Endothelium-Dependent Cerebral Artery Dilation Mediated by TRPA1 and Ca^{2+}-Activated K^+ Channels

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Abstract—Although it is well established that changes in endothelial intracellular [Ca^{2+}] regulate endothelium-dependent vasodilatory pathways, the molecular identities of the ion channels responsible for Ca^{2+} influx in these cells are not clearly defined. The sole member of the ankyrin (A) transient receptor potential (TRP) subfamily, TRPA1, is a Ca^{2+}-permeable nonselective cation channel activated by electrophilic compounds such as acrolein (tear gas), allicin (garlic), and allyl isothiocyanate (AITC) (mustard oil). The present study examines the hypothesis that Ca^{2+} influx via TRPA1 causes endothelium-dependent vasodilation. The effects of TRPA1 activity on vascular tone were examined using isolated, pressurized cerebral arteries. AITC induced concentration-dependent dilation of pressurized vessels with myogenic tone that was accompanied by a corresponding decrease in smooth muscle intracellular [Ca^{2+}]. AITC-induced dilation was attenuated by disruption of the endothelium and when the TRPA1 channel blocker HC-030031 was present in the arterial lumen. TRPA1 channels were found to be present in native endothelial cells, localized to endothelial cell membrane projections proximal to vascular smooth muscle cells. AITC-induced dilation was insensitive to nitric oxide synthase or cyclooxygenase inhibition but was blocked by luminal administration of the small and intermediate conductance Ca^{2+}-activated K^+ channel blockers apamin and TRAM34. $BaCl_2$, a blocker of inwardly rectifying K^+ channels, also inhibited AITC-induced dilation. AITC-induced smooth muscle cell hyperpolarization was blocked by apamin and TRAM34. We conclude that Ca^{2+} influx via endothelial TRPA1 channels elicits vasodilation of cerebral arteries by a mechanism involving endothelial cell Ca^{2+}-activated K^+ channels and inwardly rectifying K^+ channels in arterial myocytes. **(*Circ Res*. 2009;104:987-994.)**

Key Words: TRP channels ■ EDHF ■ AITC ■ inwardly rectifying K^+ channels

Changes in endothelial intracellular [Ca^{2+}] regulate endothelium-dependent vasodilation through diverse signaling pathways. Production of potent vasoactive substances such as nitric oxide (NO)[1] and prostacyclin (PG)I_2[2] are stimulated by increases in intracellular [Ca^{2+}]. In addition, small ($K_{Ca}2.3$) and intermediate ($K_{Ca}3.1$) conductance Ca^{2+}-activated K^+ channels hyperpolarize the endothelial cell plasma membrane and influence smooth muscle excitability, vascular tone, and arterial blood pressure by direct electric communication via myoendothelial gap junctions[3,4] or release of K^+ ions.[5] Despite the impact of endothelial cell Ca^{2+} mobilization and dynamics on critical aspects of vascular function, the molecular identities and regulation of Ca^{2+} entry channels present in these cells are poorly understood. Further characterization of the ion channels responsible for endothelial cell Ca^{2+} influx is expected to provide critical insight into the nature of endothelium-dependent vasodilation.

Ca^{2+}-permeable ion channels belonging to the transient receptor potential (TRP) superfamily[6] are present in vascular endothelial cells and likely play a major role in Ca^{2+}-dependent signaling processes.[7–9] The sole member of the ankyrin (A) TRP subfamily, TRPA1,[10] is a Ca^{2+}-permeable nonselective cation channel that is activated by electrophilic compounds such as acrolein (an active component of tear gas), allicin (found in garlic), and allyl isothiocyanate (AITC) (derived from mustard oil).[11,12] Unsaturated aldehydes produced endogenously in response to oxidative stress, such as 4-hydroxy-2-nonenal (4-HNE),[13,14] 4-oxo-nonenal (4-ONE),[13] and 4-hydroxyhexenal (4-HHE),[13] also activate TRPA1. TRPA1 is expressed by a subset of nociceptive sensory neurons and mediates inflammatory pain in response to stimuli such as topical administration of chemical irritants[15] and inhalation of cigarette smoke.[16] TRPA1 is also present in nonneuronal tissues such as basal urothelial cells[17] and prostate epithelial cells,[18] although the functional significance of the channel in these tissues is not known.

The effects of TRPA1 activity on endothelium-dependent dilation of cerebral resistance arteries was investigated. We find that endothelial cell TRPA1 channels mediate vasodilation by a novel pathway involving Ca^{2+}-activated K^+ channels in endothelial cells and inwardly rectifying K^+ (K_{IR}) channels in arterial myocytes.

Materials and Methods

Cerebral and cerebellar arteries used for these studies were isolated from male Sprague–Dawley rats (250 to 350 g; Harlan, Indianapolis, Ind). All animal use procedures were in accordance with institutional

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guidelines and approved by the Institutional Animal Care and Use Committee of Colorado State University.

For isolated vessel experiments, arteries were cannulated, pressurized with physiological saline solution, and superfused with aerated physiological saline solution at 37°C. To monitor changes in vessel wall [Ca^{2+}] (representative of smooth muscle intracellular [Ca^{2+}]), arteries were loaded with the ratiometric Ca^{2+} indicator dye fura-2AM from the abluminal surface. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix). Pressurized vessels were intermittently excited with UV light at 340 and 380 nm (10 Hz), and deep red wavelength emissions were recorded using a photomultiplier tube and expressed as the ratio of emissions during 340 nm excitation versus 380 nm excitation (340/380 ratio). For some experiments, endothelial cell function was disrupted by passage of air and distilled water through the vessel lumen. AITC-induced dilation was found to be very reproducible (Figure I in the online data supplement, available at http://circres.ahajournals.org), allowing all experiments examining the effects of endothelial disruption, NO synthase (NOS) and cyclooxygenase (COX) inhibition, or block of K^+ channels on vasodilation to be performed and analyzed using a paired design. Smooth muscle cell membrane potential was recorded in pressurized (70 mm Hg) cerebral arteries with intracellular microelectrodes using previously described methods.[19,20]

To assess TRPA1 mRNA expression in vascular tissue, enzymatically dispersed native endothelial cells were visually identified using phase-contrast microscopy and were collected using a micromanipulator controlled pipette. Total RNA was isolated from these cells and RT-PCR was performed using TRPA1-specific primers yielding a product of 500 bp. RT-PCR was performed using RNA isolated from 3 animals.

Immunostaining for TRPA1 was performed using intact cerebral arteries with the endothelium exposed by cutting the vessel lengthwise and pinning the tissue to a Silgard block. Fixed tissue was probed with anti-TRPA1 (Santa Cruz Biotechnology; 1:1000),[21] anti-$K_{Ca}3.1$, and/or anti-$K_{Ca}2.3$ (both Alomone, 1:1000) overnight at 4°C. Arteries were probed with fluorescent secondary antibodies (Texas Red, Santa Cruz Biotechnology, 1:500; or Alexa 633, Molecular Probes, 1:500), and immunofluorescence was detected using a Zeiss LSM 510 Meta laser scanning confocal microscope. Immunofluorescence was not detected in tissues probed with secondary antibodies alone.

An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Results

TRPA1 Agonists Dilate Cerebral Arteries by an Endothelium-Dependent Mechanism

To investigate the effects of TRPA1 channel activity on vasomotor tone of cerebral arteries, changes in luminal diameter and vessel wall [Ca^{2+}] were recorded when the TRPA1 agonist AITC was present in the bathing solution. For these experiments, fura-2AM was administered to the abluminal surface, preferentially loading vascular smooth muscle cells.[22] Arteries were pressurized to 70 mm Hg, and spontaneous myogenic tone was allowed to develop. AITC administration resulted in robust, persistent vasodilation of preconstricted arteries that was accompanied by a corresponding decrease in vessel wall [Ca^{2+}] (Figure 1A). Arteries contracted to their original diameter when AITC was removed from the bathing solution (Online Figure I), and subsequent AITC administration elicited equivalent dilator responses (Online Figure I), demonstrating that TRPA1-mediated vasodilation is reversible and reproducible. AITC elicited statistically significant vasodilation and reduction in vessel wall [Ca^{2+}] at concentrations as low as 3 μmol/L, and induced maximal changes in luminal diameter at a concentration of 30 to 100 μmol/L (Figure 1B and 1C). The half-maximal effective concentration (EC_{50}) for vasodilation was 16.4 μmol/L (Figure 1D). These findings show that activation of TRPA1 channels causes dilation of preconstricted cerebral arteries by reducing smooth muscle intracellular [Ca^{2+}]. During myogenic constriction, voltage-dependent Ca^{2+} channels are the primary Ca^{2+} influx pathway in cerebral artery myocytes.[22] Thus, these findings suggest that TRPA1 activity elicits dilation by hyperpolarizing the membrane potential of vascular smooth muscle cells.

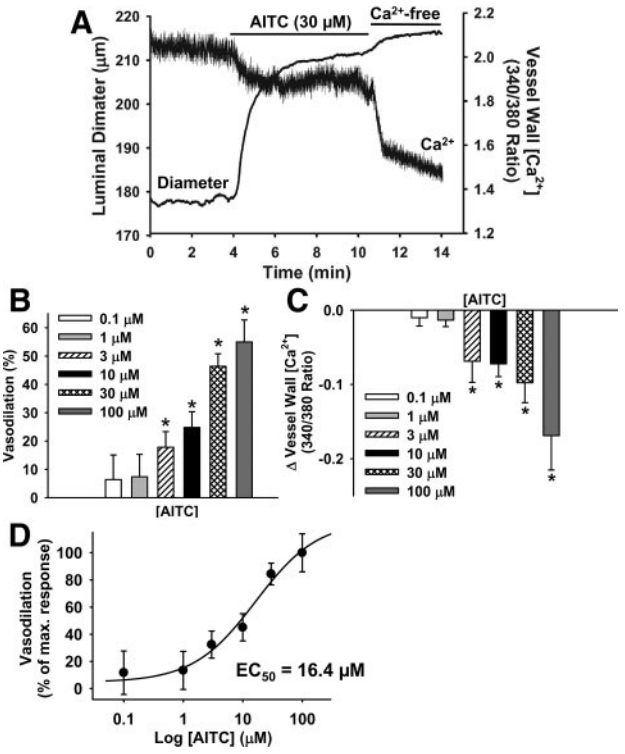

Figure 1. The TRPA1 agonist AITC elicits vasodilation of cerebral arteries. A, Representative recording of AITC (30 μmol/L)-induced dilation and decrease in vessel wall [Ca^{2+}] of an isolated cerebral artery with myogenic tone (70 mm Hg). B, Vasodilation (normalized to passive diameter recorded under Ca^{2+}-free conditions) in response to increasing concentrations of AITC (n=5 to 11 per concentration). C, Decrease in vessel wall [Ca^{2+}] (expressed as change in 340/380 ratio) in response to increasing concentrations of AITC (n=6 to 11 per concentration). D, Concentration response data normalized to maximum vasodilation. EC_{50}=16.4 μmol/L.

Activation of TRPA1 channels in any of the cell types within the vascular wall, including smooth muscle, endothelial cells, or perivascular nerve terminals, could be responsible for AITC-induced vasodilation. To determine whether the endothelium contributes to TRPA1-dependent vasodilation, endothelial cell function was disrupted by briefly perfusing isolated cerebral arteries with distilled water followed by air. Vasodilation and changes in vessel wall [Ca^{2+}] in response to AITC were recorded before and after endothelial cell disruption. Damage to the endothelium resulted in impaired AITC-induced vasodilation (Figure 2A and 2B), demonstrating that endothelial cells are involved in arterial dilation associated

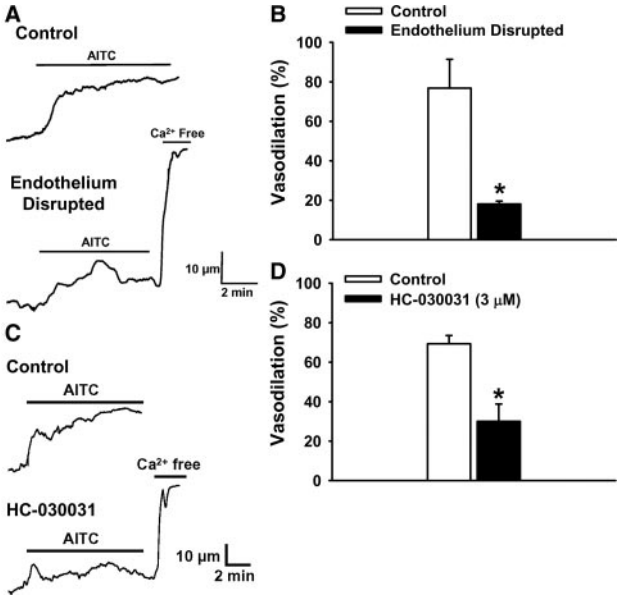

Figure 2. Endothelial cell TRPA1 channels mediate AITC-induced vasodilation. A, Vasodilation in response to AITC (100 μmol/L) before and after disruption of endothelial cell function. B, Summary data of the effects of endothelium disruption on AITC-induced vasodilation (n=3). *$P \leq 0.05$ vs control. C, Vasodilation in response to AITC (100 μmol/L) before and after luminal administration of the TRPA1 blocker HC-030031 (3 μmol/L). D, Summary data for the effects of luminal HC-030031 on AITC-induced vasodilation (n=5). *$P \leq 0.05$ vs control.

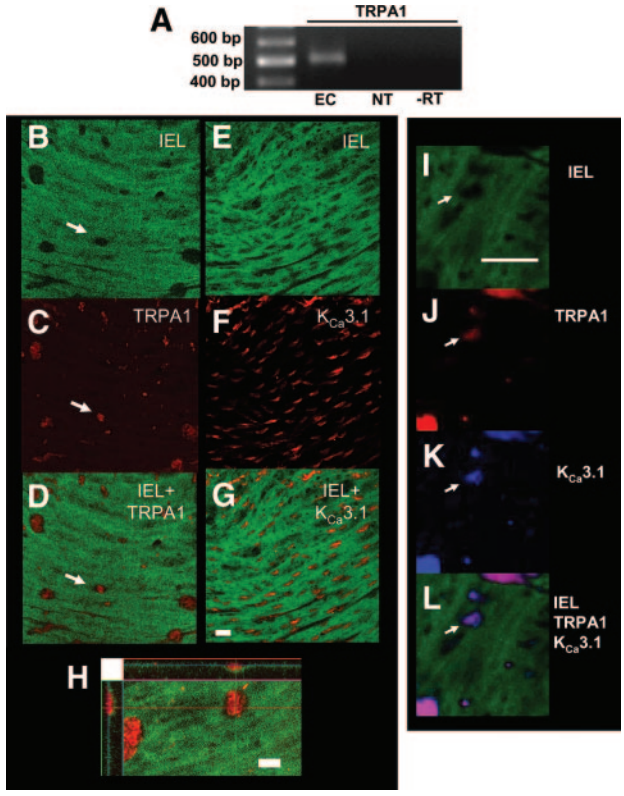

Figure 3. TRPA1 is present in endothelial cells isolated from rat cerebral arteries. A, RT-PCR for TRPA1 using total RNA from freshly isolated rat cerebral artery endothelial cells (EC). NT indicates no template control; -RT, no reverse transcriptase control. Data are representative of RNA isolated from 3 rats. B through H, Localization of TRPA1 and $K_{Ca}3.1$ channels in cerebral artery endothelial cell membrane projections. Images show immunostaining for TRPA1 (red) (C through E) and $K_{Ca}3.1$ (red) (F through H). The level of the IEL is shown in green. Black holes (arrow) in the IEL indicate endothelial cell membrane projections in the direction of vascular smooth muscle cells (C and F). Superimposed images demonstrate that TRPA1 channels and $K_{Ca}3.1$ channels are abundant in the holes in the IEL (E and H). Bar=20 μm. I, Z-stack image showing projection of TRPA1 immunostaining though the IEL. Bar=10 μm. I through L, Coimmunostaining for TRPA1 and $K_{Ca}3.1$ in cerebral artery endothelial cell membrane projections. The level of the IEL is shown in green (I). Black holes (arrow) in the IEL indicate endothelial cell membrane projections in the direction of vascular smooth muscle cells. J, Immunostaining for TRPA1 (red). K, Immunostaining for KCa3.1 (blue). L, Superimposed images demonstrate that TRPA1 channels and $K_{Ca}3.1$ channels colocalize in black holes in the IEL. Bar=25 μm. All immunostaining data are representative of arteries isolated from at least 3 animals.

with TRPA1 channel activation. In addition, AITC administration did not cause significant changes in vessel wall $[Ca^{2+}]$ in endothelium-disrupted arteries, suggesting that this agonist has no direct effect on vascular smooth muscle cells.

A recently described blocker of TRPA1 channels, HC-030031,[23] was used to further probe the role of TRPA1 channels in endothelium-dependent vasodilation. The half-maximal inhibitory concentration (IC_{50}) of HC-030031 for AITC-induced TRPA1 currents in patch clamp studies is reportedly 0.7±0.1 μmol/L.[23] HC-030031 is selective for TRPA1 channels and does not inhibit TRPV1, TRPV3, TRPV4, hERG, or $Na_V1.2$ channel activity in the concentration range used for the current study (IC_{50} for these channels >10 to 20 μmol/L).[23] To block endothelial cell TRPA1 channels, HC-030031 (3 μmol/L) was administered to the lumen of isolated cerebral arteries. In paired experiments, AITC-induced dilation was impaired in the presence of luminal HC-030031 (Figure 2C and 2D), demonstrating that activation of TRPA1 channels in vascular endothelial cells causes vasodilation of cerebral arteries. Interestingly, luminal administration of HC-030031 also caused a small, yet statistically significant increase in myogenic tone (Online Figure II). These data suggest that endothelial TRPA1 channels support a tonic vasodilatory influence that is independent of exogenous TRPA1 activators.

TRPA1 Channels Are Present in Cerebral Artery Endothelial Cells

Endothelial cell damage (Figure 2B) and luminal administration of the TRPA1 blocker HC-030031 inhibits AITC-induced vasodilation (Figure 2D), suggesting that TRPA1 channels present in vascular endothelial cells mediate this response. Consistent with these results, message encoding TRPA1 is present in total RNA extracted from native cerebral artery endothelial cells (Figure 3A).

To assess the localization of TRPA1 channels in cerebral arteries, vessels were cut longitudinally, pinned to Silgard blocks, fixed, and immunostained using an anti-TRPA1 antibody (Santa Cruz Biotechnology).[21] To localize the endothelium in this preparation, the internal elastic lamina (IEL) was identified based on its characteristic green autofluorescence (Figure 3B and 3E).[24,25] Trans-IEL endothelial cell membrane projections[24,25] are clearly visible in these images

(arrow). Immunostaining for TRPA1 (red, Figure 3C) is abundant in these membrane depressions (Figure 3D, arrow), demonstrating that the channel is expressed at high levels in these structures. Diffuse immunostaining for TRPA1 channels in the endothelium was also present outside of endothelial cell membrane projections (Figure 3C). In agreement with recent findings reported by Sandow et al.[24] and Ledoux et al,[25] $K_{Ca}3.1$ channels are also highly enriched in these membrane structures (Figure 3E through 3G). Dual-label experiments confirm colocalization of TRPA1 and $K_{Ca}3.1$ in black holes (Figure 3I through 3L). Consistent with an earlier study,[24] immunostaining for the SK_{Ca} channel $K_{Ca}2.3$ was present in cerebral arteries but did not localize to myoendothelial membrane projections (Online Figure III). A reconstructed series of images taken vertically through the depth of the tissue ("z-stack") demonstrates that TRPA1 immunostaining projects though the IEL (Figure 3H). TRPA1 immunostaining was not present following mechanical disruption of the endothelium (Online Figure IV). These findings demonstrate that TRPA1 channels are present in the endothelium and are abundant in trans-IEL membrane domains with elevated levels of $K_{Ca}3.1$ channels.

TRPA1-Mediated Vasodilation Requires Endothelial K_{Ca} and Smooth Muscle K_{IR} Channels

Stimulation of TRPA1 activity causes endothelium-dependent vasodilation. Pharmacological inhibition of the NOS and COX pathways was used to examine their contribution to TRPA1-induced vasodilation. Superfusion of arteries with the NOS inhibitor N-nitro-L-arginine (L-NNA) (300 μmol/L, 20 minutes) or a combination of L-NNA and the COX inhibitor indomethacin (10 μmol/L, 15 minutes) caused an increase in basal myogenic tone (myogenic tone: 19.4±4.4% under control condition; 25.0±7.6% in the presence of L-NNA; 27.4±10.0% in the presence of L-NNA+indomethacin; n=5). AITC-induced vasodilation was not diminished by NOS or COX inhibition (Online Figure V), demonstrating that activation of TRPA1 channels in the endothelium causes vasodilation by a pathway that is independent of NO and PGI_2 production. These data do not directly address the effects of AITC on NOS and COX activity.

Ca^{2+}-activated K^+ channels are present in vascular endothelial cells, and activation of these channels can cause vasodilation.[3,4,26] To determine whether TRPA1-dependent Ca^{2+} influx elicits dilation by activating these channels, the $K_{Ca}3.1$ inhibitor TRAM34 (1 μmol/L) or a combination of TRAM34 and the SK_{Ca} blocker apamin (1 μmol/L) was administered to the lumen of cerebral arteries that had been allowed to develop spontaneous myogenic tone (70 mm Hg). TRAM34 significantly decreased AITC-induced vasodilation (Figure 4B), whereas the combination of apamin and TRAM34 abolished sustained AITC-induced vasodilation (Figure 4A and 4C), indicating that SK_{Ca} and $K_{Ca}3.1$ channel activity contribute to TRPA1-mediated vasodilatory responses. Arteries treated with apamin and TRAM34 dilated to nearly maximal diameters (99.3±1.4% of Ca^{2+}-free diameter; n=3) in response to the ATP-sensitive K^+ (K_{ATP}) channel opener pinacidil (10 μmol/L) (Figure 4A), demonstrating that K_{Ca} channel inhibition did not block vasodilation

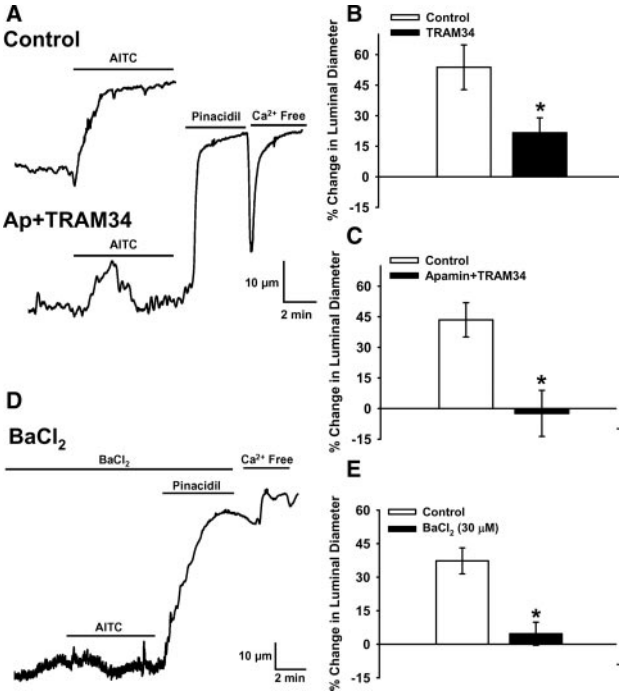

Figure 4. TRPA1-dependent vasodilation requires K_{Ca} and K_{IR} channels. A, Representative recordings of vasodilation in response to AITC (10 μmol/L) before and after luminal administration of the SK_{Ca} blocker apamin (1 μmol/L) and the $K_{Ca}3.1$ blocker TRAM34 (1 μmol/L). B, Summary of the effects of $K_{Ca}3.1$ blockade on AITC-induced dilation (n=5). C, Summary of the effects of $SK_{Ca}/K_{Ca}3.1$ blockade on AITC-induced vasodilation (n=5). *$P \leq 0.05$ vs control. D, Representative recording of vasodilation in response to AITC (10 μmol/L) in the presence of the K_{IR} blocker $BaCl_2$ (30 μmol/L). E, Summary of the effects $BaCl_2$ on AITC-induced vasodilation (n=5). *$P \leq 0.05$ vs control.

in response to a stimulus that hyperpolarizes vascular smooth muscle cells. These data show that Ca^{2+} influx via endothelial TRPA1 stimulates $SK_{Ca}/K_{Ca}3.1$ channels, causing K^+ efflux and vasodilation. Selective activation of $SK_{Ca}/K_{Ca}3.1$ versus NOS and COX pathways in response to AITC is in agreement with the hypothesis that stimulation of TRPA1 channels does not globally increase intracellular [Ca^{2+}] but instead results in localized elevation of intracellular [Ca^{2+}] in subcellular microdomains containing K_{Ca} channels. Consistent with this possibility, TRPA1 and $K_{Ca}3.1$ channels are colocalized in endothelial cell membrane domains spanning the IEL (Figure 3I through 3L).

Activation of endothelial $SK_{Ca}/K_{Ca}3.1$ channels hyperpolarizes the endothelial cell plasma membrane.[3,4,26] Myoendothelial gap junctions conduct changes in endothelial cell membrane potential to vascular smooth muscle, resulting in myocyte hyperpolarization and vasodilation.[3,4,26] In cerebral arteries, endothelium-dependent smooth muscle hyperpolarization can be amplified by the negative slope conductance of inwardly rectifying K^+ (K_{IR}) channels.[27–29] Alternatively, K^+ released from endothelial cell K_{Ca} channels could locally elevate [K^+] in the interstitial space proximal to vascular smooth muscle cells to levels (8 to 12 mmol/L) sufficient to activate K_{IR} channels present in cerebral artery myocytes.[5] Activation of K_{IR} channels in smooth muscle cells at physiologically relevant membrane potentials (−50 mV to −35

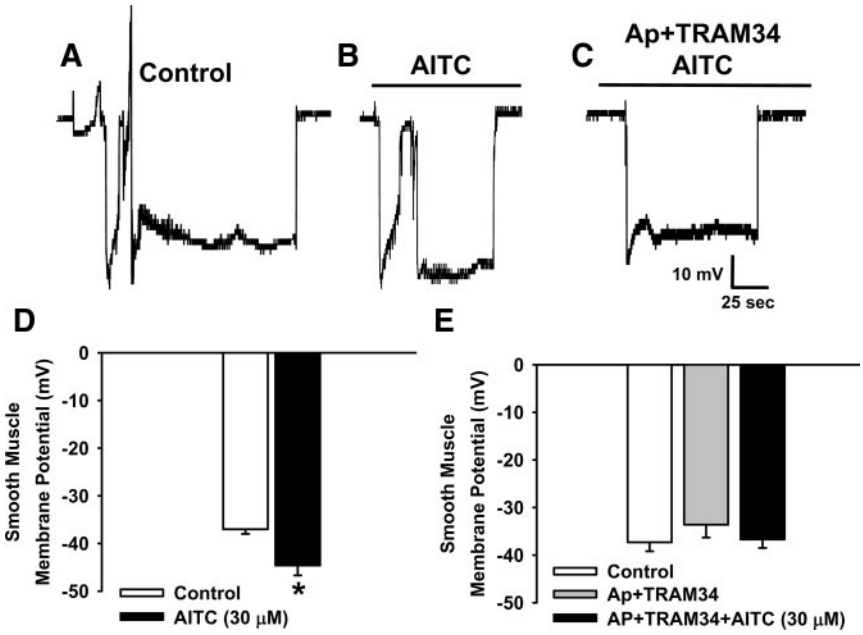

Figure 5. TRPA1 channel activation hyperpolarizes cerebral artery myocytes. A through C, Representative membrane potential recordings of smooth muscle cells in pressurized (70 mm Hg) cerebral arteries under control conditions (A), in the presence of AITC (30 μmol/L) (B), and in the presence of AITC (30 μmol/L) following luminal administration apamin (1 μmol/L) and TRAM34 (1 μmol/L) (C). D, Summary data for the effects of AITC (30 μmol/L) on smooth muscle cell membrane potential. Data are from tissue isolated from 3 animals (n=7 cells for each group). *$P \leq 0.05$ vs control. E, Summary data for the effects of AITC (30 μmol/L) on smooth muscle cell membrane potential when apamin (1 μmol/L) and TRAM34 (1 μmol/L) was present in the arterial lumen. Data are from tissue isolated from 3 animals (n=5 to 7 cells per group). There were no significant differences.

mV) causes K^+ efflux, which hyperpolarizes the sarcolemma, resulting in myocyte relaxation and vasodilation.[30] Low (micromolar) concentrations of Ba^{2+} ions are effective at selectively blocking K_{IR} channels in smooth muscle cells.[30] Extracellular $BaCl_2$ (30 μmol/L) essentially abolished AITC-induced vasodilation (Figure 4D and 4E), demonstrating that K_{IR} channel activity is required for cerebral artery dilation in response to TRPA1 activation. These experiments also show that pinacidil-induced vasodilation was not blocked by $BaCl_2$ (Figure 4D; 96.5±1% of Ca^{2+}-free diameter, n=4), indicating that inhibition of K_{IR} channels does not impair K_{ATP}-induced myocyte hyperpolarization and arterial dilation.

TRPA1 Channel Activation Hyperpolarizes Cerebral Artery Myocytes

The effects of TRPA1 channel activation on smooth muscle cell membrane potential were directly assessed using intracellular microelectrodes. The resting membrane potential of arterial myocytes in cerebral arteries pressurized to 70 mm Hg under control conditions is −37.0±1.0 mV (n=7) (Figure 5A and 5D), whereas in the presence of AITC (30 μmol/L), smooth muscle membrane potential is −44.6±2.1 mV (n=7) (Figure 5B and 5D). These data demonstrate that TRPA1 channel activity hyperpolarizes the sarcolemma. To investigate a potential role for $SK_{Ca}/K_{Ca}3.1$ channels in AITC-induced hyperpolarization, experiments were performed when apamin and TRAM34 (1 μmol/L each) were present in the arterial lumen. This treatment had no significant effect on membrane potential or myogenic tone under control conditions. In addition, smooth muscle cell membrane potential did not differ when recordings were obtained under control conditions (37.3±1.8 mV, n=7), following luminal administration of apamin and TRAM34 (33.6±2.6 mV, n=5) and when apamin and TRAM34-treated arteries were superfused with AITC (30 μmol/L) (−36.7±1.8 mV, n=7; Figure 5C and 5E). These findings show that activation of TRPA1 in the endothelium hyperpolarizes smooth muscle cells by a mechanism that requires SK_{Ca} and $K_{Ca}3.1$ channel activity (Figure 6).

Discussion

This study examined the functional consequences of TRPA1-mediated endothelial cell Ca^{2+} influx on the vasomotor activity of cerebral arteries. The major findings are: (1) the TRPA1 agonist AITC causes concentration-dependent dilation of cerebral arteries that is associated with a decrease in smooth muscle intracellular $[Ca^{2+}]$; (2) TRPA1 agonist-dependent dilation is impaired by disruption of the endothelium and by luminal administration of a TRPA1 blocking compound; (3) TRPA1 channels are present in the vascular endothelium and are localized to membrane projections that approach underlying smooth muscle cells; (4) vasodilation resulting from stimulation of TRPA1 activity is independent of NOS and COX activity but is attenuated by blockade of

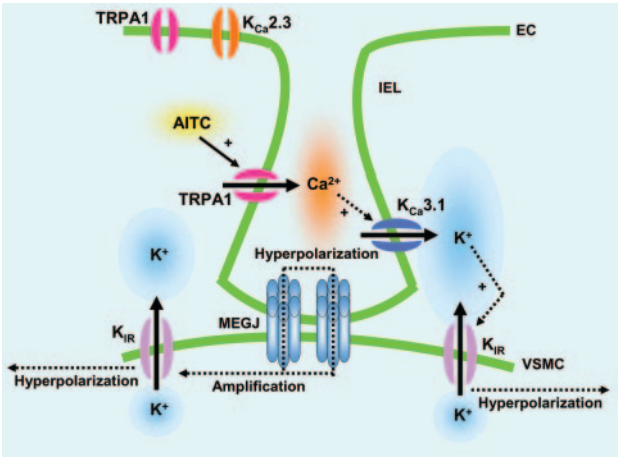

Figure 6. Proposed signaling pathway for TRPA1-mediated vasodilation of cerebral arteries. EC indicates endothelial cell; VSMC, vascular smooth muscle cell; MEGJ, myoendothelial gap junction.

SK_{Ca} and $K_{Ca}3.1$ channels; (5) inhibition of K_{IR} channel activity abolishes TRPA1-dependent vasodilation; and (6) activation of TRPA1 causes smooth muscle cell hyperpolarization by a mechanism that requires the activity of SK_{Ca} and $K_{Ca}3.1$ channels. The findings of this study demonstrate that endothelial TRPA1 channels are part of a vasodilatory signaling complex that includes K_{Ca} channels in endothelial cells and K_{IR} channels in cerebral artery myocytes (Figure 6).

Endothelium-dependent dilation of cerebral arteries following stimulation of TRPA1 channels is not altered by blockade of NOS and COX, but is sensitive to inhibition of small- and intermediate-conductance Ca^{2+}-activated K^+ channels. In addition, activation of TRPA1 results in smooth muscle cell hyperpolarization that is sensitive to K_{Ca} channel blockade. These properties are hallmarks of "endothelium-derived hyperpolarizing factor" (EDHF)-induced dilation.[31] Despite considerable effort, the identity of the underlying molecular nature of EDHF remains elusive. Compelling evidence in support of a number of mechanisms, including cytochrome P450 epoxygenase products,[32] K^+ ions,[5] hydrogen peroxide,[33] and gap junctional communication,[34,35] has been reported. The present findings demonstrate that endothelial TRPA1 channels promote EDHF-type vasodilation in cerebral arteries. These observations are in agreement with those of earlier studies demonstrating that dilation of pulmonary[36] and mesenteric arteries[37] in response to the TRPA1 agonist allicin is independent of NOS and COX activity. However, in contrast to findings presented here demonstrating a critical role for the vascular endothelium, Bautista et al showed that allicin-induced relaxation of mesenteric arteries was attenuated by pretreatment with an calcitonin gene-related peptide (CGRP) antagonist.[37] Bautista et al conclude that activation of TRPA1 channels in perivascular sensory nerve endings causes vasodilation of mesenteric arteries by stimulating release of CGRP.[37] A potential role for the endothelium in mesenteric artery relaxation in response to TRPA1 stimulation was not investigated.[37] The present findings show that disruption of the endothelium or luminal administration of a TRPA1 blocking compound eliminates approximately 75% of AITC-induced cerebral artery dilation (Figure 2B and 2D), clearly demonstrating that endothelial cells mediate changes in vascular tone in response to TRPA1 activity in this vascular bed. Transient vasodilation in response to AITC following disruption of the endothelium (Figure 2A) or in the presence of apamin and TRAM34 (Figure 4A) may result from release of CGRP or other endothelium-independent mechanisms. Although it is possible that stimulation of TRPA1 channels present in perivascular nerve endings mediates a component of the response,[37] the endothelium appears to play a major role in steady-state TRPA1-dependent vasodilation in the cerebral vasculature.

The findings of this study provide new insight into the relationship between endothelial cell Ca^{2+} dynamics and endothelium-dependent vasodilation. The TRPA1 vasodilatory response is attenuated by blockade of Ca^{2+}-activated K^+ channels (Figure 4A through 4C) but not by inhibition of the NOS/COX pathways. Because all of these vasodilatory mechanisms are activated by increases in endothelial cell $[Ca^{2+}]$, the present findings suggest that spatially distinct Ca^{2+} signals regulate particular endothelium-dependent vasodilatory pathways. This conclusion is consistent with recent work by Ledoux et al, demonstrating that localized Ca^{2+} release events ("Ca^{2+} pulsars") exist in native endothelial cells and are dependent on Ca^{2+} release from inositol trisphosphate (IP_3) receptors located on the endoplasmic reticulum.[25] These dynamic Ca^{2+} events occur in structures that project from endothelial cells through the IEL to underlying vascular smooth muscle cells.[25] Ledoux et al also report that endoplasmic reticulum elements, IP_3 receptors, and $K_{Ca}3.1$ channels colocalize with myoendothelial membrane projections in mesenteric arteries.[25] The present study demonstrates that TRPA1 channels and $K_{Ca}3.1$ channels are associated with these endothelial cell membrane projections in cerebral vessels. These findings are consistent with the hypothesis that TRPA1 channels and $K_{Ca}3.1$ channels form a vasodilatory signaling complex in endothelial cell membrane projections proximal to arterial myocytes. In this proposed pathway, localized TRPA1 Ca^{2+} signals can either directly stimulate K_{Ca} channel activity or can be amplified by causing a Ca^{2+}-dependent increase in the open probability of IP_3 receptors located in myoendothelial membrane projections,[25] which serves to elevate the frequency of Ca^{2+} pulsars and activate K_{Ca} channels.[25] A combination of the SK_{Ca} channel blocker apamin and the $K_{Ca}3.1$ channel blocker TRAM34 appears to be more effective in blocking TRPA1-induced vasodilation compared with TRAM34 alone. $K_{Ca}2.3$, the most abundant SK_{Ca} channel in cerebral artery smooth muscle, is critical for endothelium-dependent vasodilation and regulation of endothelial cell membrane potential.[4] This channel is absent from myoendothelial membrane projections that span the IEL (Online Figure III).[24] It is not clear from the present studies whether SK_{Ca} channels are directly involved in TRPA1-dependent responses or whether the effects of apamin reflect a tonic hyperpolarizing effect of SK_{Ca} channel activity on the endothelial cell membrane.[4] K_{Ca} channel activity hyperpolarizes the endothelial cell plasma membrane and serves to hyperpolarize underlying smooth muscle by charge transfer mediated by myoendothelial gap junctions present at the tip of the endothelial cell membrane projection spanning the IEL (Figure 6).

AITC-induced dilation was blocked by Ba^{2+}, demonstrating that K_{IR} channel activity is required for vasodilation in response to activation of TRPA1 channels. K_{IR} channels are prominent in smooth muscle cells and may be present in the endothelium of some vascular beds. Although the present study does not rule out the possibility that endothelial K_{IR} channels contribute to TRPA1-mediated vasodilation, prior reports provide a strong conceptual framework supporting an important role for smooth muscle K_{IR} activity in vasodilatory responses. For example, release of K^+ from K_{Ca} channels into the interstitial space between the endothelium and underlying vascular smooth muscle cells could increase $[K^+]$ from a nominal 3 mmol/L to 8 to 12 mmol/L, sufficient to activate smooth muscle K_{IR} channels and account for TRPA1-dependent vasodilation.[30] Biophysical properties of K_{IR} channels suggest another mechanism that could explain involvement of this channel in TRPA1-mediated vasodilation. K_{IR} channels composed of $K_{IR}2.1$ and 2.2 subunits have an

unusual current–voltage relationship known as "negative slope conductance."[27,28] Over a limited voltage range, outward K^+ currents increase as membrane potential becomes more hyperpolarized. This effect is apparent for membrane potentials that are physiologically relevant for vascular smooth muscle cells. Message encoding $K_{IR}2.1$ and -2.2 is present in cerebral artery smooth muscle cells and K_{IR} channels appear to facilitate endothelium-dependent vasodilation in these vessels.[29] Furthermore, using both computational models and empirical studies, Smith et al demonstrate that the negative slope conductance of K_{IR} channels serves to amplify endothelium-derived hyperpolarizing stimuli.[29] K_{IR}-dependent amplification of gap junction-mediated change transfer could also enhance smooth muscle hyperpolarization and vasodilation resulting from TRPA1 activity. This proposed signaling pathway highlights the importance of spatial and temporal relationships of ion channels and Ca^{2+} signals in endothelial and smooth muscle cells.

Although the electrophilic agonist AITC is commonly used to stimulate TRPA1 activity, recent reports suggest that TRPA1 channels may also be activated by endogenously occurring substances. 4-HNE, 4-ONE, and 4-HHE are lipid peroxidation products that potently activate TRPA1 channels expressed by HEK cells and cultured sensory neurons.[13,14] The cyclopentanone prostaglandin 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) also stimulates TRPA1 activity.[13] All of these compounds are produced in response to oxidative stress, consistent with a possible role for endothelial TRPA1 channels in vascular responses to pathophysiological conditions that increase generation of reactive oxygen species, such as ischemia/reperfusion injury, hypoxia, and inflammation.[13,14,38] Inhibition of TRPA1 activity in isolated cerebral arteries results in a small, yet statistically significant, increase in myogenic tone, providing evidence that TRPA1 channels mediate tonic relaxation of cerebral arteries in the absence of exogenous agonists. Thus, in addition to eliciting vasodilation in response to environmental factors, TRPA1 channels may play an important role in vasomotor tone regulation under normal conditions and during pathophysiological situations that result in the generation of reactive oxygen species.

Pungent compounds found in garlic and mustard oil stimulate TRPA1, and activation of TRPA1 channels in the endothelium may contribute to improvements in vascular function attributed to these foods. Additionally, TRPA1 channels are activated by compounds produced in response to oxidative stress and this mechanism could mediate vascular responses to certain pathological conditions. The findings of this study suggest that endothelial cell TRPA1 channels present an interesting and novel target for the development of anti–cardiovascular disease drugs.

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Disclosures

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