British Journal of Pharmacology (2005) 145, 775–784
www.nature.com/bjp

Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids

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1 In coronary arteries, bradykinin opens endothelial intermediate- and small-conductance Ca^{2+}-sensitive K^+ channels (IK_{Ca} and SK_{Ca}) and, additionally, releases epoxyeicosatrienoic acids (EETs) from the endothelium. To clarify the involvement of these pathways in endothelium-dependent myocyte hyperpolarization, bradykinin-induced electrical changes in endothelial cells and myocytes of porcine coronary arteries (following nitric oxide (NO) synthase and cyclooxygenase inhibition) were measured using sharp microelectrodes.

2 Hyperpolarization of endothelial cells by bradykinin (27.0 ± 0.9 mV, $n = 4$) was partially inhibited (74%) by blockade of IK_{Ca} and SK_{Ca} channels using 10 μM TRAM-39 (2-(2-chlorophenyl)-2,2-diphenylacetonitrile) plus 100 nM apamin (leaving an iberiotoxin-sensitive component), whereas the response to substance P was abolished.

3 After gap junction blockade with HEPES, (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid)) hyperpolarization of the endothelium by 100 nM bradykinin was abolished by TRAM-39 plus apamin, whereas myocyte hyperpolarization still occurred (12.9 ± 1.0 mV, $n = 4$). The residual hyperpolarizations to 100 nM bradykinin were antagonized by the EET antagonist, 14,15-EEZE (14,15-epoxyeicosa-5(Z)-enoic acid) (10 μM), and abolished by iberiotoxin. Bradykinin-induced myocyte hyperpolarizations were also reduced by 14,15-EEZE-mSI (14,15-EEZE-methylsulfonylimide) (5,6- and 14,15-EET antagonist), whereas those to exogenous 11,12-EET were unaffected.

4 These data show that bradykinin-induced hyperpolarization of endothelial cells (due to the opening of IK_{Ca} and SK_{Ca} channels) is electrotonically transferred to the myocytes *via* gap junctions. Bradykinin (but not substance P) also hyperpolarizes myocytes by a mechanism (independent of endothelial cell hyperpolarization) which involves endothelial cell production of EETs (most likely 14,15- and/or 11,12-EET). These open endothelial IK_{Ca} and SK_{Ca} channels and also activate large-conductance calcium-sensitive K^+ channels (BK_{Ca}) on the surrounding myocytes.
British Journal of Pharmacology (2005) **145,** 775–784. doi:10.1038/sj.bjp.0706256;
published online 16 May 2005

Keywords: Endothelium-dependent hyperpolarization; porcine coronary artery; bradykinin; substance P; EDHF; 5,6-EET; 11,12-EET; 14,15-EET; 14,15-EEZE; 14,15-EEZE-mSI

Abbreviations: 1-EBIO, 1-ethyl, 2-benzimidazolinone; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; 14,15-EEZE-mSI, 14,15-EEZE-methylsulfonylimide; BK_{Ca}, large-conductance calcium-sensitive K^+ channel; EDHF, endothelium-derived hyperpolarizing factor; EET, epoxyeicosatrienoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid); IK_{Ca}, intermediate-conductance calcium-sensitive K^+ channel; NO, nitric oxide; NS1619, 1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone; SK_{Ca}, small-conductance calcium-sensitive K^+ channel; TRAM-39, (2-(2-chlorophenyl)-2,2-diphenylacetonitrile)

Introduction

Autacoids act on vascular endothelial cells to lower blood vessel tone by activating several vasodilator pathways, two of which involve the release of nitric oxide (NO; Furchgott & Zawadzki, 1980) and prostacyclin (Moncada & Vane, 1979). However, after inhibition of NO synthase and cyclooxygenase, acetylcholine, bradykinin and substance P still relax blood vessels by mechanisms that involve endothelium-dependent hyperpolarization of vascular myocytes (Busse *et al*., 2002).

Vascular endothelial cells and myocytes possess different populations of functionally active calcium-sensitive K^+ channels. Thus, whereas arterial endothelial cells possess small- and intermediate-conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively) and possibly also large-conductance Ca^{2+}-activated potassium channels (BK_{Ca}; see Nilius & Droogmans, 2001), it is unlikely that any BK_{Ca} channels are functionally active in nonproliferating endothelial cells (Bychkov *et al*., 2002; Gauthier *et al*., 2002b). In contrast,

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in endothelium-denuded arteries, the pharmacology of the smooth muscle is consistent with the presence of functional BK_{Ca} channels but not SK_{Ca} or IK_{Ca} channels (Doughty *et al*., 1999; Edwards *et al*., 1999; Walker *et al*., 2001). In most arteries, endothelium-dependent hyperpolarization of the myocytes initially involves the opening of both endothelial SK_{Ca} and IK_{Ca}. This is often known as the endothelium-derived hyperpolarizing factor (EDHF) pathway (Busse *et al*., 2002). It can be blocked by a mixture of charybdotoxin, which inhibits both IK_{Ca} and large conductance, Ca^{2+}-sensitive K^+ channels (BK_{Ca}) *plus* apamin (a selective SK_{Ca} inhibitor). The selective BK_{Ca} inhibitor, iberiotoxin, cannot substitute for charybdotoxin in the toxin mixture (Busse *et al*., 2002). In large coronary arteries, however, bradykinin-induced endothelium-dependent vasodilatation also involves the generation of arachidonic acid derivatives. Produced *via* a cytochrome *P*450-dependent pathway, these metabolites are most likely to be 14,15- or 11,12-epoxyeicosatrienoic acid (EET) (Rosolowsky & Campbell, 1996; Fisslthaler *et al*., 1999; Gauthier *et al*., 2003). Such fatty acids hyperpolarize and relax vascular smooth muscle by opening BK_{Ca} channels on the myocytes (e.g. Zou *et al*., 1996; Li & Campbell, 1997; Eckman *et al*., 1998; Fisslthaler *et al*., 1999; Archer *et al*., 2003). In addition, EETs may act as second messengers in endothelial cells and be involved in initiating endothelial hyperpolarization and its conduction to surrounding myocytes. Thus, EETs modulate endothelial Ca^{2+} influx following Ca^{2+}-store depletion (Hoebel *et al*., 1997) and facilitate endothelial cell K^+ channel activation by increasing channel Ca^{2+}-sensitivity (Baron *et al*., 1997; Popp *et al*., 2002). Additionally, EETs may regulate gap-junctional communication between endothelial cells (Popp *et al*., 2002).

To date, investigations of bradykinin-activated vasodilator pathways have been hampered by the lack of *selective* inhibitors. Thus, charybdotoxin inhibits not only endothelial cell IK_{Ca} channels but also the myocyte BK_{Ca} channels that can be opened by EETs. Other blockers of IK_{Ca} such as clotrimazole, also inhibit cytochrome *P*450 epoxygenases and thereby prevent EETs synthesis (Capdevila *et al*., 1988; Jensen *et al*., 2001).

The study reported in this paper was undertaken to exploit recent and important pharmacological advances. To clarify the contribution of endothelial electrical pathways to the actions of bradykinin, IK_{Ca} channels were selectively blocked using TRAM-39 (2-(2-chlorophenyl)-2,2-diphenylacetonitrile). This clotrimazole derivative has little or no effect on either cytochrome *P*450-dependent pathways or BK_{Ca} channels (Wulff *et al*., 2001). In addition, the extent to which EET isomers are also involved was studied using the newly developed EET receptor antagonists 14,15-EEZE (14,15-epoxyeicosa-5(Z)-enoic acid) and 14,15-EEZE-mSI (14,15-EEZE-methylsulfonylimide) (Gauthier *et al*., 2002a; 2003). Using these approaches, the complexity of endothelium-dependent mechanisms underlying the vascular smooth muscle hyperpolarization to the local hormone bradykinin has been revealed.

Methods

Pig hearts were obtained from the local abattoir and transported to the laboratory in ice-cold Krebs' solution. Left anterior descending coronary arteries were dissected free of surrounding tissue and all side branches were removed while maintained in ice-cold Krebs' solution.

Electrophysiology

Small segments of the artery (4–5 mm long) of the distal portion of the main artery (diameter 2–3 mm) were opened along their longitudinal axis and pinned to the Sylgard base of a thermostatically controlled (37°C) bath. They were then superfused (3 ml min^{-1}) with Krebs' solution bubbled with 95% O_2: 5% CO_2. Unless otherwise indicated, myocytes were impaled *via* the adventitial surface using microelectrodes filled with 3 M KCl (resistance 40–80 MΩ) (Edwards *et al*., 2001). Endothelial cells were probed using similar electrodes inserted through the luminal surface. For some experiments, vessels were preincubated at room temperature (28°C) for 18 h in oxygenated HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid))-buffered Tyrode solution to inhibit gap junctions (Kilarski *et al*., 1995; Bevans & Harris 1999; Edwards *et al*., 2001) and membrane potential recordings were carried out in the same solution but at 37°C. Bradykinin, 1-EBIO (1-ethyl, 2-benzimidazolinone), 5,6-, 11,12-, and 14,15-EET, levcromakalim, (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone). NS1619 and substance P were each added as bolus injections directly into the bath in quantities calculated to give (transiently) the final concentrations indicated. Apamin, 14,15-EEZE, 14,15-EEZE-mSI, iberiotoxin and TRAM-39 were each added to the reservoir of physiological salt solution superfusing the bath. All recordings were made using a high impedance amplifier (WPI Instruments). Signals were digitized and analysed using a MacLab system (AD Instruments); 50 Hz interference was selectively removed using an active processing circuit (Humbug, Digitimer).

Drugs and solutions

The following substances were used: apamin (Latoxan, Valence, France), bradykinin, 5,6-EET ((±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid; Cayman Chemicals/Alexis Corporation, Nottingham, U.K.), 11,12-EET ((+)11,12-epoxy-eicosa-5Z, 8Z, 14Z-trienoic acid, Affiniti Research Products Ltd, Exeter, U.K.), 14,15-EET (±)14,15-epoxy-5Z,8Z,11Z-eicosatrienoic acid; Cayman Chemicals/Alexis Corporation, Nottingham, U.K.), 1-EBIO (1-ethyl-2-benzimidazolinone; Aldrich, Gillingham, U.K.), 14,15-EEZE and 14,15-EEZE-mSI were synthesized as previously described (Falck *et al*., 2003), synthetic iberiotoxin (Latoxan), indomethacin, nitro-L-arginine, NS1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone (RBI, Poole, U.K.) and substance P (RBI). Levcromakalim was supplied by Smith-Kline Beecham (Horsham, U.K.) TRAM-39 was a kind gift from Dr H. Wulff. Unless otherwise mentioned, all substances were obtained from Sigma (Poole, U.K.).

The Krebs' solution contained (mM): 118 NaCl, 3.4 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 11 glucose, 25 $NaHCO_3$ plus 10 μM indomethacin and 300 μM L-nitro-arginine. It was bubbled with 95%O_2/5%CO_2. The composition of the HEPES-buffered Tyrode solution was (mM): 140 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 11.1 glucose plus 10 μM indomethacin and 300 μM L-nitro-arginine. The pH of this

solution was adjusted at 37°C to 7.4 using NaOH and the solution was oxygenated during the incubation period.

Statistics

Data are shown as mean ± s.e.m.; *n* indicates the number of tissues in which membrane potential was recorded. Statistical analyses were performed using Student's *t*-test for paired or unpaired observations. Differences were considered to be statistically significant when *P* was less than 0.05.

Results

General

All experiments were performed in the presence of indomethacin (10 μM) and L-nitro-arginine (300 μM) to eliminate the effects of prostacyclin and NO, respectively. Previous studies have shown that the hyperpolarizing effects of bradykinin and substance P are abolished by endothelium removal (Edwards *et al*., 2000).

Endothelial cell hyperpolarization to bradykinin, substance P and 1-EBIO and effect of TRAM-39

The resting membrane potential of porcine coronary artery endothelial cells was −48.9 ± 0.2 mV ($n=8$). Under control conditions, substance P (100 nM), 1-EBIO (600 μM) and levcromakalim (10 μM) produced hyperpolarizations of the endothelial cells that reached 25.6 ± 1.0 mV ($n=4$), 18.2 ± 0.8 mV ($n=4$) and 10.7 ± 0.8 mV ($n=4$), respectively (Figure 1a, b). In the presence of 10 μM TRAM-39, the hyperpolarization to substance P was unchanged (23.9 ± 0.7 mV, $n=4$), whereas that to 1-EBIO (an opener of IK_{Ca} channels; Devor *et al*., 1996) was almost abolished (0.9 ± 0.4 mV, $n=4$). Exposure to 100 nM apamin, in the continued presence of TRAM-39, produced a small depolarization (2.5 ± 0.4 mV, $n=4$) and prevented any effect of substance P, although under these conditions there was no reduction in the hyperpolarization to levcromakalim (14.2 ± 1.8 mV, $n=4$) (Figure 1a, b).

Bradykinin (100 nM) produced an endothelial cell hyperpolarization (27.2 ± 0.9 mV, $n=4$), similar in magnitude to that induced by substance P (compare Figure 1a, b with c, d), which was significantly, but not fully, inhibited by the combination of 10 μM TRAM-39 + 100 nM apamin (7.1 ± 1.2 mV hyperpolarization in the presence of TRAM-39 + apamin, $n=4$). In the continued presence of these inhibitors, the addition of iberiotoxin (100 nM) inhibited the residual hyperpolarization to bradykinin, whereas hyperpolarization to levcromakalim was still observed (Figure 1c, d).

Use of HEPES to inhibit myo-endothelial gap junction coupling: identification of two separate pathways to bradykinin-induced myocyte hyperpolarization

HEPES is a gap junction inhibitor (Kilarski *et al*., 1995; Bevans & Harris, 1999) which allows electrical uncoupling of endothelial cells from the surrounding myocytes (Edwards *et al*., 2001). In the present study, following overnight incubation of arteries in HEPES-buffered Tyrode solution, a microelectrode was first inserted into an endothelial cell. The resting membrane potential (−50.2 ± 0.2 mV, $n=4$; Figure 2) was slightly, but significantly ($P<0.01$) increased by the HEPES exposure (compare with Figure 1). Levcromakalim (10 μM; a maximally effective concentration of this myocyte K_{ATP} opener) did not modify the membrane potential, confirming that the microelectrode did impale an endothelial

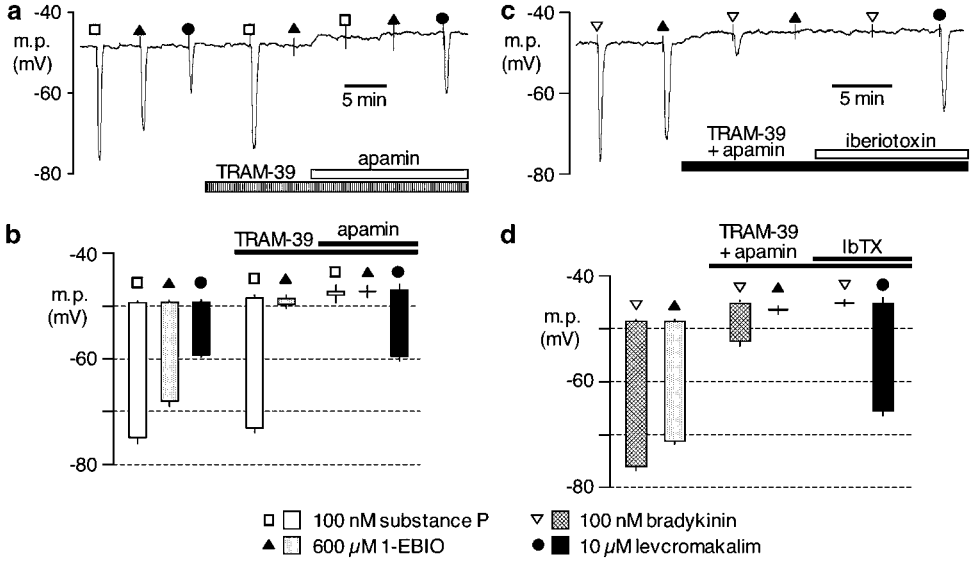

Figure 1 Effect of 10 μM TRAM-39 and 100 nM apamin on changes in endothelial cell membrane potential (m.p.) induced by substance P, bradykinin, 1-EBIO and levcromakalim in segments of endothelium-intact porcine coronary arteries in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (a, c) Typical traces showing the responses before and in the presence of TRAM-39 and/or TRAM-39 + apamin (a) and in the additional presence of 100 nM iberiotoxin (c). Graphical representation of data from four separate experiments of the types shown in (a) and (c) are shown in (b) and (d), respectively. Each column in (b) and (d) represents m.p. ± s.e.m., before and during exposure to bolus doses of substance P, bradykinin, 1-EBIO or levcromakalim calculated to give, transiently, the final bath concentrations indicated.

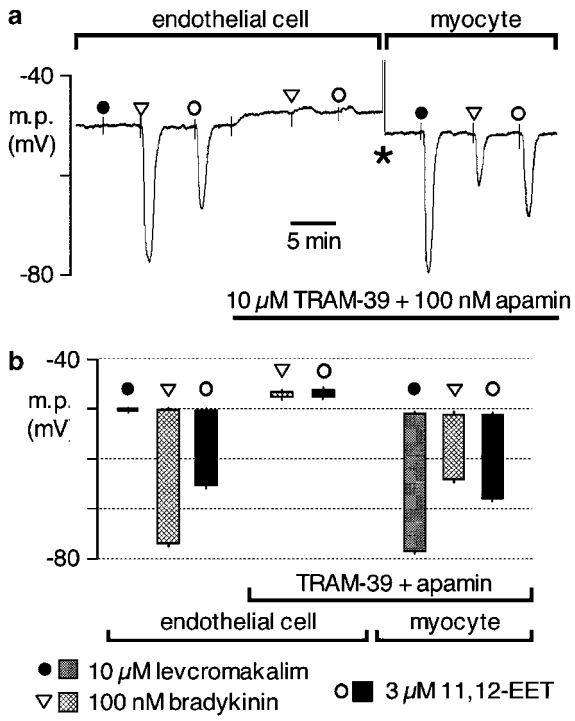

Figure 2 Effect of TRAM-39 + apamin on endothelial cell and myocyte responses to bradykinin and 11,12-EET in endothelium-intact segments of porcine coronary arteries preincubated for 18 h in HEPES-buffered Tyrode solution to inhibit gap junctions. (a) Typical trace showing endothelial cell responses in the absence and presence of 10 μM TRAM-39 + 100 nM apamin. After recording from the endothelial cell, the electrode was withdrawn (*) and a myocyte in the same artery segment was impaled. Myocyte responses to bradykinin, 11,12-EET and levcromakalim were then recorded in the continued presence of the TRAM-39 + apamin. (b) Graphical representation of data from four separate experiments of the type shown in (a); each column represents the membrane potential (m.p.) ± s.e.m., before and during exposure to bolus doses of levcromakalim, bradykinin and 11,12-EET calculated to give, transiently, the final bath concentrations indicated. Experiments were performed in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin.

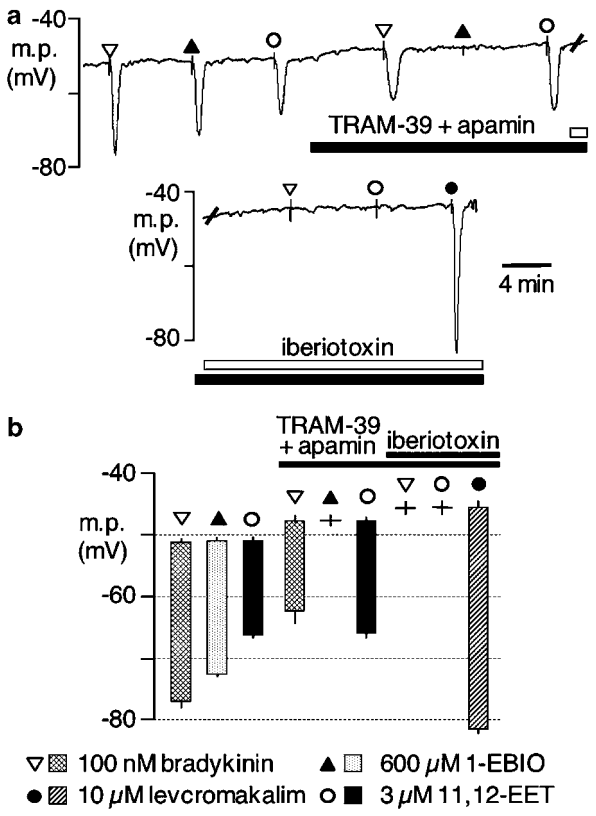

Figure 3 Effect of TRAM-39 + apamin and iberiotoxin on myocyte hyperpolarizations induced by bradykinin, 1-EBIO and 11,12-EET in endothelium-intact porcine coronary artery segments in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (a) Typical trace showing responses in the absence and presence of 10 μM TRAM-39 + 100 nM apamin and in the additional presence of 100 nM iberiotoxin. Levcromakalim was used to demonstrate the integrity of the tissue. The slanting bar shows where the continuous trace was cut for publication purposes. (b) Graphical representation of data from four separate experiments of the type shown in (a); each column represents the membrane potential (m.p.) ± s.e.m., before and during exposure to bolus doses of bradykinin, 1-EBIO, 11,12-EET and levcromakalim calculated to give, transiently, the final bath concentrations indicated.

cell (and not a myocyte) and also that the gap junction uncoupling procedure had been successful. Exposure to 100 nM bradykinin and 3 μM 11,12-EET (one of the fatty acids proposed to be responsible for some of bradykinin's effects; Fisslthaler *et al*., 1999) hyperpolarized the endothelial cells by 26.7 ± 0.7 mV ($n = 4$) and 15.0 ± 0.9 mV ($n = 4$), respectively. The magnitude of the endothelial cell hyperpolarization to bradykinin after overnight exposure to HEPES was similar ($P > 0.05$) to that of the freshly isolated arteries (hyperpolarization 27.2 ± 0.9 mV, $n = 4$) depicted in Figure 1. When the HEPES-treated artery segments were subsequently exposed to TRAM-39 + apamin (which depolarized the endothelium by 2.4 ± 0.2 mV), the hyperpolarizing actions of both bradykinin and 11,12-EET were abolished ($P < 0.05$, $n = 4$), indicating that they were generated by the opening of endothelial IK_{Ca} and SK_{Ca} channels (Figure 2).

In the continued presence of TRAM-39 + apamin, the microelectrode was pushed through the endothelial cell and the internal elastic lamina and an adjacent myocyte was then impaled. The location of the electrode within a smooth muscle cell was confirmed using levcromakalim (which hyperpolarized the myocyte to near the potassium equilibrium potential; Figure 2). Under these conditions, the myocyte membrane potential (−51.0 ± 0.2 mV) was significantly greater than that of the endothelial cells in the same segments of artery −47.5 ± 0.5 mV ($P < 0.01$, $n = 4$), but bradykinin and 11,12-EET still elicited myocyte hyperpolarizations (Figure 2). In contrast, the endothelium-dependent smooth muscle hyperpolarization to substance P was totally abolished following incubation with HEPES (Edwards *et al*., 2001). In the present study, under control conditions, myocyte hyperpolarization to substance P (22.6 ± 0.6 mV, $n = 4$) was also abolished by 10 μM TRAM-39 + 100 nM apamin.

Identification of the ion channel opened by the endothelium-derived hyperpolarizing factor

In endothelium-intact coronary artery segments, bradykinin, 11,12-EET and 1-EBIO each produced myocyte hyperpolarization (Figure 3). To eliminate the pathway involving the activation of endothelial K^+ channels, these were first blocked

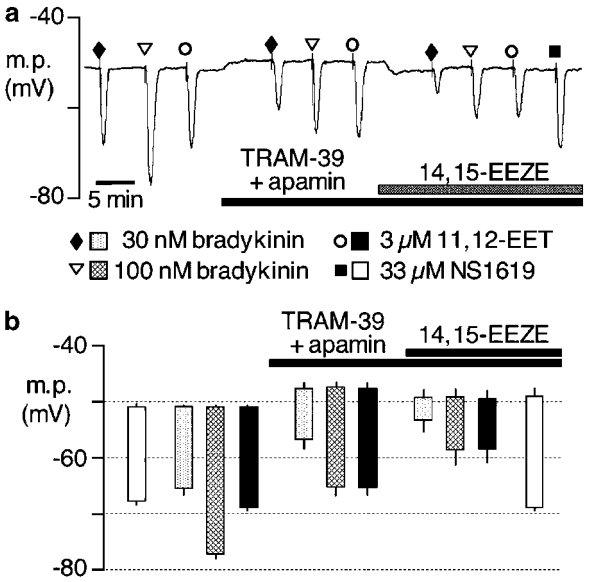

Figure 4 (a) Typical trace showing effect of 10 μM 14,15-EEZE and 100 nM iberiotoxin on 10 μM TRAM-39 + 100 nM apamin-resistant myocyte hyperpolarizations induced by bradykinin and 11,12-EET in endothelium-intact porcine coronary artery segments in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (b) Graphical representation of data from four separate experiments of the type shown in (a). Levcromakalim was used to demonstrate the integrity of the tissue. Each column represents the membrane potential (m.p.) ± s.e.m. ($n = 4$), before and during exposure to bolus doses of the agents indicated, calculated to give, transiently, the stated final bath concentrations.

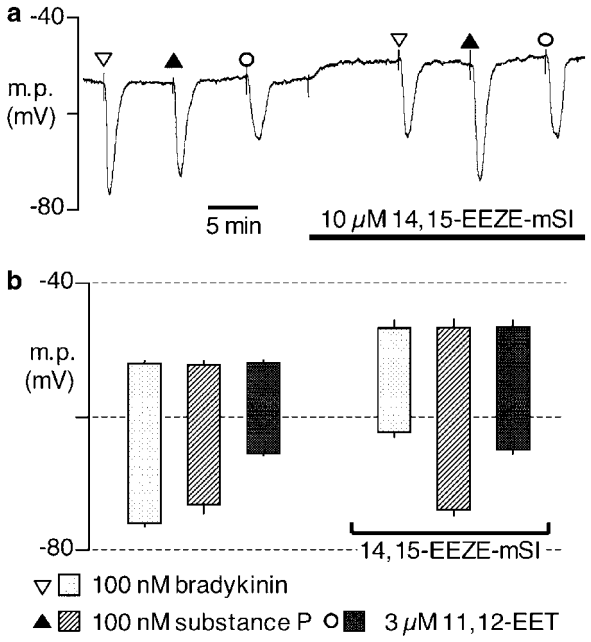

Figure 5 Effect of 14,15-EEZE-mSI on myocyte hyperpolarizations induced by bradykinin, substance P and 11,12-EET in endothelium-intact segments of porcine coronary arteries in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (a) Typical trace showing responses before and in the presence of 10 μM 14,15-EEZE-mSI. (b) Graphical representation of data from four separate experiments of the type shown in (a) in which each column represents the membrane potential (m.p.) ± s.e.m. ($n = 4$), before and during exposure to bolus doses of the agents indicated (calculated to give, transiently, the final bath concentrations indicated) in the absence and presence of 14,15-EEZE-mSI.

using 10 μM TRAM-39 + 100 nM apamin. Under these conditions, the effect of 1-EBIO was abolished ($P < 0.001$), whereas that to bradykinin was merely reduced (by approximately 50%; $P < 0.05$) and the hyperpolarization induced by 11,12-EET was unaffected ($P > 0.05$) (Figure 3). Subsequent exposure to iberiotoxin (the selective BK_{Ca} inhibitor; Galvez *et al*., 1990) abolished ($P < 0.05$) both the residual myocyte hyperpolarization to bradykinin and the effects of 11,12-EET (Figure 3).

Is an EET the endothelium-derived hyperpolarizing factor liberated by bradykinin?

To determine whether an EET was involved in part of the hyperpolarizing action of bradykinin, use was made of 14,15-EEZE which antagonizes all four regioisomers of EET (5,6-, 8,9-, 11,12- and 14,15-EET; Gauthier *et al*., 2003). With a protocol similar to that already described, the putative EET-mediated component of the action of bradykinin was isolated after exposure to TRAM-39 + apamin (Figure 4). On exposure to 14,15-EEZE in the continued presence of TRAM-39 + apamin, both the residual response to bradykinin and the effects of authentic 11,12-EET were reduced by approximately 50% ($P < 0.05$). However, the peak membrane potential induced by 33 μM NS1619 (an opener of BK_{Ca}; Olesen *et al*., 1994) in the presence of these inhibitors (-69.0 ± 0.3 mV, $n = 4$) was not significantly different from that which was induced, in a separate series of experiments, in their absence (-67.7 ± 0.7 mV, $n = 4$; $P > 0.05$; Figure 4b), indicating that 14,15-EEZE was not simply a blocker of BK_{Ca} channels.

Inhibition of response to bradykinin (but not substance P) by 14,15-EEZE-mSI

To obtain more information about the EET component of bradykinin's action, experiments were carried out using the EET antagonist 14,15-EEZE-mSI, which, in bovine coronary artery, inhibits the relaxant effects of 5,6- and 14,15-EET but, unlike 14,15-EEZE, does not inhibit relaxations to 8,9- or 11,12-EET (Gauthier *et al*., 2003).

In the first series of experiments, the effects of 14,15-EEZE-mSI on endothelium-dependent hyperpolarizations to bradykinin and substance P were assessed. The antagonist reduced ($P < 0.01$) the hyperpolarization to 100 nM bradykinin but not that to 100 nM substance P. Consistent with the findings in the bovine coronary artery (Gauthier *et al*., 2003), 14,15-EEZE-mSI had no effect on the response to 3 μM 11,12-EET in endothelium-intact porcine coronary artery segments ($P > 0.05$) (Figure 5).

Selectivity of 14,15-EEZE-mSI as an EET antagonist

In a further series of experiments, the selectivity of 14,15-EEZE-mSI was tested by examining its effects in endothelium-denuded arteries. Both 5,6- and 14,15-EET have been proposed to represent EDHF in rat and bovine coronary arteries, respectively (Fulton *et al*., 1998; Gauthier *et al*., 2003). In the present study, each hyperpolarized the myocytes in the absence of the endothelium, although in equimolar concentra-

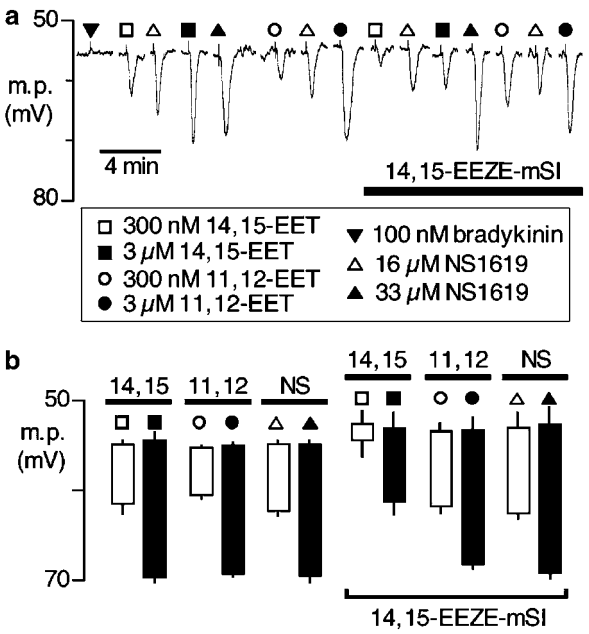

Figure 6 Effect of 10 μM 14,15-EEZE-mSI on myocyte hyperpolarizations induced by bradykinin, 11,12-EET, 14,15-EET and NS1619 in endothelium-denuded segments of porcine coronary arteries in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (a) Typical trace from a single continuous impalement but with sections between responses removed for clarity. (b) Graphical representation of data from four experiments of the type shown in (a) in which each column represents the membrane potential (m.p.) $\pm$ s.e.m. ($n=4$), before and after exposure to bolus doses of NS1619, 5,6-, 11,12- and 14,15-EET (calculated to give, transiently, the final bath concentrations indicated) in the absence and presence of 14,15-EEZE-mSI.

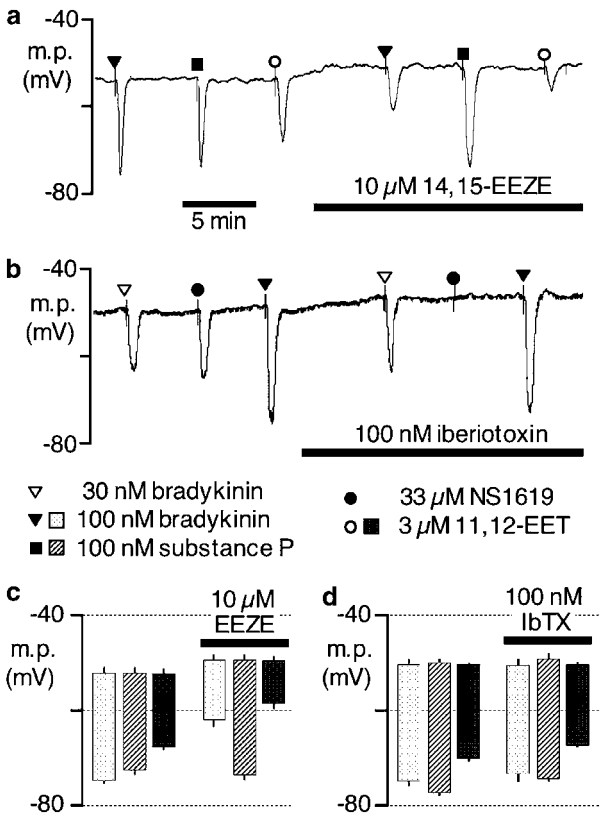

Figure 7 Typical traces comparing the effects of 14,15-EEZE (a) and iberiotoxin (b) on myocyte hyperpolarizations induced by transient exposure to bradykinin, substance P and 11,12-EET in endothelium-intact segments of porcine coronary arteries in the presence of 300 μM nitro-L-arginine and 10 μM indomethacin. (c, d) Graphical representation of data from experiments of the types shown in (a, b) in which each column represents the membrane potential (m.p.) $\pm$ s.e.m. ($n=4$), before and during exposure to bolus doses of bradykinin, substance P and 11,12-EET calculated to give, transiently, the final bath concentrations indicated.

tions (3 μM), the peak hyperpolarization to 14,15-EET (17.8 ± 0.5 mV, $n=4$) was significantly greater than that to 5,6-EET (12.4 ± 0.6 mV, $n=4$; $P<0.02$). However, the hyperpolarization to the BK_{Ca} opener NS1619 (33 μM; 17.7 ± 0.5, $n=4$) was similar to that produced by 3 μM 14,15-EET. Following exposure to the EET antagonist 14,15-EEZE-mSI, myocyte hyperpolarizations to both 5,6-EET (9.2 ± 0.6 mV, $n=4$) and 14,15-EET (7.5 ± 0.8 mV, $n=4$) were significantly reduced ($P<0.05$), whereas that to NS1619 (21.6 ± 0.4 mV, $n=4$) was increased ($P<0.02$) due to the depolarization induced by EEZE-mSI (3.4 ± 0.2 mV, $n=4$).

The lack of inhibitory effect of 14,15-EEZE-mSI on the response to 3 μM 11,12-EET (in the presence of the endothelium) or to 33 μM NS1619 (in the absence of the endothelium) could have resulted from the use of a supramaximal concentrations of agonists. Similarly, the inhibitory effect of the antagonist against 3 μM 14,15-EET may have been underestimated because of the use of a supramaximal concentration of the latter. Further experiments were thus performed to investigate the inhibitory effect of 14,15-EEZE-mSI on responses to submaximal concentrations of 14,15-EET and NS1619 (Figure 6).

In endothelium-denuded segments of artery, 11,12-EET, 14,15-EET and NS1619 produced concentration-dependent hyperpolarizations (Figure 6). Although not evident in the trace in Figure 6, 14,15-EEZE-mSI produced a small depolarization in some tissues. In the presence of this antagonist, the hyperpolarizations to 14,15-EET were substantially reduced, whereas responses to 11,12-EET and NS1619 (which in the absence of 14,15-EEZE-mSI were of similar magnitude to those of 14,15-EET) were unchanged (Figure 6).

Do bradykinin-generated EETs act on both myocytes and endothelial cells?

The studies so far described have shown that exogenous EETs can hyperpolarize not only endothelial cells (by opening IK_{Ca} and SK_{Ca}; Figure 2) but also myocytes (by opening BK_{Ca}; Figure 3). The possibility therefore arises that *endogenous* EETs generated by bradykinin exert not only a paracrine effect on the myocytes but also an autocrine action on the endothelial cells from which they are derived. An indication of the latter is apparent from the experiments shown in Figure 7. Thus, in endothelium-intact vessels, the EET antagonist 14,15-EEZE inhibited ($P<0.05$) bradykinin-induced myocyte hyperpolarizations, whereas the myocyte BK_{Ca} blocker, iberiotoxin, had no such inhibitory effect ($P>0.05$). Hyperpolarizations produced by substance P were also unaffected ($P>0.05$) by 14,15-EEZE, an indication of its antagonist selectivity.

Discussion

When the contributions of NO and prostacyclin to endothelium-dependent relaxations are eliminated using NO synthase and cyclooxygenase inhibitors, local hormones like bradykinin and substance P still relax blood vessels by a mechanism associated with myocyte hyperpolarization. An earlier study in porcine coronary artery segments (Edwards *et al*., 2001) suggested that the endothelium-dependent hyperpolarization of coronary myocytes produced by bradykinin is exerted by two separate, endothelium-dependent pathways. One of these (also activated by substance P) involved the opening of endothelial SK_{Ca} and IK_{Ca} channels and the transfer of endothelial cell hyperpolarization to surrounding myocytes *via* myo-endothelial gap junctions (Edwards *et al*., 2001; see also Busse *et al*., 2002). The other, involving EETs (Campbell *et al*., 1996; Li & Campbell, 1997; Eckman *et al*., 1998; Fisslthaler *et al*., 1999), was not activated by substance P but was totally inhibited by either iberiotoxin or 17-octadecynoic acid (Edwards *et al*., 2001). It is well established that endothelium-dependent myocyte hyperpolarization is abolished using a combination of charybdotoxin + apamin to block endothelial IK_{Ca} and SK_{Ca} channels, respectively (see Busse *et al*., 2002). However, when TRAM-39 was substituted for charybdotoxin in experiments preliminary to the present study (Weston *et al*., 2004), bradykinin-induced myocyte hyperpolarizations were only partially inhibited by TRAM-39 plus apamin. In the present investigation, the utilization of the selective IK_{Ca} inhibitor TRAM-39 (Wulff *et al*., 2000; 2001) together with the EET antagonists 14,15-EEZE and 14,15-EEZE-mSI (Gauthier *et al*., 2002a; 2003) has now clarified the mechanism of action of bradykinin in porcine coronary vessels.

Activation of endothelial cell calcium-sensitive K^+ channels

In the present study, both bradykinin and substance P induced endothelial cell hyperpolarization. In the simultaneous presence of TRAM-39 and apamin (inhibitors of IK_{Ca} and SK_{Ca} channels, respectively), the endothelial response to substance P was totally abolished indicating that it resulted from the opening of endothelial IK_{Ca} and SK_{Ca} channels. However, a residual hyperpolarization to bradykinin still occurred in endothelial cells in the presence of TRAM-39 + apamin and this response was iberiotoxin sensitive, indicating that it resulted from the opening of BK_{Ca}. It is assumed that such channels are located on the myocytes since there is little evidence for the *functional* presence of BK_{Ca} in nonproliferating endothelial cells (Bychkov *et al*., 2002; Gauthier *et al*., 2002b). However, unpublished data that we have obtained using RT–PCR and immunohistochemistry strongly suggest that the BK_{Ca} mRNA and protein *is* present in porcine coronary artery endothelial cells. Furthermore, when tested on these cells in primary culture, 5,6-, 8,9-, 11,12- and 14,15-EETs all activated BK_{Ca} channels (Baron *et al*., 1997). We thus considered the possibility that the channel could be present in endothelial cells but (possibly because it lacks a beta-subunit; Papassotiriou *et al*., 2000) is not activated by the bradykinin- or substance P-induced elevation of intracellular calcium. We considered the possibility that EETs could activate an endothelial cell BK_{Ca} and that this (rather than a channel on the myocyte) was responsible for the iberiotoxin-sensitive component of the endothelium-dependent myocyte hyperpolarization induced by bradykinin. However, the unequivocal localization of where such iberiotoxin-sensitive events are initiated in an intact vessel was not a simple task.

To determine this (in the present study), endothelial cells were electrically isolated from the surrounding myocytes by overnight incubation of artery segments in Tyrode solution containing HEPES (Edwards *et al*., 2001). HEPES reduces electrical coupling by physically blocking gap junctions (Bevans & Harris, 1999) and by reducing gap junction plaques within the membrane (Kilarski *et al*., 1995). In the porcine coronary artery, Western blot analysis suggests that overnight exposure to HEPES reduces connexin-40 protein but not that of connexins 37 and 43, the other major connexins found in this vessel (Gardener *et al*., 2003). We have not directly investigated the possibility that HEPES inhibits gap junctions within the myocyte or endothelial cell layers of the porcine coronary artery. Nevertheless, it appears that HEPES does indeed inhibit myo-endothelial cell coupling since the myocyte response to substance P was lost despite the normal hyperpolarization of the endothelium induced by this agonist. The effectiveness of the gap junction inhibition was confirmed using the K_{ATP} opener, levcromakalim (Edwards *et al*., 2001). Endothelial cells lack functional K_{ATP} channels and the endothelial hyperpolarization observed on exposure to K_{ATP} openers like levcromakalim is generated following *myocyte* hyperpolarization that is transmitted to the endothelium *via* gap junctions (Murai *et al*., 1999; White & Hiley, 2000; Edwards *et al*., 2001; Takano *et al*., 2004). In the present study, overnight incubation with HEPES abolished the electrical response to levcromakalim following the impalement of a endothelial cell whereas the myocyte responded normally to levcromakalim. This observation confirms both endothelial cell penetration by the electrode and the effectiveness of HEPES as an inhibitor of myo-endothelial cell gap junctions.

After incubation in HEPES-Tyrode, bradykinin produced endothelial cell hyperpolarizations that were abolished after inhibition of endothelial cell IK_{Ca} and SK_{Ca} channels with TRAM-39 + apamin. Unlike charybdotoxin (a previously available IK_{Ca} inhibitor), TRAM-39 does not block BK_{Ca} (Wulff *et al*., 2000; 2001). The selective inhibition of IK_{Ca} by TRAM-39 was also demonstrated in the present study, allowing the conclusion that bradykinin opens endothelial IK_{Ca} and SK_{Ca} channels without involving any endothelial cell BK_{Ca} conductances.

Bradykinin (but not substance P) releases an endothelium-derived hyperpolarizing factor that activates myocyte BK_{Ca} channels

Neither substance P nor bradykinin has a direct effect on porcine coronary artery myocytes in the absence of the endothelium (Nagao & Vanhoutte, 1992; Edwards *et al*., 2001). Results from a previous study showed that bradykinin (but not substance P) was still able to hyperpolarize the myocytes following inhibition of gap junctions using HEPES (Edwards *et al*., 2001). In the present study, these observations were extended by the finding that under similar conditions, bradykinin was still able to hyperpolarize the myocytes despite abolition of endothelial cell hyperpolarization by TRAM-39 + apamin. In both experimental situations, the action of

bradykinin was mimicked by 11,12-EET, one of the epoxyeicosatrienoic acids previously implicated in the actions of bradykinin in coronary arteries (Rosolowsky & Campbell, 1996; Fisslthaler *et al*., 1999). Furthermore, under these conditions, the effects of bradykinin and of 11,12-EET were abolished by the specific BK_{Ca} blocker, iberiotoxin. Collectively, these data strongly suggest that bradykinin releases a hyperpolarizing factor from the endothelium and that this factor opens myocyte BK_{Ca} channels, an action that it shares with 11,12-EET. Presumably, the iberiotoxin-sensitive component of bradykinin-induced endothelial cell hyperpolarization (which is lost after incubation in HEPES) reflects transfer of myocyte membrane potential changes to the endothelial cells by gap junctions. Interestingly, whereas endothelial cell hyperpolarization appears to be transferred to the myocytes without loss, the magnitude of the indirect endothelial cell hyperpolarization both to levcrokalim and to bradykinin (in the presence of TRAM-39 and apamin) were always less than the corresponding direct hyperpolarizations of the myocytes, suggesting possible rectification by the myo-endothelial cell gap junctions.

The endothelium-derived hyperpolarizing factor liberated by bradykinin is an EET that does not require endothelial hyperpolarization for release

Evidence that bradykinin liberates an EET from the vascular endothelium was initially sought using a nonselective EET antagonist, 14,15-EEZE, which was first described by Gauthier *et al*. (2002a). In endothelium-intact artery segments in which endothelial IK_{Ca} and SK_{Ca} channels had previously been blocked using TRAM-39 + apamin, the residual myocyte hyperpolarization to bradykinin was partially inhibited by 14,15-EEZE. The response to 3 μM 11,12-EET was also inhibited, to a similar extent, by 14,15-EEZE. The concentration of 11,12-EET was relatively high but, on the basis of previous tissue bath experiments using bovine coronary arteries (Gauthier *et al*., 2002a), it was unlikely to be supramaximal. Thus, we concluded that the failure of 14,15-EEZE to inhibit fully the response to 11,12-EET showed that 14,15-EEZE was not fully efficacious as an inhibitor of the epoxide, a finding similar to that reported by Gauthier *et al*. (2002a) in the bovine coronary artery. The observed partial inhibition of the bradykinin-induced hyperpolarization by 14,15-EEZE was thus consistent with the conclusions of an earlier study (Fisslthaler *et al*., 1999) that bradykinin-induced smooth muscle hyperpolarisation of porcine coronary arteries results from the release of 11,12-EET. Nevertheless, the observation in the present study was important because it indicated, for the first time, that EETs production by bradykinin did not require endothelial cell hyperpolarization since the iberiotoxin-sensitive component of myocyte hyperpolarization was observed *after* endothelial K_{Ca} channels had been blocked using TRAM-39 + apamin. Furthermore, it demonstrated that EETs were liberated from the endothelium to produce a paracrine effect by stimulating the opening of BK_{Ca} channels on the underlying smooth muscle.

To obtain more information about the specific EET regioisomer(s) generated by bradykinin, experiments were carried out using 14,15-EEZE-mSI (Gauthier *et al*., 2003). In the presence of this antagonist, there was paradoxically no significant inhibition of responses to substance P or to 11,12-EET, whereas hyperpolarizations to bradykinin and to 5,6- and 14,15-EET were significantly reduced. Such findings, coupled with the inability of 14,15-EEZE-mSI to modify the actions of the BK_{Ca} activator NS1619, provide powerful evidence that the production of 5,6- or 14,15-EET is a component of the action of bradykinin (but not of substance P). Such findings conflict with those of a previous study (Fisslthaler *et al*., 1999) in which 11,12-EET was the only regio-isomer detected in porcine coronary endothelial cell extracts. However, the data from the present study are consistent with a mass-spectrophotometric analysis of bovine coronary endothelium in which the 14,15-isomer was found to be the major EET in bovine coronary endothelium (Rosolowsky & Campbell, 1996).

Collectively, the findings of the present study, together with those of earlier investigations (Rosolowsky & Campbell, 1996; Fisslthaler *et al*., 1999), suggest that *both* 14,15- *and* 11,12-EET are the most likely of the EET regio-isomers to be involved in the actions of bradykinin but that these cytochrome *P*450 metabolites do not contribute to the response to substance P.

Bradykinin-generated EETs exert both autocrine and paracrine effects on endothelial and smooth muscle cells, respectively

When applied exogenously, EET regioisomers stimulate the opening of BK_{Ca} channels in coronary artery myocytes and endothelial cells (Baron *et al*., 1997; Li & Campbell, 1997). To determine whether bradykinin-generated endogenous EETs exerted both autocrine and paracrine actions, the ability of 14,15-EEZE and iberiotoxin to antagonize bradykinin was

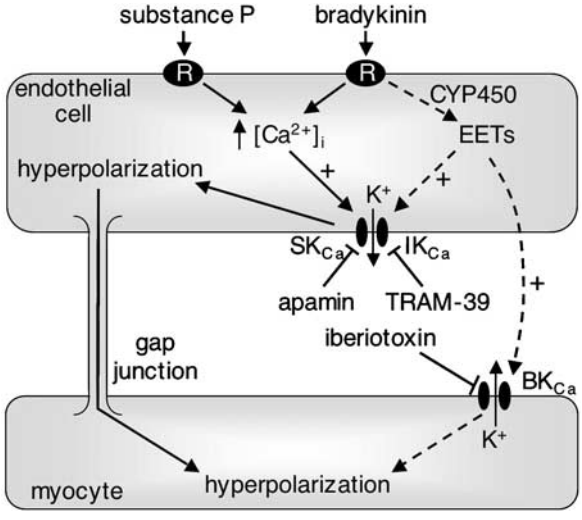

Figure 8 Bradykinin produces endothelium-dependent myocyte hyperpolarization in coronary arteries *via* two pathways. One of these (solid lines) involves the opening of endothelial SK_{Ca} and IK_{Ca} channels that can be blocked with apamin and TRAM-39, respectively. Substance P activates only this pathway. The other (dashed lines) involves the generation of epoxyeicosatrienoic acids (EETs) *via* a cytochrome *P*450 (CYP450)-dependent mechanism. EETs not only activate endothelial SK_{Ca} and IK_{Ca} channels but also open myocyte BK_{Ca} channels sensitive to iberiotoxin. This component is usually masked by the hyperpolarization resulting from the opening of endothelial SK_{Ca} and IK_{Ca} channels. Neither the generation of EETs nor their effect on BK_{Ca} requires endothelial cell hyperpolarization.

compared. If the generated EETs acted solely as paracrine factors on the myocytes, it was reasoned that any antagonism of bradykinin by 14,15-EEZE should be matched by a similar degree of antagonism in the presence of iberiotoxin. In fact, the ability of 14,15-EEZE to antagonise bradykinin contrasted with the *ineffectiveness* of iberiotoxin. These results thus suggest that EETs exert not only an autocrine action on the endothelial cells from which they are derived but also that this action is dominant. Any paracrine effects of these fatty acids are uncovered only when the key autocrine component involving endothelial K_{Ca} channels is blocked using TRAM-39 + apamin.

Conclusions

The development of selective IK_{Ca} blockers (Wulff *et al*., 2000; 2001) and of EET antagonists (Gauthier *et al*., 2002a; 2003) coupled with precise intracellular microelectrode recordings has allowed the endothelium-dependent, myocyte hyperpolarizing actions of bradykinin in porcine coronary arteries to be separated into two parallel pathways (Figure 8). One of these functions to open endothelial cell IK_{Ca} and SK_{Ca} channels, an action that hyperpolarizes the myocytes *via* gap junctions. This aspect of the action of bradykinin is thus identical to that of substance P, another autacoid that produces endothelium-dependent myocyte hyperpolarizations in several vessels (Edwards *et al*., 2001; Busse *et al*., 2002). However, unlike substance P, bradykinin also activates another pathway that is not associated with any change in endothelial cell membrane potential and involves the generation of EETs. The most likely candidates are 14,15- and 11,12-EET although 5,6- and 8,9-EET cannot be totally excluded. These fatty acids exert an autocrine action on the endothelial cells from which they are derived and open endothelial IK_{Ca} and SK_{Ca} channels. In addition, EETs are liberated from the endothelium to activate BK_{Ca} channels on the surrounding myocytes. Characterization of the intracellular pathways that enable bradykinin, but not substance P, to generate EETs is the subject of an ongoing study.

This study was supported by grants from the British Heart Foundation (GE, AHW), the National Institutes of Health (Hl-51055 and GM31278) (WBC and JRF) and the Robert A. Welch Foundation (JRF).

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(Received January 14, 2005
Revised March 18, 2005
Accepted April 4, 2005
Published online 16 May 2005)