Evidence for two-pore domain potassium channels in rat cerebral arteries

Robert M. Bryan, Jr.,1,2,3 Junping You,1 Sharon C. Phillips,1 Jon J. Andresen,1 Eric E. Lloyd,1 Paul A. Rogers,4 Stuart E. Dryer,5 and Sean P. Marrelli1,3

Departments of 1Anesthesiology, 2Medicine (Cardiovascular Sciences), and 3Molecular Physiology and Biophysics, Baylor College of Medicine, 4Department of Biochemistry and Biological Sciences, University of Houston, Houston, Texas; and 5Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, Louisiana

Submitted 28 December 2005; accepted in final form 7 March 2006

Bryan, Robert M., Jr., Junping You, Sharon C. Phillips, Jon J. Andresen, Eric E. Lloyd, Paul A. Rogers, Stuart E. Dryer, and Sean P. Marrelli. Evidence for two-pore domain potassium channels in rat cerebral arteries. Am J Physiol Heart Circ Physiol 291: H770–H780, 2006. First published March 24, 2006; doi:10.1152/ajpheart.01377.2005.—Little is known about the presence and function of two-pore domain K+ (K2P) channels in vascular smooth muscle cells (VSMCs). Five members of the K2P channel family are known to be directly activated by arachidonic acid (AA). The purpose of this study was to determine 1) whether AA-sensitive K2P channels are expressed in cerebral VSMCs and 2) whether AA dilates the rat middle cerebral artery (MCA) by increasing K+ currents in VSMCs via an atypical K+ channel. RT-PCR revealed message for the following AA-sensitive K2P channels in rat MCA: tandem of P domains in weak inward rectifier K+ (TWIK-2), TWIK-related K+ (TREK-1 and TREK-2), TWIK-related AA-stimulated K+ (TRAAK), and TWIK-related halothane-inhibited K+ (THIK-1) channels. However, in isolated VSMCs, only message for TWIK-2 was found. Western blotting showed that TWIK-2 is present in MCA, and immunohistochemistry further demonstrated its presence in VSMCs. AA (10–100 μM) dilated MCAs through an endothelium-independent mechanism. AA-induced dilation was not affected by inhibition of cyclooxygenase, epoxygenase, or lipoxygenase or inhibition of the AA metabolic pathways or blockade of classical K+ channels. AA-induced currents were not affected by inhibitors of the AA metabolic pathways or blockade of classical K+ channels. RT-PCR revealed message for the following AA-sensitive K2P channels in rat cerebral arteries: Kir, KATP, KCa, and Kv channels are collectively termed “classical” K+ channels; those that are not classical are referred to as “atypical” K+ channels.

Recently, a newly discovered family of K+ channels with four transmembrane-spanning domains and two pore regions for each protein subunit has been identified. This new family, two-pore domain K+ (K2P) channels, has ~12 known functional members (5, 14, 20, 25, 38). There is evidence that the K2P channels are located in vascular tissues, and one of these channels, TASK-1, may be involved with changes in vascular tone as a function of pH (15, 17). Although other K2P channels may be expressed in vascular tissues (15, 22, 34, 41), no functional data have been published about any of these.

Five members of the K2P channel family are directly activated by unmetabolized arachidonic acid (AA). The AA-sensitive K2P channels are TWIK-2 (tandem of P domains in weak inward rectifier K+), TREK-1 and TREK-2 (TWIK-related K+), TRAAK (TWIK-related AA-stimulated K+), and THIK-1 (TWIK-related halothane-inhibited K+) channels. Because there are no selective inhibitors for the AA-sensitive K2P channels, functional identification of these channels in smooth muscle cells will be challenging. As a step toward determining whether functional AA-sensitive K2P channels exist on vascular smooth muscle, we have asked the following questions: 1) Are AA-sensitive K2P channels expressed in smooth muscle of rat middle cerebral artery (MCA)? 2) Does unmetabolized AA, not a metabolite of AA, dilate MCAs and increase K+ currents in vascular smooth muscle cells (VSMCs)? 3) Do the dilations and increased K+ currents elicited by AA involve an atypical K+ channel?

METHODS

Isolated MCA preparation. The Animal Protocol Review Committee at Baylor College of Medicine approved the experimental protocol. Male Long-Evans rats (~300 g body wt) were anesthetized with 3% isoflurane and decapitated. (The Long-Evans strain was developed in 1915 by cross-breeding Wistar females with a wild gray male

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The pipette buffer consisted of (in mM) 110 gluconate (K
forge (model MF-830, Narishige). Pipette resistances were 5–6 M
pipette puller (model PP-830, Narishige) and polished with a micro-
tubing (catalog no. 64-0819, Warner Instruments) in two stages by a
divided by pulse voltage. Patch electrodes were pulled from glass
calculate membrane capacitance (pF): charge produced during pulses
currents are reported in picoamperes. pCLAMP software was used to
calculate currents after they were warmed to 37°C and pressurized. Each MCA was
magnified ×450, displayed on a video monitor, and archived on
videotape. Changes in the diameters were measured using image
analysis software (version 5.1, Optimas, Bothell, WA).

MCA diameters were measured after addition of AA, palmitic acid,
or 5,8,11,14-eicosatetraynoic acid (ETYA) to the luminal perfusate. In
preliminary studies, we demonstrated identical AA-induced dilations
with AA was added to the luminal perfusate and with AA added to the
extraluminal bath. In all studies involving pressurized MCAs, nitric
oxide synthase and cyclooxygenase (COX) were inhibited with 10
μM N-nitro-l-arginine methyl ester (l-NAME) and 10 μM indometh-
acin, respectively.

Isolation of VSMCs from rat MCA. Single VSMCs were enzymat-
ically isolated using a modification of a previously described protocol
(18). Rat MCAs from both sides of the brain were harvested as
described above, cleaned of connective tissue, and placed in digestion
buffer (135 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 0.42 mM Na2HPO4, 0.44 mM NaH2PO4, 4.2 mM NaHCO3, 10 mM HEPES,
and 1 mg BSA/ml, with pH adjusted to 7.25 with NaOH). The MCA was
cut into <1-mm-long pieces, which were digested with 18 U/ml
papain and 1 mg/ml dithioerythritol in digestion buffer for 35 min at
37°C. The tissue was washed with digestion buffer and further
digested with 1.2 mg/ml collagenase II, 0.8 mg/ml soybean trypsin
inhibitor, and 60 U/ml elastase in digestion buffer for 10 min at 37°C.
The tissue was washed several times with digestion buffer and
triturated with a pipette that had been coated with BSA by wash with
digestion buffer. The cells were placed on ice and used within 8 h.

Electrophysiological measurements. The VSMCs were placed in a
chamber on the stage of an inverted microscope (Olympus IX 71) and
continually superfused with buffer. Fatty acids and reagents were
applied from separate gravity-fed reservoirs. VSMCs were exposed to
fatty acids no more than once during the course of an experiment.

An integrating patch-clamp amplifier (Axopatch 200B) and
pCLAMP 9.2 software (Axon Instruments, Union City, CA) were
used to measure whole cell currents in individual VSMCs. Data were
filtered at 1 kHz with a four-pole Bessel filter, digitized at 5 kHz, and
stored on a hard disk. There was no compensation for cellular
capacitance, series resistance, or leak current. The liquid junction
potential was calculated using pClamp and corrected. Currents are
expressed as current density (pA/pF) to normalize for differences
between cell membrane areas, except in Figs. 9 and 10, where
currents are reported in picocanampere.

Since currents were reported in picocanampere, pCLAMP software was used to
calculate membrane capacitance (pF): charge produced during pulses
divided by pulse voltage. Patch electrodes were pulled from glass
tubing (catalog no. 64-0819, Warner Instruments) in two stages by a
pipette puller (model PP-830, Narishghe). Pipette resistances were 5–6 MΩ.
The pipette buffer consisted of (in mM) 110 gluconate (K+ salt), 30
KCl, 1 MgCl2, 2.2 CaCl2, 3 EGTA, 3 Na2ATP, and 10 HEPES; pH
was adjusted to 7.2 with KOH. The calculated free Ca2+
concentration was 420 nM. The bath buffer contained (mM) 140 NaCl, 4.2 KCl,
3 NaHCO3, 1.2 KH2PO4, 2 MgCl2, 0.1 CaCl2, 10 glucose, and 10
HEPES; pH was adjusted to 7.4 with NaOH. In one study, K+
concentration was increased to 30, 100, and 140 mM. In each case,
Na+ concentration was reduced in an equimolar amount.

After the patch was ruptured using negative pressure, whole cell
currents were measured using a ramp or a pulse protocol. In all cases,
VSMCs were held at ~62 mV before initiation of the protocol. For the
ramp protocol, the potential was shifted to ~72 mV for 60 ms and
~132 mV for 20 ms and then ramped to +88 mV over 1 s. For the
pulse protocol, the potential was shifted to ~82 mV for 0.5 s followed
by progressive 8-mV voltage steps (500 ms, 5-s intervals) from ~82
to 46 mV. In one study, the current-clamp mode was used to measure
differences in membrane potential with addition of AA to VSMCs.

RT-PCR. All materials for RNA isolation were certified RNase
free, and the instruments were wiped with RNaseZap (Ambion). MCAs were quickly harvested from the brain, stripped of connective
tissue, and minced. Extraction of total RNA with the RNeasy Micro
Kit (Qiagen) was performed according to the manufacturer’s instruc-
tions. For brain RNA, a section containing striatum and cortex was removed. Total RNA was extracted using TRIzol reagent
(Invitrogen) according to the manufacturer’s instructions. RNA
samples were treated with DNase I to reduce the potential of genomic
dNA contamination. Total RNA was evaluated by UV spectropho-
томetry for concentration and purity. RNA with a 260/280 ratio of
absorbance at 260 nm to absorbance at 280 nm was deemed accept-
able for further use.

Random hexamers were used for first-strand synthesis with the
Superscript II RT kit (Invitrogen). RT-PCR was performed with an
Eppendorf thermocycler. PCR was performed for 35–40 cycles using
the following temperature protocol: 94°C (30 s), 53–58°C (1 min),
and 72°C (60 s). The optimum annealing temperature was determined for
each set of primers. Gene-specific primer pairs were as follows:
CCATAGGGATTGAAAAACTCCTCCAC (forward) and CAAT-
CAGGCTCAGAAGACGTGCAAG (reverse) for TREK-1, GCTG-
GCATAAACATTCAGGAAATG (forward) and GTTGTCCTGA-
GAAGGCCCTTG (reverse) for TREK-2, TTTTCTTCTGGGACATCATCAC
(forward) and AGGCTAGCAGGCAAAAAGAGTCCAGAAC (reverse)
for TRAAK, and CTTGAAAACTGGTGTTATG (forward) and
GCTCCACAGGAGATGGCT (reverse) for THIK-1. Primer pairs were used for the detection of endothelial nitric oxide synthase (eNOS)
and SM22α, markers for endothelium and vascular smooth muscle,
respectively, were used to determine the composition of the
VSMC sample. Primer pairs were ATGGATGAGCAACACTGAG (for-
ward) and CCACTCGTCTTGGGACAT (reverse) for eNOS and
GGCAGCTGAGATGGTCAAG (forward) and CTTGCGCTCC-
CTTCTAC (reverse) for SM22α (33). Products were size frac-
tioned by electrophoresis in agarose gels (1.5%), and ethidium
bromide was used for visualization. The product was initially identi-
fied by the presence of a single band of the predicted size. For final
confirmation, the PCR product was sequenced (Baylor College of
Medicine Core Sequencing Facilities) and compared with known
sequences (BLAST).

In addition to intact MCAs, 100–150 VSMCs were digested and
dispersed as described above and collected in a pipette with gentle
suction. RT-PCR was conducted using a modification of a previously
published protocol (23).

Western blot analysis. MCA, basilar artery, and brain were minced
on ice and homogenized in buffer containing 1% SDS (Bio-Rad), 10
mM EDTA, and protease inhibitor cocktail (Complete Mini, Roche).
Samples were boiled for 15 min and centrifuged at 15,000 × g for 15
min, and the supernatant was collected for protein analysis using the
DC protein assay (Bio-Rad). Protein samples were size fractionated
by electrophoresis at 150 V for 45 min at room temperature with a
4–20% SDS polyacrylamide gel (Ready Gel, Gel, Bio-Rad). The amount
of protein loaded per lane ranged from 33 to 75 μg. The immunoblot
in Fig. 2 shows 33 μg of MCA protein for TWIK-2 and TRAAK, 33
μg of basilar artery protein for TRAAK, 75 μg of MCA protein for
TREK-1, and 51 μg of brain protein for TREK-1. Proteins were transferred to supported nitrocellulose membranes (Bio-Rad) at 250

AJP-Heart Circ Physiol • VOL 291 • AUGUST 2006 • www.ajpheart.org

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mA for 2.5 h on ice and visualized using Ponceau S solution (Sigma). Immediately before exposure to primary and secondary antibodies, the blots were blocked with 5% nonfat dry milk and 1% BSA (Sigma) in PBS (Invitrogen) for 1 h at room temperature. Primary antibodies directed against TREK-1, TREK-2, and TRAAK (Santa Cruz Biotechnology) were used at dilutions of 1:50 and 1:100. Primary antibodies directed against TREK-1 and TREK-2 (Alomone) were used at dilutions of 1:50 and 1:100. The primary antibody directed against TWIK-2 (Alomone) was used at a dilution of 1:100. After three washes in PBS, the blots were blocked and exposed to horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Anti-rabbit secondary antibodies (1:10,000 dilution; Pierce) were used against Alomone primary antibodies, and anti-goat secondary antibodies (1:90,000 dilution; Sigma) were used against Santa Cruz Biotechnology primary antibodies. Blots were incubated in chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity, Pierce) for 1 min at room temperature before exposure to film (Hyperfilm ECL, Amersham Biosciences).

Immunohistochemistry. Rats were anesthetized with 3% isoflurane and decapitated. The brain was removed from the skull, and wedges of brain containing MCAs were dissected and snap frozen in 2-methylbutane that had been previously chilled to −50°C in dry ice. Each tissue wedge was mounted in a cryostat (−20°C) and cut into 10-μm sections. The tissue was oriented to produce cross sections of the MCA when cut. The sections were picked up on slides (Superfrost Plus, Fisher), dried, and stored at −80°C.

The sections were washed in PBS and fixed with 4% formaldehyde for 40 min at 4°C. After a second wash, the sections were blocked and permeabilized for 30 min at room temperature with PBS containing 0.5% BSA, 0.1% Tween 20, and 10% serum from the species (donkey or goat) used to generate the secondary antibody. Primary antibodies were suspended in the block/permeabilization solution described above and added to the sections. Tissue sections were incubated overnight with TREK-1 antibodies (Santa Cruz Biotechnology) in a humidifying chamber at 4°C. TREK-1, TREK-2, and TWIK-2 (Alomone) were incubated at a concentration of 6 and 12 μg/ml for 2 h at room temperature.

The sections were washed and exposed to the secondary antibody for 20 min. For antibodies from Santa Cruz Biotechnology, the secondary antibody was rhodamine-donkey anti-goat IgG (15 μg/ml; Jackson Laboratories); for antibodies from Alomone, the secondary antibody was Alexa Fluor 594-goat anti-rabbit IgG (4 μg/ml; Molecular Probes). After a final wash, the sections were treated with Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories) and protected with a coverslip. Controls for each antibody consisted of replacement of the primary antibody with nonimmune IgG from the host species of the primary antibody.

Immunohistochemistry was also conducted on dispersed VSMCs. After digestion of rat MCAs, aliquots of VSMCs were suspended in a droplet of PBS on a microscope slide. After the VSMCs were allowed to settle and adhere for 30 min, they were fixed in 4% formaldehyde for 15 min. The subsequent steps for detection of TWIK-2 were similar to those described above for tissue sections. A mouse monoclonal antibody directed against smooth muscle α-actin was used as a marker for smooth muscle. The antibody, which was conjugated to FITC, was administered simultaneously with the secondary antibodies directed against TWIK-2.

Drugs and reagents. Antibodies directed against TREK-1, TREK-2, and TWIK-2 were purchased from Alomone; antibodies directed against TREK-1 (N-20), TREK-2 (S-14), and TRAAK (C-13 and A-20) from Santa Cruz Biotechnology: FITC-conjugated antibody directed against smooth muscle α-actin, AA (as the Na^+ salt), ETYA, AA, t-NAME, indomethacin, miconazole [(1-[2,4-dichlorobenzyl]-(oxy)phenethyl]imidazole), 6-(2-propargyl-oxophenyl)hexanoic acid (PPOH), palmitic acid (as the Na salt), BSA, papain, dithioerythritol, and the K^+ channel inhibitors tetraethylammonium (TEA), glibenclamide, BaCl2, 4-aminopyridine (4-AP), charybotoxin, and apamin from Sigma; baicalein (5,6,7-trihydroxyflavone) and nordihydroguaiaretic acid (NDGA) from Biomol; collagenase II and soybean trypsin inhibitor from Worthington; and elastase from Calbiochem.

Indomethacin was dissolved in a 15 mM Na2CO3 solution. AA, PPOH, NDGA, and miconazole were dissolved in ethanol. Baicalein was dissolved in DMSO. Other reagents were dissolved in distilled water. The composition of the PSS was as previously described (4).

Data analysis. Values are means ± SE. Dilations of the MCAs are expressed as percentage of the maximum diameter according to the following equation

\[ \% \text{ maximum diameter} = \left( \frac{D_{\text{max}} - D_{\text{base}}}{D_{\text{max}} - D_{\text{base}}} \right) \times 100 \]

where \( D_{\text{base}} \) is diameter after luminal administration of a fatty acid (AA, palmitic acid, or ETYA), \( D_{\text{base}} \) is baseline diameter before

Fig. 1. A: RT-PCR of arachidonic acid (AA)-sensitive 2-pore domain K^+ (K2P) channels in homogenates of rat brain and middle cerebral artery (MCA). B: RT-PCR of AA-sensitive K2P channels in vascular smooth muscle cells (VSMCs) collected after digestion and dispersion. Message for SM22-α and endothelial nitric oxide synthase (eNOS) was used for markers of smooth muscle and endothelial cells, respectively. Intact MCAs showed message for SM22-α and eNOS, and VSMCs showed message for SM22-α.
addition of fatty acid, and $D_{\text{max}}$ is maximum diameter at 85 mmHg, which was obtained in the presence of Ca$^{2+}$-free PSS containing EGTA.

For statistical analysis, one-way ANOVA or two-way repeated-measures ANOVA was followed by Tukey’s test for individual comparisons when appropriate. $P < 0.05$ defined the acceptable level of significance.

**RESULTS**

RT-PCR results for the five members of the $K_{2p}$ channel family, which are activated by AA, are shown in Fig. 1A. mRNA for all five channels, TREK-1, TREK-2, TWIK-2, TRAAK, and THIK-1, were present in brain and MCA. Bands were consistent with the predicted sizes for TREK-1 (452 bp), TREK-2 (465 bp), TWIK-2 (454 bp), TRAAK (454 bp), and
THIK-1 (327 bp). PCR products were sequenced at the Baylor College of Medicine Core Sequencing Facilities and confirmed for each of the K<sub>2p</sub> channels by comparison with known sequences (BLAST). Because MCAs consist of endothelium, vascular smooth muscle, and perivascular nerves, the amplified product of the five K<sub>2p</sub> channels could be present in any or all of these cell types. Therefore, further RT-PCR studies were conducted on VSMCs that were collected after digestion and dispersion. VSMCs showed only mRNA for TWIK-2 (Fig. 1B); mRNA for TREK-1, TREK-2, TRAAK, and THIK-1 were not found. Message for SM22-α (325 bp) and eNOS (356 bp) was used for markers of smooth muscle and endothelial cells, respectively. Intact MCAs showed message for SM22-α and eNOS (Fig. 1B, left), but dispersed VSMCs were positive only for SM22-α.

Immunoblots for TWIK-2, TREK-1, and TRAAK are shown in Fig. 2. In MCA, a single distinctive band for TWIK-2 was present near the predicted size of 37 kDa (41). The immunoblot for TREK-1 (N-20 antibody, Santa Cruz Biotechnology) showed a band at ~45 kDa in brain, but not MCA, homogenates. This band in brain is consistent with the predicted size of 45 kDa for TREK-1 (31). A second TREK-1 antibody (Alomone) also lacked a band near 45 kDa in MCAs (data not shown). Bands for TRAAK were present near the predicted size of 43 kDa (21) in MCA and basilar artery. An immunoblot for TREK-2 is not shown, because we were unable to obtain a suitable band for spleen, a tissue that is rich in TREK-2 (2), although we tried the TREK-2 antibody from Alomone and the S-14 TREK-2 antibody from Santa Cruz Biotechnology. An immunoblot for THIK-1 is not provided, because an antibody is not available.

Immunofluorescence images of TWIK-2, TREK-1, and TREK-2 in MCA and brain are shown in Fig. 3. An immunofluorescence image for TRAAK was omitted, because the
results were inconsistent with the RT-PCR analysis. Message for TRAAK was not found in VSMCs; however, immunofluorescence indicated the presence of TRAAK in vascular smooth muscle. The immunofluorescence could have been due to nonspecific binding. Note the dark band at ~120 kDa in Fig. 2, which is larger than the predicted size for TRAAK. In controls, nonimmune IgG from the primary antibody host was substituted for the primary antibody. In all cases, a positive signal for each K2P channel is indicated by red fluorescence, the internal elastic lamina by green autofluorescence, and nuclei by blue fluorescence. TWIK-2 was detected in VSMCs of the MCA and pia and in brain structures adjacent to the MCA (Fig. 3A). TWIK-2 is also likely in endothelium (on the luminal side of the internal elastic lamina); however, more studies are needed to confirm this possibility. All or part of the TWIK-2 fluorescence in brain appears to originate from capillaries. TREK-1 was found in brain tissue surrounding the MCA but not in the MCA (Fig. 3C). This finding is consistent with the immunoblot analysis of TREK-1. TREK-2 was not detected in any part of the MCA or surrounding brain (Fig. 3E). Although the TREK-2 antibodies were not effective for immunoblotting in our hands, we did conduct immunohistochemical studies, because antibodies can be useful for one application, and not for the other.

Antibodies directed against smooth muscle α-actin (green) and TWIK-2 (red) were used to conduct immunofluorescence studies in dispersed VSMCs (Fig. 4). Green and red fluorescence and an overlay of red and green channels, along with 4′,6-diamidino-2-phenylindole staining (blue) for nuclei, are shown in Fig. 4. When the antibodies for smooth muscle α-actin and TWIK-2 were given simultaneously, the overlay showed a yellowish color, indicating colocalization of the antibodies. (Three-dimensional reconstructions of TWIK-2 and smooth muscle α-actin can be viewed in the online version of this article, which contains supplemental data.)

The next series of experiments was conducted to determine the effects of AA on isolated, pressurized, and luminally perfused MCAs. All studies involving intact MCAs were conducted in the presence of l-NAME and indomethacin (10 μM each) to inhibit nitric oxide synthase and COX, respectively. Charybdotoxin (CHTX, 100 nM) is an inhibitor of large- and intermediate-conductance Ca2+-activated K+ (KCa) channels; apamin (apa, 1 μM) is an inhibitor of small-conductance KCa channels. Tiron (10 mM) scavenges reactive oxygen species. Inhibition of dilations by 50 mM K+ indicates K+ channel involvement. *P < 0.05 vs. control.
tively. Luminal application of AA dilated rat MCAs ($n = 4$, $P < 0.001$; Figs. 5A and 6). The dilation in response to $10^{-4}$ M AA was close to the maximum diameter obtained by removal of Ca$^{2+}$. Removal of the endothelium had no effect on the response to AA ($n = 5$). ETYA, an analog of AA that inhibits further metabolism of AA, dilated rat MCAs but was not as potent as AA ($P < 0.001$; Figs. 5B and 6). Palmitic acid had no dilator effects on MCAs (Fig. 5B).

Combined inhibition of the epoxygenase and lipoxygenase pathways with $10^{-5}$ M baicalein and $30^{-6}$ M miconazole had no effect on the dilation induced by $10^{-4}$ M AA ($n = 5$) compared with control MCAs ($n = 12$; Fig. 7). Similarly, inhibitors of classical K$^+$ channels had no effect on the dilation in response to $10^{-4}$ M AA. A cocktail consisting of $100^{-6}$ M Ba$^{2+}$, $10^{-5}$ M glibenclamide, and $3^{-3}$ M 4-AP had no effect on dilations in response to $10^{-4}$ M AA ($n = 5$; Fig. 7). The combination of charybdotoxin (100 nM) and apamin (1 $\mu$M) had no effect on dilations induced by $10^{-4}$ M AA ($n = 4$; Fig. 7). Tiron (10 mM, $n = 3$), a scavenger of reactive oxygen species, had no effect on AA-induced dilations. However, 50 mM K$^+$ abolished the dilations induced by $10^{-4}$ M AA ($P < 0.05$, $n = 5$; Fig. 7). Inhibition of the AA-induced dilations with 50 mM K$^+$ indicates involvement of a K$^+$ channel (1).

The current-clamp mode was used to measure changes in membrane potential on addition of AA to freshly isolated VSMCs. Figure 8 shows that $10^{-5}$ M AA hyperpolarized a VSMC that had been pretreated with 10 mM TEA. On addition of 140 mM K$^+$ to the bath, the cell depolarized to near 0 mV. TEA (10 mM) was added to ensure that large-conductance K$_{Ca}$ channels, the activation of which could be confused with that of an atypical K$^+$ channel, were inhibited. In the presence of 10 mM TEA, VSMCs hyperpolarized 31 ± 6 mV ($P < 0.003$, $n = 7$) on addition of $10^{-5}$ M AA to the bath. Thus AA significantly hyperpolarized VSMCs at physiological membrane potentials.

In freshly isolated smooth muscle cells from rat MCAs, voltage ramps in the whole cell configuration showed very little current at potentials more negative than $-20$ mV. However, as the membrane depolarized, outward current could be detected. A representative trace in a freshly isolated VSMC is shown in Fig. 9A. With addition of 10 mM TEA, current density decreased from $-120$ to $-10$ pA/pF at 80 mV (Fig. 9A). Addition of $10^{-5}$ M AA in the presence of 10 mM TEA resulted in a large increase in whole cell current (Fig. 9A). The response to a pulse protocol (see METHODS) for a single smooth muscle cell is shown in Fig. 9B. In the control state, outward current occurred at the more positive potentials. Addition of 10
mM TEA abolished most of this current, and addition of $10^{-5}$ M AA in the presence of TEA dramatically increased the outward current. In the presence of AA, the current reached a plateau, at which it remained for the duration of the pulse (500 ms).

Figure 9C shows summary data obtained using the pulse protocol for control ($n = 13$), 10 mM TEA ($n = 12$), and $10^{-5}$ M AA in the presence of 10 mM TEA ($n = 10$). After the cell was washed with buffer containing 10 mM TEA ($n = 3$), the current density returned to the level before addition of AA (Fig. 9C). In a single VSMC, the measured currents at a given membrane potential were similar whether the ramp or pulse protocol was used.

To determine whether K+ was carrying the current, the ramp protocol was used to measure reversal potential after addition of $10^{-5}$ M AA in the presence of 10 mM TEA at 5.4, 30, 100, and 140 mM external K+ (Fig. 10). Na+ concentration was reduced appropriately for each K+ solution to achieve a constant osmolality. Raw data from three different smooth muscle cells are shown in Fig. 10A. Figure 10B depicts summary data for reversal potential plotted as a function of the logarithm of extracellular K+ concentration ($n = 14, 6, 6, and 3$ for 5.4, 30, 100, and 140 mM, respectively, $P < 0.001$ by ANOVA). The increases in the reversal potential with increasing extracellular K+ demonstrate that K+ is the current carrier.

Metabolism of AA through the COX, epoxygenase, or lipoxygenase pathway could produce products responsible for the increased whole cell current when AA was added to the cells. Ω-Hydroxylation can also metabolize AA, but its products are associated with decreased K+ channel activity. Figure 11A shows current densities when $10^{-5}$ M ETYA was added in the presence of 10 mM TEA ($n = 5$). ETYA, an analog of AA, is capable of directly activating K+ channels while inhibiting further metabolism of AA. The response to $10^{-4}$ M ETYA ($n = 5$) was similar to the response to $10^{-5}$ M AA ($n = 10$). In the presence of 10 μM NDGA, 30 μM PPOH, and 10 μM indomethacin, $10^{-5}$ M AA ($n = 8$) increased the current density to the same degree as in the absence of these inhibitors ($n = 10$; Fig. 11B). Figure 11A also demonstrates that ethanol ($n = 5$), the vehicle for AA and ETYA, had no effect on whole cell current. Similarly, the saturated fatty acids arachidic acid (100 μM, $n = 5$) and palmitic acid (100 μM, $n = 3$; data not shown) had no effect on the current density.

Blockers of conventional K+ channels did not significantly affect whole cell current densities in response to $10^{-5}$ M AA (Fig. 12). The whole cell current responses to $10^{-5}$ M AA in the presence of 10 mM TEA ($n = 3$) were not significantly affected by addition of 3 mM 4-AP ($n = 5$) or a cocktail containing 100 μM Ba2+ and 10 μM glibenclamide ($n = 3$).

**DISCUSSION**

AA has been reported to dilate a variety of arteries and arterioles after conversion to dilator metabolites. The metabolites, which can be synthesized through one or more pathways, activate K+ channels (mostly large-conductance Ca2+-activated K+ channels) on vascular smooth muscle to elicit the dilation. In the present study, we report that AA-mediated dilations in the isolated rat MCA involve a previously undescribed mechanism. AA elicited dilation without having to be further metabolized, and these dilations involved atypical K+ (i.e., not Ktr, KATP, KCa, or Kv) channels. With the possible exception of intermediate-conductance Ca2+-activated K+ channels, the above-mentioned K+ channel types are known to be expressed by vascular smooth muscle and to dilate vessels when activated. We speculate that the atypical channel involved with dilations to AA in rat MCA belongs to the recently discovered K2P channel family.

AA elicits dilation and whole cell current without having to be further metabolized. Application of AA to pial arterioles and arteries in vivo elicited concentration-dependent dilations and could be inhibited by indomethacin, implicating the COX pathway (6, 11, 24, 43). Interestingly, the major dilatory effect was not due to a metabolite derived from AA; rather, reactive oxygen species generated in the COX-catalyzed reaction were...
responsible for the dilation (6, 11, 24, 43). In the basilar artery in vivo, AA dilations were not affected by indomethacin but, rather, were attenuated 50–70% by inhibitors of the lipoxygenase pathway (12). The dilations in basilar and pial arterioles were inhibited by TEA or iberiotoxin, indicating involvement of large-conductance KCa channels involved in the response to AA (12, 43).

In isolated peripheral arteries, AA administration elicited concentration-dependent dilations involving the COX and/or lipoxygenase pathway (35, 36, 45). In addition to the COX and lipoxygenase pathways, metabolism of AA in endothelium through the epoxygenase pathway can produce epoxygenoids, which are endothelium-derived hyperpolarizing factors in some peripheral vessels (7). As in some cerebral vessels, dilations in peripheral vessels involved activation of the K_\text{Ca} channel (7, 35).

Contrary to other studies, the dilations we observed in the isolated rat MCAs did not involve metabolites of AA and were elicited independent of KCa channels. Two observations provide evidence that AA was the dilating agent: 1) inhibitors of the COX, lipoxygenase, and epoxygenase pathways had no effect on the dilator response (Fig. 7), and 2) ETYA also elicited dilation in the MCAs (Figs. 5B and 6). ETYA is an analog of AA that inhibits further metabolism of AA and, consequently, is often used to determine whether unmetabolized AA, instead of a metabolite, is the signaling molecule.

We also conclude that AA was acting directly on smooth muscle, because removal of the endothelium had no effect on the dilator response (Fig. 5A).

The whole cell currents in isolated smooth muscle cells from the rat MCA further support the idea that unmetabolized AA was responsible for the observed effect. For instance, inhibitors of the COX, lipoxygenase, and epoxygenase pathways had no effect on the currents elicited by AA, and ETYA elicited increased currents similar to that of AA (Fig. 11).

Dilations and increased whole cell current elicited by AA involve an atypical K\textsuperscript{+} channel. The dilations to AA appear to involve an atypical K\textsuperscript{+} channel, because inhibition of classical K\textsuperscript{+} channels had no effect on the response. The combination of glibenclamide, Ba\textsuperscript{2+}, and 4-AP did not significantly affect the AA-induced dilations. Glibenclamide is an inhibitor of the K_\text{ATP} channel, Ba\textsuperscript{2+} is an inhibitor of the K_\text{ir} channel, and 4-AP is an inhibitor of the K_\text{o} channel. Furthermore, a combination of charybdotoxin and apamin had no effect on the dilator response to AA. Charybdotoxin is an inhibitor of large- and intermediate-conductance K_\text{Ca} channels, and apamin is an inhibitor of small-conductance K_\text{Ca} channels. However, 50 mM K\textsuperscript{+} completely inhibited the dilations to AA, implicating the
involvement of K⁺ channels. At high K⁺ concentrations, the reversal potential for K⁺ becomes equal to the membrane potential. Thus, under high-K⁺ conditions, opening of a K⁺ channel produces no net movement of K⁺ across the membrane, no hyperpolarization of the vascular smooth muscle, and, thus, no dilation. High K⁺ is often used to determine whether a K⁺ channel might be involved (1).

The electrophysiological data further support the idea that an atypical K⁺ channel is involved. The addition of AA increased K⁺ current across the membrane of individual smooth muscle cells from the rat MCA. Inhibition of KCa, Kv, and KATP channels with TEA, 4-AP, glibenclamide, and Ba²⁺ had no effect on the whole cell current induced by AA. The vessel data and the electrophysiological data indicate that unmetabolized AA activated an atypical K⁺ channel to hyperpolarize smooth muscle and dilate the MCAs.

Activation of K₂P channels by AA. We speculate that the recently discovered K₂P family of channels is involved with the dilations through increased whole cell current elicited by AA in the rat MCAs. The K₂P channels are a family of K⁺ channels with four transmembrane-spanning domains and two pore domains in each subunit. In the mid- and late 1990s, K₂P channels were cloned and studied in expression systems (for reviews see Refs. 5, 14, 20, 25, 26, 38, 40, 44). Depending on the individual family member, K₂P channels can be regulated by one or more of the following: temperature, mechanical perturbation, anesthetics, pH, lysophosphatidylcholine, cAMP, cGMP, G proteins, PKC, diacylglycerol, and phosphatidylinositol 4,5-bisphosphate (5, 8, 9, 16, 19, 29, 30, 38, 40). There are no selective inhibitors or activators for any of the K₂P channels. TREK-1, TREK-2, TRAAK, and THIK-1 have been reported to be activated by AA (2, 13, 39, 41, 42). In addition to AA, TREK-1, TREK-2, and TRAAK are also activated by other polyunsaturated, but not saturated, fatty acids (2, 13, 39, 41, 42). The response to polyunsaturated fatty acids, other than AA, and saturated fatty acids has not been tested in TWIK-2 or THIK-1.

AA-sensitive K₂P channels are located throughout the body, with the brain being an especially rich source. Little attention has been directed at determining whether K₂P channels, in general, and AA-sensitive K₂P channels, in particular, are located in the vasculature. Message for TWIK-2 has been found in the aorta and pulmonary artery in rat and human (34, 34, 41). TREK-1 message has been found in the portal vein and pulmonary artery in mouse (22). TREK-2 message was found in the pulmonary artery, but not in the portal vein, in mouse (22).

Although we found message for all five AA-sensitive K₂P channels in the rat MCA (Fig. 1), our RT-PCR studies indicate that, of the five K₂P channels, only TWIK-2 is expressed in VSMCs. Our immunoblots (Fig. 2) and immunohistochemical studies (Figs. 3 and 4) reveal that TWIK-2 protein is present in the MCA and, specifically, in VSMCs. Our results for TRAAK were inconsistent. Although no message was found in VSMCs, immunohistochemistry was positive for TRAAK in vascular smooth muscle. However, given that there was a dark band for TRAAK in the immunoblot (Fig. 2) that was not the predicted size, it is likely that the antibody may be giving false-positive results in the immunofluorescence analysis. We found no evidence for the presence of TREK-1 or TREK-2 on MCAs. Because we were unable to obtain suitable immunoblots from spleen, a tissue that is rich in TREK-2 (2), any conclusion regarding TREK-2 would be premature. However, we did not find mRNA for TREK-2 in isolated VSMCs. Contrary to our results, Blondeau et al. (3), using an antibody that is not commercially available, observed protein for TREK-1, in addition to message, in rat (and mouse) cerebral arteries but not in the carotid or femoral arteries. We have no explanation for the disparity with TREK-1 in rat cerebral arteries.

In the present study, we demonstrate that unmetabolized AA dilates rat MCAs by activating an atypical K⁺ channel on cerebrovascular smooth muscle. Although the specific K⁺ channel responsible for this effect is uncertain, we speculate that it is a member of the K₂P channel family that is directly activated by AA. Because we have found that TWIK-2 expressed in cerebrovascular smooth muscle and TWIK-2 is activated by AA, it is a good candidate for the increased K⁺ current in response to AA. Because there are no selective inhibitors for the AA-sensitive K₂P channels, functional identification of these channels in native cells is a challenge that requires future studies. The present study indicating the potential for an AA-sensitive K₂P channel in cerebrovascular smooth muscle extends previous studies which provide evidence that TASK-1, an AA-insensitive K₂P channel, may be involved with changes in vascular tone as a function of pH in mesenteric and pulmonary arteries (15, 17).

REFERENCES


