TRPV4 Forms a Novel Ca\(^{2+}\) Signaling Complex With Ryanodine Receptors and BK\(_{\text{Ca}}\) Channels

Scott Earley, Thomas J. Heppner, Mark T. Nelson, Joseph E. Brayden

Abstract—Vasodilatory factors produced by the endothelium are critical for the maintenance of normal blood pressure and flow. We hypothesized that endothelial signals are transduced to underlying vascular smooth muscle by vanilloid transient receptor potential (TRPV) channels. TRPV4 message was detected in RNA from cerebral artery smooth muscle cells. In patch-clamp experiments using freshly isolated cerebral myocytes, outwardly rectifying whole-cell currents with properties consistent with those of expressed TRPV4 channels were evoked by the TRPV4 agonist 4\(\alpha\)-phorbol 12,13-didecanoate (4\(\alpha\)-PDD) (5 \(\mu\)mol/L) and the endothelium-derived arachidonic acid metabolite 11,12 epoxyeicosatrienoic acid (11,12 EET) (300 nmol/L). Using high-speed laser-scanning confocal microscopy, we found that 11,12 EET increased the frequency of unitary Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks) via ryanodine receptors located on the sarcoplasmic reticulum of cerebral artery smooth muscle cells. EET-induced Ca\(^{2+}\) sparks activated nearby sarcolemmal large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{\text{Ca}}\)) channels, measured as an increase in the frequency of transient K\(^+\) currents (referred to as “spontaneous transient outward currents” [STOCs]). 11,12 EET–induced increases in Ca\(^{2+}\) spark and STOC frequency were inhibited by lowering external Ca\(^{2+}\) from 2 mmol/L to 10 \(\mu\)mol/L but not by voltage-dependent Ca\(^{2+}\) channel inhibitors, suggesting that these responses require extracellular Ca\(^{2+}\) influx via channels other than voltage-dependent Ca\(^{2+}\) channels. Antisense-mediated suppression of TRPV4 expression in intact cerebral arteries prevented 11,12 EET–induced smooth muscle hyperpolarization and vasodilation. Thus, we conclude that TRPV4 forms a novel Ca\(^{2+}\) signaling complex with ryanodine receptors and BK\(_{\text{Ca}}\) channels that elicits smooth muscle hyperpolarization and arterial dilation via Ca\(^{2+}\)-induced Ca\(^{2+}\) release in response to an endothelium-derived factor. (Circ Res. 2005;97:1270-1279.)

Key Words: Ca\(^{2+}\) sparks ■ Ca\(^{2+}\) transients ■ eicosanoids ■ ion channels ■ ryanodine receptor ■ vascular smooth muscle ■ vasodilation

Diffusible factors produced by the vascular endothelium are vital for the regulation of smooth muscle membrane potential, arterial tone, blood pressure, and blood flow. Epoxyeicosatrienoic acids (EETs), the cytochrome P450 epoxygenase products of arachidonic acid,\(^{1}\) cause vasodilation and account for endothelium-derived hyperpolarizing factor (EDHF) activity in some vascular beds.\(^{2,\text{a}}\) Critical components of signaling pathways activated by these compounds are not fully understood.\(^{\text{a}}\) Although evidence supportive of a receptor-dependent smooth muscle hyperpolarization mechanism has been presented,\(^{4}\) the molecular identity of the “EET receptor” has not been reported. Vanilloid transient receptor potential (TRPV) channels are important sensors of biochemical and physiological stimuli.\(^{5}\) Therefore, we tested the hypothesis that TRPV channels are involved in transducing endothelial signals to underlying vascular smooth muscle. EETs promote Ca\(^{2+}\) influx in HEK cells overexpressing TRPV4\(^{\text{a}}\) and increase intracellular [Ca\(^{2+}\)] in cultured smooth muscle cells.\(^{7}\) In addition, activation of TRPV4 with the phorbol compound 4\(\alpha\)-phorbol 12,13-didecanoate (4\(\alpha\)-PDD) increases intracellular [Ca\(^{2+}\)] in airway smooth muscle.\(^{9}\) An elevation in intracellular [Ca\(^{2+}\)] is usually associated with vasoconstriction. Thus, these reports pose a paradox: How does EET-induced Ca\(^{2+}\) influx cause vasodilation? A number of studies suggest involvement of large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{\text{Ca}}\)) channels in EET-induced smooth muscle hyperpolarization and vasodilation.\(^{1,2}\) Under physiological conditions, BK\(_{\text{Ca}}\) channels in smooth muscle cells are activated by elementary Ca\(^{2+}\)-release events (Ca\(^{2+}\) sparks) via ryanodine receptors (RyRs) located on the sarcoplasmic reticulum (SR). Ca\(^{2+}\) sparks activate nearby BK\(_{\text{Ca}}\) channels to cause transient membrane hyperpolarization and vasodilation.\(^{10}\) Thus, EET-induced dilation could be explained by Ca\(^{2+}\) sparks activating BK\(_{\text{Ca}}\) channels. We further hypothesized that Ca\(^{2+}\) influx through TRPV4 preferentially stimulates RyRs in the SR, generating Ca\(^{2+}\) sparks that signal adjacent BK\(_{\text{Ca}}\) channels to open and cause membrane hyperpolarization and vasodilation.\(^{10}\) Consistent with this possibility, the open probability (NP\(_{o}\)) of SR RyRs is increased by both elevated cytoplasmic [Ca\(^{2+}\)]\(^{11}\) and enhanced SR Ca\(^{2+}\)
loading, which augments Ca\(^{2+}\) spark activity in arterial smooth muscle. Thus, Ca\(^{2+}\) influx via TRPV4 could stimulate SR RyRs, either directly or through increased SR Ca\(^{2+}\) loading, or both, to generate Ca\(^{2+}\) sparks and enhance BK\(_{Ca}\) channel activity, thereby causing smooth muscle hyperpolarization and relaxation. We propose that TRPV4 forms a novel Ca\(^{2+}\) signaling complex with RyRs and BK\(_{Ca}\) channels that elicits smooth muscle hyperpolarization and arterial dilation via Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR).

**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. Total RNA was extracted from whole brain, isolated cerebral arteries, and freshly dispersed cerebral artery smooth muscle cells, and RT-PCR was used to determine whether TRPV4 mRNA is present in these tissues. Forward and reverse primers specific for TRPV4 were TRPV4F (5′-GGGCAGCTCCCCAAGTAGAA-3′) and TRPV4R (5′-GTGCTTGGGCCCAAGAAG-3′). These primers yield a 578-bp PCR product.

Whole-cell patch-clamp experiments were used to determine whether ion channels with biophysical properties similar to TRPV4 were present in freshly isolated cerebral artery myocytes. Currents were recorded during voltage ramps between −120 and +80 mV before and after administration of 4α-PDD or 11,12 EET and were normalized to membrane capacitance (pA/pF). Isolated smooth muscle cells were loaded with the rapid Ca\(^{2+}\) indicator dye fluo-4 (Molecular Probes), and the effects of 11,12 EET on Ca\(^{2+}\) spark frequency were evaluated using high-speed (30 to 60 frames/s) laser-scanning confocal microscopy. Cerebral myocytes were also patch clamped in the perforated-patch configuration to examine the effects of 11,12 EET on the frequency of transient macroscopic BK\(_{Ca}\) currents (“spontaneous transient outward currents” [STOCs]).

Downregulation of TRPV4 expression in isolated cerebral arteries was accomplished with antisense oligonucleotides. The sequence of the TRPV4 antisense oligonucleotide used for these studies was 5′-CATCACCAGGTCTGCCGATCTGCTG-3′ (Operon Biotechnologies Inc). The complementary sequence was used as the sense (control) oligonucleotide. Sense and antisense oligonucleotides were introduced into intact cerebral arteries using a reversible permeabilization procedure. Following reversal of permeabilization, arteries were organ cultured for 2 to 3 days in DMEM/F-12 medium without serum to allow for TRPV4 downregulation. Semiquantitative RT-PCR was used to evaluate the effects of antisense treatment on TRPV4 mRNA levels. Smooth muscle cells isolated from cultured arteries were patch clamped in whole-cell or perforated-patch con-
TRPV4-Like Channels Are Present in Cerebral Artery Smooth Muscle

Message encoding TRPV4 was found in rat brain, cerebral arteries, and isolated cerebral artery smooth muscle cells (Figure 1a, inset). PCR products were not detected when template cDNA was omitted from the PCR (Figure 1a) or when reverse-transcription reactions were performed in the absence of reverse transcriptase (not shown). Because a number of transient receptor potential channels, including TRPC1,19 TRPC3,10–21 TRPC4,19 TRPC6,19,21 and TRPM4,22 are also present in cerebral artery myocytes, conventional whole-cell patch-clamp experiments were performed to determine whether ion channels with biophysical properties similar to those of cloned TRPV4 channels were present in these cells. The TRPV4 agonist 4α-PDD (5 μmol/L) activated an outwardly rectifying current in these cells (Figure 1a and 1b). 11,12 EET (300 nmol/L) also rapidly (≤1 minute) evoked a current with similar current–voltage characteristics (Figure 1c). Activation of whole-cell currents by 11,12 EET (300 nmol/L) was blocked by prior administration of the TRPV channel inhibitor ruthenium red (RR) (1 μmol/L) (Figure 1d and 1e). The rapid activation and inactivation of 11,12 EET–evoked currents in cerebral myocytes (Figure 1f) was similar to the reported time course of EET–induced Ca2+ influx and whole-cell currents in cultured cells overexpressing TRPV4.6 Activation of inward, but not outward, currents by 11,12 EET (300 nmol/L) was absent when N-methyl-D-glucamine (NMDG) was substituted for cations in the extracellular solution (Figure 1g and 1h). Thus, properties of this current in cerebral myocytes, such as current–voltage relationships, activation by 4α-PDD and 11,12 EET, and block by RR, are consistent with previously described characteristics of cloned TRPV4 channels6,8,23 and, combined with expression of TRPV4 mRNA in these cells, strongly suggests that functional TRPV4 channels are present in cerebral artery smooth muscle.

EETs Increase Ca2+ Spark and Transient BKCa Activity in Cerebral Myocytes

EETs, which activate TRPV4 channels,6 have also been shown to induce membrane potential hyperpolarization and relaxation of arteries, which is prevented by blocking BKCa channels.2 We, therefore, examined the effects of EETs on intracellular Ca2+ dynamics in cerebral artery smooth muscle cells loaded with the rapid Ca2+ indicator dye fluo-4. 11,12 EET (300 nmol/L) increased Ca2+ spark frequency nearly 2-fold in freshly isolated cerebral myocytes (Figure 2a and 2b). Previous reports demonstrate that Ca2+ sparks stimulate nearby sarcolemmal BKCa channels to cause transient K+ currents (STOCs).10 11,12 EET (100 nmol/L) increased the frequency of STOCs in voltage-clamped (0 mV) arterial smooth muscle cells (Figure 2c and 2d). 11,12 EET (300 nmol/L) also significantly (P<0.05) increased STOC frequency (0.31±0.01 versus 0.73±0.14 Hz; n=3) for cells voltage clamped at −40 mV, a membrane potential in the physiological range for smooth muscle cells in pressurized cerebral arteries. STOC activity was not altered by the vehicle for 11,12 EET (DMSO, 0.1%) (Figure 2d) or by the metabolic precursor of 11,12 EET, arachidonic acid (1 μmol/L; 101.4±27% of control; n=5). STOC activity in the presence of 11,12 EET was greatly diminished by ryanodine (5 μmol/L; 0.55±0.08 versus 0.12±0.07 Hz; n=3) and the
specific BK<sub>Ca</sub> channel blocker iberiotoxin (100 nmol/L; 0.33±0.11 versus 0.05±0.02 Hz; n=3). In addition, 11,12 EET (300 nmol/L) did not increase the open probability (NPo) of BK<sub>Ca</sub> channels in membrane patches obtained from cerebral artery myocytes (online Figure I). These findings strongly support the concept that EETs signal RyRs to increase Ca<sup>2+</sup> sparks, which in turn activate BK<sub>Ca</sub> channels.

**EET–Induced Increases in Ca<sup>2+</sup> Sparks and Transient BK<sub>Ca</sub> Activity Requires Ca<sup>2+</sup> Influx**

TRPV4 channels are Ca<sup>2+</sup> permeable, consistent with the possibility that Ca<sup>2+</sup> entry through this channel could trigger Ca<sup>2+</sup> sparks in arterial myocytes. In the presence of an L-type voltage-dependent Ca<sup>2+</sup> channel (VDCC) blocker (30 µmol/L diltiazem), 11,12 EET (300 µmol/L) significantly elevated Ca<sup>2+</sup> spark frequency when external [Ca<sup>2+</sup>]<sub>e</sub> was maintained at physiological levels (2 mmol/L) (Figure 3a). In contrast, when external [Ca<sup>2+</sup>]<sub>e</sub> was reduced to 10 µmol/L while VDCC inhibition was maintained, STOC activity was not altered by 11,12 EET administration (Figure 2b and 2c). These findings demonstrate that EET-induced increases in Ca<sup>2+</sup> sparks and STOC frequency are dependent on Ca<sup>2+</sup> entry via a non-VDCC channel.

**EET–Induced Increases in Transient BK<sub>Ca</sub> Activity Are TRPV4 Dependent**

TRPV4 channels are activated by EETs, consistent with the possibility that these channels could mediate EET-induced hyperpolarization in cerebral artery smooth muscle cells. The TRPV antagonist RR (1 µmol/L), applied externally, blocked 11,12 EET–induced increases in STOC frequency without altering basal STOC activity (Figure 4a and 4b). In addition, RR (1 µmol/L) reversed 11,12 EET–induced increases in STOC frequency to control levels (Figure 4c and 4d). The TRPV4 agonist 4a-PDD (5 µmol/L) also elevated STOC frequency in cerebral myocytes, and this response was inhibited by RR (1 µmol/L) (Figure 4c and 4d).

To examine further the relationship between EET-induced, TRPV4-mediated Ca<sup>2+</sup> influx and smooth muscle hyperpolarization, increased STOC frequency when external [Ca<sup>2+</sup>]<sub>e</sub> was 2 mmol/L and VDCCs were blocked (1 µmol/L/RR), whereas when bath [Ca<sup>2+</sup>]<sub>e</sub> was reduced to 10 µmol/L while VDCC inhibition was maintained, STOC activity was not altered by 11,12 EET administration (Figure 3b and 3c). These findings demonstrate that EET-induced increases in Ca<sup>2+</sup> sparks and STOC frequency are dependent on Ca<sup>2+</sup> entry via a non-VDCC channel.

**Figure 3.** EET-induced increases in Ca<sup>2+</sup> spark and STOC frequency require extracellular Ca<sup>2+</sup>. a, Effect of normal and reduced extracellular [Ca<sup>2+</sup>]<sub>e</sub> on 11,12 EET–induced (300 nmol/L) increases in Ca<sup>2+</sup> spark frequency in the presence of the VDCC blocker diltiazem (30 µmol/L). *P<0.05 vs all other groups. n=8 to 16. b, Perforated patch–clamp recording showing the effects of normal and reduced external [Ca<sup>2+</sup>]<sub>e</sub> on 11,12 EET–induced increases in STOC frequency in the presence of the VDCC blocker nisoldipine (1 µmol/L). c, Effect of normal and reduced extracellular [Ca<sup>2+</sup>]<sub>e</sub> on 11,12 EET–induced (300 nmol/L) increases in STOC frequency during VDCC inhibition. *P<0.05 vs all other groups, †P<0.05 vs 11,12 EET–treated cells in normal external [Ca<sup>2+</sup>]<sub>e</sub> solution. n=5 for all groups. Data are mean±SE.

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**Earley et al. TRPV4 Mediates Smooth Muscle Hyperpolarization**

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larization, TRPV4 expression was suppressed in intact vessels using antisense oligonucleotides. Cerebral arteries were permeabilized, were exposed to TRPV4 sense or antisense oligonucleotides and, following reversal of permeabilization, were organ cultured for 2 to 3 days to allow TRPV4 downregulation. Semiquantitative RT-PCR was used to examine the efficacy of these procedures, and TRPV4 mRNA levels were found to be diminished in antisense- versus sense-treated arteries (Figure 5a, inset).

11,12 EET (300 nmol/L) activated an outwardly rectifying current in smooth muscle cells from sense-treated vessels that had a similar current–voltage relationship, current density,

Figure 4. The TRPV antagonist RR blocks 11,12 EET– and 4α-PDD–induced increases in STOC frequency. a, Example patch-clamp recording demonstrating that RR (1 μmol/L) blocks 11,12 EET–induced increases in STOC frequency. b, Summary data (n=4 for each group). There were no significant differences. c, Example patch-clamp recording demonstrating that RR (1 μmol/L) reverses 11,12 EET–induced (300 nmol/L) increases in STOC frequency. d, Summary data (n=5 for each group). *P<0.05 vs all other groups. e, Example patch-clamp recording demonstrating that the TRPV4 agonist 4α-PDD (5 μmol/L) increases STOC frequency. This response was blocked by RR (1 μmol/L). f, Summary data (n=4 to 5 for each group). *P<0.05 vs all other groups. Data are mean±SE.
time course, and reversal potential as 11,12 EET– and 4α-PDD–induced currents in cerebral myocytes from freshly isolated arteries (Figure 5a and 5e). However, 11,12 EET–activated currents were significantly reduced in smooth muscle cells from antisense-treated vessels (Figure 5b through 5d).

To further elucidate the consequences of TRPV4 activation, the effects of 11,12 EET on Ca$^{2+}$ sparks were measured in isolated, pressurized cerebral arteries loaded with the Ca$^{2+}$ indicator dye fluo-4. 11,12 EET (300 nmol/L) increased Ca$^{2+}$ spark frequency in pressurized (60 mm Hg) sense-treated cerebral arteries (Figure 6a and 6b), whereas Ca$^{2+}$ spark frequency in antisense-treated vessels was not altered by 11,12 EET administration (Figure 6a and 6b). Consistent with these observations, 11,12 EET (300 nmol/L) elevated STOC frequency in cerebral myocytes isolated from sense-treated,
but not antisense-treated, arteries (Figure 6c and 6d). These findings demonstrate that TRPV4 is a critical mediator of 11,12 EET–induced increases in Ca\(^{2+}\)/H\(_{11001}\) spark–activated transient BK\(_{Ca}\) currents in cerebral artery smooth muscle.

**TRPV4 Mediates EET–Induced Smooth Muscle Hyperpolarization and Vasodilation**

An elevation of Ca\(^{2+}\) spark frequency can dilate cerebral arteries through activation of BK\(_{Ca}\) channels. Therefore, the relationships between TRPV4 downregulation, EET-induced changes in Ca\(^{2+}\) spark frequency, and alterations in smooth muscle membrane potential and vasomotor responses were examined. 11,12 EET (300 nmol/L) caused a smooth muscle membrane potential hyperpolarization of approximately 10 mV in TRPV4 sense-treated vessels (Figure 7a and 7b). In contrast, membrane potential of cerebral myocytes in antisense-treated vessels was not significantly altered by 11,12 EET (Figure 7a and 7b). TRPV4 sense- and antisense-treated vessels constricted to the same extent in response to a depolarizing concentration of KCl (60 mmol/L) or pressure (60 mm Hg), suggesting that membrane depolarization–induced Ca\(^{2+}\) influx through VDCCs was unaffected. Vasodilation in response to the K\(_{ATP}\) channel agonist pinacidil (10 μmol/L) also did not differ between groups (sense, 83.3±3.7% of passive diameter; antisense, 86.7±5.5%; n=3 for each group). In contrast, 11,12 EET–induced vasodilator responses were significantly inhibited by TRPV4 downregulation (Figure 7c and 7d), whereas TRPV4 sense-treated arteries dilated to an extent similar to that of freshly isolated arteries (Figure 7c and 7d). In the presence of ryanodine (10 μmol/L), freshly isolated cerebral arteries exhibited only slight, statistically insignificant dilation (6.9±2.5 μm; n=3) in response to 11,12 EET (300 nmol/L), providing further evidence for a Ca\(^{2+}\) spark–dependent mechanism in this response. These findings demonstrate a role for TRPV4 and RyRs in EET-induced smooth muscle hyperpolarization and vasodilation.

**Discussion**

The major findings of this study are as follows: (1) functional TRPV4 ion channels are present in cerebral artery smooth muscle; (2) activation of TRPV4 in cerebral myocytes with 11,12 EET elevates Ca\(^{2+}\) spark and transient BK\(_{Ca}\) channel...
activity; (3) increases in Ca\(^{2+}\) spark and transient BK\(_{Ca}\) channel activity elicited by 11,12 EET are unaffected by inhibition of VDCCs but are absent when extracellular [Ca\(^{2+}\)] is lowered; and (4) antisense-mediated TRPV4 downregulation in isolated cerebral arteries blocks EET-induced increases in Ca\(^{2+}\) spark and transient BK\(_{Ca}\) channel activity as well as membrane potential hyperpolarization and vasodilation. Thus, our findings show that in cerebral artery myocytes, Ca\(^{2+}\) influx through TRPV4, stimulated by 11,12 EET,\(^6\) increases Ca\(^{2+}\) spark frequency and BK\(_{Ca}\) channel activity, resulting in cerebral myocyte hyperpolarization. Consistent with an important functional role for this pathway, suppression of TRPV4 expression in intact cerebral arteries prevents 11,12 EET–induced smooth muscle hyperpolarization and vasodilation.

Cytochrome P450 epoxygenase products are recognized as potent vasodilatory factors produced by the vascular endothelium.\(^3\),\(^24\) Elucidation of the intracellular signaling pathways responsible for smooth muscle cell hyperpolarization by these compounds has been the subject of considerable effort. An important role for K\(^+\) channel activation in hyperpolarizing and vasodilatory response has been demonstrated in a number of previous reports. Although evidence suggesting activation of K\(_{ATP}\) channels by EETs has been presented,\(^25\) most studies support a role for BK\(_{Ca}\) channels in EET-induced hyperpolarization. The simplest explanation for the role of this channel in vascular responses is direct activation of BK\(_{Ca}\) by EETs.\(^6\) Although data supporting this mechanism have been reported,\(^26\) our findings (online Figure I) are in agreement with a number of previous reports\(^4,\),\(^27,\)^28 and demonstrate no direct effect of 11,12 EET on BK\(_{Ca}\) NPo in isolated membrane patches. These data suggest that, rather than acting directly on the channel, EETs activate intracellular signaling pathways that ultimately cause smooth muscle hyperpolarization via BK\(_{Ca}\) channels. Recently reported findings that TRPV4-dependent currents are activated by EETs\(^6\) prompted...
properties of a number of tissues, including sensory neurons, pancreatic β cells, and cardiac myocytes, as well as many types of smooth muscle. Thus, in addition to providing novel information regarding regulation of vascular tone, our observations suggest the possibility that TRPV4 (and perhaps other TRPV channels) can alter the function of many types of excitable cells by modulating complex Ca²⁺ events. Furthermore, as TRPV channels are activated by a number of stimuli, such as changes in osmolarity, temperature (in the physiological range), and fatty acids, this mechanism may constitute a fundamental way in which environmental factors influence excitable cells through a local Ca²⁺ signaling mechanism involving CICR.

Acknowledgments

This work was supported by NIH grant F32HL075995 and American Heart Association Grant 0535226N (to S.E.); NIH grants RO1HL44455, RO1HL63722, RO1DK53832, and RO1DK065947 (to M.T.N.); NIH grant RO1HL58231 (to J.E.B.); and the Totman Medical Trust. The Noran confocal microscope used for these studies is housed in the Neuroscience Centers of Biomedical Research Excellence (COBRE) Imaging/Physiology core facility (NIH grant P20 RR16435 from the COBRE Program of the National Center for Research Resources) and supported by a National Science Foundation grant. We thank Katherine Lutz and Emily R. Levy for technical assistance; Adrian Bonev, PhD, for insightful comments and design of custom software for the analysis of dynamic Ca²⁺ events; and Stephen V. Straub, PhD, for comments on the manuscript.

References

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Circ Res. 2005;97:1270-1279; originally published online November 3, 2005;
doi: 10.1161/01.RES.0000194321.60300.d6
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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 (300-400 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 [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 MOPS-buffered saline 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 0.3 mg/ml papain (Worthington) and 0.3 mg/ml dithioerythritol for 15 min, followed by 15 min incubation in 0.67 mg/ml type F collagenase (Sigma), 0.33 mg/ml type H collagenase (Sigma), 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.
**RNA Preparation and RT-PCR**

RNA was prepared from whole rat brain, cerebral artery homogenates, and isolated smooth muscle cells. Smooth muscle cells (~1000) were visually identified under phase contrast microscopy (600X) and harvested using a micropipette directed by a micromanipulator. Following total RNA extraction (RNeasy mini kit; Qiagen Inc.), first strand cDNA was synthesized using the superscript reverse transcriptase (RT) kit (Qiagen Inc). 5 µl of each first strand cDNA reaction was subsequently placed in a PCR reaction solution (50 µl) containing forward and reverse primers, dNTPs, 2 x reaction buffer “J” (Epicenter) and 2.5 U Fail-safe DNA polymerase (Epicenter). PCR was performed for ~40 cycles of 94°C for 60 s, 58°C for 90 s, and 72°C for 60 s. Primer sequences were 5’-GGGCAGCTCCCCAAAGTAGAA-3’ (forward) and 5’-GTGCCTGGGCCCAAGAAA-3’ (reverse). Controls consisted of PCR reactions that did not contain template cDNA (No template, NT) and reactions that contained template from RT reactions that were performed in the absence of RT (no RT). Furthermore, PCR primers were designed to span intron exon boundaries in the TRPV4 gene to minimize the possibility of amplification of contaminating genomic DNA. Reaction products were resolved on 1.0% agarose gels.

**Ca²⁺ Imaging**

Ca²⁺ sparks were recorded according to previously described methods¹. Briefly, dispersed cells and pressurized cerebral arteries were loaded with the fast Ca²⁺ sensitive dye fluo-4 and were imaged with a laser scanning confocal
system (OZ; Noran Instruments) mounted in an Diaphot inverted microscope with a 60× water immersion objective. Dispersed arterial myocytes were studied in a bath solution consisting of (in mM): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES pH 7.4 at room temperature. Isolated vessels were superfused and pressurized (60 mmHg) with the same solution warmed to 37°C. Images were acquired at a rate of 30/sec for dispersed cell experiments and 60/sec for pressurized vessel experiments. Ca²⁺ spark recordings were analyzed with the aid of a custom software application.

**Patch-Clamp Experiments**

Patch clamp experiments were performed using an Axopatch 200B amplifier equipped with a CV203BU headstage (Axon Instruments). Recording electrodes (resistance, ~6 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 9.0, and Clampfit version 9.0 (Axon Instruments) were used for data acquisition and analysis. For whole-cell recordings, the bathing solution contained (in mM): 142 NaCl, 10 Glucose, 10 HEPES, 2 CaCl₂, 6 KCl, 1 MgCl₂, pH 7.4. The pipette solution was: 100 Na-glutamate, 20 NaCl, 1 MgCl₂, 1 EGTA, 10 HEPES, 4 Na₂ATP, pH 7.2. Currents were recorded during voltage ramps from -120 to +80 mV. Ramps were repeated every 15 sec for at least 1 min prior to drug administration, and, after administration, for every 15 sec until maximal current activation. Cells were held at 0 mV between voltage ramps. To examine the effects of ruthenium red on
11,12 EET-induced currents, whole cell currents were first recorded under basal conditions and RR (1 µM) was administered. Currents were first recorded in the presence of RR, and then 11,12 EET (300 nM) was administered in the presence of RR. Ramp currents were recorded every 15 seconds for at least 3 min. Additional experiments were performed in which NMDG was substituted for all monovalent cations in the bathing solution. For all experiments, currents were normalized to membrane capacitance and liquid junction potential offset in pipette potential was subtracted during analysis.

For perforated patch recordings of transient BKCa currents (STOCs), the bathing solution contained (in mM) 134 NaCl, 6 KCl, 1 MgCl2, 2 CaCl2, 10 glucose and 10 HEPES pH 7.4 and the pipette solution contained: 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl2, 5 EGTA, 10 HEPES pH 7.2 and 200-250 µg/ml amphotericin B. STOCs were recorded at a holding potential of 0 mV. STOCs were defined as transient current events that were greater than three times the expected unitary current for BKCa channels at the membrane potential for which the recordings were obtained. For a membrane potential of 0 mV, the threshold current was set at 24 pA. All events greater than the threshold current were counted and STOC frequency was calculated by dividing the number of events by the duration of the recording. All recordings were at least 1 minute in duration. All patch clamp experiments were performed at room temperature (~22ºC).
TRPV4 Antisense Procedures

The sequence of the antisense oligonucleotide used for these studies (previously shown to suppress TRPV4 expression\(^2\)) was 5’-CATCACCAGGATCTGCCATACTG-3’. The last three bases on the 5’ and 3’ ends of the oligonucleotide were phosphorothioated to limit degradation by cellular nucleases. Oligonucleotides were dissolved at a concentration of 2 mM in nuclease-free water and were introduced into intact cerebral arteries using a reversible permeabilization procedure as previously described\(^3,4\). Arteries were first incubated for 20 minutes at 4°C in (in mM): KCl, 120; MgCl\(_2\), 2; EGTA, 10; Na\(_2\)ATP, 5; TES, 20; pH 6.8. These vessels were then placed in a similar solution containing oligonucleotides (200 µM) for 90 minutes at 4°C and then in a similar oligonucleotides –containing solution with elevated MgCl\(_2\) (10 mM). Permeabilization was reversed by placing the arteries in MOPS buffered solution containing (in mM): NaCl, 140, KCl, 5, MgCl\(_2\), 10, glucose, 5, MOPS, 2; pH 7.1, 22°C. The [Ca\(^{2+}\)] of this solution was then gradually (~45 min) increased from nominally Ca\(^{2+}\)-free to 0.01, 0.1, and 1.8 mM. Arteries were organ cultured in D-MEM/F-12 media supplemented with L-glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 ug/ml) in a humidified incubator. CO\(_2\) was maintained at 5%.

Isolated Vessel Experiments

Isolated vessel procedures were performed essentially as previously described\(^3\). Briefly, arterial segments were cleaned and transferred to a vessel chamber. The proximal end of the vessel was cannulated with a glass
micropipette and secured, the lumen was gently rinsed, and the distal end of the vessel cannulated and secured. The endothelium was removed by passage of an air bubble through the lumen. Vessels were pressurized to 60 mmHg with physiological saline solution (PSS) and superfused with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O₂, 5% CO₂, balance N₂) and allowed to develop spontaneous myogenic tone. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix) as increasing concentrations of 11,12 EET (Cayman Chemical) were added to the superfusion solution.

**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 abluminal wall with glass intracellular microelectrodes (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 Axotape 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 approximately 0 mV after the electrode was retracted from the cell.
Calculations and Statistics

All data are mean ± SE. Values of n refer to number of cells for experiments involving isolated cerebral myocytes or number of animals for experiments employing isolated arteries. Comparisons involving two experimental groups were made by unpaired Student’s t-tests. Comparisons involving multiple groups were made by one-way or two-way ANOVA or repeated measures two-way ANOVA, as appropriate, followed by Student-Newman-Keuls post hoc test. A level of $P \leq 0.05$ was accepted as statistically significant for all experiments.

REFERENCES


Figure S1: a: Single BK$_{Ca}$ channel recordings of membrane patches from cerebral artery smooth muscle cells before and after 11,12 EET (300 nM) was administered. C = closed state of the channel. b: Channel open probability (NP$_O$) as a function of membrane potential ($V_M$) for untreated cells and cells treated with 11,12 EET (300 nM). n = 5-7 at each membrane potential. There were no significant differences.