EETs relax airway smooth muscle via an EpDHF effect: BK$_{Ca}$ channel activation and hyperpolarization

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Benoit, Catherine, Barbara Renaudon, Dany Salvail, and Eric Rousseau. EETs relax airway smooth muscle via an EpDHF effect: BK$_{Ca}$ channel activation and hyperpolarization. Am J Physiol Lung Cell Mol Physiol 280: L965–L973, 2001.—Epoxyeicosatrienoic acids (EETs) are produced from arachidonic acid via the cytochrome P-450 epoxygenase pathway. EETs are able to modulate smooth muscle tone by increasing K$^+$ conductance, hence generating hyperpolarization of the tissues. However, the molecular mechanisms by which EETs induce smooth muscle relaxation are not fully understood. In the present study, the effects of EETs on airway smooth muscle (ASM) were investigated using three electrophysiological techniques. 8,9-EET and 14,15-EET induced concentration-dependent relaxations of the ASM precontracted with a mucuscine agonist (carbamylcholine chloride), and these relaxations were partly inhibited by 10 nM iberiotoxin (IbTX), a specific large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel blocker. Moreover, 3 μM 8,9- or 14,15-EET induced hyperpolarizations of −12 ± 3.5 and −16 ± 3 mV, with EC$_{50}$ values of 0.13 and 0.14 μM, respectively, which were either reversed or blocked on addition of 10 nM IbTX. These results indicate that BK$_{Ca}$ channels are involved in hyperpolarization and participate in the relaxation of ASM. In addition, complementary experiments demonstrated that 8,9- and 14,15-EET activate reconstituted BK$_{Ca}$ channels at low free Ca$^{2+}$ concentrations without affecting their unitary conductance. These increases in channel activity were IbTX sensitive and correlated well with the IbTX-sensitive hyperpolarization and relaxation of ASM. Together these results support the view that, in ASM, the EETs act through an epithelium-derived hyperpolarizing factorlike effect.

The presence of CYP450 epoxygenase and EET production in epithelial and Clara cells of the airways has been verified (11). Recently, Scarborough et al. (35) also reported the expression of a CYP2J2 isoform in non-Clara cells from human and rat lung epithelium. Investigation of the possible roles of EETs in airway smooth muscle (ASM) revealed that 5,6- and 8,9-EET relax precontracted rabbit bronchial tissues (43) and can directly activate BK$_{Ca}$ channels (12) and inhibit Cl$^-$-selective channels (33). Despite this, few studies have focused their attention on the efficiency of EETs as relaxing agents in the vascular system (32).

Despite the reported presence of cytochrome P-450 monooxygenases in liver (21, 22, 39), kidney (21, 31), and lung (11, 23, 43) of many species such as human, rabbit, mouse, hamster, and guinea pig, the role of the third AA pathway is less understood. This pathway involves ω-hydroxylase and the NADPH-dependent epoxyeicosatrienoic enzymes, which respectively produce hydroxyeicosatetraenoic acids (HETEs) and the four epoxyeicosatrienoic acid regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET) (8, 9, 19, 28, 36). The physiological roles and modes of action of these bioactive compounds have yet to be fully elucidated. Recent studies in vascular smooth muscle (VSM) have shown that EETs can dilate renal, cerebral, and coronary arteries by acting as an endothelium-derived relaxant factor (EDRF) (7, 16, 29, 30, 40). The four EET regioisomers produce vasorelaxation by causing membrane potential hyperpolarization in VSM cells (3, 13, 14). Hyperpolarizing effects do not involve either COX or NOS-dependent pathways but rather CYP450 epoxyeicosatrienoic acid metabolites (15). Indeed, there is good evidence that these effects could be mediated in the vascular bed by an EET-dependent mechanism. The activation of large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channels by the EETs (17) reveals their possible roles as an endothelium-derived hyperpolarizing factor (EDHF).

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reports demonstrating variations in the cellular responses to the different isomers (44), the rationale for the present work was based on the observations made by this laboratory that the EETs might have a different order of potency in the airways than in the vascular bed (6).

This study was aimed at assessing the electrophysiological effects of EETs on ASM at the tissue, cellular, and molecular levels. Three complementary approaches were used: 1) tension measurements of relaxation on epithelium-denuded guinea pig ASM precontracted with a muscarinic agonist, 2) membrane potential measurements using the classical microelectrode technique and quantification of the pharmacological effects of the EETs, and 3) analyses of the direct activating effects of two EETs on unitary BKCa channels reconstituted into planar lipid bilayer. We demonstrate that 8,9- and 14,15-EET induced concentration-dependent and iberiotoxin (IbTX)-sensitive relaxations as well as a hyperpolarization of the resting membrane potential of ASM from tracheae of rabbits. These effects were correlated with a direct activation of BKCa channels from ASM cells as shown by single-channel analysis.

MATERIALS AND METHODS

Tension measurements. Albino guinea pigs (Hartley, weighing 300–350 g) were anesthetized with a lethal dose of pentobarbital sodium (50 mg/kg). The trachea and lungs were quickly removed and placed in an oxygenated (95% O2-5% CO2) Krebs-bicarbonate solution at room temperature. The trachea was rapidly removed and placed in an oxygenated (95% O2-5% CO2) Tyrode solution at room temperature (22°C), pH 7.4. The tissues were quickly removed and placed in an oxygenated Tyrode solution for several hours. The tracheal smooth muscle was then cut into longitudinal and 5 mm wide. The strips were affixed with the ASM facing up in the middle chamber (capacity 3 ml) of a tricompartament system in which the temperature (37°C) and solution level were strictly controlled as previously described (33, 36). The tissues were perfused at a constant flow rate of 2 ml/min with standard (or modified whenever needed) Tyrode solution and were allowed to equilibrate for 20 min followed by another 20 min with 5 μM wortmannin to prevent smooth muscle cell contractures at the time of impalement. Nonexperimental tissues were conserved at room temperature in oxygenated Tyrode solution for several hours. The transmembrane potential was measured by ASM cell impalement from the luminal side using conventional intracellular borosilicate microelectrodes filled with 3 M KCl. Their electrical resistance ranged from 20 to 50 MΩ. The microelectrodes were connected via a Ag-AgCl2 pellet to the head stage of an amplifier mounted on a Narishige No13004 micromanipulator. Measurements were performed with a KS-700 amplifier from World Precision Instruments. Electrical signals were continuously monitored on an oscilloscope (Tektroniks, TDS 310). The membrane potentials were recorded using a Digitdata 1200B interface and Axoscope 7.0 software (Axon Instruments). Data were stored on disk for further analysis.

Preparation of tracheal smooth muscle microsomal fractions and channel reconstitution. Preparation of bovine crude ASM microsomal fractions and planar lipid bilayers (PLB) was carried out exactly as described previously (12, 34). Two muscle strips were cut, denuded of epithelium by facing the ASM luminal face of the trachea. The epithelial cells were mechanically removed. Each tissue was placed in a 4-ml jacketed chamber, which was connected to the head stage of voltage-clamp amplifier (model 8900; Dagan, Minneapolis, MN), whereas the trans chamber was connected to virtual ground, both by means of low-resistance electrodes (MERE 2; World Precision Instruments, Sarasota, FL). To facilitate the fusion, the experimental chambers contained the following solution (in mM) 250 KCl cis-50 KCl trans plus 20 K-HEPES and 0.01 free Ca++ (109 μM CaCl2 + 100 μM K-EGTA), pH 7.4. However, most current traces were recorded in symmetrical condition, if not specified otherwise. The unitary currents were filtered at 1 kHz and recorded on videotape (DAS/VR 900 Toshiba, Unidrome) and then digitized at 4 kHz for storage in 3-min files on hard disk. All reconstitution studies were performed at a room temperature (22 ± 2°C). BKCa channel activities were analyzed in terms of current amplitudes and channel open probability (Po) values with pCLAMP 6 (Axon instruments).

Drugs and chemical reagents. CCh, IbTX, and wortmannin were all purchased from Sigma (St Louis, MO). 5,6-EET dissolved in 100% acetonitrile (ACN) and 11,12-, 8,9- and 14,15-EET dissolved in 100% ethanol were purchased from Cayman Chemical (Ann Arbor, MI) and stored as 312 μM stock solutions. All phospholipids were obtained from Avanti Polar Lipids (Albaster, AL). For microelectrode measurements, EETs and IbTX were dissolved in Tyrode solution at the required concentration, and wortmannin was dissolved and stored in DMSO. For each experimental procedure, the vehicles were tested separately at the maximal concentration and stored in DMSO. For each experimental procedure, the vehicles were tested separately at the maximal concentration and stored in DMSO. All reconstitution studies were performed at a room temperature (22 ± 2°C). BKCa channel activities were analyzed in terms of current amplitudes and channel open probability (Po) values with pCLAMP 6 (Axon instruments).

Data analysis and statistics. The concentration-response curves were fitted to the Hill equation

\[ y = \frac{M_{\text{ref}} + \left|M_{\text{max}} - M_{\text{ref}}\right|}{1 + EC_{50}^{x \times n_H}} \]

where \(M_{\text{ref}}\) is the reference membrane potential under control condition, \(M_{\text{max}}\) is the maximal membrane potential under a specific compound, \(x\) is the concentration tested, \(EC_{50}\) is the concentration that produces half of the maximal effect, and \(n_H\) is the Hill coefficient. Results are expressed as means ± SE. Statistical analyses were performed using un-
paired Student’s t-tests. Values of $P < 0.05$ were considered significant. $N$ represents the number of tissues obtained from different rabbits and $n$ indicates the number of impalements. Bar histograms and curve fittings were performed using Sigma Plot 4.0 program from SPSS-Science (Chicago, IL).

RESULTS

Relaxing effect of EETs. Epithelium-denuded guinea pig bronchial smooth muscle spirals were used to assess the relative and putative effects of the four EETs on ASM tension. The tissues were initially precontracted with 0.2 μM CCh, and once the plateau phase was reached, cumulative concentrations of EETs were added. As shown in Fig. 1, all EET regioisomers had relaxing effects, with 5,6-EET being the most potent isomer. However, since the EETs were dissolved in either ACN (5,6-EET) or ethanol (8,9-, 11,12-, and 14,15-EET) and considering the concentration-dependent effect of ACN (Fig. 1, inset), the specific effect of the 5,6-EET was relatively small after the solvent effect was subtracted. Hence 8,9- and 14,15-EET exhibited greater net relaxing effects, inducing relaxation of 42% and 82% at the maximal 10 μM concentration tested (Fig. 1, solid symbols).

IbTX sensitivity of EET-induced relaxations. Figure 2A shows the effects of pretreating the bronchial strips with 10 nM IbTX, a specific blocker of BK Ca channels on ASM responsiveness to EETs. The ASM spirals were pretreated with 10 nM IbTX before muscarinic stimulation (CCh), which resulted in a significant inhibition of the EET-induced relaxation. More specifically, the relaxations induced by 3 μM 8,9-, 11,12-, and 14,15-EET were reduced by 45, 45, and 47%, respectively (Fig. 2B). Note that IbTX had partial (17%) inhibitory effects on the relaxation induced by 3 μM 5,6-EET. The IbTX sensitivity of the relaxations induced by the other EET regioisomers appeared very similar, which strongly supports the role of BK Ca channels in the control of the relaxation process induced by EETs. Thus the present results confirm and complement the observations reported previously on the same preparation (12).

Effect of EETs on ASM membrane potential. The effects of 8,9- and 14,15-EETs were tested on membrane potential. Impaled microelectrodes were used with tracheal epithelium-denuded rabbit ASM. Because of tissue vibrations, cell depolarization, and/or rupture on impalement, continuous recordings were very difficult to obtain and a multi-impalement protocol consisting of repetitive penetrations in single ASM cells was designed. In control conditions, the experimental chamber was continuously perfused with normal Tyrode solution (37°C), and the mean membrane potential of epithelium-denuded cells was measured at $-50 \pm 1.7$ mV. The addition of 8,9-EET consistently induced a hyperpolarization of the membrane potential (Fig. 3A). Concentration-response curves (Fig. 3B) were obtained on cumulative addition of EETs (0.01–5 μM); 8,9- and 14,15-EET had maximal hyperpolarizing effects of $-12 \pm 3.5$ and $-16 \pm 3.0$ mV, respectively. Corresponding EC50 values of 0.13 and 0.14 μM were derived from the sigmoidal response curves obtained. These EET-induced hyperpolarizing effects were fully reversible on washout of the exogenous EETs with normal Tyrode solution for 20 min. The vehicle (ethanol) had basically no effect on the membrane potential when tested at the same concentration used in the presence of the EETs except at the higher concentration tested (Fig. 3B, solid squares). Overall, ASM membrane potential was much more sensitive to the actions of EETs than tissue isometric tension.

![Fig. 1. Concentration-response curves for the four epoxyeicosatrienoic acid (EET) regioisomers on guinea pig airway smooth muscle (ASM) relaxation. Epithelium-denuded ASM spirals were precontracted with 0.2 μM carbamylcholine chloride (CCh) and then challenged with increasing concentrations of EETs dissolved in either acetonitrile (ACN) or ethanol (EtOH) as indicated ($n = 20–30$). Inset: effects of both vehicles, ACN and EtOH, used on precontracted ASM with CCh. The vehicle concentrations tested were 0.1, 0.3, 1.0, and 1.5% corresponding to 18.95, 56.85, 189.5, and 284.25 mM of ACN and 17.12, 51.36, 171.2, and 256.8 mM of EtOH, respectively. 1% ACN had a marked and larger effect than EtOH on ASM relaxation. Taking into account the effect of the solvent, 8,9- and 14,15-EET were deemed the most efficient relaxing compounds.](http://ajplung.physiology.org/content/early/2017/09/30/ajplung.00907.2017/Fig.1.png)
**IbTX sensitivity of hyperpolarizations induced by EETs.** To test for the involvement of BKCa channels on the robust hyperpolarizations induced by both EETs tested, two protocols were used: IbTX pretreatment before EET challenges and IbTX reversal of EET-induced hyperpolarizations. IbTX alone had no significant effect on the membrane potential (−49.9 ± 2 mV) as illustrated in Fig. 4A. The data reveal that 10 nM IbTX completely prevented the hyperpolarizations normally induced by the two EET isomers; subsequent addition of the EC50 doses of 8,9- and 14,15-EET to the perfusion chamber did not cause any hyperpolarization of the membrane potential. On the other hand, 8,9- and 14,15-EET (3 μM) induced membrane potential hyperpolarizations that were completely reversed by subsequent addition of 10 nM IbTX to the perfusion medium (Fig. 4B). Both IbTX effects attest to the role of BKCa channels in the EET-induced hyperpolarizations. However, IbTX effects on ASM relaxation by the EETs demonstrate that BKCa activation by the latter only accounted for one-half of the relaxation, suggesting the involvement of other pathways in the control of the relaxation process elicited by EETs.

**Effect of EETs on activation of reconstituted BKCa channels in PLB.** To assess the effects of 8,9- and 14,15-EET on channel activity, BKCa channels were reconstituted in a symmetrical (250/250 mM) KCl...
buffer system containing, initially, 10 μM free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{trans}}\]). [Ca\(^{2+}\)]\(_{\text{trans}}\) was reduced on addition of EGTA to the trans chamber (cytoplasmic side of the channel), and channel open probability (P\(_o\)) decreased due to its Ca\(^{2+}\) sensitivity. Figure 5A illustrates representative single BK\(_{\text{Ca}}\) channel recordings in control conditions (+30 mV, 0.6 μM free [Ca\(^{2+}\)]) and following sequential additions of increasing concentrations of 8,9-EET in the cis chamber. These current recordings show that increasing concentrations of 8,9-EET enhanced BK\(_{\text{Ca}}\) activity (Fig. 5A). Similar observations were made with 14,15-EET (Fig. 6A), which demonstrated the capacity of this eicosanoid to also activate BK\(_{\text{Ca}}\) channels in a concentration-dependent manner but with a lower effectiveness. The lowest concentration displaying a significant effect was 1.5 μM in each case, which nearly doubled the P\(_o\) of the channel (Fig. 6B). At the maximal concentration tested (10 μM), 8,9- and 14,15-EET caused an increase in P\(_o\) from nearly zero to 0.70 ± 0.09 and 0.09 ± 0.03, respectively. The activating effects of 8,9- and 14,15-EET were not voltage dependent over the range of potentials tested (−60 to 60 mV; data not shown). They were observed at negative potentials compatible with the resting membrane potential of ASM under physiological conditions. For instance, at −50 mV, in control conditions (low free [Ca\(^{2+}\)]), the P\(_o\) value increased from 0.07 to 0.32 in the

Fig. 4. Effects of 8,9- and 14,15-EET on ASM membrane potential were either prevented or reversed by IbTX. A: 10 nM IbTX pretreatment prevented the hyperpolarization induced by either 0.14 μM 8,9-EET or 0.13 μM 14,15-EET. Note that 10 nM iberiotoxin does not influence the resting membrane potential (n = 60). B: 10 nM IbTX reversed the hyperpolarization induced by 0.14 μM 8,9-EET (7.33 mV) and 0.13 μM 14,15-EET (9.09 mV). Each bar represents the average of 35 impalements derived from 3 distinct tissue preparations.

Fig. 5. Effect of 8,9-EET on single-channel recordings and concentration-dependent activation of reconstituted BK\(_{\text{Ca}}\) channels. A: representative traces of a unitary current obtained in symmetrical 250 mM KCl buffer (top trace), at +30 mV in high free Ca\(^{2+}\) concentration in the trans compartment ([Ca\(^{2+}\)]\(_{\text{trans}}\), 10 μM, second trace), and after the addition of two concentrations of 8,9-EET in the cis compartment (bottom trace). B: quantitative analysis of 8,9-EET effects in low 0.6 μM free [Ca\(^{2+}\)]\(_{\text{trans}}\) (control, third trace), and after the addition of two concentrations of 8,9-EET in the cis compartment (bottom trace). C: current-voltage curves in control conditions (symmetrical KCl buffer plus 0.6 μM free Ca\(^{2+}\)) and in the presence of increasing 8,9-EET concentrations. 8,9-EET had no effect on either current amplitude or unitary conductance of BK\(_{\text{Ca}}\) channels. All data are means ± SE; n = 7.
The presence of 6.0 \text{ M} 8.9\text{-EET} (data not illustrated).

Current-voltage curves obtained in control conditions and in the presence of increasing concentrations of 8.9- or 14,15- \text{ EET} (Figs. 5 \text{ C} and 6 \text{ C}) reveal that \text{ EETs had no effect on current amplitudes and unitary conductances.}

\text{IbTX sensitivity of EET activation of the BK}_{\text{Ca}} \text{ channel.} Figure 7A shows single-channel recordings with a low \( P_{o} \) (0.02) in the presence of 0.5 \text{ M free Ca}^{2+}. Subsequent addition of 3 \text{ M} 8.9\text{-EET} enhanced the activity of this channel, an effect that was completely blocked on addition of 10 nM IbTX on the extracellular side of the channel. Similar results were obtained in the presence of 6 \text{ M} 14,15\text{-EET and 10 nM IbTX (Fig. 7B)}. Thus both \text{ EETs activate the BK}_{\text{Ca}} \text{ channel at low free [Ca}^{2+} \text{], an effect completely blocked by IbTX.}

\text{Effects of \text{ EETs in the presence of NOS and COX inhibitors.} Figure 8 shows the effects of 100 \text{ M} \text{N}^\text{G}-\text{nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) and 1 \text{ M indomethacin (a COX inhibitor) used alone and combined. There is basically no significant effect of these inhibitors on the membrane potential of epithelium-denuded ASM cells. In the presence of both inhibitors, the addition of either 8.9- or 14,15- \text{ EET (3 \text{ M}) induced a hyperpolarization of } \text{-5 and } \text{-8 mV, respectively. These results demonstrate that, in the presence of NOS and COX inhibitors, exogenous \text{ EETs have electrophysiological effects with the hallmark of epithelium-derived hyperpolarizing factor (EpDHF). However, we must consider that the effects of both isomers were smaller than their effects measured at the same concentration (3 \text{ M}) in the absence of inhibitors (Fig. 3B). Together these results suggest that the}

\text{Fig. 7. IbTX blocks the BK}_{\text{Ca}} \text{ channel activation induced by either 8.9- or 14,15- \text{ EET.} A: representative current from a single reconstituted BK}_{\text{Ca}} \text{ channel obtained in symmetrical 250 mM KCl buffer (top trace), at } +30 \text{ mV in 0.6 \text{ M free [Ca}^{2+}]_{\text{trans (control, second trace) and after addition of cumulative concentrations of 14,15- \text{EET on the cis side (bottom traces)}}. B: quantification of the effects of 14,15- \text{EET in the presence 0.6 \text{ M free [Ca}^{2+}]_{\text{trans on channel P}o. Significant P}_{o} \text{ increases were obtained on addition of 14,15- \text{EET concentrations } \geq 1.5 \text{ M. * } \text{P < 0.05. C: current-voltage curves in control conditions (symmetrical KCl buffer plus 0.6 \text{ M [Ca}^{2+}] \text{)) and in the presence of increased 14,15- \text{EET concentrations. 14,15- \text{EET had no effect on BK}_{\text{Ca}} \text{ current amplitude or unitary conductance. All data are means } \pm \text{ SE; } n = 5.}

\text{Fig. 6. Effect of 14,15- \text{EET on single-channel current and concentration-dependent activation of reconstituted BK}_{\text{Ca}} \text{ channels.} A: representative traces of a unitary current obtained in symmetrical 250 mM KCl buffer (top trace), at +30 mV in 0.6 \text{ M free Ca}^{2+} \text{ (control, second trace) and after addition of cumulative concentrations of 14,15- \text{EET on the cis side (bottom traces)}). B: quantification of the effects of 14,15- \text{EET in the presence of increased concentrations of 8.9- or 14,15- \text{EET (Figs. 5C and 6C) reveal that \text{ EETs had no effect on current amplitudes and unitary conductances.} \text{IbTX sensitivity of EET activation of the BK}_{\text{Ca}} \text{ channel.} Figure 7A shows single-channel recordings with a low \( P_{o} \) (0.02) in the presence of 0.5 \text{ M free [Ca}^{2+}]. Subsequent addition of 3 \text{ M} 8.9\text{-EET enhanced the activity of this channel, an effect that was completely blocked on addition of 10 nM IbTX on the extracellular side of the channel. Similar results were obtained in the presence of 6 \text{ M} 14,15\text{-EET and 10 nM IbTX (Fig. 7B). Thus both \text{ EETs activate the BK}_{\text{Ca}} \text{ channel at low free [Ca}^{2+}], an effect completely blocked by IbTX.}

\text{Effects of \text{ EETs in the presence of NOS and COX inhibitors.} Figure 8 shows the effects of 100 \text{ M} \text{N}^\text{G}-\text{nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) and 1 \text{ M indomethacin (a COX inhibitor) used alone and combined. There is basically no significant effect of these inhibitors on the membrane potential of epithelium-denuded ASM cells. In the presence of both inhibitors, the addition of either 8.9- or 14,15- \text{EET (3 \text{ M}) induced a hyperpolarization of } \text{-5 and } \text{-8 mV, respectively. These results demonstrate that, in the presence of NOS and COX inhibitors, exogenous \text{ EETs have electrophysiological effects with the hallmark of epithelium-derived hyperpolarizing factor (EpDHF). However, we must consider that the effects of both isomers were smaller than their effects measured at the same concentration (3 \text{ M}) in the absence of inhibitors (Fig. 3B). Together these results suggest that the}
EETs might also influence other intracellular enzymatic systems.

**DISCUSSION**

In this work, we investigated the ability of four EET regioisomers to relax tissues precontracted by muscarinic stimulation. This relaxation was associated with a hyperpolarization of ASM cells via an IbTX-sensitive mechanism. Using complementary experimental procedures, we have shown that 8,9- and 14,15-EET induced cellular hyperpolarizations, which in turn resulted from BKCa channel activation and were completely blocked or reversed by the application of 10 nM IbTX. These results obtained on ASM are consistent with those from other reports on VSM in which the EETs have hyperpolarizing effects induced by both EETs, BKCa channel activation appears to be a key determinant in the hyperpolarization process. However, the difference in IbTX sensitivity between hyperpolarization and relaxation measurements is consistent with the involvement of other mechanisms in the regulation of muscle tone. In VSM, charybdotoxin (a less selective BKCa channel blocker) has been shown to inhibit hyperpolarizations induced by bradykinin, which corresponded to an EDHF effect, since it was observed in the presence of L-NAME and indomethacin, specific inhibitors of the NOS and COX pathways, respectively (2). We are now reporting that, in ASM cells, EETs have hyperpolarizing effects (Fig. 8) in the presence of NOS and COX inhibitors. In human coronary arterioles, the EDHF effect was insensitive to 1 μM glibenclamide (an ATP-sensitive K+ channel blocker) and to 0.1 μM apamin (a small-conductance KCa channel blocker) (27). These observations support the fact that BKCa channels are major effectors of EDHF and therefore probably of EpDHF (27).

On the other hand, membrane reconstitution of BKCa channels allowed the evaluation of direct effects of EETs on the BKCa single-channel properties. 8,9-EET and 14,15-EET were added to the extracellular side of the channel to mimic their physiological release by epithelial cells and/or other lung cells (4). Moreover, the sidedness of the effects of EETs on BKCa channels, reconstituted in PLB, was previously assessed by our
group and revealed that EETs had no effect on the channel when added to the trans side (intracellular face) of the channel (12). Both EETs induced a concentration-dependent activation of BK_{Ca} channels, without affecting current amplitudes and unitary channel conductances. This partial activation of the reconstituted channels proves that the bioactive molecules tested herein had a direct effect on BK_{Ca} channels. One has to consider that the trans chamber (intracellular face) did not contain G_{i/o} protein that modulates channel gating (25). However, these EET-induced BK_{Ca} channel activations by intracellular mechanisms remain controversial; a recent study in VSM showed that EETs could modulate the channel activity via a cAMP-dependent kinase (PKA)-dependent signaling pathway (20). In ASM it has been shown that BK_{Ca} channels could be activated by either PKA-dependent or cGMP-dependent protein kinase-dependent phosphorylations (1, 24, 34). However, to date, our results do not demonstrate the existence of a specific intracellular mechanism explaining BK_{Ca} channel activation by EETs. Such a possibility could yield a plausible explanation for the differences in EET sensitivity at the tissular and molecular levels; the results reported here suggest the existence of specific binding sites on cell membrane proteins and/or interactions with membrane lipid components. The existence of specific binding sites for 14(R),15(S)-EET have been reported in mononuclear cells (41) and for 11,12-EET in cell membrane of cerebral vascular beds (26). Further investigations will be required to verify the existence of an EET receptor on ASM cells. For practical purposes, we used three different animal species; there is no evidence that the role of EET in modulating ASM tone is significantly different in these mammalian species. In fact, the evidence gathered from various reports has clearly shown that EETs have similar effects in several species (12, 43).

Aside from the mechanisms by which the EETs induce ASM relaxation, another aim of this study was to assess whether or not the EETs could act as an EpDHF. Based on the accepted definition of an EDHF (18), a candidate molecule must be able to modulate smooth muscle tone via a hyperpolarization of the membrane potential through BK_{Ca} channel activation, which should be independent from NO and prostaglandin production. The results obtained in the present work reveal that EET isomers induce ASM relaxation mainly via membrane hyperpolarizations induced by direct BK_{Ca} activation. Thus the mechanistic characteristics of EET relaxation, hyperpolarization, and BK_{Ca} activation are consistent with a role of the EETs as EpDHF in the airways.

In summary, both EET isomers induced concentration-dependent relaxing effects on ASM, which were related to hyperpolarizations and were likely due to BK_{Ca} channel activation. These eicosanoids were able to directly activate reconstituted channels, although the quantitative analysis, derived from the partial lBTX-inhibitory effects on tension measurements, suggests that a second intracellular mechanism could also be operable. Altogether, our data support the view and bring new evidence that, in the airway, EET regioisomers may behave as EpDHF-like agents.

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REFERENCES


