3 currents, recombinant TRPC3 channels and ANG II AT1 receptors were coexpressed in HEK cells. Transfected cells were patch clamped in the
9-Phenanthrol Does Not Block BKCa, Kv, or KIR Channels in TRPM4 Currents.

Currents at a concentration sufficient to nearly eliminate TRPM4 channels but has no effect on TRPC3 or TRPC6 experiments demonstrate that 9-phenanthrol effectively blocks that TRPC6 channels are not blocked by this compound. These experiments demonstrate that 9-phenanthrol effectively blocks TRPM4 channels but has no effect on TRPC3 or TRPC6 currents at a concentration sufficient to nearly eliminate TRPM4 currents.

9-Phenanthrol Does Not Block BKCa, Kv, or KIR Channels in Smooth Muscle Cells

To further examine the utility of 9-phenanthrol for investigations into the role of TRPM4 channels in freshly isolated vascular smooth muscle, we examined the effects of the compound on the major K+(BKCa, Kv, and KIR) channels involved in the function of these cells. STOCs were recorded from smooth muscle cells using the perforated patch voltage-clamp configuration. 9-Phenanthrol (30 μM) had no effect on STOC frequency or amplitude (Fig. 3, A–C), demonstrating that the compound does not alter BKCa channel activity or the ryanodine receptor-dependent SR Ca2+ release events (Ca2+ sparks) that generate STOCs.

The conventional whole cell patch-clamp configuration was used to examine the effects of 9-phenanthrol (30 μM) on Kc and KIR channel activity. Kc currents evoked by voltage steps did not differ between control and 9-phenanthrol-treated cells (Fig. 3, D and E), indicating that the compound is without effect on these channels. KIR channels were isolated using BaCl2, according to a method described by Smith et al. (20). Ba2+-sensitive currents did not differ when recorded in the presence or absence of 9-phenanthrol (30 μM), demonstrating that the compound does not alter the activity of KIR channels (Fig. 3, F–H).

Voltage-Dependent Ca2+ Channels Are Not Blocked by 9-Phenanthrol

VDCCs are the major Ca2+ influx pathway in cerebral artery myocytes. Conventional whole cell patch clamp was used to investigate the effects of 9-phenanthrol on VDCC activity in native cerebral artery smooth muscle cells. To evoke VDCC currents, cells were voltage clamped at −70 mV and then stepped to +30 mV. Whole cell Ca2+ currents recorded (ICa) under these conditions did not differ between vehicle and 9-phenanthrol (30 μM)-treated cells (Fig. 4, A and B), demonstrating that the compound is without effect on VDCCs at this concentration.

9-Phenanthrol Hyperpolarizes Vascular Smooth Muscle Cells

Knockdown of TRPM4 expression diminishes pressure-induced smooth muscle depolarization (6), but the acute effects of TRPM4 inhibition on resting membrane potential have not been reported. 9-Phenanthrol was used to examine the contri-

Fig. 2. TRPC3 and TRPC6 currents are not blocked by 9-phenanthrol. A; current (I) vs. voltage (V) relationship of baseline and angiotensin II (ANG II)-induced currents in human embryonic kidney (HEK) cells overexpressing TRPC3 recorded in absence and presence of 9-phenanthrol (9-phen; 30 μM). Note that the current recorded in the presence of 9-phenanthrol is obscured by the current recorded in the presence of vehicle. B; summary data of average current density at +85 mV before and following ANG II (10 nM) stimulation and after administration of 9-phenanthrol (30 μM) in the presence of ANG II; n = 4. C; I vs. V relationship of whole cell currents recorded from nontransfected HEK cells and TRPC6-expressing cells in absence and presence of 9-phenanthrol (30 μM). D; summary data of average current density at +85 mV for nontransfected cells and TRPC6-expressing cells in absence and presence of 9-phenanthrol (30 μM); n = 6.
bution of TRPM4-dependent current to the resting membrane potential of vascular smooth muscle cells in isolated cerebral and cerebellar arteries. Arteries were pressurized to physiological levels (70 mmHg), and smooth muscle membrane potential was measured using intracellular microelectrodes before and after 9-phenanthrol (30 μM) was administered via the superfusion bath, and again after the drug was washed out of the recording chamber. Before 9-phenanthrol administration, the resting membrane potential of arterial myocytes was $-39.0 \pm 1.9$ mV ($n = 4$) (Fig. 5, A and B). In the presence of 9-phenanthrol, the membrane potential was significantly hyperpolarized ($-73.1 \pm 8.3$ mV; $n = 4$) (Fig. 5, A and B). Following washout of the drug, resting membrane potential did not differ from that recorded before 9-phenanthrol administration ($-36.4 \pm 1.2$ mV; $n = 3$), demonstrating that the effects of the drug are reversible (Fig. 5, A and B). In theory, 9-phenanthrol-induced membrane hyperpolarization could result from inhibition of an inward cation current or from activation of an outward K$^+$ current. However, we find that 9-phenanthrol does not alter K$^+$ currents in smooth muscle cells, indicating that the compound hyperpolarizes the membrane by blocking an inward cation current. 9-Phenanthrol does not block TRPC6 or TRPC3 currents at this concentration, suggesting that a block of a TRPM4-dependent Na$^+$ current is responsible for hyperpolarization of the smooth muscle membrane. These data demonstrate that TRPM4 is an important depolarizing influence in cerebral artery smooth muscle cells.
9-Phenanthrol blocks TRPM4 currents and hyperpolarizes vascular smooth muscle cells in pressurized cerebral arteries. Hyperpolarization of the arterial myocyte membrane causes vasodilation by closing VDCCs and decreasing intracellular \([\text{Ca}^{2+}]\). To determine the effects of 9-phenanthrol on smooth muscle contractility, isolated cerebral arteries were pressurized to 70 mmHg and allowed to develop spontaneous myogenic tone. Luminal diameter was continuously recorded as 9-phenanthrol was added to the superfusion bath. Administration of 9-phenanthrol (30 \(\mu\text{M}\)) significantly dilated pressurized cerebral arteries (Fig. 6, A–C). Myogenic tone decreased by \(\sim 75\%\) in the presence of 30 \(\mu\text{M}\) 9-phenanthrol and was essentially eliminated by a concentration of 100 \(\mu\text{M}\) (Fig. 6, A–C). The effects of the drug were reversible, and myogenic tone redeveloped after 9-phenanthrol was washed from the superfusion bath (Fig. 6, A and B). The IC\(_{50}\) for 9-phenanthrol-induced dilation of cerebral arteries with myogenic tone is 2.6 \(\mu\text{M}\) (Fig. 6C), comparable with the IC\(_{50}\) of this compound for block of sustained TRPM4 currents in smooth muscle cells (10.6 \(\mu\text{M}\)) (Fig. 1B).

We also found that administration of 9-phenanthrol (30 \(\mu\text{M}\)) did not alter vasoconstriction in response to elevated levels of extracellular KCl (60 mM) (Fig. 6D). This finding provides further evidence that the compound does not block depolarization-induced \(\text{Ca}^{2+}\) influx. Furthermore, these data suggest that 9-phenanthrol does not interfere with membrane depolarization resulting from diminished \(K^+\) efflux or directly inhibit smooth muscle contraction in isolated resistance arteries. Our findings indicate that 9-phenanthrol causes arterial dilation by blocking depolarizing TRPM4 currents to hyperpolarize smooth muscle cell membrane potential and decrease voltage-dependent \(\text{Ca}^{2+}\) influx.

DISCUSSION

The current study examined the specificity of the TRPM4 blocker 9-phenanthrol and investigated the effects of the compound on the resting membrane potential and contractility of cerebral artery smooth muscle cells. The major findings of this study are 1) 9-phenanthrol inhibits sustained TRPM4 currents in cerebral artery vascular smooth muscle cells; 2) at a concentration that nearly abolishes sustained TRPM4 currents, 9-phenanthrol does not alter the activity of TRPC3, TRPC6, K\(_{\text{IR}}\), BK\(_{\text{Ca}}\), or VDCC channels; 3) 9-phenanthrol reversibly hyperpolarizes the smooth muscle cell membrane potential in pressurized cerebral arteries; and 4) 9-phenanthrol reversibly dilates cerebral arteries with myogenic tone. These findings demonstrate for the first time that 9-phenanthrol blocks native TRPM4 channels in cerebral artery myocytes without affecting the activity of other channels that are important for smooth muscle function. More importantly, these findings show that TRPM4-dependent cation currents are a primary depolarizing influence in cerebral artery smooth muscle cells under physi-
ological conditions and are critical for establishment and maintenance of myogenic tone.

Selective pharmacological inhibitors are invaluable tools for elucidating the functional impact of the many ion channels present in vascular smooth muscle cells. Few TRP channels inhibitors with significant specificity are currently available, and studies of these channels have relied on molecular techniques, such as antisense (4–6) or siRNA-mediated (7) down-regulation of channel expression. Although these methods are useful, limitations, such as phenotypic changes during culture and possible off-target effects of the inhibitory molecules, complicate interpretation of the data. It is also impossible to examine the acute effects and reversibility of channel inhibition using these methods. Antisense-mediated downregulation experiments suggest that TRPM4 is an important mediator of pressure-dependent (6) and protein kinase C-dependent (5) vasconstriction and autoregulation of cerebral blood flow in vivo (18). To better understand the significance of this channel, we sought a pharmacological inhibitor that was without significant off-target effects. Prior studies demonstrate that 9-phenanthrol effectively blocks TRPM4 under whole cell (10), inside-out (10), and amphotericin B perforated patch configurations (7). Grand et al. (10) also report that 9-phenanthrol does not affect the function of the CFTR Cl⁻ transporter or TRPM5 channels. However, the effects of the compound on cation, K⁺, and Ca²⁺ channels that are important regulators of smooth muscle cell membrane potential and contractility have not been previously reported. Current findings demonstrate that 9-phenanthrol does not inhibit TRPC3 or TRPC6 channels at a concentration (30 μM) that essentially abolishes TRPM4 currents in smooth muscle cells. Furthermore, this concentration of the compound does not alter STOC activity in cerebral myocytes, demonstrating that 9-phenanthrol is without effect on BKCa channels and Ca²⁺ sparks generated by release of Ca²⁺ from ryanodine receptors on the SR. We also find that 9-phenanthrol does not alter the activity of Kᵥ or KIR currents. Our findings also show that 9-phenanthrol does not block VDCC activity. These data are in agreement with the lack of effect of the compound on arterial constriction in response to elevated extracellular K⁺, demonstrating that the compound does not interfere with voltage-dependent Ca²⁺ influx in intact arteries. In addition, this experiment shows that 9-phenanthrol does not interfere with myosin light chain kinase activity or other components of the vascular smooth muscle contractile apparatus. Taken together, these findings demonstrate that 9-phenanthrol is a useful reagent for studying TRPM4 function in cerebral artery smooth muscle cells.

Contractility of arterial smooth muscle is largely regulated by graded changes in smooth muscle cell membrane potential (13). Membrane depolarization activates VDCCs, stimulating Ca²⁺ influx and enhancing contractility by increasing the activity of myosin light chain kinase (13). The resting membrane potential of cerebral artery smooth muscle cells (~−40 mV) under physiological levels of intraluminal pressure (~70 mmHg) (13) is depolarized compared with the K⁺ equilibrium potential (~−90 mV) and is hyperpolarized compared with the equilibrium potentials for cations (E_Na at 37°C ~+60 mV, E_Ca2+ ~+150 mV) and Cl⁻ (~−30 mV) (16). Thus, cerebral arterial membrane potential and contractility are ultimately determined by the relative magnitudes of hyperpolarizing and depolarizing currents. Increases in outward K⁺ currents via BKCa, Kᵥ, and KIR channels hyperpolarize the smooth muscle plasma membrane and cause arterial relaxation (16). Cl⁻ efflux could theoretically have a depolarizing effect on the arterial myocyte plasma membrane, but there is no convincing evidence demonstrating that Cl⁻ currents are significant for pressure-dependent regulation of cerebral artery smooth muscle membrane potential or arterial tone. Furthermore, Ca²⁺-activated Cl⁻ channels are not present in cerebral artery smooth muscle (23), and swelling-activated mechanosensitive currents in these cells are dependent on cation channel activity (25). In contrast, significant evidence suggests that TRPC3, TRPC6, and TRPM4 cation currents contribute to agonist and pressure-induced membrane potential depolarization and constriction of cerebral arteries (6, 18, 19, 24). In the presence of 9-phenanthrol, smooth muscle membrane potential in pressurized cerebral arteries is significantly hyperpolarized and myogenic tone is nearly abolished. 9-Phenanthrol does not activate K⁺ currents or inhibit TRPC3, TRPC6, or VDCC channels, suggesting that membrane hyperpolarization and loss of myogenic tone resulting from 9-phenanthrol administration is due to inhibition of TRPM4 cation currents. Treatment with 9-phenanthrol (30 μM) hyperpolarized smooth muscle cells in cerebral arteries pressurized to 70 mmHg to ~−70 mV, whereas, following antisense-mediated downregulation of TRPM4, smooth muscle cell membrane potential in cerebral arteries pressurized to 80 mmHg expression was ~−50 mV (6). Differences in membrane potential between arteries exposed to TRPM4 antisense and 9-phenanthrol may be due to incomplete knockdown in antisense-treated arteries. Previous work from our lab using reversible permeabilization suggests that ~80% of smooth muscle cells take up TRPM4 siRNA (7), and the cells that do not take up TRPM4 siRNA exhibit normal levels of TRPM4 expression (7). Taken together, these findings indicate that TRPM4 currents are a major depolarizing influence in cerebral artery smooth muscle cells and likely influence contractility by increasing Ca²⁺ influx via VDCC. It is also possible that TRPM4-mediated Na⁺ influx could decrease Ca²⁺ efflux by the Na⁺/Ca²⁺ exchanger, increasing intracellular [Ca²⁺] and force generation. In either case, our findings demonstrate that TRPM4 channels significantly influence myocyte contractility and arterial tone.

TRPM4 and TRPC6 have been linked to pressure-dependent smooth muscle membrane depolarization and myogenic vasoconstriction of cerebral arteries (6, 24). The current findings shed light on the relative importance of these channels in this important physiological response (1). We find that a concentration of 9-phenanthrol (30 μM) that inhibits ~75% of TRPM4 activity under perforated patch-clamp conditions but does not block TRPC6 activity considerably hyperpolarizes the smooth muscle cell membrane and nearly abolishes myogenic tone. These data suggest that TRPM4 is the primary mediator of pressure-induced responses in cerebral artery smooth muscle cells and that TRPC6 channels play a secondary or regulatory role. It is possible that TRPC6 channels could act upstream of TRPM4 to mediate myogenic constriction by influencing intracellular Ca²⁺ dynamics. TRPM4 requires high levels of intracellular Ca²⁺ for activation (14, 17). We recently reported that in native cerebral artery smooth muscle cells, TRPM4 channels are activated by release of Ca²⁺ from SR stores through IP₃ receptors (7). TRPC6 is permeable to Ca²⁺, and it is possible that Ca²⁺ influx through the channel could poten-
tiate IP3-dependent Ca2+ signals required for TRPM4 activity (7) by a Ca2+-induced-Ca2+ release mechanism. Interestingly, TRPC6 channels may be inherently mechanosensitive (21) and could be directly activated by increases in intraluminal pressure. However, a report from another lab did not find TRPC6 channels to be mechanosensitive (9), and further experiments are required to test this hypothesis and clarify the role of TRPC6 in the development of myogenic tone. While the involvement of TRPC6 remains uncertain, the findings of the current study clearly demonstrate that TRPM4 channels are required for the maintenance as well as the development of myogenic tone. The prior work using antisense oligonucleotides to silence TRPM4 prevented the development of myogenic tone and autoregulation of cerebral blood flow (6, 18), but because of the limitations of the molecular approach, did not investigate the role of the channel in maintaining arterial tone. The current findings show that 9-phenanthrol reverses spontaneous myogenic tone when applied to pressurized arteries and that tone redevelops when the drug is removed. Thus, we conclude that TRPM4 activity is required for both the development and maintenance of myogenic tone in cerebral arteries and is a central regulator of vascular function.

In summary, our findings demonstrate that 9-phenanthrol is useful for investigating the functional significance of TRPM4 channels in vascular smooth muscle cells. Using this compound, we find that TRPM4 is a major depolarizing influence regulating smooth muscle cell membrane potential and that activity of the channel is necessary for the development and maintenance of myogenic tone. Our findings may stimulate the development of new therapeutic strategies for the treatment of cardiovascular diseases, such as systemic and pulmonary hypertension, that are associated with smooth muscle cell membrane depolarization. It is possible that derivatives of 9-phenanthrol or other selective TRPM4 inhibitors may reverse this effect and prove useful in controlling these conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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