# Epoxyeicosatrienoic acids activate a high-conductance, $Ca^{2+}$-dependent $K^+$ channel on pig coronary artery endothelial cells

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1. Epoxyeicosatrienoic acids (EETs) have been described as endothelium-derived hyperpolarizing factors (EDHFs), based on their stimulatory effects on smooth muscle $K^+$ channels. In order to reveal a putative autocrine effect of EETs on endothelial channels, we have studied the effects of the four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET) on the high-conductance, $Ca^{2+}$-dependent $K^+$ ($BK_{Ca}$) channel recorded in inside-out patches of primary cultured pig coronary artery endothelial cells. Currents were recorded in the presence of either 500 nM or 1 $\mu$M free $Ca^{2+}$ on the cytosolic side of the membrane.

2. In 81 % of experiments, EETs at < 156 nM, applied on the cytosolic side of the membrane, transiently increased $BK_{Ca}$ channel open state probability ($P_o$) without affecting its unitary conductance, thus providing evidence for direct action of EETs, without involvement of a cytosolic transduction pathway.

3. The four EET regioisomers appeared to be equally active, multiplying the $BK_{Ca}$ channel $P_o$ by a mean factor of $4{\cdot}3 \pm 0{\cdot}6$ ($n = 15$), and involving an increase in the number and duration of openings.

4. The EET-induced increase in $BK_{Ca}$ channel activity was more pronounced with low initial $P_o$. When the $BK_{Ca}$ channel was activated by 500 nM $Ca^{2+}$, application of EETs increased the initial $P_o$ value of below 0·1 by a factor of 5. When the channel was activated by 1 $\mu$M $Ca^{2+}$, application of EETs increased the initial $P_o$ value by a factor of 3.

5. Our results show that EETs potentiate endothelial $BK_{Ca}$ channel activation by $Ca^{2+}$. The autocrine action of EETs on endothelial cells, which occurs in the same concentration range as their action on muscle cells, should therefore fully participate in the vasoactive effects of EETs, and thus be taken into account when considering their putative EDHF function.

When stimulated by blood mediators, endothelial cells release powerful vasoactive agents including hyperpolarizing factors called EDHFs (endothelium-derived hyperpolarizing factors; Bény & Brunet, 1988). Produced from arachidonic acid by cytochrome P450 mono-oxygenase, epoxyeicosatrienoic acids (EETs) have recently been described as being EDHFs. EETs, formed by the vascular endothelium in response to vasodilator mediators like bradykinin, relax vessels and hyperpolarize vascular smooth muscle by opening $K^+$ channels (Bauersachs, Hecker & Busse, 1994; Hecker, Bara, Bauersachs & Busse, 1994; Campbell, Gebremedhin, Pratt & Harder, 1996).

In endothelial cells, most agonists that increase intracellular $Ca^{2+}$ concentration, like bradykinin or thrombin, activate phospholipase $A_2$, thus leading to arachidonic acid release from membrane phospholipids (Buckley, Barchowsky, Dolor & Whorton, 1991). Endothelial cells can metabolize arachidonic acid via the cyclo-oxygenase, lipoxygenase and cytochrome P450 pathways (Harder, Campbell & Roman, 1995). Four EET regioisomers are formed by cytochrome P450: 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET, which generally appear to be equally active, hyperpolarizing and relaxing precontracted coronary artery with an $EC_{50}$ around 1 $\mu$M (Rosolowsky, Falck, Willerson & Campbell, 1990; Hu & Kim, 1993; Rosolowsky & Campbell, 1993; Harder *et al.* 1995; Campbell *et al.* 1996).

The involvement of EETs during vasodilatation has been mostly studied with regard to their stimulatory effects on smooth muscle $K^+$ channels. The activation of endothelial cells by a vasoactive mediator generally involves a rise in cytosolic $Ca^{2+}$ and a hyperpolarization, and several endothelial $Ca^{2+}$-dependent $K^+$ channels have been

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described, among which the $BK_{Ca}$ channel appears to play a major hyperpolarizing role (Adams, 1994). In the present study, we were interested in putative autocrine actions of EETs on endothelial $BK_{Ca}$ channels, which appear to share the same functional and pharmacological profile as smooth muscle $BK_{Ca}$ channels. Using porcine coronary artery primary cultured endothelial cells, we have studied the effects of EETs on unitary $BK_{Ca}$ currents previously characterized as being activated by bradykinin (Baron, Frieden, Chabaud & Bény, 1996).

## METHODS

### Endothelial cell primary culture

Left anterior descending branches of coronary arteries from freshly killed domestic pigs, *Sus scrofa*, were obtained at the slaughterhouse. The endothelial cells were collected by gentle rubbing of the internal face of the vessel with a scalpel, and centrifuged at 800 *g* for 8 min in culture medium (M199 medium) supplemented with 20% fetal calf serum, 2 mM glutamine, non-essential amino acids (mg $l^{-1}$: 8·9 L-Ala, 13·2 L-Asp, 13·3 L-Asn, 14·7 L-Glu, 7·5 L-Gly, 11·5 L-Pro, 10·5 L-Ser), minimal essential medium (MEM) vitamin solution (mg $l^{-1}$: 1 D-calcium pantothenate, 1 choline chloride, 1 folic acid, 2 *m*-inositol, 1 nicotinamide, 1 pyridoxal HCl, 0·1 riboflavin, 1 thiamine) and 50 mg $l^{-1}$ gentamicin (all from Gibco). The cell pellet was resuspended in culture medium and plated on collagen-coated glass coverslips. Cells were cultured at 37 °C under 5% $CO_2$. Culture medium was changed 3 times a week. Cells were used after 2–7 days of primary culture. Endothelial cells were identified by their morphology, fusiform growing cells forming islets within 4–5 days, and a monolayer of polygonal cells (cobblestone-like) after 5 days of culture.

### Single channel recording and analysis

Endothelial cells were observed on an inverted microscope (TMS; Nikon, Tokyo, Japan). Unitary membrane currents were measured using the standard patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) in the inside-out configuration, using a patch-clamp amplifier EPC7 (List Medical). Currents were monitored on a digital oscilloscope (Gould, DSO 1604). Borosilicate glass patch pipettes pulled with a BB-CH-PC puller (Mecanex SA, Nyon, Switzerland) had a resistance of 2–4 MΩ. Currents were filtered with a low-pass filter at 1 kHz, digitized by an IT16 interface (Instrutech Corp., Greatneck, NY, USA) and sampled at 16 kHz by a Macintosh II vx computer (Pulse, HEKA Electronik, Lambrecht, Germany).

Experiments were performed at room temperature (20–22 °C). The standard pipette solution contained 130 mM KCl or NaCl and 10 mM Hepes (pH adjusted to 7·45 with KOH or NaOH). The standard bathing solution contained (mM): 130 NaCl, 5·6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 8 Hepes (pH adjusted to 7·5 with NaOH).

EETs were applied on the cytosolic side of inside-out patches. To activate $BK_{Ca}$ channels, bathing solutions with calculated free $Ca^{2+}$ concentration of 500 nM and 1 μM were used, with $Ca^{2+}$ buffered by 5 mM EGTA.

Channel event detection and open time duration histograms were performed using MacTAC software (Instrutech Corp., and SKALAR Instr., Inc., Seattle, WA, USA). The threshold of event detection was usually set as 50% of unitary current amplitude. The probability of a channel being open, $P_o$, was expressed as the time spent in the open state ($T_o$) divided by the total recording time ($T$): $P_o = T_o/T$. $P_o$ was usually calculated each 15 s on 10 sweeps of 1 s each. When several ($N$) identical channels are simultaneously recorded in the same patch, the probability of one channel being open was calculated as follows: $P_o = (T_{o1} + 2T_{o2} + 3T_{o3} + ... + NT_{oN})/NT$, with $T_{oN}$ the time spent by a channel at the open level $N$.

### Chemicals and drugs

EGTA was purchased from Sigma. Bradykinin was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Epoxyeicosatrienoic acids dissolved in ethanol were purchased from ICN (Costa Mesa, CA, USA) and used 1000 times diluted (156 nM) in the standard bath solution. Since these compounds have a short half-life in aqueous solution (Proctor, Falck & Capdevila, 1987), the dilution was made as late as possible before rapid perfusion into the experimental chamber. The concentration of EET reaching the recorded cell was thus probably less than the initial concentration of 156 nM, and is thus given as < 156 nM.

### Statistics

Experimental data were expressed as means ± S.E.M., with $n$ referring to the number of measurements. Student's $t$ test was used to compare results.

## RESULTS

On coronary artery endothelial cells in primary culture, we have previously shown that bradykinin, an endothelium-dependent vasodilator, activates a high-conductance, $Ca^{2+}$-dependent $K^+$ ($BK_{Ca}$) channel recorded in cell-attached patch-clamp mode (Baron *et al.* 1996). In order to test for a direct effect of EETs on this channel, the $BK_{Ca}$ current was recorded in inside-out patches, the channel being activated by the $Ca^{2+}$ of the bathing solution. In this condition, the current showed a linear 285 pS conductance in symmetrical high-KCl solution (Fig. 1*A*, ●) and, according to the Nernst equilibrium for $K^+$, the reversal potential was shifted from 0 mV towards −80 or +80 mV when the KCl was replaced by NaCl in the pipette (Fig. 1*A*, ▲) or in the bathing solution (Fig. 1*A*, ◆), respectively. In order to be sure that the current recorded represented $BK_{Ca}$ channels, experiments were often done in the presence of high NaCl in the pipette, or sometimes, when the pipette was filled with high KCl, a high-NaCl bathing solution was perfused into the experimental chamber at the end of the experiment.

The $BK_{Ca}$ channel is regulated by membrane potential, its $P_o$ increasing with depolarization (Blatz & Magleby, 1987; Baron *et al.* 1996). In order to study a putative stimulatory effect of EETs, it was necessary to find experimental conditions allowing recording of high-amplitude currents with an intermediate $P_o$ value. With a high-NaCl pipette solution, the membrane potential should be above −30 mV to allow easy measurement of unitary currents. In the presence of 2 mM $Ca^{2+}$ on the cytosolic side of the membrane to activate the channel, the $P_o$ was maximal (near 0·9) around −20 mV (Fig. 1*B*, ◇). Consequently, $BK_{Ca}$ currents were recorded in the presence of low $Ca^{2+}$ concentration, close to physiological levels. The activity of $BK_{Ca}$ channels is variable in excised patches, probably due to the loss of intracellular regulation, which can affect the sensitivity to $Ca^{2+}$

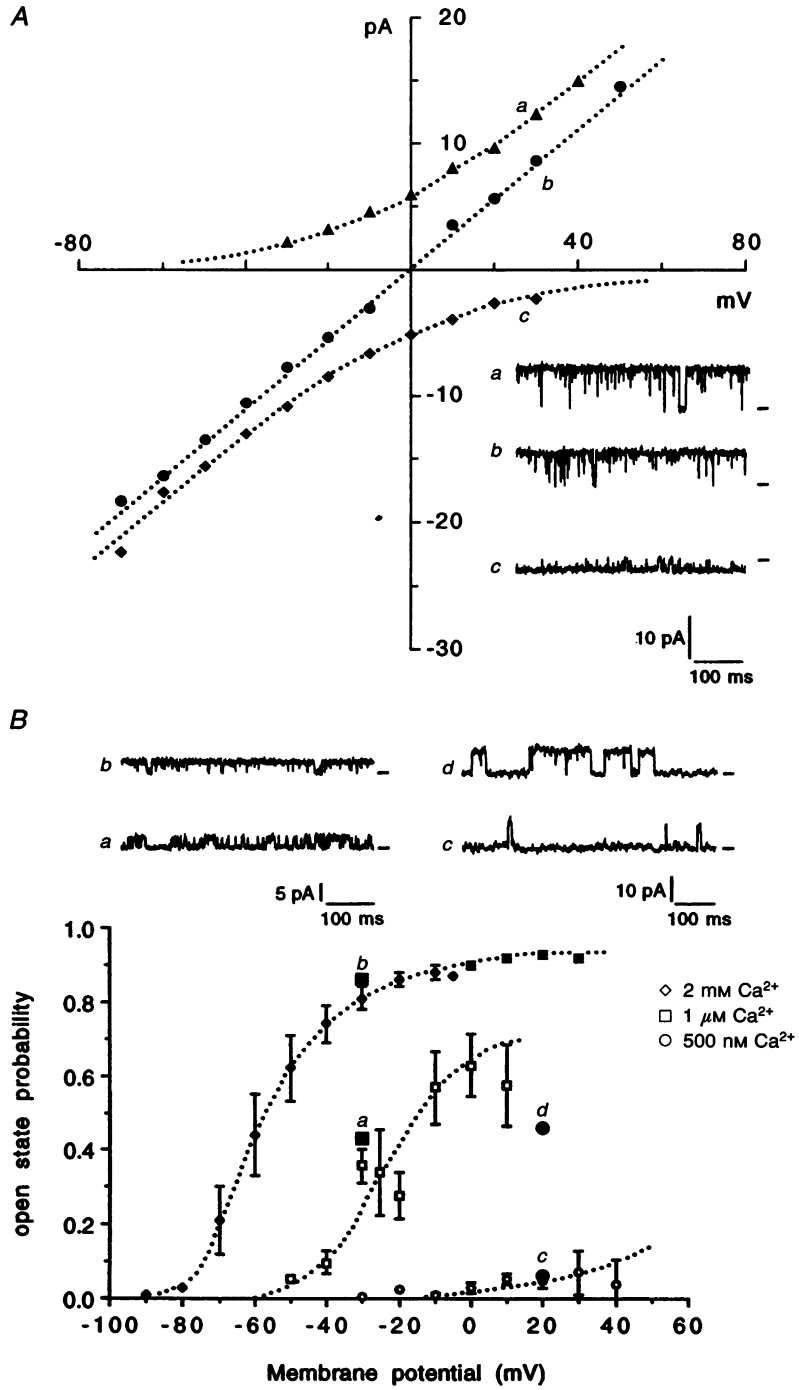


**Figure 1. Endothelial $BK_{Ca}$ channel activation by EETs**

*A*, $K^+$ selectivity of coronary artery endothelial $BK_{Ca}$ channel recorded in inside-out patches. Mean current–voltage relationships obtained in inside-out patches in the presence of 130 mM KCl in the pipette and 130 mM NaCl in the bath (◆), in the presence of high symmetrical 130 mM KCl (●) and in the presence of 130 mM NaCl in the pipette and 130 mM KCl in the bath (▲). The bathing solution contained 2 mM $CaCl_2$. Unitary current traces, recorded at +30 mV, are shown, the horizontal bar on the right indicating the closed channel level. Each point represents the mean of 2–21 experiments, S.E.M. (0·15–1·59 pA) not shown. *B*, $Ca^{2+}$ and potential dependence of the $BK_{Ca}$ channel and potentiation by EETs. Open state probability ($P_o$) expressed as a function of holding potential in the presence of various intracellular $Ca^{2+}$ concentrations: 2 mM (◇), 1 μM (□), and 500 nM (○). Each point is the mean ± S.E.M. of 3–17 experiments. Filled symbols represent $P_o$ values from single experiments during which EETs (< 156 nM) were applied. 14,15-EET was applied in the presence of 1 μM $Ca^{2+}$ in the bath (■, *a*, control; *b*, 14,15-EET), 5,6-EET was applied in the presence of 500 nM $Ca^{2+}$ in the bath (●, *c*, control; *d*, 5,6-EET). Corresponding original current traces are shown, the horizontal bar on the right indicating the closed channel current level. Currents were recorded in the presence of 130 mM NaCl in the pipette and 130 mM KCl in the bath.

and potential, associated with a run-down phenomenon (Baron *et al.* 1996). Depending on the channel basal activity, bathing solutions containing 500 nM or 1 $\mu$M $Ca^{2+}$ were used. In the presence of 1 $\mu$M $Ca^{2+}$ on the cytosolic side of the membrane, open state probabilities around 0·4 could be obtained at potentials between −30 and −10 (Fig. 1*B*, □), whereas, in the presence of 500 nM $Ca^{2+}$, the channel $P_o$ stayed below 0·1, even for positive potentials (Fig. 1*B*, ○).

In the presence of 500 nM $Ca^{2+}$ (membrane potential, +20 mV), < 156 nM 5,6-EET applied on the cytosolic side of an inside-out patch increased the $BK_{Ca}$ channel $P_o$ from a control value of 0·06 to 0·46 (Fig. 1*B*, ●; *c*, control; *d*, 5,6-EET). On a different patch, with currents recorded in the presence of 1 $\mu$M $Ca^{2+}$ (membrane potential, −30 mV), 14,15-EET showed a similar effect (Fig. 1*B*, ■; *a*, control; *b*, 14,15-EET), increasing the channel $P_o$ from 0·43 to 0·86. Unitary $BK_{Ca}$ current amplitude was not modified in the presence of EET, as shown by the original current recordings. EETs were effective in increasing $BK_{Ca}$ channel $P_o$ in seventeen of twenty-one experiments (81 %).

Figure 2 shows the effect of 5,6-EET and 14,15-EET on the channel $P_o$, mean open time and number of openings. In this experiment, the two EETs were applied successively to the same $BK_{Ca}$ channel recorded at +20 mV in the presence of 500 nM $Ca^{2+}$. 5,6-EET at < 156 nM increased the $P_o$ from a control value around 0·08 to a maximal value of 0·45 which could be correlated with an increase in both channel mean open time and number of openings. After a 3 min wash-out, during which the $P_o$ decreased towards control values, 14,15-EET at < 156 nM induced a similar effect, with a maximal $P_o$ of 0·5, also involving an increase in the number and duration of channel openings. Both effects of 5,6- and 14,15-EET appeared to be transient, possibly due to the degradation of these compounds in aqueous solution and also to biochemical reactions occurring in excised patches and affecting the channel function or regulation.

The four regioisomers of EETs increased the endothelial $BK_{Ca}$ channel $P_o$ with a similar pattern of action, involving an increase in the number and duration of openings. To compare EET actions independently of experimental conditions and variable open state probabilities, multiplying factor values have been represented as histograms in Fig. 3. The channel $P_o$, mean open time and number of openings were respectively increased by 2·8 ± 0·6, 3·6 ± 0·5 and 2·5 ± 0·7 in the presence of 14,15-EET, by 4·0 ± 1·2, 2·9 ± 0·8 and 3·3 ± 1·0 in the presence of 5,6-EET, 4·2 ± 1·5, 2·6 ± 0·7 and 3·4 ± 0·8 in the presence of 11,12-

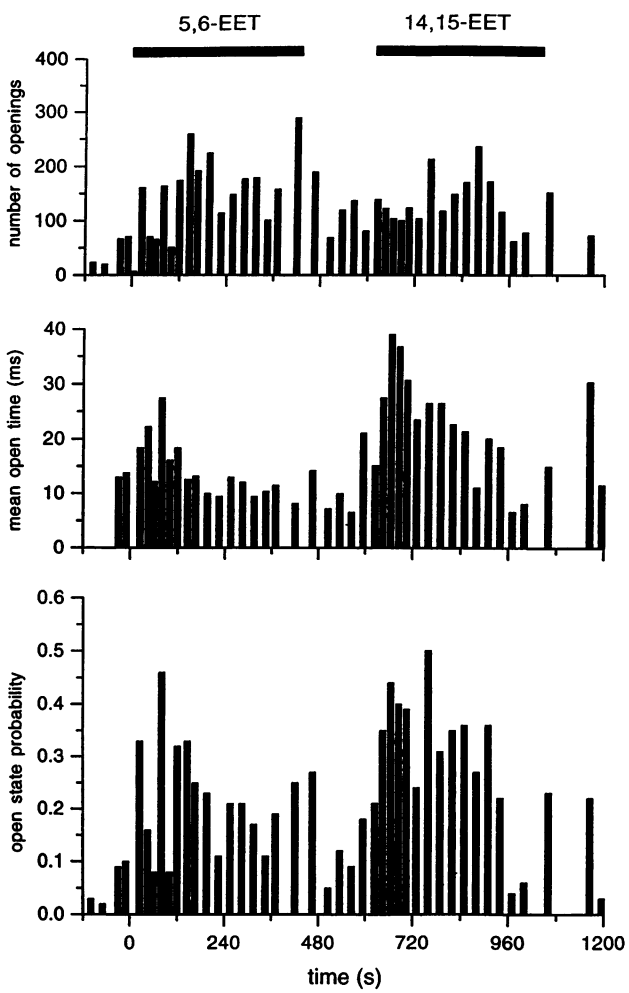


**Figure 2. Effects of EETs on the endothelial $BK_{Ca}$ channel open state probability, mean open time and number of openings**

Results obtained on an inside-out patch, 5,6-EET and 14,15-EET (< 156 nM) being applied in the high-KCl bathing solution containing 500 nM $Ca^{2+}$. Membrane potential, +20 mV. From top to bottom, number of openings, mean open time and open state probability are expressed as a function of time of EET perfusion. Each bar represents a mean value calculated over 10 s.

EET, and by $4{\cdot}4 \pm 1{\cdot}5$, $2{\cdot}8 \pm 0{\cdot}1$ and $3{\cdot}5 \pm 0{\cdot}2$ in the presence of 8,9-EET (Fig. 3*A*, $n$ from 2 to 5 for each value). These values refer to the mean maximal increase over a 10 s interval. Considering all data, the four EETs appeared to be equally active, multiplying the channel $P_o$ by a mean factor of $4{\cdot}3 \pm 0{\cdot}6$ ($n = 15$). However, the effect of EETs depended on the experimental conditions: the channel $P_o$ was multiplied by $4{\cdot}9 \pm 0{\cdot}8$ ($n = 10$) in the presence of 500 nM $Ca^{2+}$, but only by $3{\cdot}0 \pm 0{\cdot}9$ ($n = 3$, $P < 0{\cdot}1$) in the presence of 1 $\mu$M $Ca^{2+}$ (Fig. 3*B*). Thus, the increase in $BK_{Ca}$ channel $P_o$ produced by EETs was greater the smaller the initial $P_o$, probably because of the ceiling imposed by the maximal $P_o$ of around 0·9 (Fig. 1*B*).

## DISCUSSION

Our results show that EETs increase the activity of endothelial $BK_{Ca}$ channels, recorded in inside-out patches, by increasing their frequency and duration of opening, without any effect on the channel unitary conductance. The four regioisomers appear to be equally effective in stimulating $BK_{Ca}$ channels. EETs are particularly effective when the $BK_{Ca}$ channel $P_o$ is low, i.e. with a cytosolic $Ca^{2+}$ concentration around 500 nM. In the presence of this concentration, EETs can increase the $BK_{Ca}$ channel $P_o$ by a factor of 5, compared with a factor of 3 in the presence of 1 $\mu$M $Ca^{2+}$.

Similar effects of EETs have been already documented on vascular smooth muscle cells, thus supporting the involvement of EETs as EDHFs. In several vessels including porcine coronary artery, the four EETs activated a smooth muscle $BK_{Ca}$ channel with an $EC_{50}$ of around 100 nM (Hu & Kim, 1993; Harder *et al.* 1995; Campbell *et al.* 1996; Zou *et al.* 1996; Popp, Bauersachs, Hecker, Fleming & Busse, 1996), which produced a membrane hyperpolarization and vasodilatation of the vessel. Generally, these effects were observed on cell-attached patches, thus leading to the hypothesis of a membrane receptor and a cytosolic transduction pathway activated by EETs. On several vascular

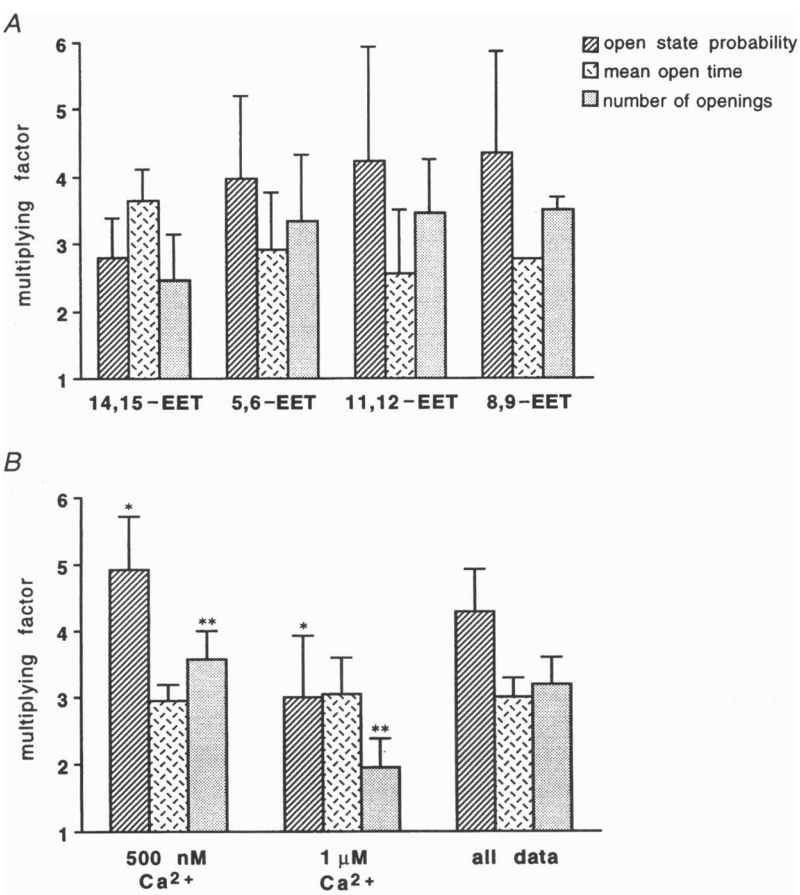


**Figure 3. Mean multiplying factors of increase in $BK_{Ca}$ channel open state probability, mean open time and number of openings induced by EETs**

*A*, effects of the four EET regioisomers. Data are considered independently of $Ca^{2+}$ concentration or membrane potential. *B*, $BK_{Ca}$ channel activation by EETs as a function of $Ca^{2+}$ concentration. Data are considered independently of EET regioisomer and membrane potential. From left to right, effect of EETs in the presence of 500 nM $Ca^{2+}$, in the presence of 1 $\mu$M $Ca^{2+}$, all data. S.E.M. are figured, $n$ ranging from 2–5 for each value. * $P < 0{\cdot}1$, ** $P < 0{\cdot}05$.

myocytes, Hu & Kim (1993) showed that the four EETs produced little or no potentiation of the $BK_{Ca}$ channel activity in inside-out compared with cell-attached patches. However, Campbell *et al.* (1996) proposed a direct action of EETs on ionic channels, because no changes in cytosolic second messenger concentrations could be detected.

Our results, obtained using inside-out patches, provide evidence for a direct action of EETs on $BK_{Ca}$ channels in endothelial cells. However, the time course of the action of EETs, as well as the amplitude of increase in channel activity could be highly dependent on experimental conditions. A direct interaction between EETs and ionic channels could explain why EETs appear to be equally active from one cell type to another (Hu & Kim, 1993). In arterial smooth muscle cells, long-chain fatty acids, like arachidonic acid, have been shown to activate a $Ca^{2+}$-dependent $K^+$ channel when applied to either the extracellular surface of the membrane, in whole-cell mode, or to the cytosolic side of an inside-out patch (Ordway, Singer & Walsh, 1991).

Similar to the effect we report on endothelial $BK_{Ca}$ channels, activation of smooth muscle $BK_{Ca}$ channels by EETs involved an increase in $P_o$ without any change in single channel conductance (Gebremedhin, Ma, Falck, Roman, VanRollins & Harder, 1992; Hu & Kim, 1993; Campbell *et al.* 1996; Zou *et al.* 1996). When analysed, this effect could be explained by an increase in both the frequency of opening and the mean open time. Zou *et al.* (1996) reported a 2-fold increase in the number of $BK_{Ca}$ channel openings induced by 11,12-EET in renal artery.

In bovine coronary artery myocytes, where both 14,15-EET and 11,12-EET increased the probability of $BK_{Ca}$ channel opening in a concentration-related manner, 1 and 100 nM EET induced a 2- and 2·5-fold increase, respectively (Campbell *et al.* 1996). In pig coronary artery endothelial cells, we found that EETs at $< 156$ nM increase the channel $P_o$ by a mean factor of 4·3 (range from 3 to 5, depending on the control value). Multiplying factors of 30–60 have been reported for 8,9-EET and 11,12-EET in arterial smooth muscle, the $BK_{Ca}$ channel showing very low initial $P_o$ (below 0·005). In this case, the major part of the effect was due to an increase in the channel opening frequency, even though a rise in opening duration also occurred (Gebremedhin *et al.* 1992).

The effects of EETs on muscle $BK_{Ca}$ channels thus appear to be highly similar to those we have described for endothelial cells. The concentrations at which EETs are active also appear to be similar in both vascular cell types, between 1 nM and 1 μM. These concentrations fit with physiological levels of plasma EETs, which can rise to 1 μM upon release by endothelial cells or platelets from a basal plasma level of around 1 nM (Rosolowsky, Falck & Campbell, 1991; Zhu, Schieber, McGiff & Balazy, 1995). The autocrine action of EETs on endothelial cells, which occurs in the same concentration range as their effects on muscle cells, should therefore fully participate in the vasoactive effects of EETs.

Endothelial cell activation by an endothelium-dependent vasodilator involves two major phenomena, membrane hyperpolarization and cytosolic $Ca^{2+}$ increase. These phenomena generally appear to be needed to induce subsequent relaxation of underlying smooth muscle, even if their respective roles still remain to be defined (Adams, 1994). In pig coronary artery endothelial cells, bradykinin increases cytosolic $Ca^{2+}$ from 80 nM to 1·3 μM, thus inducing the opening of $BK_{Ca}$ channels (Baron *et al.* 1996). In response to bradykinin and an increase in cytosolic $Ca^{2+}$, endothelial cells have also been shown to release EETs (Buckley *et al.* 1991) which, acting as autocrine factors, could potentiate $BK_{Ca}$ channel activation by cytosolic $Ca^{2+}$. EET-induced potentiation of $BK_{Ca}$ channels should play a significant role in cellular effects triggered by low mediator concentrations, which involve a moderate increase in cytosolic $Ca^{2+}$. This supports the physiological relevance of $BK_{Ca}$ channels in endothelial hyperpolarization, despite their apparent low sensitivity to $Ca^{2+}$.

Another second messenger function has been reported for EET in endothelial cells. Without any depletion of internal $Ca^{2+}$ stores, 5,6-EET increases $Ca^{2+}$ influx into human umbilical vein and bovine coronary artery endothelial cells. The similarity between the $Ca^{2+}$ influx induced by EET and that induced by bradykinin suggested that 5,6-EET could be a second messenger triggering the agonist-induced $Ca^{2+}$ influx (Graier, Simecek & Sturek, 1995). The hyperpolarizing effect of $K^+$ channel activation, associated with the stimulated $Ca^{2+}$ influx would therefore lead to a stimulation of autacoid release.

In addition to their stimulatory effect on muscle $K^+$ channels, which supports their function as EDHFs, EETs also activate $Ca^{2+}$-dependent $K^+$ channels in vascular endothelial cells, thus contributing to membrane hyperpolarization and cellular activation. Such autocrine effects of EETs must be taken into account when considering modulation of vascular tone by circulating mediators.

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