Critical Role for Transient Receptor Potential Channel TRPM4 in Myogenic Constriction of Cerebral Arteries

Scott Earley, Brian J. Waldron, Joseph E. Brayden

Abstract—Local control of cerebral blood flow is regulated in part through myogenic constriction of resistance arteries. Although this response requires Ca\(^{2+}\) influx via voltage-dependent Ca\(^{2+}\) channels secondary to smooth muscle cell depolarization, the mechanisms responsible for alteration of vascular smooth muscle (VSM) cell membrane potential are not fully understood. A previous study from our laboratory demonstrated a critical role for a member of the transient receptor potential (TRP) superfamily of ion channels, TRPC6, in this response. Several other of the approximately 22 known TRP proteins are also present in cerebral arteries, but their functions have not been elucidated. Two of these channels, TRPM4 and TRPM5, exhibit biophysical properties that are consistent with a role for control of membrane potential of excitable cells. We hypothesized that TRPM4/TRPM5-dependent currents contribute to myogenic vasoconstriction of cerebral arteries. Cation channels with unitary conductance, ion selectivity and Ca\(^{2+}\)-dependence similar to those of cloned TRPM4 and TRPM5 were present in freshly isolated VSM cells. We found that TRPM4 mRNA was detected in both whole cerebral arteries and in isolated VSM cells whereas TRPM5 message was absent from cerebral artery myocytes. We also found that pressure-induced smooth muscle cell depolarization was attenuated in isolated cerebral arteries treated with TRPM4 antisense oligodeoxynucleotides to downregulate channel subunit expression. In agreement with these data, myogenic vasoconstriction of intact cerebral arteries administered TRPM4 antisense was attenuated compared with controls, whereas KCl-induced constriction did not differ between groups. We concluded that activation of TRPM4-dependent currents contributed to myogenic vasoconstriction of cerebral arteries.

(Key Words: TRP channels ■ cerebral circulation ■ cation channels ■ vasoconstriction)

Small arteries and arterioles supplying blood to the brain constrict in response to increasing intraluminal pressure and dilate in response to decreasing pressure. This vital regulatory mechanism, known as the vascular myogenic response, ensures that blood flow remains nearly constant during moment-to-moment changes in arterial pressure. Although initially described in vivo, myogenic constriction also occurs in blood vessels studied in isolation, demonstrating that mechanisms inherent to the vascular wall are sufficient to induce this response. Disruption of the endothelium does not impair pressure-induced constriction, suggesting that sensor and effector mechanisms responsible for myogenic reactivity both reside at the level of smooth muscle cells. Increased intravascular pressure causes depolarization of the arterial myocyte cell membrane, thereby activating voltage-dependent Ca\(^{2+}\) channels, resulting in Ca\(^{2+}\) influx and subsequent vasoconstriction. Despite the essential nature of this response, signaling pathways responsible for pressure-induced smooth muscle depolarization are poorly understood. Improved comprehension of this mechanism could have significant therapeutic potential because impaired myogenic responsiveness and cerebral blood flow autoregulation are associated with a number of pathological conditions, including systemic hypertension, diabetes mellitus, stroke, and head trauma.

Under resting conditions, vascular smooth muscle (VSM) membrane potential is a consequence of ionic homeostasis. Disruption of this equilibrium via activation of mechanosensitive ion channels could account for altered membrane potential during pressure elevation. Consistent with this hypothesis, a previous study from our laboratory demonstrated a critical role for the transient receptor potential (TRP) channel TRPC6 in pressure-induced smooth muscle depolarization and vasoconstriction of cerebral arteries. TRPC6 is a member of the TRP superfamily of cation channels, comprising at least 22 separate genes. Although message for other TRPs is present in blood vessels, potential functions of the majority of these proteins in vascular tissues have not been reported. The current study focused on TRPM4 and TRPM5, members of the melastatin TRP subfamily, which exhibit distinguishing biophysical characteristics suggesting a potential role for regulation of Ca\(^{2+}\) homeostasis of excitable cells. When expressed in HEK cells, these channels are selective for monovalent cations, Ca\(^{2+}\)-impermeant, and are...
activated by intracellular \(\text{Ca}^{2+}\). Interestingly, the unitary conductance of these two TRPs (=25 pico Siemens [pS]) is similar to that reported for mechanosensitive cation channels expressed by smooth muscle cells.16–19 We, therefore, hypothesized that TRPM4 and/or TRPM5 contribute to VSM cell depolarization and vasoconstriction associated with increases in intraluminal pressure. Here we report the presence of a channel with biophysical properties similar to TRPM4 and TRPM5 in cerebral artery smooth muscle cells. Message for TRPM4 but not TRPM5 was detected in these cells. Consistent with our hypothesis, we found that downregulation of TRPM4 expression in isolated vessels impairs pressure-induced depolarization and vasoconstriction, suggesting a key role for this channel in cerebral blood flow regulation.

### Materials and Methods

Cerebral and cerebellar arteries used for these studies were isolated from male Sprague-Dawley rats (250 to 350 g; Charles River Laboratories; St. Constant, Quebec, Canada). All animal use procedures were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of the University of Vermont. To determine whether TRPM4 or TRPM5 like-channels are present in cerebral artery myocytes, the biophysical properties of Ca\(^{2+}\)-dependence and the single-channel conductance and current-voltage relationship of cation channels expressed by these cells were investigated. Inside-out membrane patches were obtained from enzymatically dispersed smooth muscle cells. The bathing solution (intracellular face) contained (in mmol/L) 110 NaCl, 10 HEPES (pH 7.4), and 1.5 MgCl\(_2\). CaCl\(_2\) was added to the bath solution to achieve the concentration required for specific protocols. The pipette solution contained (in mmol/L) 110 NaCl, 10 HEPES (pH 7.4), 1.5 MgCl\(_2\), 2 CaCl\(_2\), 60 mannitol, and 300 mmol/L iiberotoxin. These solutions result in reversal potentials for Na\(^{+}\) of \(-1\) mV, for Cl\(^{-}\) of \(-79\) mV, and for Ca\(^{2+}\) of \(+76\) mV (when bath [Ca\(^{2+}\)] = 100 mmol/L). For some experiments, cells were treated with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) (0.5 mmol/L) for at least 10 minutes before membrane patches were excised. RT-PCR was used to determine whether TRPM4 and TRPM5 mRNA were present in cerebral arteries. Total RNA was extracted from isolated vessels or dispersed smooth muscle cells and first-strand cDNA was synthesized. Forward and reverse primers specific for TRPM4 were as follows: TRPMF, 5'-GTCATCGTGACAAGATGAGA-3'; and TRPM4R, 5'-GTCACCTCTGGGAGCTGTC-3'. These primers yield a 707-bp PCR product. Forward and reverse primers specific for TRPM5 were TRPM5F 5'-CAAGTGATGACATTGCGCATC-3' and TRPM5R 5'-GTCAGGTTGACGGACAGAGT-3', yielding a 639-bp PCR product. PCR products were resolved on 1% agarose gels.

### Results

**TRPM4-/TRPM5-Like Channel Is Present in Rat Cerebral Arteries**

Patch-clamp studies were performed to determine whether channels with biophysical properties similar to TRPM4 and TRPM5 were present in cerebral artery smooth muscle cells. In some inside-out membrane patches obtained from freshly isolated cells (9 of 72 cells; 12.5%; n = 10 rats), channels were observed that exhibited inward currents at negative holding potentials, reversed near 0 mV, and became outward at positive
Characterization of this channel was initially protracted because of the low frequency of observation. However, it was recently reported that the PKC activator PMA increases the frequency of observation of a TRPM4-like cation channel recently reported that the PKC activator PMA increases the frequency of observation of a TRPM4-like cation channel recently reported that the PKC activator PMA increases the frequency of observation of a TRPM4-like cation channel.

To date, selective inhibitors of TRP channels are not available. Therefore, to investigate a potential functional role for increasing Ca\(^{2+}\) concentration (Figure 2A and 2B), demonstrating Ca\(^{2+}\)-dependence of the \(\approx 24\) pS cation channel. [Ca\(^{2+}\)] for half-maximal channel activity was \(\approx 200\) \(\mu\)mol/L.

The Ca\(^{2+}\)-dependence, unitary conductance, and ion permeability of this cerebral artery myocyte channel were consistent with those reported for cloned TRPM4\(^{14,15}\) and TRPM5\(^{23-26}\). To determine whether message encoding these channels was expressed by rat cerebral arteries, we performed RT-PCR for TRPM4 and TRPM5. Message for TRPM4 was present in RNA isolated from both whole cerebral arteries as well as smooth muscle cells isolated from these vessels (Figure 3). TRPM5 message was also present in RNA from whole cerebral arteries (Figure 3). However, we found that TRPM5 was absent in RNA extracted from isolated smooth muscle cells (Figure 3). These findings indicate that the \(\approx 24\) pS Ca\(^{2+}\)-dependent cation channel identified in cerebral artery myocytes could be TRPM4 but not TRPM5.

**Figure 2.** Effects of bath [Ca\(^{2+}\)] on channel activity. A, Example single channel recordings at bath [Ca\(^{2+}\)] of 0.1, 10, 100, and 1000 \(\mu\)mol/L. \(V_{th}=-40\) mV; c indicates closed state. B, Open probability (NP\(_o\)) as a function of bath [Ca\(^{2+}\)]; \(n=4\) at each concentration. Data were fitted to a Boltzman function.

**Figure 3.** RT-PCR for TRPM4 and TRPM5 for RNA extracted from whole cerebral artery (CA) or isolated smooth muscle cells (SMC). NT indicates no template control.
TRPM4 in cerebral arteries we suppressed expression of the channel using antisense technology. We found that the fluorescence of arteries that were permeabilized and exposed to fluorescein-labeled TRPM4 antisense ODNs (Figure 4A) was much greater than that of arteries that were exposed to ODNs but not permeabilized (Figure 4B) or untreated arteries (Figure 4C). Fluorescently labeled ODNs appeared to be present within smooth muscle of permeabilized arteries (Figure 4A), suggesting efficient delivery to these cells.

Semiquantitative RT-PCR was used to evaluate the effects of antisense ODNs on TRPM4 mRNA levels. We found that the intensity of TRPM4 bands in reactions using cDNA derived from antisense-treated arteries was less than that of sense-treated vessels (Figure 4D), suggesting that antisense ODNs suppress TRPM4 expression. The band intensity of a housekeeping gene (GAPDH) did not differ between groups (Figure 4D). We also examined the effects of TRPM4 antisense on mRNA levels of TRPC6, previously shown to contribute to cerebral artery function. As an additional control, we assessed the effects of TRPC6 antisense on TRPM4 mRNA levels in cerebral arteries. TRPM4 expression was not altered by TRPC6 antisense (Figure 4F), whereas TRPC6 mRNA levels were diminished in these arteries (Figure 4G).

To further examine the possibility that the \( \sim 24 \) pS channel is TRPM4, we studied the effects of TRPM4 antisense on the frequency of observation of the channel in patch-clamp experiments. Inside-out membrane patches were obtained from smooth muscle cells isolated from TRPM4 sense and antisense treated arteries after PMA administration. Channels (\( \sim 24 \) pS) were observed in cells obtained from TRPM4-sense treated arteries at a frequency (53%) similar to that of cells from untreated arteries (47%). In contrast, these channels were less frequently (10%) observed in cells from arteries treated with TRPM4 antisense ODNs (Table). The number of cells exhibiting TRPM4-like channels was significantly less for antisense compared with sense-treated vessels (Table). These findings further support the hypothesis that the \( \sim 24 \) pS \( \text{Ca}^{2+} \)-activated monovalent-selective cation channel expressed by cerebral artery myocytes is TRPM4.

### Functional Role of TRPM4 in Cerebral Arteries

The effects of TRPM4 downregulation on pressure-induced depolarization and myogenic constriction were examined using isolated, pressurized cerebral blood vessels treated with TRPM4 sense and antisense ODNs. We found that VSM cells in TRPM4-sense treated arteries were more depolarized when the vessels were pressurized at 80 Torr compared with 20 Torr (Figure 5A and 5B). Smooth muscles cells in these vessels were also depolarized when the purinergic receptor agonist UTP (30 \( \mu \text{mol/L} \)) was administered. The degree of pressure and agonist-induced depolarization observed in these experiments was consistent with those reported for freshly isolated arteries. In contrast, smooth muscle membrane potential of antisense-treated arteries did not depolarize when pressure was elevated from 20 or 80 Torr (Figure 5C and 5D). Furthermore, VSM cells in antisense-treated arteries

<p>| Frequency of Observation of TRPM4-Like Channels in Cells from Sense- and Antisense-Treated Arteries |
|-------------------------------------------------|-------------------------------------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Cells Without TRPM4-Like Channels</th>
<th>No. of Cells With TRPM4-Like Channels</th>
<th>Total No. of Cells per Group</th>
<th>Cells With TRPM4-Like Channels, %</th>
</tr>
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<tbody>
<tr>
<td>Sense</td>
<td>7</td>
<td>8</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>Antisense</td>
<td>18</td>
<td>2</td>
<td>20</td>
<td>10*</td>
</tr>
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* \( \text{P}<0.01 \) vs sense-treated.

n (rats) = 5 for sense-treated arteries; n = 6 for antisense-treated arteries.
pressurized to 80 Torr were hyperpolarized compared with sense-treated vessels at this pressure (Figure 5D). Administration of UTP (30 μmol/L) induced depolarization of smooth muscle in antisense-treated vessels equal to that in sense-treated arteries (Figure 5D). These findings show that TRPM4 contributes to pressure-, but not UTP-, dependent smooth muscle cell depolarization in cerebral arteries.

In subsequent studies, we found that arterial constriction resulting from administration of 60 mmol/L KCl did not differ between sense and antisense-treated arteries (Figure 6A), suggesting that TRPM4 downregulation does not impair K+ depolarization-induced vasoconstriction of these vessels. However, myogenic constriction of TRPM4 antisense-treated vessels was much less than that of sense-treated arteries (Figure 6B through 6C). Pressure-induced constriction of TRPM4 antisense-treated vessels was significantly (P<0.05) less than that of sense-treated arteries at intraluminal pressures ≥60 Torr. These findings demonstrate an important functional role for TRPM4 in pressure-induced depolarization and constriction of cerebral blood vessels.

Discussion
Although the properties of cloned TRPM4 and TRPM5 are consistent with possible roles for these channels in the regulation of membrane potential and Ca2+-homeostasis of excitable cells, their function in smooth muscle has not previously been reported. The objective of this study was to determine whether these channels were present within the cerebral vasculature and how they influence arterial function. Our major findings were as follows: (1) a monovalent selective, Ca2+-dependent cation channel with unitary conductance of ~24 pS was present in cerebral artery smooth muscle cells; (2) TRPM4 message was present in cerebral artery myocytes, whereas mRNA encoding TRPM5 was not detected in these cells; (3) suppression of TRPM4 expression in isolated cerebral arteries decreased the frequency of observation of the ~24 pS channel and impaired pressure-induced smooth muscle depolarization; and (4) myogenic constriction of cerebral arteries was diminished by downregulation of TRPM4. Thus, our findings show that functional TRPM4 is present in VSM and suggest a critical role for this channel in the regulation of cerebral artery tone.

The biophysical characteristics of TRPM4 and TRPM5 are distinct from those of other TRPs. For example, other members of the TRP superfamily are nonselective or Ca2+-selective cation channels, whereas TRPM4 and TRPM5 exhibit specificity for monovalent cations. In addition, activation of TRPM4 and TRPM5 is dependent on intracellular [Ca2+], whereas other TRPs are either indifferent to or inhibited by intracellular Ca2+. Cloned TRPM4 channels expressed in HEK cells are also voltage-dependent. Our data show that cation channels with unitary conductance, ionic selectivity, and Ca2+-dependence similar to those of TRPM4 and TRPM5 are present in cerebral artery myocytes. Unlike cloned TRPM4, these channels did not exhibit voltage dependence. Although the reason for differences in voltage sensitivity between expressed TRPM4 and the arterial smooth muscle cation channel are unclear, this may reflect differential tissue or species-specific properties of the channel, alternative splicing, or heteromultimerization of TRP channel proteins. Interestingly, a recent study demonstrates that cloned TRPM4 is not voltage dependent in the presence of decavanadate. This activity of decavanadate was found to be dependent on a cluster of positively charged amino acid residues in the C terminus of TRPM4. These findings suggest the possibility that the voltage dependence of TRPM4 may be modulated in native cells via this C-terminal domain. Despite the apparent differences in voltage dependence, our findings that message for TRPM4, but not TRPM5, was present in isolated cerebral artery myocytes and that the frequency of observation of the ~24 pS cation channel was reduced when TRPM4 expression was suppressed strongly suggests that that the Ca2+-dependent, monovalent-selective cation channel identified in cerebral artery smooth muscle cells is TRPM4.
Further experiments examined the functional role of TRPM4 in the cerebral vasculature. Deformation of the smooth muscle cell plasma membrane resulting in activation of mechanosensitive ion channels may contribute to depolarization associated with elevation of intraluminal pressure. In support of this possibility, a number of studies demonstrate stretch-activation of cation channels in smooth muscle cells.\textsuperscript{16–19} The unitary conductance for Na\textsuperscript{+}/H\textsuperscript{+} ions of these stretch-activated channels is \( \approx 25 \) pS, and the channels are inhibited by trivalent ions such as Gd\textsuperscript{3+}. These properties are consistent with those of cloned TRPM4 and TRPM5 expressed in HEK cells.\textsuperscript{14,15} To test the hypothesis that the molecular identity of these previously described, stretch-activated channels is TRPM4, we successfully downregulated expression of this TRP protein in isolated cerebral arteries. We found that decreased TRPM4 expression is specifically associated with attenuated pressure-induced smooth muscle cell depolarization and myogenic constriction of these vessels. These findings demonstrate that TRPM4 is an important determinant of pressure-induced smooth muscle cell depolarization and vasoconstriction, suggesting a key role for this channel in the control of cerebral blood flow.

A previous study from our laboratory has demonstrated a critical role for TRPC6 in myogenic constriction of cerebral arteries,\textsuperscript{11} whereas the current study shows that TRPM4 is also very important in this response. These findings could be the result of nonselectivity of the antisense technology used to suppress TRP expression. For example, a recent study found that suppression of TRPM7 expression also downregulates TRPM2 in primary neuron cultures, suggesting transcriptional interdependency among TRP channels in these cells.\textsuperscript{29} To examine the possibility that a similar phenomenon was responsible for our observations, we evaluated the effects of TRPM4 antisense on TRPC6 expression and found that TRPC6 mRNA levels were unaltered by this treatment. We also examined the effects of TRPC6 antisense on TRPM4 expression and found TRPM4 levels to be unchanged when TRPC6 mRNA levels were decreased. These findings support the specificity of antisense procedures used for the current and previous\textsuperscript{11} studies and suggest that expression of both TRPM4 and TRPC6 is necessary for myogenic constriction of cerebral arteries. Given the diversity of biophysical properties and complex molecular biology of the TRP superfamily,\textsuperscript{12} a number of potential mechanisms for interaction between these two channels is possible. For example, activation of TRPM4 during increases in intraluminal pressure may occur as a result of TRPC6-dependent Ca\textsuperscript{2+} influx. Another possibility may be the formation of channels that comprised both TRPC6 and TRPM4 protein subunits. Previous studies have shown that heteromeric channels with novel characteristics can form when multiple TRPC proteins are coexpressed.\textsuperscript{30–32} Although heteromization of channels between different TRP families has not been reported, this could account for our observations. Of course, activation of TRPM4 during increases in intraluminal pressure may occur as a result of TRPC6-dependent Ca\textsuperscript{2+} influx. Another possibility may be the formation of channels that comprised both TRPC6 and TRPM4 protein subunits. Previous studies have shown that heteromeric channels with novel characteristics can form when multiple TRPC proteins are coexpressed.\textsuperscript{30–32} Although heteromization of channels between different TRP families has not been reported, this could account for our observations. Of course, activation of TRPC6 and TRPM4 channels could contribute to pressure-induced depolarization of cerebral VSM, independent of one another. The relative importance of the two channels in this response has not yet been determined, but studies of this type will provide important insights about the integrated functions and diversity of physiologic role of TRP channels in smooth muscle.

We report here that suppression of TRPM4 expression attenuates pressure-induced smooth muscle depolarization. This finding suggests that a TRPM4-dependent depolarizing current is activated by increased intravascular pressure, although the mechanism responsible for activation of this channel in smooth muscle cells is still unknown. Among TRP channels, Ca\textsuperscript{2+}-dependence is unique to TRPM4 and TRPM5, suggesting that intracellular [Ca\textsuperscript{2+}] may be an important regulatory stimulus under physiological conditions. However,
considerable disagreement regarding the [Ca\textsuperscript{2+}] required to open TRPM4 has been reported. Initially, Launay et al. reported half-maximal P\textsubscript{0} of the cloned channel in whole cell experiments at intracellular [Ca\textsuperscript{2+}] of \(\approx 0.4 \mu\text{mol/L,}^{15}\) whereas a study by Nilius et al. reported that this value was 370 \(\mu\text{mol/L for inside-out membrane patches.}^{33}\) Our finding of half-maximal activation at \(\approx 200 \mu\text{mol/L in cerebral artery smooth muscle cells is more in agreement with the latter study. The reason for the discrepancy in Ca\textsuperscript{2+} sensitivity of}

the channel is not known, but may be attributable to differences in experimental conditions used. Our findings suggest that the level of Ca\textsuperscript{2+} required to activate TRPM4 is greater than the physiological range (0.1 to 0.4 \(\mu\text{mol/L}) for global intracellular [Ca\textsuperscript{2+}] normally encountered in smooth muscle cells. However, local [Ca\textsuperscript{2+}] can exceed 10 \(\mu\text{mol/L during transient Ca\textsuperscript{2+} events, such as Ca\textsuperscript{2+} sparks.}^{34}\) Our findings show that this level of Ca\textsuperscript{2+} is sufficient to activate TRPM4 in smooth muscle cells. Transient Ca\textsuperscript{2+} events play a critical role in the regulation of K\textsuperscript{+} channel function in arterial smooth muscle.\(^{35}\) It is possible that such events also modulate TRPM4 activity, should the appropriate localization of TRPM4 channels and Ca\textsuperscript{2+} release sites be present in VSM.

Similar to a previous report using cardiac myocytes,\(^{21}\) we found that administration of a potent PKC activator increases the frequency of observation of a TRPM4-like channel in inside-out membrane patches from VSM cells. In addition, a PROSITE (http://us.expasy.org/prosite/) search of the mouse TRPM4 amino acid sequence identified several PKC activation sites on projected intracellular domains of the channel. These findings suggest that TRPM4 activity can be enhanced by PKC-dependent phosphorylation. Interestingly, an earlier study reports pressure-dependent increases in PKC translocation to the plasma membrane,\(^{36}\) whereas the current study demonstrates a critical role for TRPM4 in pressure-induced smooth muscle cell depolarization. These observations raise the possibility that pressure-dependent PKC activity contributes to activation of depolarizing currents mediated by TRPM4 in VSM cells. Although not directly addressed by the current study, this possibility is in agreement with a number of early reports demonstrating impaired myogenic constriction following PKC inhibition.\(^{37-39}\) Whereas evidence supporting Ca\textsuperscript{2+}-independent pathways for PKC-induced vasoconstriction has been reported,\(^{40-41}\) other studies show that smooth muscle constriction induced by PKC activation is dependent on extracellular Ca\textsuperscript{2+} and can be blocked by antagonist of voltage-dependent Ca\textsuperscript{2+} channels.\(^{42-45}\) Furthermore, administration of PMA depolarizes both airway\(^{46}\) and cerebral artery\(^{47}\) smooth muscle, and this effect is blocked by PKC inhibition. Future investigations will examine the hypothesis that pressure-induced, PKC-dependent phosphorylation of TRPM4 constitutes a novel mechanism for myogenic depolarization and constriction of cerebral arteries.

In summary, the current study demonstrates an important role for TRPM4 in myogenic constriction of cerebral vessels. Considering the findings of our previous study show a similar role for TRPC6,\(^{11}\) we conclude that multiple TRP channels expressed by arterial smooth muscle participate in the regulation of cerebral blood flow.

Acknowledgments

This work was supported by National Heart, Lung, and Blood Institute grants F32HL075995 (to S.E.) and R01HL58231 (to J.E.B.). We thank Johann Patlak for technical assistance, Drs Adrian Bonev and Thomas Heppner for advice on patch-clamp methodology, and Drs Kevin Thorneelo and Mark T. Nelson for critical comments on the manuscript.

References

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_Circ Res._ 2004;95:922-929; originally published online October 7, 2004;
doi: 10.1161/01.RES.0000147311.54833.03

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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EXPANDED MATERIALS and METHODS

Animals

Male Sprague-Dawley rats (250-350 g; Charles River Laboratories; St. Constant, Quebec, Canada) were used for these studies. Animals were deeply anesthetized with pentobarbital sodium (50 mg. i.p.) and euthanized by exsanguination according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont. Brains were isolated in ice-cold MOPS-buffered saline (MBS) [3 mmol/L MOPS (pH 7.4), 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO4, 2.5 mmol/L CaCl2, 1 mmol/L KH2PO4, 0.02 mmol/L EDTA, 2 mmol/L pyruvate, 5 mmol/L glucose and 1% bovine serum albumin]. Cerebral and cerebellar arteries were dissected from the brain, cleaned of connective tissue, and stored in MBS prior to further manipulation.

Cerebral Artery Smooth Muscle Cell Preparation

To isolate VSM cells, vessels were cut into 2-mm segments, and placed in the following cell isolation solution (in mmol/L): 60 NaCl, 80 Na glutamate, 5 KCl, 2 MgCl2, 10 glucose, and 10 HEPES; pH 7.2. Arterial segments were initially incubated at 37°C in 0.5 mg/ml papain, 0.5 mg/ml dithioerythritol, and 1mg/ml BSA for 10 min, followed by 7 min incubation in 0.67 mg/ml type F collagenase, 0.33 mg/ml type H collagenase, 1.0 mg/ml hyaluronidase, and 100 μmol/L CaCl2. The digested segments were then washed three times in ice-cold isolation solution and triturated to release VSM cells. Cells were stored on ice in isolation solution for use the same day.
Single-Channel Patch Clamp Studies

Single-channel currents were recorded from inside-out membrane patches obtained from freshly-isolated arterial myocytes using an Axopatch 200B amplifier equipped with a CV203BU headstage (Axon Instruments). Recording electrodes (resistance, 6-10 MΩ) were pulled from borosilicate glass (1.5 mm OD, 1.17 mm ID; Sutter Instrument, Novato, CA) and coated with wax to reduce capacitance. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. pCLAMP version 8.1, and Clampfit versions 8.1 and 9.2 (Axon Instruments) were used for data acquisition and analysis. Patches were initially held at a membrane potential of 0 mV and single channel currents were recorded at holding potentials between -80 and +80 mV. All recordings were performed at room temperature (22°C). The bathing solution (intracellular face) contained (in mmol/L) 110 Na-glutamate, 5 NaCl, 10 HEPES (pH 7.2), and 60 mannitol. CaCl₂ was added to the bath solution to achieve the concentration required for specific protocols. For experiments with bath [Ca²⁺] = 100 nmol/L, EGTA (5 mmol/L) was added to the bath solution and the appropriate amount of CaCl₂ as calculated using SLIDERS 2.10, (www.stanford.edu/~cpatton.maxc.html) was added to achieve a free [Ca²⁺] of 100 nmol/L. The pipette solution contained (in mmol/L) 110 NaCl, 10 HEPES (pH 7.4), 1.5 MgCl₂, 2 CaCl₂, 60 mannitol, and 300 nmol/L iberiotoxin. These solutions result in reversal potentials for Na⁺ ≅ -1 mV, for Cl⁻, ≅ -79 mV, and for Ca²⁺, ≅ 76 mV (when bath [Ca²⁺] = 100 µmol/L). For some experiments, cells were treated with the protein kinase C (PKC) activator phorbol 12-myristate 13-
acetate (PMA; 0.5 µmol/L) for at least 10 minutes before membrane patches were excised.

Cloned TRPM4 channels are highly selective for Na⁺ vs. Ca²⁺ ions. To examine the ionic specificity of the ~24 pS channel observed in smooth muscle cells, recordings were obtained from inside-out membrane patches from PMA-treated cells in bathing solution in which Na⁺ was replaced with Ca²⁺. The bathing solution (intracellular face) for these experiments contained (in mmol/L) 100 CaCl₂, 13.5 NaCl, 10 HEPES (pH 7.2), and 60 mannitol. Relative Ca²⁺ vs. Na⁺ permeability (Pₐ/Cₐ) was calculated using the Fatt-Ginsburg equation¹;

\[
\frac{E_{rev}}{\frac{RT}{2F}} = \ln \left( \frac{P_{Ca} \cdot [Ca^{2+}]}{P_{Na} \cdot [Na^{+}]} \right)
\]

Where:

\(E_{rev}\) = observed reversal potential

\(P_{Ca}\) = Ca²⁺ permeability

\(P_{Na}\) = Na⁺ permeability

\([Ca^{2+}]\) = intracellular (bath) Ca²⁺ concentration

\([Na^{+}]\) = extracellular (pipette) Na⁺ concentration

To examine the effects of extracellular (bath) [Ca²⁺] on Nₚ₀ of the ~24 pS channel, membrane patches were excised from PMA treated cells into bath solution containing the 0.1 to 1000 µmol/L free Ca²⁺. Cloned TRPM4 channels exhibit a rapid “rundown” of channel activity following excision of the membrane patch². A similar rundown effect was observed for the ~24 pS channel expressed by arterial smooth muscle cells. Therefore, channel activity was recorded for only a single Ca²⁺
concentration per patch for approximately 1 minute following membrane excision. Multiple conductance states were not observed during these experiments, suggesting that each patch contained only a single ~24 pS channel.

Channel open probability ($NP_o$) was calculated using the following equation:

$$NP_o = \sum_{j=1}^{N} \left( \frac{t_j}{T} \right)^j$$

Where:

$t_j$ = time spent in seconds with $j = 1,2,....N$ channels open

$N$ = max number of channels observed

$T$ = duration of measurement.

**RT-PCR for TRP mRNA**

RNA was prepared from arteries or isolated smooth muscle cells using the RNeasy kit (Qiagen). First-strand cDNA was prepared from 300 ng total RNA using the Omniscript Reverse Transcriptase kit (Qiagen). PCR was performed using Failsafe PCR Buffer J (Epicentre). PCR cycling conditions were 39-45 cycles of 94ºC for 30 s, 58ºC (55ºC for TRPC6) for 60 s, and 72ºC for 60 s. Primers were designed to span intron-exon boundaries to eliminate potential amplification of contaminating DNA. All reaction products were resolved on 1% agarose gels. All PCR products were sequenced to confirm identity. Primer sequences and expected PCR product sizes are shown in Table 1.
ODN Sequences and Reverse Permeabilization of Cerebral Arteries

Antisense ODNs for TRPM4 were designed based on a published sequence (GI = 20269878). Two antisense ODNs were used for these studies: TRPM4 AS-1: 5’-GTGTGCATCGCTGTCCCACA-3’ and TRPM4 AS-2: 5’-CTGCGATAGCActGCTGCCCACA-3’. Sense and antisense ODNs sequences for TRPC6 were identical to those used in a prior study 3. The last three bases on the 5’ and 3’ ends of the ODNs were phosphorothioated to limit degradation by cellular nucleases and fluorescein was added to the 5’ end of each ODN to allow assessment of cellular location. ODNs were obtained from Qiagen and were dissolved at a concentration of 2 mM in nuclease-free water. ODNs were introduced into intact cerebral arteries using a reversible permeabilization procedure 4. To permeabilize the arteries, segments were first incubated for 20 minutes at 4°C in the following solution (in mmol/L): 120 KCl; 2 MgCl₂; 10 EGTA; 5 Na₂ATP; 20 TES; pH 6.8. Arteries were then placed in a similar solution containing ODNs (2 µmol/L) for 90 minutes at 4°C and then in a similar ODN-containing solution with elevated MgCl₂ (10 mmol/L). Permeabilization was reversed by placing the arteries for 30 minutes in a MOPS buffered physiological solution containing (in mmol/L): 140 NaCl; 5 KCl; 10 MgCl₂; 5 glucose; 2 MOPS; pH 7.1, 22°C. Ca²⁺ was gradually increased in the latter solution from nominally calcium free to 0.01, 0.1, and 1.8 mmol/L over a 45 minute period. Following the reversible permeabilization procedures, arteries were organ cultured for 2.5 days in D-MEM/F-12 culture media supplemented with L-glutamine (2 mmol/L).
penicillin (50 units/ml) and streptomycin (50 µg/ml). Smooth Muscle Cell

Membrane Potential

For measurement of smooth muscle cell membrane potential, cerebral arteries were isolated and pressurized, and VSM cells were impaled through the adventia with glass intracellular microelectrodes (tip resistance 100-200 MΩ). A WPI Intra 767 amplifier was used for recording membrane potential (\(E_m\)). Analog output from the amplifier recorded using Axotape software (sample frequency 20 Hz). Criteria for acceptance of \(E_m\) 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 approximately 0 mV after the electrode was retracted from the cell. VSM cell \(E_m\) was recorded for TRPM4 sense and antisense treated arteries at intraluminal pressures of 20 and 80 Torr, and following administration of UTP (30 µmol/L) to vessels pressurized to 20 Torr.

Assessment of Myogenic Responsiveness

Arterial segments were cleaned and transferred to a vessel chamber (University of Vermont Instrumentation Facility). The proximal end of the vessel was cannulated with a glass micropipette and secured, blood was gently rinsed from the lumen, and the distal end of the vessel cannulated and secured. Vessels were pressurized to 20 Torr with PSS and superfused (5 ml/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O₂, 6% CO₂, balance N₂). Following a 15-minute equilibration period, intraluminal pressure
was slowly increased to 100 Torr, vessels were stretched to remove bends, and pressure was reduced to 20 Torr for an additional 15-minute equilibration period. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix). To assess depolarization-induced constrictor responses, arteries were exposed to isotonic PSS containing 60 mmol/L KCl. Following a 15 minute wash period, vessels were subjected to a series of pressure steps between 20 and 100 Torr and spontaneous myogenic tone was allowed to develop at each step for 3 min. After completion of the pressure-response curve, intraluminal pressure was maintained at 20 Torr and vessels were superfused with Ca^{2+}-free PSS (129.8 mmol/L NaCl, 5.4 mmol/L KCl, 0.83 mmol/L MgSO_{4}, 19 mmol/L NaHCO_{3}, 5.5 mmol/L glucose and 3 mmol/L EGTA). The pressure-response curve was repeated under Ca^{2+}-free conditions to obtain passive responses. Myogenic tone was calculated as the percent difference in diameter observed for Ca^{2+}-containing vs. Ca^{2+}-free PSS at each pressure. KCl-induced vasoconstriction was calculated as the percent change in diameter relative to the passive diameter at 20 Torr (i.e., % of Passive). KCl-induced constriction and myogenic tone were determined for TRPM4 sense and antisense-treated arteries (n=6 for both groups). A single artery was studied from each rat, thus for isolated vessel experiments, values of n refer to the number of animals used for a particular experimental group.
Calculations and Statistics

All data are mean ± SE. Comparisons of the frequency of observation of a ~24 pS channel between cells from sense and antisense-treated vessels were made using Chi-square analysis (Fisher's Exact Probability test). KCl-induced and myogenic constriction between TRPM4 sense and antisense-treated arteries were compared by unpaired Student's t-tests. Comparisons of membrane potentials between TRPM4 sense and antisense-treated arteries pressurized to 20 or 80 Torr or treated with UTP were made by two-way ANOVA followed by Student-Newman-Keuls post hoc test. A level of $P \leq 0.05$ was accepted as statistically significant for all experiments.
Table 1: PCR Primer Sequences and Expected Product Sizes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size</th>
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<tbody>
<tr>
<td>TRPM4</td>
<td>5'-GTCATCGTGAGCAAGATGATGAA -3'</td>
<td>5'-GTCCACCTTCTGGGACGTGC -3'</td>
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<td>TRPC6</td>
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<td>5'-CTGGATCTTCACTGGACAAT-3'</td>
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<tr>
<td>GAPDH</td>
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<td>5'-CATGTTAGGCCATGAGTGCCACCAC-3'</td>
<td>1000</td>
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REFERENCES


