2,2,2-Trichloroethanol Activates a Nonclassical Potassium Channel in Cerebrovascular Smooth Muscle and Dilates the Middle Cerebral Artery^[S]

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ABSTRACT

Trichloroacetaldehyde monohydrate [chloral hydrate (CH)] is a sedative/hypnotic that increases cerebral blood flow (CBF), and its active metabolite 2,2,2-trichloroethanol (TCE) is an agonist for the nonclassical two-pore domain K⁺ (K_{2P}) channels TREK-1 and TRAAK. We sought to determine whether TCE dilates cerebral arteries in vitro by activating nonclassical K⁺ channels. TCE dilated pressurized and perfused rat middle cerebral arteries (MCAs) in a manner consistent with activation of nonclassical K⁺ channels. Dilation to TCE was inhibited by elevated external K⁺ but not by an inhibitory cocktail (IC) of classical K⁺ channel blockers. Patch-clamp electrophysiology revealed that, in the presence of the IC, TCE increased whole-

cell currents and hyperpolarized the membrane potential of isolated MCA smooth muscle cells. Heating increased TCE-sensitive currents, indicating that the activated channel was thermosensitive. Immunofluorescence in sections of the rat MCA demonstrated that, like TREK-1, TRAAK is expressed in the smooth muscle of cerebral arteries. Isoflurane did not, however, dilate the MCA, suggesting that TREK-1 was not functional. These data indicate that TCE activated a nonclassical K⁺ channel with the characteristics of TRAAK in rat MCA smooth-muscle cells. Stimulation of K⁺ channels such as TRAAK in cerebral arteries may therefore explain in part how CH/TCE increases CBF.

Trichloroacetaldehyde monohydrate [chloral hydrate (CH)] is a sedative/hypnotic with a variety of uses ranging from sedation of agitated neonates to treatment of elderly patients who have trouble sleeping (Pershad et al., 1999; Gauillard et al., 2002; Twite et al., 2004). CH is also used in veterinary medicine and in experimental animals, although its use in humans and animals is declining (Cabana and Gessner, 1970; Gessner and Cabana, 1970; Silverman and Muir,

1993). The active metabolite of CH is 2,2,2-trichloroethanol (TCE), and both it and CH are environmental pollutants (Beland, 1999; Gauillard et al., 2002; National Toxicology Program, 2002; Merdink et al., 2008). CH is rapidly metabolized to TCE in hepatocytes and erythrocytes, but it is only slowly excreted by the kidneys (Cabana and Gessner, 1970; Gessner and Cabana, 1970; Beland, 1999; Gauillard et al., 2002; National Toxicology Program, 2002; Merdink et al., 2008). Thus, because of potentially life-threatening central nervous system depression, CH/TCE overdose is of concern (Gessner and Cabana, 1970; Levine et al., 1985; Jones and Singer, 2008).

The effects of CH/TCE on cerebral blood flow (CBF) are incompletely understood; however, CH seems to increase CBF. By use of autoradiography, it was recently found that CH increased CBF and decoupled brain glucose metabolism from CBF (Uematsu et al., 2009). In addition, measurements of regional CBF using a tissue oxygen and glucose biosensor demonstrated that CH increased local CBF (Lowry and Fil-

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ABBREVIATIONS: CH, chloral hydrate; TCE, 2,2,2-trichloroethanol; IC, inhibitory cocktail; CBF, cerebral blood flow; VSMC, vascular smooth muscle cell; MCA, middle cerebral artery; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; RT, room temperature.

lenz, 2001). It is possible that the effects of CH/TCE on CBF are mediated by direct actions on cerebral arteries.

By controlling the diameter of cerebral arteries, potassium (K^+) channels are determinants of CBF (Nelson and Quayle, 1995; Faraci and Heistad, 1998). Vascular smooth muscle cells (VSMCs) express a variety of K^+ channels, including classical and nonclassical types, the latter of which comprise the two-pore domain K^+ (K_{2P}) channel family (Nelson and Quayle, 1995; Faraci and Heistad, 1998; Lesage and Lazdunski, 2000; Lotshaw, 2007). Activation of K^+ channels in VSMC hyperpolarizes the cells promoting VSMC relaxation and thus vasodilation by closure of voltage-dependent calcium channels (Nelson and Quayle, 1995; Faraci and Heistad, 1998).

It is noteworthy that TCE is an agonist for the K_{2P} channels TREK-1 (KCNK2) and TRAAK (KCNK4), which are both expressed in cerebral arteries (Harinath and Sikdar, 2004; Bryan et al., 2006; Blondeau et al., 2007). TREK-1 is found throughout the wall of the mouse basilar artery (Blondeau et al., 2007), whereas the distribution of TRAAK in cerebral arteries has not been reported. Activation of heterologously expressed human TREK-1 by TCE was transient because of autoinhibition, whereas stimulation of human TRAAK was sustained (Harinath and Sikdar, 2004). Although TCE activates K^+ channels that are expressed in cerebral arteries, the effect of TCE on cerebrovascular function has until now remained unexplored.

The purpose of this study was to examine the possibility that TCE is a vasodilator and to determine the role of K^+ channels in the response to TCE. To address this, we studied in vitro pressurized and perfused rat middle cerebral arteries (MCAs) and found that TCE dilated these arteries in a manner consistent with activation of a nonclassical K^+ channel. In addition, we used patch-clamp electrophysiology to examine whole-cell currents in freshly dissociated rat MCA smooth muscle cells. TCE stimulated nonclassical K^+ currents in and hyperpolarized the membrane potential of MCA smooth muscle cells. Thus, our novel findings demonstrate that TCE activated a nonclassical K^+ channel in cerebrovascular smooth muscle, with characteristics of a K_{2P} channel, and dilated cerebral arteries.

Materials and Methods

Animals and Reagents. Male Long-Evans rats (250–350 g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether (isoflurane; Abbott Laboratories, North Chicago, IL) and decapitated before tissue harvesting. The Animal Protocol Review Committee at Baylor College of Medicine or the Animal Care and Use Committee at the University of Missouri-Kansas City approved all protocols. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Western Blotting. Rat tissue samples, including the aorta, carotid arteries, cerebral arteries (MCA+basilar pooled from three rats), and heart were homogenized in protein extraction buffer [1% SDS, 10 mM EDTA, and complete mini protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)]. Samples were heated to 85°C for 15 min and centrifuged at 15,000g for 15 min. Protein concentration of the supernatants was determined by use of the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples were diluted with 6× Laemmli buffer (30% glycerol, 50 mM EDTA, 0.25% bromphenol blue, and 10% β-mercaptoethanol) and heated to 85°C before loading 15 μg into wells of 4 to 20% polyacrylamide gels

(Invitrogen, Carlsbad, CA). After room temperature electrophoresis at a constant 150 V, proteins were transferred to nitrocellulose membranes by use of the iBlot dry blotting system (Invitrogen) running program 3 for 8 min. Blots were blocked for 1 h by use of blocking solution [0.5% nonfat dry milk (Bio-Rad), 1% bovine serum albumin (BSA), and 0.01% polysorbate 20 (Tween 20) in PBS (Invitrogen)] and then incubated overnight at 4°C with a primary goat polyclonal IgG directed against the C terminus of human TRAAK (C-13; Santa Cruz Biotechnology, Santa Cruz, CA), which was diluted 1:100 in blocking solution.

Blots were then rinsed for 15 min three times at room temperature by use of 0.05% Tween 20 in PBS. After blocking for 1 h blots were incubated for another hour with a fluorescently tagged (Alexa Fluor 555; Invitrogen) rabbit anti-goat secondary antibody diluted 1:1000 in blocking solution. Blots were rinsed as before, and the bands were detected by use of an Ettan DIGE fluorescent imager (GE Healthcare Life Sciences, Piscataway, NJ).

Immunohistochemistry. Deeply anesthetized rats were decapitated; the brain was removed and frozen in isopentane at −40°C, and sectioned at 8 μm by use of a cryostat. Sections were mounted two to a slide and fixed in 4% methanal (formaldehyde) for 10 min at room temperature. Slides were washed with 0.1% Triton X-100 in PBS (PBS-T), followed by blocking with 2% normal donkey serum and 10% BSA for 1 h. Sections were incubated overnight at 4°C with either a rabbit anti-TRAAK antibody (BIOMOL Research Laboratories, Plymouth Meeting, PA) at 8 ng ml^{−1} in blocking buffer or an equivalent amount of rabbit nonimmune IgG (Affinity Biologicals, Ancaster, ON) in blocking buffer as a control. On the 2nd day, sections were washed three times with PBS-T for 10 min each time, and then blocked for 1 h at room temperature. Sections were then incubated with a fluorescently tagged DyLight donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:1000 in blocking buffer for 1 h at room temperature. After this, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml in blocking buffer) for 5 min. Before being coverslipped, sections were washed three times in PBS-T and dehydrated with increasing concentrations of ethanol. Sections were dried and coverslipped using VECTASHIELD HardSet mounting medium (Reactivolab SA, Servion, Switzerland).

Sections were imaged by epifluorescence microscopy using an IX51 inverted microscope (Olympus America Inc., Center Valley, PA), Orca-ERGA CCD camera (Hamamatsu Corporation, Bridgewater, NJ), Brightline DAPI and Texas Red filter sets (Semrock, Rochester, NY), and X-cite 120 metal halide light source (Exfo Life Sciences Division, Mississauga, ON). Images were processed by SlideBook imaging software (Intelligent Imaging Innovations, Denver, CO).

Isobaric Vessel Studies. Rat brains were quickly removed and placed in ice-cold Hank's buffered saline solution (Invitrogen). The MCAs were carefully dissected away and studied in a pressurized artery myograph as described previously (Andresen et al., 2006). In brief, isolated vessels were mounted on glass micropipettes, pressurized, and lumenally perfused with Krebs' buffer (119 mM NaCl, 4.7 mM KCl, 24 mM NaHCO₃, 1.18 mM KH₂PO₄, 1.19 mM MgSO₄, 5.5 mM glucose, and 1.6 mM CaCl₂) bubbled with 20% O₂/5% CO₂/bal. N₂ at 37°C, pH = 7.4. Endothelial denudation and verification was performed as described previously (Andresen et al., 2006). In these and the following experiments, elevated external K^+ solutions were made isotonic by replacement of Na⁺ with K⁺ on an equimolar basis. In addition, blockers of classical K^+ channels—10 mM TEA to block large conductance calcium-activated K^+ (BK_{Ca}) channels, 100 μM BaCl₂ to block inwardly rectifying (K_{ir}) channels, 3 mM 4-pyridinamine (4-aminopyridine) to block voltage-gated K^+ (K_v) channels, and 10 μM 5-chloro-N-(4-[N-(cyclohexylcarbonyl)sulfamoyl]phenethyl)-2-methoxybenzamide (glibenclamide) to block ATP-sensitive K^+ (K_{ATP}) channels—were added as an inhibitory cocktail (IC) 30 min before determining responses to various agonists in these and in subsequent experi-

ments. Thus, by using these common, well accepted blockers, all types of classical K^+ channels should have been inhibited in our preparations (Nelson and Quayle, 1995). Any activation of a K^+ channel would therefore have had to be due to a nonclassical K^+ channel, possibly a K_{2P} channel. All compounds, including TCE and the IC, were added to the luminal and abluminal perfusates.

MCA Digestion and Smooth Muscle Cell Isolation. Rat MCAs were removed from the brain and minced in digestion buffer (135 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 0.42 mM Na_2PO_4 , 0.44 mM NaH_2PO_4 , 4.2 mM $NaHCO_3$, 10 mM HEPES, and 1 mg/ml BSA with pH adjusted to 7.2 by use of 1.0 N NaOH). Enzymatic digestion started with a 37-min incubation at 37°C in digestion buffer containing 18 U/ml papain. After washing with digestion buffer, the digestion was completed by 8 min of incubation at 37°C in digestion buffer containing 1.2 mg/ml collagenase II (Worthington Biochemicals, Lakewood, NJ), 0.8 mg/ml soybean trypsin inhibitor (Worthington Biochemicals), and 60 U/ml elastase (EMD Chemicals, Gibbstown, NJ). Dissociated tissue was twice washed in digestion buffer and then triturated by passing the digest 10 times through the tip of a 200- μ l pipette tip precoated with digestion buffer containing BSA. The MCA tissue digest was stored on ice until use during the same day.

Electrophysiology. Aliquots (~20 μ l) of the rat MCA smooth muscle cell suspension were placed in the well of a laminar perfusion chamber (Bioscience Tools, San Diego, CA) on the stage of an IX71 inverted microscope (Olympus), and the cells were allowed to attach to the glass for 10 min before buffer flow was initiated. Smooth muscle cells were superfused with bath buffer (140 mM NaCl, 4.2 mM KCl, 3 mM $NaHCO_3$, 1.2 mM KH_2PO_4 , 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES with pH adjusted to 7.4 by use of 1.0 N NaOH) at ~1 ml/min. As is typical for patch-clamp studies, experiments were performed at room temperature (RT) unless otherwise noted, and solutions were not aerated. Micropipettes were made from 8520 glass capillaries (Warner Instruments, Hamden, CT) by use of a PC-10 gravity puller (Narishige, East Meadow, NY). Micropipettes were polished to 3 to 5 M Ω resistance with a MF-830 microforge (Narishige) and filled with pipette buffer (140 mM KCl, 1.0 mM $MgCl_2$, 2.2 mM $CaCl_2$, 3 mM EGTA, resulting in ~0.42 μ M free Ca^{2+} , and 10 mM HEPES with pH adjusted to 7.2 using 1.0 N KOH). Osmolarity of bath and pipette solutions was measured by use of a VAPRO5520 vapor pressure osmometer (Wescor, Logan, UT) and adjusted to 285 mOsmol/liter if necessary.

Gentle suction was used to form giga-seals on individual smooth muscle cells, and further suction ruptured the membrane inside

the pipette tip to form the whole-cell patch-clamp configuration. A MultiClamp 700B computer-controlled microelectrode amplifier and DigiData1440A low-noise digitizer run by pCLAMP 10 software (v10.2.0.14; Axon Instruments, Union City, CA) were used to acquire and measure the whole-cell currents. Holding potential was -20 mV. Current-voltage (I-V) plots were generated from a voltage-step protocol that increased membrane potential from -80 to +80 mV in 10-mV steps. Three consecutive traces were averaged to generate each I-V profile. The temperature of the recording chamber was changed with use of a TC-1 temperature controller (Bioscience Tools), which received feedback from a thermocouple placed in the recording chamber. RT averaged ~22°C; and for heat experiments, bath temperature was raised to 33°C in less than 1 min. For experiments involving K^+ channel blockade, the IC of classical K^+ channel blockers was added to the bath buffer, and the pH was adjusted to 7.4 with 1.0 N NaOH. Voltage ramps (-100 mV to +100 mV over 700 ms) were used to probe reversal potential (E_{rev}) changes in solutions of varying K^+ concentration. As above, three consecutive traces were averaged to generate each I-V profile. In solutions with elevated external K^+ ($[K^+]_{out}$), an equal amount of Na^+ was removed to maintain osmotic balance. The pH of 140 mM K^+ was adjusted to 7.4 with 1.0 N KOH. Membrane potentials were recorded in current-clamp mode. For all experiments, liquid junction potential adjustments were made during data analysis.

Statistics. Data are plotted and expressed as means $\pm$ S.E.M. The n values are detailed in the legends to the figures where each n unit represents data from one animal. In isobaric arterial studies, one artery was studied from each animal. Changes in the diameter of pressurized and perfused arteries were calculated as described previously (Andresen et al., 2006). Electrophysiological data represent an average of at least three cells per animal (range, 3–6 cells/animal). Statistics were computed by comparing responses across animals. Two-factor ANOVA was used to determine differences between concentration-response curves. One-factor ANOVA with Tukey's multiple comparison post hoc tests, or a paired Student's t test as appropriate, were used to compare peak outward currents. Changes in membrane potential under current-clamp were compared with a paired Student's t test. Where appropriate, data normality was examined with D'Agostino and Pearson tests, and homogeneity of variance was determined with Bartlett's test. To determine differences between I-V relationships, the data were fitted with third-order polynomial functions and the best-fit lines compared by extra sum-of-squares F test. Data were plotted and statis-

Fig. 1. Dilatation of the rat MCA to TCE. Top, raw traces of arterial diameter. Bottom, summary data. A, in the control condition (●), denuded, pressurized, and perfused rat MCAs dilated in response to increasing concentrations of TCE ($n = 5$). B, replacing the control Krebs' buffer with a buffer containing isotonic 60 mM KCl (■) impaired the response to TCE ($n = 8$). C, inclusion of an inhibitory cocktail of classical K^+ channel blockers (○) did not alter the dilation caused by TCE ($n = 4$). Summary data are means $\pm$ S.E.M.

tics computed with Graphpad Prism (v5.0b; San Diego, CA). Significance was accepted at $p \leq 0.05$.

Results

Vascular Responses to TCE and Isoflurane. Initial experiments demonstrated that TCE dilated intact rat MCAs and that endothelial denudation did not alter the dilation (data not shown). Thus, to examine just the effect of TCE on smooth muscle, experiments were conducted on denuded MCAs. Figure 1 (top) shows raw traces illustrating the response of pressurized and perfused rat MCAs to increasing concentrations of TCE. Under control conditions, TCE caused concentration-dependent ($p < 0.0001$) dilation of rat MCA (Fig. 1A). Figure 1B demonstrates that elevating $[K^+]_{out}$ to 60 mM substantially impaired ($p < 0.0001$) the dilation to TCE. We next added the IC of classical K^+ channel blockers to explore the identity of the K^+ channel(s) involved in the dilation to TCE. As can be seen in Fig. 1C, the IC did not alter ($p = 0.7821$) the dilation to TCE in the rat MCA. In separate experiments, application of increasing concentrations (10^{-5} to 10^{-1} M) of isoflurane totally failed to dilate denuded, pressurized, and perfused rat MCAs (data not shown).

In addition to the MCA, we found that TCE also dilated pressurized and perfused rat carotid arteries. Supplemental Fig. 1 shows that TCE concentration-dependently ($p < 0.0001$) dilated the rat carotid and that, like the MCA, elevated $[K^+]_{out}$ but not the IC impaired ($p < 0.0001$) the dilation.

TRAAK Expression. We found an immunopositive band for TRAAK near 43 kDa (predicted TRAAK size) in the rat MCA by Western blot (Fig. 2A). In addition, we found that TRAAK was expressed in hearts, aortas, and the carotid arteries of rats (Fig. 2A). We further investigated the expression of TRAAK in the vascular wall of the rat MCA by immunohistochemistry. In 8- μ m rat brain sections containing the MCA TRAAK immunoreactivity was visible in neurons and in the MCA (Fig. 2B). At high magnifications, TRAAK expression seemed to be restricted to the smooth muscle layers of the MCA.

Electrophysiology of MCA Smooth Muscle: Response to TCE under Control Conditions. Figure 3 shows raw traces of the responses of individual rat MCA vascular smooth muscle cells responding to a voltage-step stimulus protocol at baseline and after exposure to TCE. In both control myocytes and in those treated with the IC, inward current was characteristically small for rat MCA smooth muscle cells (Bryan et al., 2006). Under control conditions, TCE increased whole-cell currents primarily in the outward direction at depolarizing potentials (currents going upward in Figs. 3 and 4, B and C). Control outward current at 60 mV was significantly greater ($p = 0.0005$) than baseline only at 10^{-2} M TCE (Fig. 4A), yet TCE concentration-dependently [$p < 0.0001$ (the entire curve)] increased overall whole-cell currents in the control condition (Fig. 4B). Subtracting out the baseline response to generate difference currents revealed the currents due only to TCE (I_{TCE}). In the control condition, I_{TCE} (Fig. 4C) reversed at approximately -45 mV for both 10^{-3} and 10^{-2} M TCE, which was hyperpolarized compared with E_{rev} at baseline (approximately -36 mV). Peak I_{TCE} at 10^{-2} M TCE was 9-fold greater than I_{TCE} at 10^{-3} M TCE (Fig. 4C).

Fig. 2. TRAAK protein expression. A, Western blot showing TRAAK immunopositive bands at 43 kDa in rat cardiovascular tissues. Cerebral arteries = MCA+basilar. Positive (+) control is IMR-32 human neuroblastoma cell lysate (sc-2409, Santa Cruz Biotechnology). B, sections of rat brain and MCA imaged at three levels of magnification (10 $\times$, 40 $\times$, and 60 $\times$ oil immersion) using epifluorescence microscopy. Tissue slices (8 μ m) were incubated with a primary antibody directed toward TRAAK (right) or an equal amount of a control nonimmune IgG (left). Red fluorescence is TRAAK; blue is DAPI staining of nuclei.

Electrophysiology of MCA Smooth Muscle: Response to TCE after Blockade of Classical K^+ Channels. Currents at 60 mV stimulated by TCE in the control condition were reduced by 80% after addition of the IC, suggesting that classical K^+ channels were responsible for much of the control response to TCE (Figs. 3 and 4B). Unlike the control condition, after addition of the IC, inward currents were increased by TCE (Figs. 3A and 4B). In the presence of the IC, 10^{-2} M, but not 10^{-3} M, TCE increased ($p = 0.0144$) outward current at 60 mV over baseline levels (Fig. 4A). Likewise, only 10^{-2} M TCE increased ($p < 0.0001$) the total response (entire I-V curve) over baseline levels in the presence of the IC (Fig. 4B). Subtracting out the baseline response revealed the currents due only to TCE in the presence of the IC ($I_{TCE(IC)}$). As expected from the summary data in Fig. 4B, the difference currents demonstrated that $I_{TCE(IC)}$ for 10^{-3} M TCE was essentially zero at every

Fig. 3. Electrophysiology of isolated rat MCA smooth muscle cells. Whole-cell patch-clamp current recordings of freshly dissociated rat MCA smooth muscle cells. In response to a voltage-step protocol (signal, -80 to 80 mV, 60 -ms pulses separated by 10 mV), TCE elicited concentration-dependent increases in current (mostly in the outward direction) under control conditions (left) and after inhibition of classical K^+ channels with the IC (right). Note the difference in scale: Control is -400 to 3000 pA; inhibitory cocktail is -400 to 500 pA.

voltage, meaning that it was indistinguishable from baseline currents. $I_{TCE(IC)}$ at 10^{-2} M TCE was, however, significantly ($p < 0.0001$) non-zero and did not display voltage sensitivity (Fig. 4C). E_{rev} for $I_{TCE(IC)}$ at 10^{-2} M TCE was approximately -24 mV (Fig. 4C). Although not a large shift, this was hyperpolarized compared with baseline (E_{rev} , approximately -21 mV). To address this more carefully, we switched to current-clamp mode, which allowed for direct determination of the cell membrane potential. In the presence of the IC, 10^{-2} M TCE hyperpolarized ($p = 0.0085$) the membrane potential by 12 ± 3 mV (-25 ± 1.2 to -37 ± 3.4 mV, $n = 5$).

Changes in Reversal Potential with Increased Extracellular Potassium. Experimental data from rat MCA smooth muscle cells demonstrated a positive shift in E_{rev} with increasing $[K^+]_{out}$, as expected if the charge carrier was K^+ (Fig. 5). In normal bath buffer (5.4 mM $[K^+]_{out}$), currents stimulated by 10^{-2} M TCE in the presence of the IC reversed around -32 mV. Increasing $[K^+]_{out}$ to 54 and 140 mM shifted E_{rev} to -17 and -1 mV, respectively. Figure 5 (bottom) shows the best-fit lines through the summary data of our experimental results in addition to a simulated data set based on a hypothetical pure K^+ current. The slope of the best-fit line for the experimental data was 19.98 ± 2 mV/decade, which is less ($p < 0.0001$) than the predicted 59.65 ± 2 mV/decade for a pure K^+ current.

Effect of Heat on the TCE Response. In the presence of the IC, simply heating the patch chamber did not increase baseline whole-cell currents (Fig. 6). Heating to 33°C in the presence of 10^{-3} M TCE and the IC, however, increased ($p <$

0.0001) whole-cell currents above those present at RT such that the outward current at 60 mV and 33°C was 1.7 -fold greater ($p = 0.0195$) than RT levels (Fig. 7, A–C). Examining the difference currents ($I_{TCE(IC)}$) revealed that TCE currents at 33°C were greater ($p = 0.0001$, entire curve) than those at RT (Fig. 7D). In addition, $I_{TCE(IC)}$ at 10^{-2} M TCE and RT was indistinguishable ($p = 0.2842$) from $I_{TCE(IC)}$ stimulated by just 10^{-3} M TCE at 33°C (Fig. 7E).

Discussion

For the first time, we have observed that TCE elicited endothelium-independent dilation of the rat MCA in a manner consistent with the activation of a nonclassical K^+ channel. We found that the TCE-sensitive K_{2P} channel TRAAK was expressed in the smooth muscle layers of the MCA. In isolated MCA smooth muscle cells, TCE caused hyperpolarization and increased whole-cell currents apparently by stimulating a nonclassical K^+ channel. In addition, heat potentiated the TCE response in MCA smooth muscle cells in the presence of the IC, which is compatible with activation of a thermosensitive channel such as TRAAK. Thus, it seems that TCE causes vasodilation by activating nonclassical K^+ channels, possibly the K_{2P} channel TRAAK.

Vasomotor Function. In control MCAs, 10^{-3} M TCE caused 13% dilation, and, at $10^{-1.5}$ M TCE, 100% dilation was reached. The dilation we observed to TCE was endothelium-independent and largely inhibited by elevated extracellular K^+ . Elevated extracellular K^+ alters the equilibrium potential for K^+ , thereby reducing the driving force for K^+

Fig. 4. Summary data of currents activated by TCE in rat MCA smooth muscle cells. A to C, control cells ($n = 5$ animals) are on the left, and cells treated with the IC of classical K^+ channel blockers ($n = 5$ animals) are on the right. A, at 10^{-2} M, TCE increased peak outward current in both control cells and cells treated with the IC. In B and C, $\bullet$, I-V curves of baseline currents are shown; $\circ$, currents stimulated by TCE at 10^{-3} M; $\blacksquare$, 10^{-2} M TCE. B, under control conditions, TCE increased whole-cell currents at both 10^{-3} and 10^{-2} M. Inclusion of the IC reduced overall whole-cell currents, and increases over baseline were only seen to 10^{-2} M TCE. C, subtracting out the baseline responses generated difference currents representing the currents due only to TCE (I_{TCE}). Although the I_{TCE} was nonzero for both concentrations of TCE under control conditions, I_{TCE} at 10^{-2} M was greater than at 10^{-3} M. In the presence of the IC, however, only 10^{-2} M TCE was sufficient to generate currents that were nonzero, or different from baseline. Data are means $\pm$ S.E.M. *, statistically significant difference ($p \leq 0.05$).

movement across the cell membrane. Thus, these data indicated that some type of K^+ channel was involved in the response. Addition of the IC did not, however, impair the response of the rat MCA or carotid artery to TCE, indicating involvement of a nonclassical K^+ channel, possibly a K_{2P} channel, because they are refractory to known K^+ channel blockers. These data are similar to previous work showing that the rat MCA dilated to the AA by activation of a non-classical K^+ channel in an endothelium-independent manner (Bryan et al., 2006).

Because TREK-1 and -2 are expressed in the rat MCA, it was necessary to evaluate TREK-1 as a possible mediator of the dilatory response to TCE, because TCE can activate both TREK-1 and -2 and TRAAK. The TREK channel agonist and inhalation anesthetic, isoflurane, failed to elicit any dilation of rat MCAs, suggesting that TREK-1 and -2 were not functionally present in our preparations. Furthermore, although TCE is an agonist for TREK-1 (there are no published data

Fig. 5. Changes in reversal potential with increased extracellular potassium. Top, traces of an rat MCA smooth muscle cell treated with the IC of classical K^+ channel blockers and 10^{-2} M TCE as the extracellular K^+ concentration is raised from 5.4 mM to 54 and 140 mM. Bottom, summary of experimental data collected from rat MCA smooth muscle cells (red circles, $n = 4$ animals), and simulated pure K^+ currents (black squares). Regression lines were fitted to the experimental (red) and simulated (blue) data to determine the slope of the response. Solid lines, best-fit line; dashes, 95% confidence interval of the best-fit line. Data are means $\pm$ S.E.M. *, statistically significant difference ($p \leq 0.05$).

Fig. 6. Baseline response of rat MCA smooth muscles cell to heat. Whole-cell current traces of a MCA smooth muscle cell responding to a voltage-ramp protocol (stimulus, -100 to $+100$ mV in 700 ms) in the presence of the IC to block classical K^+ channels. Heating the preparation from room temperature (22°C) to 33°C did not alter the baseline response of the cell.

for TREK-2) and TRAAK, activation of TREK-1 by TCE is transient because of rapid channel inhibition (Harinath and Sikdar, 2004). The dilatory response to TCE, however, was sustained, further supporting the involvement of TRAAK rather than TREK-1 channels in pressurized and perfused arteries.

TRAAK Expression. Initially, TRAAK message was found only in the brain, spinal cord, and retina (Fink et al.,

Fig. 7. Effect of heat on the TCE response in rat MCA smooth muscle cells. **A**, an example of a whole-cell voltage-clamp recording of an isolated rat MCA smooth muscle cell treated with 10^{-3} M TCE in the presence of the IC to block classical K^{+} channels. In response to a voltage-step protocol (inset) currents at 33°C were augmented over those at room temperature (22°C). **B**, summary data ($n = 5$ animals) showing that I-V curves of MCA smooth muscle cells at room temperature (22°C) at baseline ($\bullet$), and after addition of 10^{-3} M TCE ($\circ$) were similar, but that simply heating to 33°C ($\blacksquare$) enhanced the currents. **C**, summary of peak outward currents at room temperature and at 33°C in the presence of the IC and 10^{-3} M TCE. **D**, subtracting out the baseline I-V response revealed that the currents attributable to 10^{-3} M TCE ($I_{\text{TCE}(-3)}$) at 33°C were greater than zero, meaning that they were different from the baseline currents. **E**, I-V plot of difference currents due to 10^{-2} M TCE at room temperature ($I_{\text{TCE}(-2)} \text{ RT}$; $\circ$) and to 10^{-3} M TCE at 33°C ($I_{\text{TCE}(-3)} \text{ } 33^{\circ}$; $\bullet$). Data are means $\pm$ S.E.M. *, statistically significant difference ($p \leq 0.05$).

1998; Reyes et al., 2000). Subsequently, we found TRAAK message and protein in rat cerebral arteries (Bryan et al., 2006). The present study extends these findings by demonstrating that TRAAK protein is expressed in rat carotid arteries, aortas, and heart. Because it was also found in rat mesenteric, but not in pulmonary arteries, TRAAK seems to be generally expressed in the cardiovascular system, at least in the systemic circulation (Gardener et al., 2004). The expression of TRAAK in the wall of the rat MCA seemed to be restricted to the smooth muscle layers because we did not observe TRAAK immunofluorescence in endothelial cells. Although our images do not totally exclude the possibility that TRAAK is expressed in endothelium, our functional data both in the present work, and in our previous study, suggest that TRAAK is functionally present only in vascular smooth muscle.

Electrophysiology. The currents stimulated by TCE in control conditions were large and had a distinct voltage-dependent component. Addition of the IC substantially reduced the overall response to TCE but, in so doing, revealed voltage-independent currents attributable to a nonclassical K^{+} channel. Given that the large voltage-dependent current stimulated by TCE was IC-sensitive, it is most likely that large conductance calcium-activated K^{+} channels (BK_{Ca}) carried these currents. Although potentially interesting, we did

not pursue this further because our pressurized and perfused in vitro artery data did not support a role for BK_{Ca} channels in the response to TCE.

To examine whether K^{+} carried I_{TCE} , we increased $[K^{+}]_{\text{out}}$ to determine E_{rev} as a proxy for the cell membrane potential. In the presence of 10^{-2} M TCE the shifts in E_{rev} caused by 54 and 140 mM $[K^{+}]_{\text{out}}$ were as expected if K^{+} was the current carrier, whereas in the normal buffer (5.4 mM $[K^{+}]_{\text{out}}$) E_{rev} was not as hyperpolarized as one might expect if there was sole activation of only a K^{+} -selective channel. This suggests that TCE activated not only K^{+} currents, but also other cationic currents that tended to shift E_{rev} to more positive values. It was, however, a priori unlikely that TCE would be totally specific for a particular ion channel. For example, TCE altered calcium handling in rat submandibular acinar cells, and inhibited *N*-methyl-D-aspartate receptors in neurons (Scheibler et al., 1999; Fischer et al., 2000; Pochet et al., 2002). Even though TCE apparently activated cation channels other than K^{+} channels, the net effect of TCE in the MCA was vasodilation, which is best explained by a predominance of K^{+} channel activation. The present results, however, are similar to the ~ 38 mV/decade slope we demonstrated previously in isolated rat MCA smooth muscle cells responding to 10^{-5} M AA (Bryan et al., 2006).

By use of the current-clamp mode to determine the membrane potential directly, we found that 10^{-2} M TCE hyper-

polarized MCA smooth muscle cells by 12 mV. Again, this is best explained by activation of a K^+ channel, because under the experimental conditions K^+ is the only ion whose equilibrium potential lay negative to the resting membrane potential. A hyperpolarization of 12 mV is large enough to cause substantial relaxation of the rat MCA (Knot and Nelson, 1998; Marrelli et al., 2003). Thus, these data support the conclusion that TCE dilates the MCA by stimulating a nonclassical K^+ channel.

We found that heat increased TCE-sensitive whole-cell currents 2-fold. Indeed, heating to 33°C in the presence of 10^{-3} M TCE elicited currents virtually identical to those stimulated by 10^{-2} M TCE at RT. This is exactly the type of enhanced response one might expect if two or more stimuli converged on a common target. In COS-7 cells overexpressing recombinant TRAAK, heating above 25°C stimulated TRAAK channel currents to reach a maximum activation at 42°C (Kang et al., 2005). These data, therefore, support the conclusion that TCE activated a thermosensitive nonclassical K^+ channel, possibly TRAAK, in isolated rat MCA smooth muscle cells. Although whole-cell patches of MCA smooth muscle cells became unstable above 33°C, it is possible that elevating the temperature beyond this point would have further increased TCE-sensitive currents.

Summary and Significance. Doses of CH that do not cause sedation can result in plasma TCE concentrations of $\sim 30 \mu\text{M}$, whereas sedative doses may elevate TCE into the millimolar range (Beland, 1999; Pochet et al., 2002; Merdink et al., 2008). Thus, stimulation of nonclassical K^+ channels to cause vasodilation may be clinically relevant. Although we are limited by a lack of specific pharmacology, and the current unavailability of TRAAK knockout mice, based on current knowledge of the ion channels expressed in vascular smooth muscle, and on the activation pattern of the channels stimulated by our interventions, it is possible that TRAAK may be capable of modulating the diameter of cerebral arteries.

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