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The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK α channel subunit in rodents

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17,18-Epoxyeicosatetraenoic acid (17,18-EETeTr) stimulates vascular large-conductance K^+ (BK) channels. BK channels are composed of the pore-forming BK α and auxiliary BK β1 subunits that confer an increased sensitivity for changes in membrane potential and calcium to BK channels. Ryanodine-sensitive calcium-release channels (RyR3) in the sarcoplasmic reticulum (SR) control the process. To elucidate the mechanism of BK channel activation, we performed whole-cell and perforated-patch clamp experiments in freshly isolated cerebral and mesenteric artery vascular smooth muscle cells (VSMC) from Sprague–Dawley rats, BK β1 gene-deficient (−/−), BK α (−/−), RyR3 (−/−) and wild-type mice. The 17,18-EETeTr (100 nM) increased tetraethylammonium (1 mM)-sensitive outward K^+ currents in VSMC from wild-type rats and wild-type mice. The effects were not inhibited by the epoxyeicosatrienoic acid (EET) antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (10 μM). BK channel currents were increased 3.5-fold in VSMC from BK β1 (−/−) mice, whereas a 2.9-fold stimulation was observed in VSMC from RyR3 (−/−) mice (at membrane voltage 60 mV). The effects were similar compared with those observed in cells from wild-type mice. The BK current increase was neither influenced by strong internal calcium buffering (Ca^{2+}, 100 nM), nor by external calcium influx. The 17,18-EETeTr did not induce outward currents in VSMC BK α (−/−) cells. We next tested the vasodilator effects of 17,18-EETeTr on isolated arteries of BK α-deficient mice. Vasodilatation was largely inhibited in cerebral and mesenteric arteries isolated from BK α (−/−) mice compared with that observed in wild-type and BK β1 (−/−) arteries. We conclude that 17,18-EETeTr represents an endogenous BK channel agonist and vasodilator. Since 17,18-EETeTr is active in small arteries lacking BK β1, the data further suggest that BK α represents the molecular target for the principal action of 17,18-EETeTr. Finally, the action of 17,18-EETeTr is not mediated by changes of the internal global calcium concentration or local SR calcium release events.

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Cytochrome P450 (CYP) enzymes can produce vasoactive metabolites of arachidonic acid (AA; 20:4 n-6) and, as demonstrated more recently, also of ω-3 long-chain polyunsaturated fatty acids (n-3 PUFA). Epoxyeicosatrienoic acids (EETs) are AA metabolites found in various vascular beds (Spector & Norris, 2006). The EETs are potent dilators of coronary, renal, cerebral and mesenteric arterioles (Ellis *et al.* 1990; Campbell & Harder, 1999; Fisslthaler *et al.* 1999; Ye *et al.* 2005) and may play a role in local regulation of blood flow and systemic blood pressure (Roman *et al.* 2000; Roman, 2002; Imig, 2006; Spector & Norris, 2006). Dietary fish oil or purified (n-3) long-chain polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA), have beneficial effects on vascular function (Connor, 2000; Simopoulos, 2003). The underlying mechanisms are incompletely

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understood (Lawson *et al.* 1991; Engler *et al.* 2000; Omura *et al.* 2001; Nyby *et al.* 2005). Eicosapentaenoic acid and other (n-3) PUFA possibly compete with AA for enzymatic conversion by CYP enzymes. This competition may lead to reduced formation of vasoactive AA metabolites (e.g. 20-epoxyeicosatrienoic acid (20-HETE), EETs) while alternative metabolites originating from EPA are increased. We showed earlier that rat cerebral artery vascular smooth muscle cells (VSMC) express the CYP isoforms 4A1 and 4A3 (Lauterbach *et al.* 2002). We found that both CYP 4A isoforms, which are known to metabolize AA (Nguyen *et al.* 1999), also accepted EPA as an efficient substrate. Cytochrome P450 4A1 showed the highest activity and produced 17,18-epoxyeicosatetraenoic acids (17,18-EETeTr), primarily 17(*R*),18(*S*)-EETeTr (Lauterbach *et al.* 2002). Similarly, Cyp4a12a, the mouse 20-HETE synthase, showed significant epoxygenase activity when converting EPA and produced 17(*R*),18(*S*)-EETeTr as a main metabolite (Muller *et al.* 2006). An additional potential source for 17(*R*),18(*S*)-EETeTr are endothelial CYP isoforms of the 2C and 2J subfamilies that otherwise produce EETs from AA (Fisslthaler *et al.* 1999; Nguyen *et al.* 1999; Zeldin, 2001; Barbosa-Sicard *et al.* 2005).

In patch clamp experiments on freshly isolated rat cerebral artery smooth muscle cells, we demonstrated that 17(*R*),18(*S*)-EETeTr stimulates the K^+ outward current. The effect was completely abolished by tetraethylammonium (TEA), but remained unaffected in the presence of 4-aminopyridine, showing the characteristics typical for BK channel activation (Lauterbach *et al.* 2002). Only the 17(*R*),18(*S*)-enantiomer was effective; the 17(*S*),18(*R*)-enantiomer was not. These findings suggested that 17(*R*),18(*S*)-EETeTr may be a novel hyperpolarizing factor in the vessel wall, targeting the BK channel. However, the mechanism of channel stimulation remained unknown and vasodilatory effects have not been demonstrated. We have now characterized the effects of 17,18-EETeTr on VSMC BK channels and arterial tone. The BK channels are composed of the pore-forming BK α and auxiliary BK β1 subunits that confer an increased sensitivity for changes in membrane potential and calcium to BK channels (Pluger *et al.* 2000; Lohn *et al.* 2001*b*). Ryanodine-sensitive calcium-release channels (RyR3) in the sarcoplasmic reticulum (SR) control the process (Lohn *et al.* 2001*a*). In VSMC, BK β1 deficiency produces an abnormal coupling between local Ca^{2+} signals, such as Ca^{2+} sparks, and BK channels (Pluger *et al.* 2000), whereas RyR3 deficiency produces an increased BK channel activity (Lohn *et al.* 2001*a*). The purpose of the present study was to determine the BK channel subunit requirements for the activity of 17,18-EETeTr on BK currents. Furthermore, we determined whether or not local Ca^{2+} release signals are involved in the principal actions of 17,18-EETeTr. We performed whole-cell and perforated-patch clamp experiments in freshly isolated VSMC from Sprague–Dawley rats, BK β1 gene-deficient (−/−) mice, RyR3 (−/−) mice and wild-type mice. We also performed vascular studies in isolated arteries from BK β1 (−/−) mice and from mice lacking the BK α pore-forming subunit (Sausbier *et al.* 2005).

Methods

Whole-cell and perforated-patch clamp experiments

All experiments were carried out according to the guidelines laid down by our institution's animal welfare committee and the government of Berlin. The requirements correspond to those of the American Physiological Society. Single VSMC were isolated from adult male Sprague–Dawley rat (250–300 g) basilar or posterior cerebral arteries, from second-order branches of the superior mesenteric artery of wild-type BK α (+/+) or BK β1 (+/+) mice and from basilar or posterior cerebral arteries of wild-type, BK β1 (−/−), BK α (−/−) and RyR3 (−/−) mice (age, 10–12 weeks; Gollasch *et al.* 1998; Pluger *et al.* 2000; Lohn *et al.* 2001*a*). Animals were decapitated under anaesthesia. If not otherwise indicated, K^+ channel currents were recorded in the perforated-patch configuration with amphotericin B (250 μg ml^{-1}) as previously described (Gollasch *et al.* 1996). In most cells, currents were recorded from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV or 500 ms step pulses to different potentials (holding potential, −80 mV). The bath solution (2 mmol l^{-1} $[Ca^{2+}]_o$) contained (in mmol l^{-1}): 6 KCl, 134 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes and 10 glucose (pH adjusted to 7.4 with NaOH). The patch pipette was filled with a solution containing (in mmol l^{-1}): 30 KCl, 110 potassium aspartate, 10 NaCl, 1 $MgCl_2$, 0.05 EGTA and 10 Hepes (pH adjusted to 7.2 with KOH). In some experiments, K^+ currents were recorded using the whole-cell configuration of the patch clamp technique. In these experiments, the pipette solution contained (in mmol l^{-1}): 30 KCl, 10 NaCl, 110 potassium aspartate, 1 $MgCl_2$, 10 EGTA, 6.14 $CaCl_2$ and 10 Hepes (pH adjusted to 7.2 with KOH; $[Ca^{2+}]_{free} = 0.1$ μM, using the program 'CaBuf' supplied by G. Droogman, Department of Physiology, KU Leuven, Belgium). The 0.1 μM $[Ca^{2+}]_o$ bath solution contained (in mmol l^{-1}): 6 KCl, 134 NaCl, 4 $MgCl_2$, 5 EGTA, 2.81 $CaCl_2$, 10 Hepes and 10 glucose (pH adjusted to 7.4 with NaOH; $[Ca^{2+}]_{free} = 0.1$ μM, using 'CaBuf'). Currents were recorded using an EPC 7 amplifier (List, Darmstadt, Germany), digitized at 5 kHz using a CED 1401 series interface (Cambridge Electronic Design Ltd, Cambridge, UK), and analysed using CED Patch and Voltage Clamp Software Version 6.08. All experiments were performed at approximately 22°C. All values are given

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as means ± S.E.M. Student's paired and unpaired *t* tests or non-parametric Wilcoxon tests were used as appropriate. $P < 0.05$ was considered statistically significant. The term '*n*' represents the number of cells tested.

Isolation and preparation of small isolated arteries

Wild-type, BK β1 (−/−) and BK α (−/−) mice (age, 10–12 weeks) were decapitated under anaesthesia followed by a mid-line laparotomy to expose and isolate the mesentery. Briefly, with the use of microscissors, forceps and an operating microscope (Nikon SMZ 654), second- and third-order branches of the superior mesenteric artery were removed from the mesenteric vascular bed and carefully cleaned of fat and surrounding connective tissue in ice-cold Krebs–Henseleit buffer solution (PSS) containing (mM): 119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1 Mg_2SO_4, 1.2 KH_2PO_4, 0.026 EDTA, 2.5 $CaCl_2$ and 11.1 dextrose, pH 7.4. Then, arterioles (200–260 μm in diameter, 1–2 mm in length) were mounted between two borosilicate glass micropipettes secured with 10–0 silk opthalmic suture in a 3 ml perfusion chamber. The inflow pipette was connected to a pressure servo-control (Living System Instrumentation Burlington, VT, USA), and the distal end was connected to a three-way stopcock and closed after removal of blood elements. The lumen of the vessel was filled with PSS through the micropipettes, and intraluminal pressure was maintained at 60 mmHg (no flow) superfused continuously with PSS saturated with 95% O_2 and 5% CO_2 at 37°C, pH 7.4 throughout experiments. These vessels contained intact endothelium and were allowed to equilibrate for 45–60 min before starting experiments. After equilibration, viability was confirmed by constriction with U46619, a thromboxane A_2 agonist, or 60 mM KCl, and acetylcholine (ACh). Vessels were discarded if they failed to constrict by 30–50% in response to 60 mM KCl or failed to dilate with ACh. The 17,18-EETeTr was added abluminally, and cumulative dose–response was determined with 3–5 min intervals between doses. Vessels were constricted by 30–60% of baseline diameter using U46619, and the vasodilator effects of 17,18-EETeTr were examined over a concentration range of 0.01–1.00 μM. Inner and outer vascular diameters were measured by video-microscopy using a microscope (Nikon Diaphot, Düsseldorf, Germany) connected to a PC with appropriate software for detection of changes in vessel inner and outer diameter (Lohn *et al.* 2001*a*,*b*). All values are given as means ± S.E.M. Data were compared with unpaired Student's *t* test or ANOVA ($P < 0.05$). The term '*n*' represents the number of arteries tested.

Chemicals

Racemic 17,18-EETeTr and 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE) were prepared according to published procedures (Gauthier *et al.* 2002; Lauterbach *et al.* 2002). U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), solubilized in ethanol as a 1 mM stock solution and stored at −20°C. All other drugs were obtained from Sigma Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Results

Effects of 17,18-EETeTr on rat cerebral artery BK currents and role of $[Ca^{2+}]$

We showed earlier that 17,18-EETeTr (100 nmol l^{-1}) stimulates BK currents in perforated-patch recordings, with 17(*R*),18(*S*)-EETeTr as the active enantiomer (Lauterbach *et al.* 2002). In the present study, we investigated the effects of 17,18-EETeTr using the whole-cell configuration of the patch clamp technique in order to explore a possible role of $[Ca^{2+}]_i$. This configuration allows 'clamping' of the $[Ca^{2+}]_i$ using the pipette solution (Gollasch *et al.* 1996). Control K^+ currents in whole-cell configuration with $[Ca^{2+}]_i$ 'clamped' at 100 nmol l^{-1} showed outward rectification with relatively large noise at membrane potentials positive to +60 mV (Figs 1*A*, *B* and *C*, control traces). The 17,18-EETeTr (100 nmol l^{-1}) induced large, relatively noisy, non-inactivating currents (Fig. 1*A*, $n = 6$). The reversal potential was not affected by 17,18-EETeTr and was approximately −80 mV. As reported for experiments in the perforated-patch configuration (Lauterbach *et al.* 2002), these currents were almost completely blocked by TEA (1 mmol l^{-1}, Fig. 1*A*) but relatively insensitive to the K_v channel blocker 4-aminopyridine (2 mmol l^{-1}), demonstrating that the outward K^+ current is mainly carried by BK channels (not shown, $n = 6$ each).

To test a possible involvement of Ca^{2+} influx through L-type channels (Marrion & Tavalin, 1998), we applied the calcium channel blocker verapamil (Hashimoto *et al.* 2006). Verapamil (50 μmol l^{-1}, $n = 5$) had no effect on the 17,18-EETeTr-induced BK channel current in perforated-patch recordings (Fig. 1*B*). Similar effects were observed with nimodipine (1 μM, $n = 2$, not shown). In another set of whole-cell recordings ($n = 5$), we tested the effects of 17,18-EETeTr on BK currents in the presence of different values of extracellular $[Ca^{2+}]$ (Fig. 1*C*). The free $[Ca^{2+}]_i$ was fixed at 100 nmol l^{-1} by the pipette solution. After the typical current increase induced by 17,18-EETeTr (100 nmol l^{-1}) was observed (Fig. 1*C*, left panel), we replaced the 2 mmol l^{-1}-containing external bath solution by a 100 nmol l^{-1}-containing bath solution (Fig. 1*C*, right panel). This manoeuvre had no effect on the 17,18-EETeTr-induced BK channel current. These results suggest that neither L-type channels nor other Ca^{2+} influx pathways are involved in BK channel stimulation by 17,18-EETeTr.

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Furthermore, the effects of 17,18-EETeTr were not reduced by pretreatment of cells with the epoxyeicosatrienoic acid antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE, 10 μM, Fig. 2). In these experiments, 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ current amplitude 2.4-fold at +60 mV in untreated cells ($n = 6$, Fig. 2*A*), whereas it increased the K^+ current amplitude 2.6-fold at +60 mV in 14,15-EEZE-pretreated cells ($n = 6$, Fig. 2*B* and *E*). The 14,15-EEZE had no effect on control BK currents ($n = 12$); however, the stimulatory effect of 11,12-epoxyeicosatrienoic acid (11,12-EET)> (100 nM) on BK currents (Fig. 2*C*) was completely abolished by pretreatment of cells with 14,15-EEZE (10 μM, Fig. 2*D*; current increase by a factor of 1.11 ± 0.05 at +60 mV, $n = 5$; n.s.). These findings rule out a major role of receptors for epoxyeicosatrienoic acid (Gauthier *et al.* 2002) in the action of 17,18-EETeTr on BK channels.

Effects of 17,18-EETeTr on mouse BK β1 (−/−) and RyR3 (−/−) VSMC

Wild-type, BK β1 (−/−) and RyR3 (−/−) cerebral artery VSMC exhibited large BK channel currents with outward rectification and relatively large noise at membrane potentials positive to +60 mV (Fig. 3*A*–*C*, control traces; Pluger *et al.* 2000; Lohn *et al.* 2001*a*). The current amplitudes were not different among wild-type, BK β1 (−/−) and RyR3 (−/−) cells. Addition of 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ outward current amplitude 3.4-fold at +60 mV in wild-type VSMC (Fig. 3*A* and *D*). In BK β1 (−/−) VSMC, 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ current amplitude 3.5-fold at +60 mV ($n = 5$, Fig. 3*B*). The reversal potential was not affected by 17,18-EETeTr and was approximately −80 mV. The stimulatory effect was not statistically

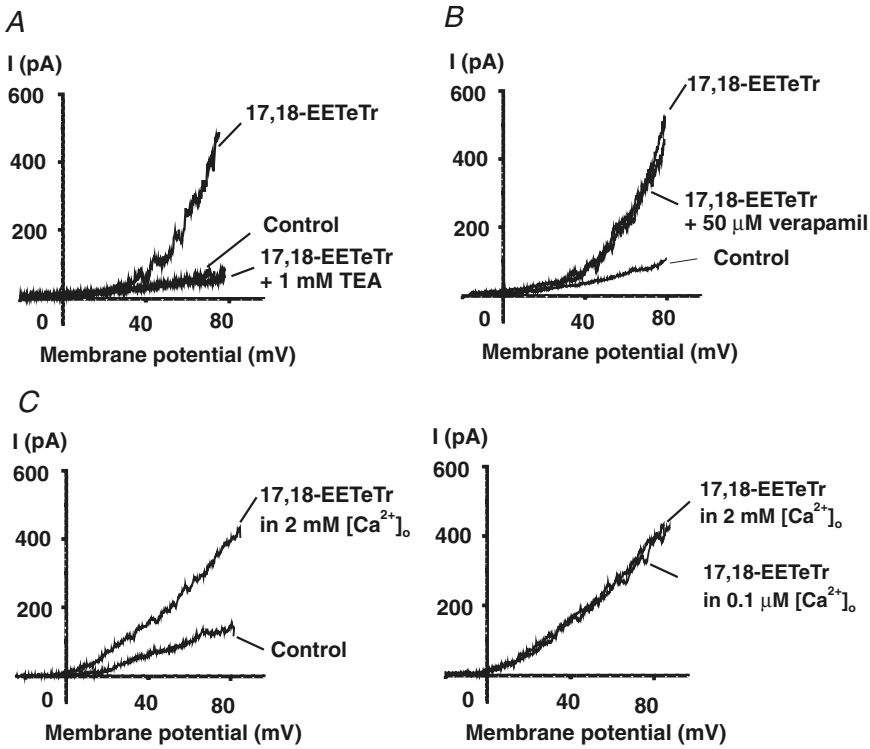

Figure 1. Stimulatory effects of 17,18-EETeTr on rat cerebral artery BK channel currents
A, currents were recorded before (control), after application of 100 nmol l^{-1} 17,18-EETeTr (40 min) and after application of 1 mmol l^{-1} TEA (+5 min) in the presence of 100 nmol l^{-1} 17,18-EETeTr in a representative cell. Currents were recorded in the whole-cell configuration from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV. *B*, currents were recorded before (control), after application of 100 nmol l^{-1} 17,18-EETeTr (40 min) and after application of 50 μmol l^{-1} verapamil (+5 min) in the presence of 100 nmol l^{-1} 17,18-EETeTr in a representative cell. Currents were recorded in the perforated-patch configuration from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV. *C*, the BK current increase was neither influenced by strong internal calcium buffering (Ca^{2+}, 100 nM) nor by external calcium influx. Currents were recorded in the whole-cell configuration from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV in a representative cell. The cell was dialysed with the pipette solution containing 0.1 μM free [Ca^{2+}]. In the left panel, currents were recorded before (control) and after application of 100 nmol l^{-1} 17,18-EETeTr (40 min) in a representative cell. In the right panel, the 17,18-EETeTr-induced K^+ current of the cell shown in the left panel was not changed after replacement of the external bath solution with a bath solution equalizing the external and internal free [Ca^{2+}] to 0.1 μM.

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different compared with wild-type cells ($n = 6$), suggesting that BK α but not the auxiliary BK β1 subunit is the target of principal action of 17,18-EETeTr. In accord, 17,18-EETeTr (100 nmol l^{-1}) did not increase the K^+ current amplitude in cerebral artery VSMCs of BK α (−/−) mice, compared with VSMCs of littermate wild-type mice (Fig. 4*A–C*, $n = 4$ each). In RyR3 (−/−) cerebral artery cells, 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ current amplitude 2.9-fold at +60 mV ($n = 5$, Fig. 3*C* and *D*). The reversal potential was not affected by 17,18-EETeTr and was approximately −80 mV. The stimulatory effect was not different compared with wild-type cells ($n = 6$),

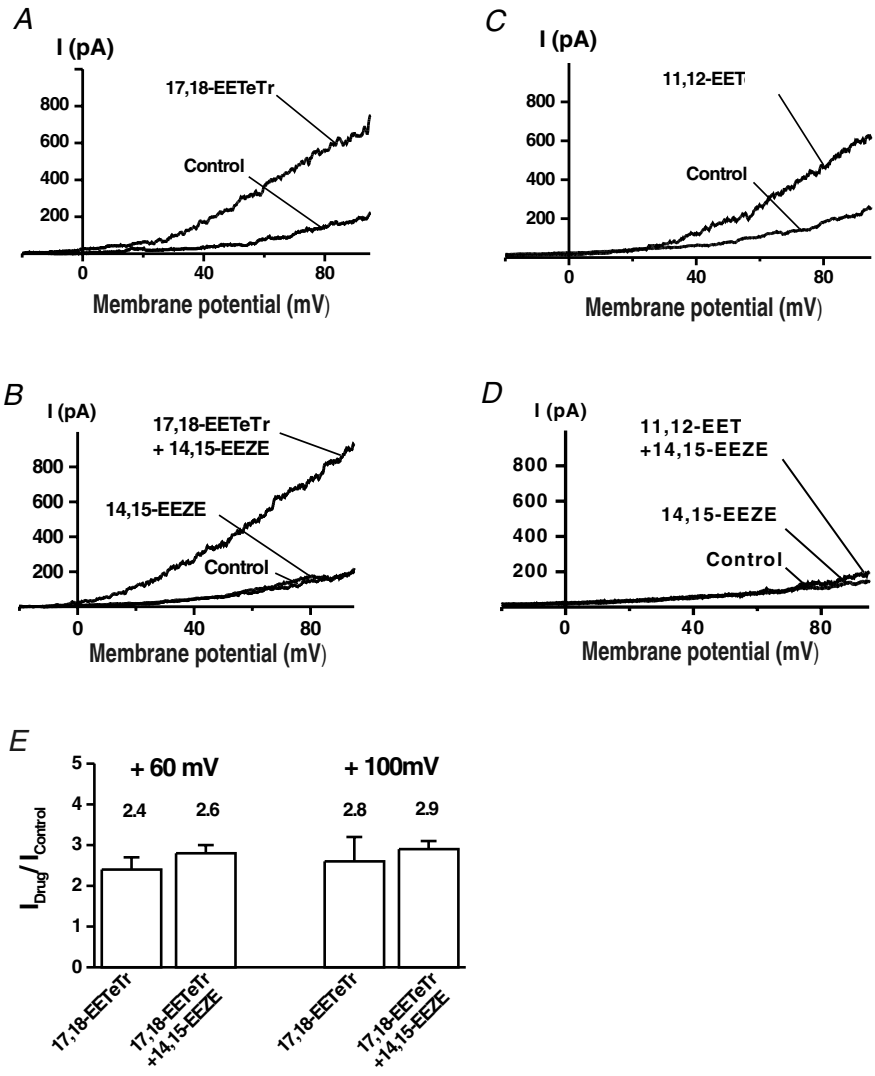

Figure 2. 14,15-EEZE inhibits the effects of 11,12-EET but not of 17,18-EETeTr on BK channel currents in rat cerebral artery

A, currents were recorded before (control) and after application of 100 nmol l^{-1} 17,18-EETeTr (40 min) in a representative cell. *B*, currents were recorded before (control), after application of 10 μmol l^{-1} 14,15-EEZE (5 min) and after application of 100 nmol l^{-1} 17,18-EETeTr (+40 min) in the presence of 10 μmol l^{-1} 14,15-EEZE in a representative cell. Currents were recorded in the perforated-patch configuration from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV. *C*, currents were recorded before (control) and after application of 100 nmol l^{-1} 11,12-EET (40 min) in a representative cell. *D*, currents were recorded before (control), after application of 10 μmol l^{-1} 14,15-EEZE (5 min) and after application of 100 nmol l^{-1} 11,12-EET (+ 40 min) in the presence of 10 μmol l^{-1} 14,15-EEZE in a representative cell. Currents were recorded in the perforated-patch configuration from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV. *E*, data show the quotient of currents after 35–50 min of 17,18-EETeTr application and before application in the absence or presence of 14,15-EEZE at +60 mV (left bars) and at +100 mV (right bars). The 14,15-EEZE did not affect the voltage-independent stimulatory effect of 17,18-EETeTr on BK channel currents. Currents were recorded from a holding potential of −40 mV during linear voltage ramps at 0.63 V s^{-1} from −100 to +100 mV. The concentrations of 17,18-EETeTr and 14,15-EEZE were 100 nmol l^{-1} and 10 μmol l^{-1}, respectively.

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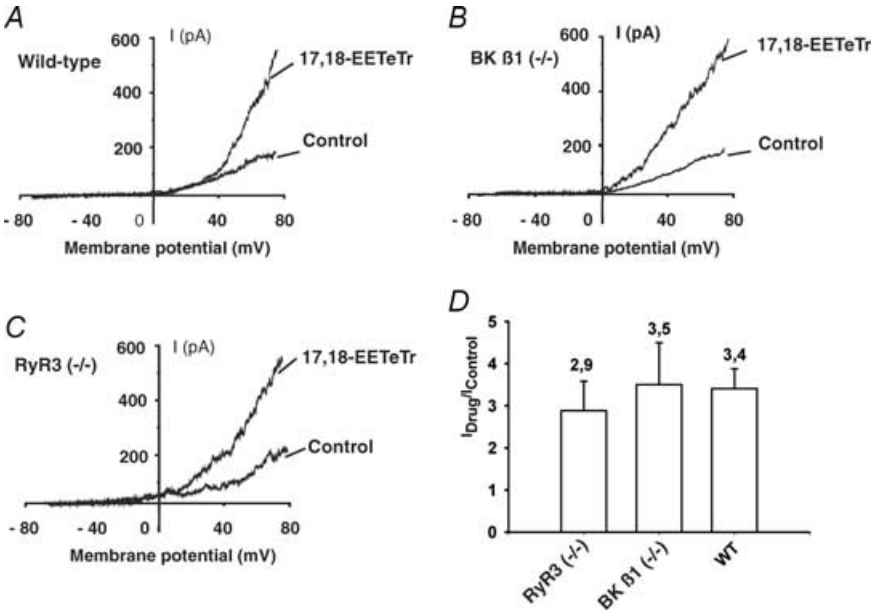

Figure 3. The effects of 17,18-EETeTr on mouse cerebral artery BK channel currents are independent of BKβ1 and RyR3
Currents were recorded in wild-type (*A*), BK β1 (−/−) (*B*) and RyR3 (−/−) cells (*C*). *A*–*C*, currents were recorded before (control) and after application of 100 nmol l^{-1} 17,18-EETeTr (40 min) in representative cells. *D*, data show the quotient of currents after 35–50 min of drug application and before application at +60 mV. Currents were recorded from a holding potential of −40 mV during linear voltage ramps at 0.53 V s^{-1} from −80 to +80 mV. The concentration of 17,18-EETeTr was 100 nmol l^{-1}. The solvent ethanol concentration was $\leq$ 0.1%; the effects were not different compared with control conditions. WT, wild-type.

suggesting that 17,18-EETeTr-mediated stimulation of BK channels is independent of local SR Ca^{2+} release controlled by RyR3 in cerebral artery smooth muscle cells.

Figure 4*D* shows that 17,18-EETeTr increased the K^+ current amplitude in mesenteric artery VSMCs of wild-type mice (2.2 ± 0.6-fold increase by 1 μM 17,18-EETeTr in 7 of 11 cells at +100 mV). The 17,18-EETeTr-induced current was inhibited by 1 mM TEA ($n = 4$).

Effects of 17,18-EETeTr in pressurized arteries of BK α (−/−), BK β1 (−/−), and wild-type mice

To further determine the significance of the activity of BK channel subunits, we tested the vasodilatory effects of 17,18-EETeTr in second- and third-order mesenteric arteries of BK α (−/−), BK β1 (−/−) and wild-type mice pressurized to 60 mmHg. The arteries were preconstricted with U46619. The 17,18-EETeTr produced dose-dependent vasodilatations (Fig. 5). At 0.01, 0.1 and 1 μM,

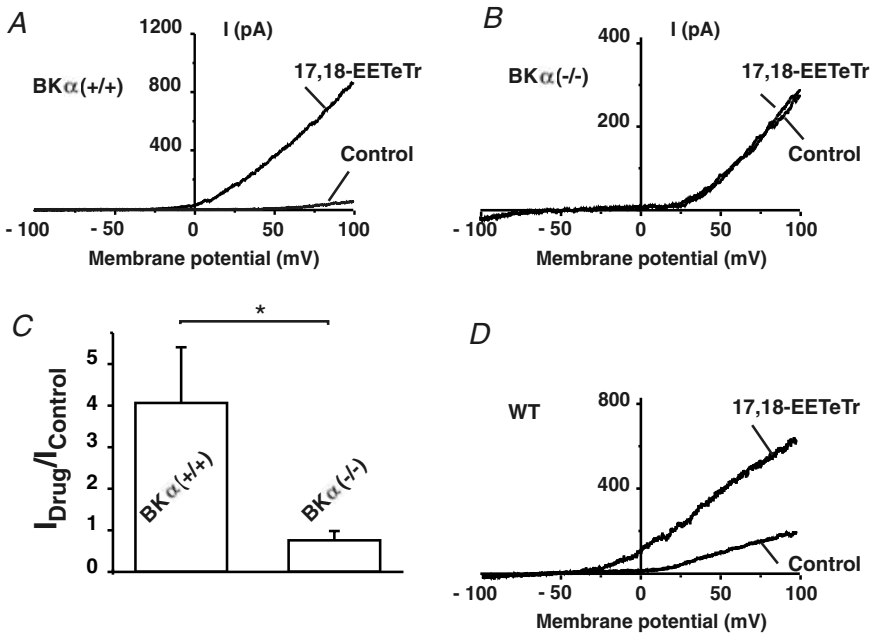

Figure 4. Lack of stimulatory effect of 17,18-EETeTr on outward K^+ currents in BK α (−/−) VSMCs
A and *B*, currents were recorded in cerebral artery BK α (+/+) and BK α (−/−) VSMCs. The holding potential was −40 mV. Linear voltage ramps at 0.53 V s^{-1} from −100 to +100 mV were applied. Currents were recorded before (control) and after application of 100 nmol l^{-1} 17,18-EETeTr (40 min). *C*, data show the quotient of currents after 35–50 min of drug application and before application at +100 mV. *D*, currents were recorded in a representative mesenteric artery wild-type cell during linear voltage ramps at 0.53 V s^{-1} from −100 to +100 mV from a holding potential of −40 mV. Currents were recorded before (control) and after application of 1 μmol l^{-1} 17,18-EETeTr (40 min).

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17,18-EETeTr induced vasodilatation in BKβ1 (−/−) and wild-type arteries (Fig. 5*A*, *C* and *D*). The corresponding relaxations were 32.4 ± 8.6, 41.3 ± 7.6 and $57.7 \pm 9.2\%$ ($n = 6$), and 16.6 ± 2.5, 31.2 ± 2.9 and $38.3 \pm 4.3\%$ ($n = 4$), respectively. However, vasodilatation was almost completely absent in BK α (−/−) arteries; 17,18-EETeTr at 0.01, 0.1 and 1 μM produced vasodilatation by -0.2 ± 4, 11.4 ± 3.7 and $14 \pm 5.4\%$, respectively ($n = 4$, Fig. 5*B*, *C* and *D*). Similar results were obtained in cerebral arteries (Fig. 5*E*). These results indicate that the BK α subunit is crucial in 17,18-EETeTr-induced vasodilatation.

Discussion

We demonstrated previously that 17,18-EETeTr was a potent vasodilator and stimulated the outward BK channel current in VSMCs of rats (Lauterbach *et al.* 2002) and mice. Only the 17(*R*),18(*S*)-enantiomer was effective, while the 17(*S*),18(*R*)-enantiomer was not (Lauterbach *et al.* 2002). 17,18-Epoxyeicosatetraenoic acid shares these properties with 11,12-epoxyeicosatrienoic acid, the compound that has been proposed as endothelium-derived hyperpolarizing factor (EDHF) in a number of vascular beds (Campbell & Harder, 1999; Fisslthaler *et al.* 1999; Quilley & McGiff, 2000; Roman *et al.* 2000) and other regioisomeric epoxides derived from n-3 PUFAs in coronary arterioles (Zhang *et al.* 2001; Ye *et al.* 2002). Our findings suggest that 17,18-EETeTr may be a novel hyperpolarizing factor in the vessel walls of rats, mice and probably other mammals. The effects of 17,18-EETeTr were not affected by 14,15-EEZE, suggesting receptor signalling mechanisms distinct from AA epoxides (Gauthier *et al.* 2002) and a number of other fatty acids that have been supposed to directly bind and interact with the BK channel protein (Denson *et al.* 2000). The BK channel stimulation is independent of intracellular calcium and

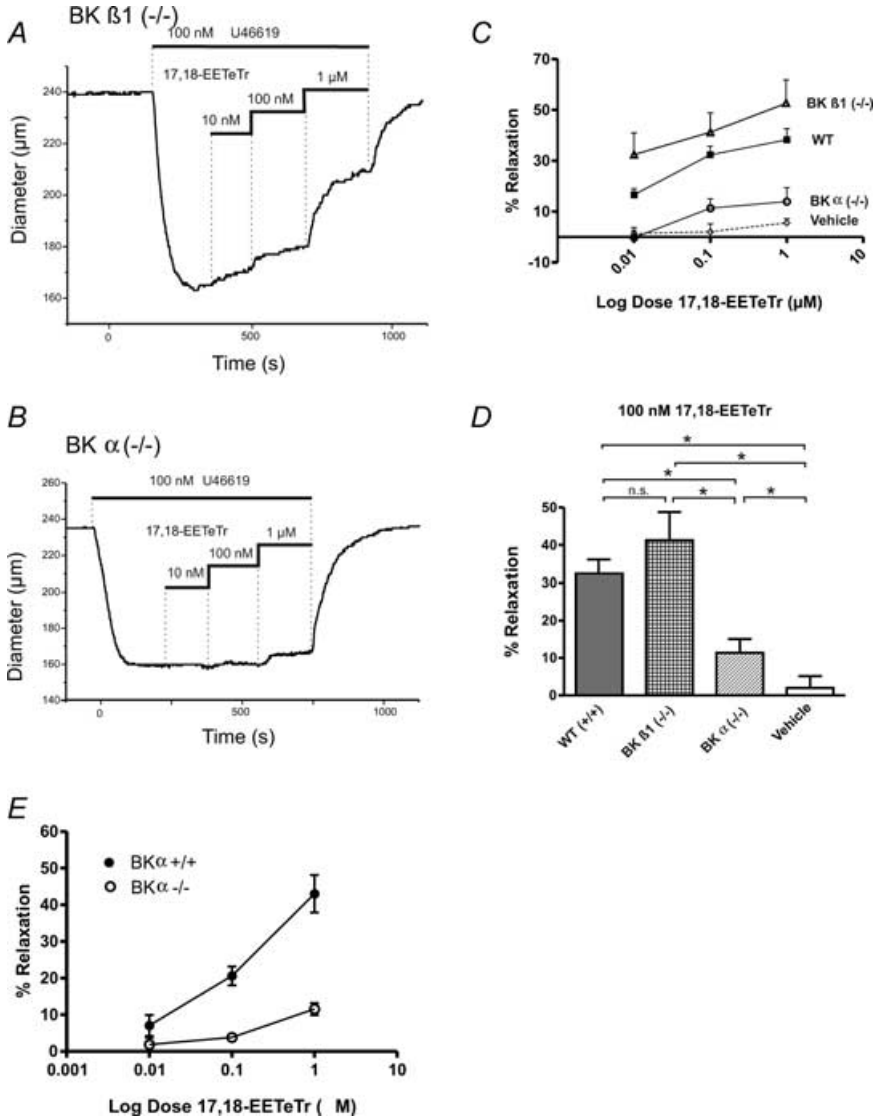

Figure 5. Vascular effects of 17,18-EETeTr on mesenteric arteries of BK α (−/−), BK β1 (−/−) and wild-type mice (*A–D*) and on cerebral arteries of of BK α (−/−) and wild-type mice (*E*)
Vasodilator responses to 17,18-EETeTr in vessels preconstricted 30–60% of baseline diameter with U46619 (100 nM) were tested. *A* and *B*, representative traces of changes in diameter following application of 17,18-EETeTr at 0.01, 0.1 and 1 μM in arteries of BK β1 (−/−) mice (*A*) and BK α (−/−) (*B*). Traces are representative of 5–6 observations per group. *C*, dose–response relationship for 17,18-EETeTr at 0.01, 0.1 and 1 μM. Vasodilatation in response to 17,18-EETeTr was significantly reduced in BK α (−/−) but not in BK β1 (−/−) and wild-type (WT) arteries. *D*, percentage vasodilatation in response to 17,18-EETeTr at 100 nM in BK α (−/−), BK β1 (−/−) and wild-type arteries, compared with vehicle control. $^*P < 0.05$. *E*, dose–response relationship for 17,18-EETeTr at 0.01, 0.1 and 1 μM. Vasodilatation in response to 17,18-EETeTr was significantly reduced in BK α (−/−) ($n = 3$) compared with wild-type BK α (+/+) arteries ($n = 5$).

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may be explained by direct activation of the pore-forming BK α channel subunit.

To study the role of intracellular Ca^{2+} concentration, we analysed the effects of 17,18-EETeTr on BK channels in strongly buffered cells with 100 nmol l^{-1} $[Ca^{2+}]_i$ using the whole-cell configuration of the patch clamp technique. We found that 17,18-EETeTr was able to stimulate BK channel currents under these conditions, suggesting that the BK current increase is not mediated by an elevation of intracellular Ca^{2+} concentration. We also showed that the 17,18-EETeTr-induced BK current was not inhibited by the calcium channel blocker verapamil. The current increase was not affected by replacement of the Ca^{2+}-containing external solution with a solution having a Ca^{2+} concentration that was equal to the intracellular Ca^{2+} concentration, i.e. 100 nmol l^{-1}. Thus, our data suggest that stimulation of BK channels by 17,18-EETeTr is independent of Ca^{2+} influx.

Next, we studied the putative role of intracellular SR calcium signals that control BK channel activity via local, subcellular Ca^{2+} release events such as calcium sparks and other unitary events (Gollasch *et al.* 1998; Lohn *et al.* 2001*a*). The RyR3 is part of the SR calcium release unit and is required to tune the release of SR calcium signals specifically to the needs of arterial smooth muscle cells, thereby enabling BK channel regulation of arterial tone (Lohn *et al.* 2001*a*). Interestingly, several vasodilators and second messenger pathways have been identified as using local SR Ca^{2+} release to activate BK channels (Bychkov *et al.* 1998). To test a putative role of local SR Ca^{2+} release in the stimulatory action of 17,18-EETeTr on BK channels, experiments were conducted in RyR3 (−/−) cells, which lack ryanodine type 3 receptors (Lohn *et al.* 2001*a*). The 17,18-EETeTr was found to stimulate BK channel currents in RyR3 (−/−) cells. The current stimulation was not significantly different from that observed in wild-type cells. Thus, our data suggest that local Ca^{2+} release signals are not involved in the principal action of 17,18-EETeTr.

We determined the BK channel subunit requirements for the activity of 17,18-EETeTr on BK currents. These experiments were conducted in BK β1 (−/−) cells, which lack the auxiliary β subunit of vascular BK channels (Pluger *et al.* 2000). As reported previously, the BK α subunit in BK β1 (−/−) arterial VSMC is functional, and the cells exhibit voltage-dependent outward K^+ currents. In symmetrical 140 mM K^+ solution, lack of BK β1 decreases the apparent Ca^{2+}/voltage sensitivity of BK channels (Brenner *et al.* 2000). However, a detailed analysis in physiological solutions has not been performed. Nonetheless, these currents were also sensitive to 17,18-EETeTr. In contrast, 17,18-EETeTr did not induce outward K^+ currents in BK α (−/−) VSMC. Therefore, the BK α subunit probably represents the molecular target for the principal action of 17,18-EETeTr. This suggestion is based on two considerations. First, 17,18-EETeTr stimulates currents through BK α subunits with a factor that was not significantly different from that found with multisubunit, complex BK channels. Second, the stimulatory effect was independent of intracellular and extracellular Ca^{2+} changes, in agreement with the biophysical properties of pure BK α channels in the absence of BK β1. How 17,17-EETeTr might stimulate the BK α subunit has not been characterized. Possibly, the hydrophobicity of 17,18-EETeTr causes binding at the channel entrances or at a transmembrane segment in the plasma membrane. In this respect, patch clamp experiments suggested that several unsaturated free fatty acids may bind to the BK channel directly (Denson *et al.* 2000). However, since the epoxyeicosatrienoic acid antagonist 14,15-EEZE had no effect on 17,18-EETeTr-dependent channel stimulation, we believe that 17,18-EETeTr does not use the same putative binding site or signalling molecules mediating epoxyeicosatrienoic acid-induced activation of the BK channels, such as G_s proteins and cyclic adenosine diphosphate-ribose (cADPR) (Li *et al.* 2002). Although the present study clearly indicates that BK α represents the molecular target for the principal action of 17,18-EETeTr, it remains unknown whether or not 17,18-EETeTr binds directly to BK α or activates it via a signalling pathway after binding to a specific receptor. Further studies are needed to clarify this point.

Finally, we evaluated the vasodilatory effects of 17,18-EETeTr with respect to presence or absence of BK α or BK β1 subunits. In both wild-type and BK β1 (−/−) arteries, 17,18-EETeTr induced similar dose-dependent dilatations. However, in BK α (−/−) vessels, the vasodilator effects of 17,18-EETeTr were markedly reduced, suggesting that the α subunit is required for activation. The fact that we observed some residual vasodilatation in the BK α (−/−) vessels may indicate heterogeneity of mechanisms associated with EETs; although here we report that the main molecular target for 17,18-ETeTr is the BK α subunit. The residual dilatation may be through ATP-sensitive K^+ (K_{ATP}) channels (Ye *et al.* 2005), which play important roles in the regulation of vascular tone (Nelson & Brayden, 1993; Standen & Quayle, 1998; Miki *et al.* 2002).

Elucidation of the role of 17,18-EETeTr as an *in vivo* modulator of ion channels and as a novel candidate for EDHF may provide new insight into the control of blood vessel function under physiological and pathophysiological conditions. A diet rich in EPA could shift the profile of CYP-dependent metabolites to an increased formation of alternative CYP metabolites such as 17,18-EETeTr.

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