Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals

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1. In addition to nitric oxide (NO) and prostacyclin (PGI_2) an as yet unidentified endothelium-derived hyperpolarizing factor (EDHF) contributes to the dilator effect of bradykinin in different vascular beds. We have investigated the nature and mechanism of action of this factor in freshly isolated bovine and porcine coronary artery segments which were preconstricted with the thromboxane mimetic U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$, 10–30 nM).
2. The concentration–response curve of bradykinin was significantly shifted to the right after inhibition of NO synthesis with N^G-nitro-L-arginine (L-NNA, 30 μM), whereas cyclo-oxygenase blockade with diclofenac (1 μM) had no effect. Preconstriction of the segments with potassium chloride (40–60 mM) completely abrogated the NO/PGI_2-independent dilator response to bradykinin. In sandwich bioassay experiments, both the luminal and abluminal release of NO, but not that of EDHF, was readily detectable.
3. Inhibitors of Ca^{2+}-activated K^+ channels (K^+_{Ca}), such as apamin (1 μM) and tetrabutylammonium (TBA, 3 mM), strongly attenuated the EDHF-mediated bradykinin-induced relaxation, while glibenclamide (3 μM), an inhibitor of K^+_{ATP} channels, had no effect.
4. These relaxations were also significantly inhibited by the phospholipase A_2 inhibitor, quinacrine (30 μM), and the cytochrome P450 inhibitors, SKF525a (30–100 μM) and clotrimazole (100 μM). Moreover, incubation of endothelium-denuded coronary artery rings with a cytochrome P450-derived arachidonic acid metabolite, 11,12-epoxyeicosatetraenoic acid, elicited a concentration-dependent (1–10 μM) dilatation which was abolished both in the presence of TBA (3 mM) and following preconstriction of the segments with potassium chloride instead of U46619.
5. These findings suggest that EDHF released by bradykinin is a cytochrome P450-derived arachidonic acid metabolite, presumably an epoxide. This factor seems to hyperpolarize the underlying smooth muscle cell layers by opening K^+_{Ca} channels.

The vascular endothelium is capable of generating three potent vasodilator autacoids, nitric oxide (NO), prostacyclin (PGI_2), and an endothelium-derived hyperpolarizing factor (EDHF). The release of this factor from the endothelium is triggered by receptor-dependent agonists, such as acetylcholine, bradykinin, histamine or substance P, and has been demonstrated in various arteries from different species (Chen, Yamamoto, Miwa & Suzuki, 1991; Cowan & Cohen, 1991; Kauser & Rubanyi, 1992; Suzuki, Chen & Yamamoto, 1992; Holzmann, Kukovetz, Windischhofer, Paschke & Graier, 1994). Although in principle this endothelium-dependent hyperpolarization could be elicited by an agonist-induced hyperpolarization of the endothelium spreading to the underlying smooth muscle via gap junctions, the release of a diffusible endothelial factor which causes an opening of K^+ channels in the smooth muscle cell layers of the media seems to be more likely (Suzuki *et al.* 1992; Bény & Pacicca, 1994). The validity of this hypothesis, however, still remains to be established. This is hampered by the fact that the release of EDHF could not be demonstrated by conventional bioassay techniques which readily detect NO

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released from endothelium-intact donor segments (Kauser & Rubanyi, 1992). Although the release of an EDHF-like factor, whose action is sensitive to ouabain, has been described in canine coronary arteries (Hoeffner, Feletou, Flavahan & Vanhoutte, 1989), the majority of endothelium-dependent hyperpolarizations appears to be ouabain insensitive (Suzuki *et al.* 1992), hence arguing against a major role of the electrogenic Na^+–K^+ pump in EDHF-induced vasodilatation.

The aim of the present study was to characterize the nature and mechanism of action of EDHF in more detail in freshly isolated porcine and bovine coronary artery segments.

METHODS

Superfusion and organ bath studies

Porcine and bovine hearts were obtained from a local slaughterhouse, placed immediately into ice-cold Krebs–Henseleit solution (see below), and transported to the laboratory. Both the right and left coronary arteries were dissected, cleaned of adventitial adipose and connective tissue, and cut into rings of 3–4 mm width which were mounted between K30 force transducers (Hugo Sachs Elektronik, March, Germany) and a rigid support for measurement of isometric force. Four rings were simultaneously incubated in 10 ml organ baths (Schuler-Organbad, kindly made available by Hugo Sachs Elektronik) containing warmed (37 °C), oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit solution, pH 7·4 (composition (in mmol l^{-1}): Na^+, 144·0; K^+, 5·9; Cl^-, 126·9; Ca^{2+}, 1·6; Mg^{2+}, 1·2; $H_2PO_4^-$, 1·2; SO_4^{2-}, 1·2; HCO_3^-, 25·0; D-glucose, 11·1) to which the cyclo-oxygenase inhibitor diclofenac was added at a concentration of 1 μM. In another series of experiments, four ring segments were simultaneously superfused at 3·0 ml min^{-1} with the same salt solution. Passive tension was adjusted over a 30 min equilibration period to 5 g; thereafter the segments were preconstricted with 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619, 10–30 nM) to 10 g of tension. In some experiments, the endothelium was removed mechanically, and its absence confirmed by the lack of a relaxant response to bradykinin.

In addition to the superfusion and organ bath studies, some experiments were also performed with luminally perfused bovine or porcine coronary artery segments, as described in detail elsewhere (Hecker, Mülsch, Bassenge & Busse, 1993). The segments were preconstricted with U46619 (30–300 nM) added to the organ bath. To monitor the release of EDHF from these segments, they were pretreated for 30 min with N^G-nitro-L-arginine (L-NNA, 30 μM).

Sandwich bioassay

An endothelium-intact and a denuded ring segment of porcine coronary artery were cut open and mounted between a force transducer and rigid support with the aid of small metal clips. The strips were separately superfused with Krebs–Henseleit solution at 3 ml min^{-1}, and their responses to bradykinin were tested. Thereafter, the endothelial surface of the donor tissue was brought into contact with the smooth muscle surface of the detector tissue by placing the endothelium-intact segment on top of the denuded segment at an angle of 90 deg (experimental set-up to study the luminal release of NO and EDHF). To study the abluminal release of EDHF, the adventitia and outer layers of the media were removed from the endothelium-intact segments by splitting the media, and the smooth muscle surface of these segments was placed into close apposition with the smooth muscle surface of the denuded detector tissues. L-NNA was added to the superfusate at a concentration of 100 μM to distinguish between the release of NO and EDHF from the donor tissue.

Statistics

Unless indicated otherwise, all data in the figures and text are expressed as means ± S.E.M. of n experiments (with four ring segments) from different hearts. Statistical evaluation was performed by one-way analysis of variance followed by a Bonferroni t test with a P value $< 0·05$ considered statistically significant.

Materials

Bradykinin (acetate salt) was purchased from Bachem (Heidelberg, Germany); diclofenac (Voltaren® I.M. injection solution) from Ciba-Geigy (Wehr, Germany); N^G-nitro-L-arginine (free acid) from Serva (Heidelberg, Germany); calmidazolium, clotrimazole, 7-ethoxyresorufin, NDGA (nordihydroguaretic acid), octanol, quinacrine, sodium nitroprusside and tetrabutylammonium from Sigma (Germany); apamin and charybdotoxin from Alomone Laboratories (Jerusalem, Israel); 11,12-epoxy-5,8, 14-eicosatrienoic acid (11,12-EET) from Paesel (Frankfurt am Main, Germany); arachidonic acid from Larodan (Malmö, Sweden); and genestein from Calbiochem (Germany). Glibenclamide and Hoe 140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin) were kindly provided by Hoechst (Frankfurt am Main, Germany), and glyceryl trinitrate by Pohl-Boskamp (Hohenlockstedt, Germany). SKF525a (proadifen), U46619 and Iloprost (methyl prostacyclin) were generous gifts from Cassella (Frankfurt am Main, Germany), UpJohn (Ann Arbor, MI, USA) and Schering (Berlin, Germany), respectively.

RESULTS

Characterization of the coronary dilator response to bradykinin

In endothelium-intact ring segments of porcine coronary arteries preconstricted with U46619, bradykinin induced a concentration-dependent dilator response (Fig. 1) which was completely abrogated either by removal of the endothelium ($n = 7$) or pretreatment with the bradykinin (B_2) receptor antagonist Hoe 140 (0·1 μM, $n = 11$) (Hock *et al.* 1991). The bradykinin-induced relaxations were not altered when the cyclo-oxygenase inhibitor diclofenac (1 μM) was omitted from the Krebs–Henseleit solution ($n = 6$). Moreover, the PGI_2 mimetic, Iloprost, in concentrations of up to 100 μmol l^{-1}, had no effect on the tone of the preconstricted coronary artery segments ($n = 3$), hence ruling out an involvement of PGI_2 in the dilator response to bradykinin.

In the presence of the NO synthase inhibitor L-NNA (30 μM) the concentration–response curve of bradykinin was significantly shifted to the right (Fig. 1) with the ED_{50} increasing from 10 ± 2 to 71 ± 16 nM ($P < 0·01$). However, the

maximum dilator effect remained virtually unchanged. This L-NNA/diclofenac-insensitive relaxation was abolished when the segments were preconstricted with potassium chloride (40–60 mM) to the same level of tone as with U46619 (Fig. 1), suggesting that it was mediated by an endothelium-dependent hyperpolarization. Preconstriction of the segments with potassium chloride in the absence of L-NNA also shifted the concentration–response curve of bradykinin to the right (ED_{50} 37 ± 8 nM; $P < 0.05$), and decreased its maximum dilator effect (Fig. 1). Virtually identical results were also obtained with bovine coronary artery segments (data not shown).

In an additional set of experiments, the effect of the gap junction uncoupler (White, Spray, Campos de Carvalho, Wittenberg & Bennett, 1985) octanol (1 mM) on the L-NNA/diclofenac-insensitive dilator response to bradykinin was investigated in luminally perfused porcine coronary artery segments. However, the bradykinin-induced relaxation was not different in the presence or absence of octanol (48 ± 5 and 44 ± 11% relaxation, respectively; $n = 4$).

NO but not EDHF release can be monitored by bioassay

By using two different sandwich bioassay techniques, we attempted to monitor the release of EDHF from the coronary endothelium both in the luminal and abluminal direction. While the release of NO was readily detected with both set-ups ($n = 8$ for the luminal release; $n = 3$ for

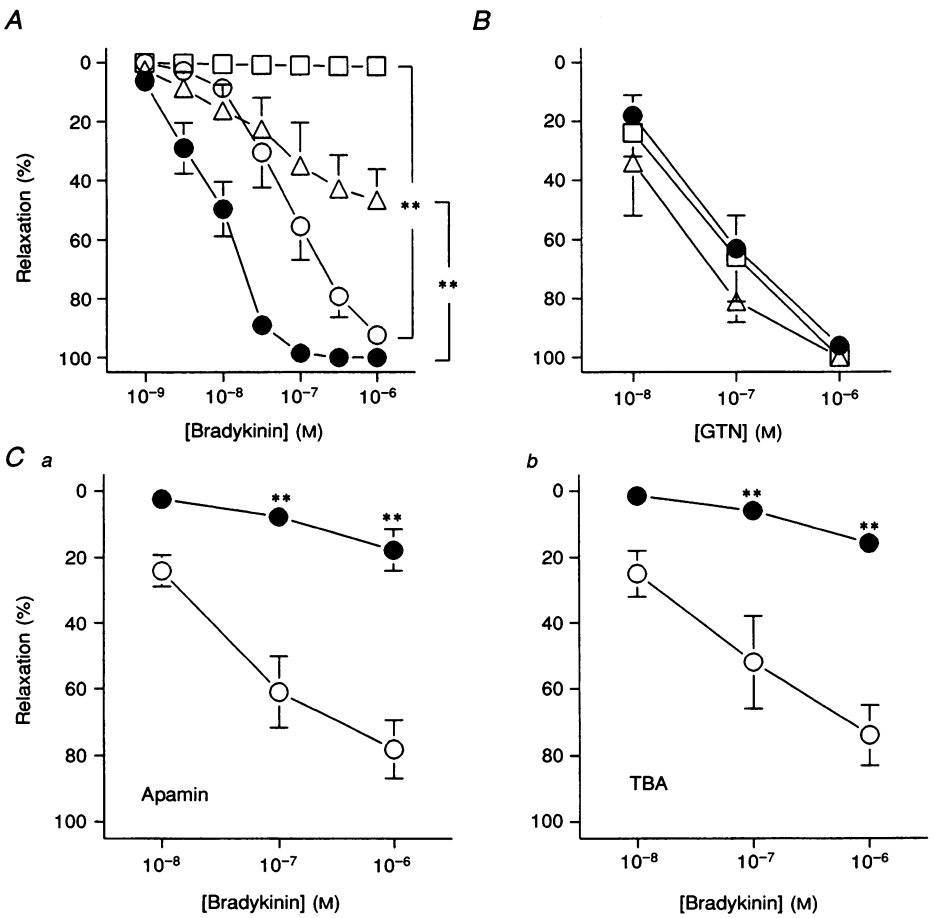

Figure 1. The NO/PGI_2 -independent dilator response to bradykinin involves the activation of K^+_{Ca} channels

A, concentration-dependent relaxation by bradykinin of porcine coronary artery segments incubated in 10 ml organ baths and preconstricted with U46619 or potassium chloride (40–60 mM) in the absence (●, U46619; Δ, KCl; $n = 8$) or in the presence of L-NNA (30 μM; ○, U46619; □, KCl; $n = 8$; $^{**}P < 0.01$ as indicated). *B*, effects of apamin (1 μM, □, $n = 3$) and TBA (3 mM, Δ, $n = 3$) on the endothelium-independent dilator response to glyceryl trinitrate (GTN, ●, $n = 9$). *C*, effects of apamin (1 μM, ●, $n = 6$) (*a*) and TBA (3 mM, ●, $n = 5$) (*b*) on the dilator responses to bradykinin of L-NNA/diclofenac-treated segments incubated in 10 ml organ baths and preconstricted with U46619 (○, $^{**}P < 0.01$ *vs.* control).

the abluminal release, Fig. 2), no dilator response was detected following inhibition of NO synthesis with L-NNA.

The endothelium-dependent smooth muscle cell hyperpolarization is brought about by the opening of Ca^{2+}-activated K^+ (K^+_{Ca}) channels

We next investigated the type of K^+ channel involved in the endothelium-dependent hyperpolarization elicited by bradykinin. Pretreatment with apamin (1 μM) and tetrabutylammonium (TBA, 3 mM) strongly reduced the L-NNA/diclofenac-insensitive dilator responses to bradykinin (Fig. 1). At 0·3 mM, the inhibitory effect of TBA already reached a maximum (from 25 ± 7, 52 ± 14 and 74 ± 9% relaxation at 0·01, 0·1 and 1 μM bradykinin to 7 ± 4, 17 ± 7* and 20 ± 10%**, respectively; $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$, $n = 5$). In contrast to apamin and TBA, charybdotoxin (10 nM), another K^+_{Ca} channel inhibitor, had no effect ($n = 3$). Similarly, the K^+_{ATP} channel inhibitor glibenclamide (3 μM) did not attenuate the L-NNA/diclofenac-insensitive dilator response to bradykinin (100 nM) in luminally perfused porcine coronary artery segments (81 ± 12 and 75 ± 9% relaxation, respectively, $n = 4$). Comparable findings, suggesting an involvement of apamin-sensitive K^+_{Ca} channels in the EDHF-mediated relaxation induced by bradykinin, were also obtained with bovine coronary artery segments (data not shown). None of the K^+ channel inhibitors tested affected the dilator response to glyceryl trinitrate (Fig. 1).

Role of arachidonic acid metabolites

To investigate the possibility that non-prostanoid arachidonic acid metabolites are involved in the EDHF-mediated dilation, inhibitors of these pathways were employed. While phospholipase A_2 inhibition with quinacrine (10–30 μM) significantly attenuated the L-NNA/diclofenac-insensitive relaxant response to

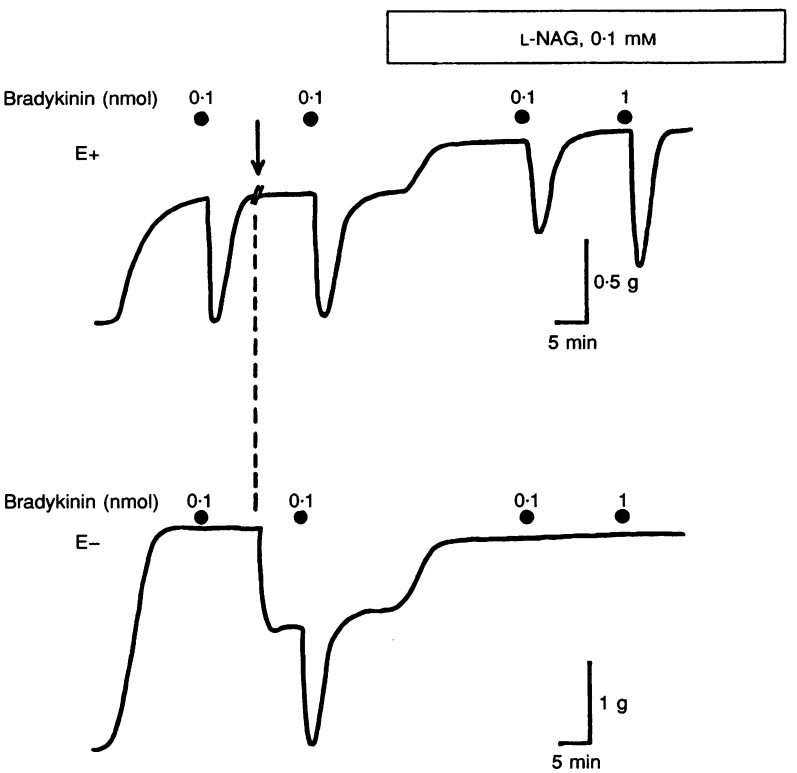

Figure 2. Detection of the abluminal release of NO, but not EDHF, from a superfused endothelium-intact segment (E+) of porcine coronary artery

Typical trace representative of 3 experiments performed in the same manner. After separately testing the relaxant response of both tissues to bradykinin (100 pmol bolus application), the endothelium-intact donor tissue (E+) was placed on top of the endothelium-denuded (E−) detector tissue at an angle of 90 deg with the smooth muscle layers facing each other (indicated by the arrow and the dashed line). After stabilization, bradykinin was applied again and induced a marked and virtually simultaneous relaxation of both tissues. Following administration of N^G-nitro-L-arginine (0·1 μM), there was a significant increase in tone both of the donor and detector segment, indicative of a loss of basal NO release. The dilator response of the donor segment to bradykinin was only partially reduced, whereas bradykinin no longer induced a relaxation of the detector tissue, even at a dose of 1 nmol.

bradykinin (Fig. 3), blockade of lipoxygenase activity with NDGA (10–30 μM) had no effect ($n = 5$).

Since these findings pointed to the involvement of a cytochrome P450-derived arachidonic acid metabolite, we next examined the effects of various cytochrome P450 inhibitors. Clotrimazole (100 μM) and SKF525a (100 μM; Fig. 3), but not 7-ethoxyresorufin (1 μM, $n = 3$), strongly attenuated the bradykinin-induced dilation. Neither cytochrome P450 inhibitor affected the endothelium-independent relaxant response to glyceryl trinitrate ($n = 3$). Unlike SKF525a, which significantly ($P < 0.01$) shifted the concentration–response curve of bradykinin to the right, clotrimazole mainly reduced the amplitude of the dilator response to the peptide. A weak inhibitory effect of clotrimazole was already apparent at a concentration of 10 μM ($n = 3$). The inhibition by SKF525a was less pronounced at 30 μM, but still significant (from 14 ± 4, 65 ± 8 and 90 ± 6% relaxation at 0·01, 0·1 and 1 μM bradykinin to 3 ± 1, 24 ± 5** and 70 ± 7%, respectively; **$P < 0.01$, $n = 4$). Concentrations of the two cytochrome P450 inhibitors exceeding 100 μM were not employed, since they started to precipitate in the Krebs–Henseleit solution. In bovine coronary artery rings incubated in organ baths, clotrimazole and SKF525a also strongly attenuated the NO/PGI_2-independent relaxant response to bradykinin (data not shown).

Characterization of the relaxant effect of 11,12-EET

Since these findings supported the notion that a cytochrome P450-derived arachidonic acid metabolite mediates the NO/PGI_2-independent relaxation of the coronary smooth muscle, we investigated the relaxant effect of such a metabolite (11,12-EET) (Rosolowsky & Campbell, 1993). The epoxide elicited a concentration-

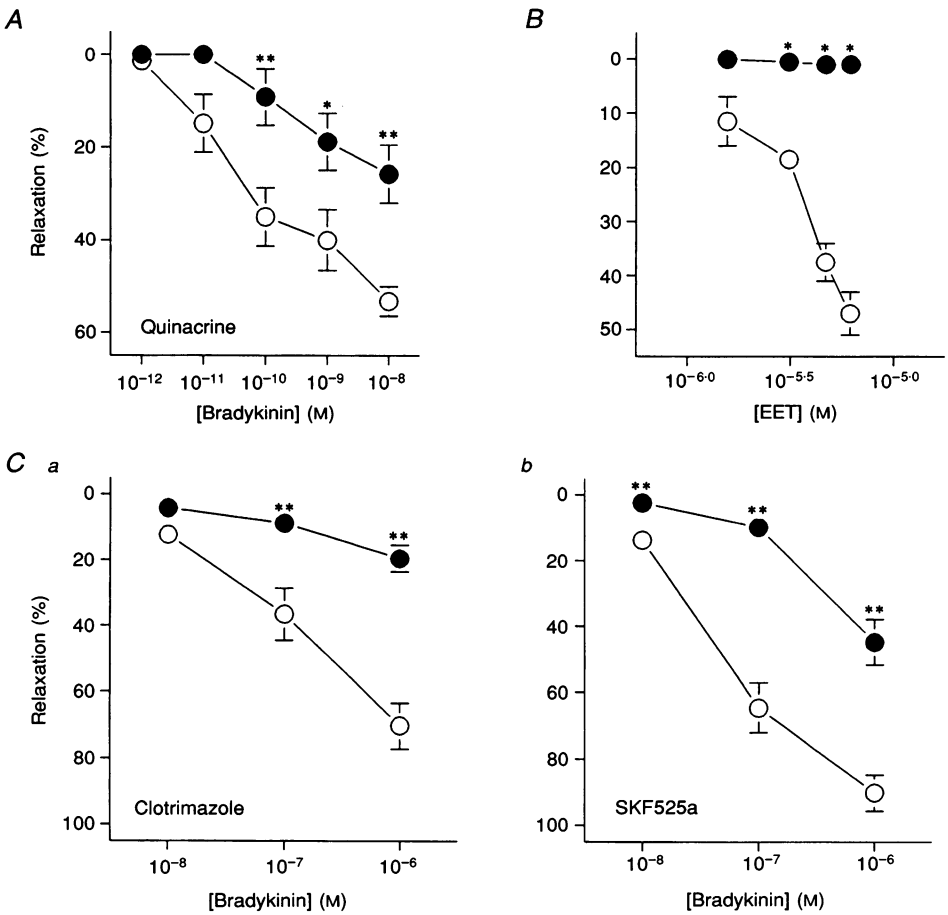

Figure 3. The NO/PGI_2-independent dilator response to bradykinin is mediated by a cytochrome P450-derived arachidonic acid metabolite

A, inhibition by quinacrine (30 μM, ●, $n = 6$) of the L-NNA/diclofenac-insensitive dilator response to bradykinin (○) of superfused porcine coronary artery segments (*$P < 0.05$, **$P < 0.01$ *vs.* control). *B*, inhibition by TBA (3 mM, ●, $n = 3$) of the dilator response to 11,12-EET (○) of endothelium-denuded rings segments of porcine coronary artery preconstricted with U46619 (**$P < 0.01$ *vs.* control). *C*, effects of clotrimazole (100 μM, ●, $n = 6$) (*a*) and SKF525a (100 μM, ●, $n = 5$) (*b*) on the dilator responses to bradykinin of L-NNA/diclofenac-treated segments incubated in 10 ml organ baths and preconstricted with U46619 (○, **$P < 0.01$ *vs.* control).

dependent (1–10 μM) relaxation of endothelium-denuded porcine coronary artery segments preconstricted with U46619, and these relaxations were blocked by TBA (3 mM; Fig. 3). Significantly, 11,12-EET failed to relax rings preconstricted with depolarizing concentrations (40–60 mM) of potassium chloride ($n = 4$). Since the epoxide was dissolved in ethanol, concentrations exceeding 10 μM (final ethanol concentration 1% v/v) could not be employed due to the constrictor effect of the solvent. In contrast with 11,12-EET, arachidonic acid (0·1–100 μM) had no effect on the tone of the endothelium-denuded segments ($n = 4$).

DISCUSSION

The findings described above demonstrate that, in isolated porcine and bovine coronary arteries, the NO/PGI_2-independent dilator response to bradykinin is mediated by EDHF. This conclusion is based on the finding that high K^+ concentrations (40–60 mM) abolished, and K^+_{Ca} channel inhibitors strongly attenuated, the L-NNA/diclofenac-insensitive bradykinin-induced relaxation.

Although the nature of EDHF has not yet been elucidated, recent studies suggest that the release of a diffusible substance from the endothelium rather than an electrical signal transmitted from endothelial to smooth muscle cells mediates the vasodilatation (Bény & Pacicca, 1994). Moreover, the number of myoendothelial junctions through which the agonist-induced hyperpolarization of the endothelium could be transmitted to the smooth muscle is rather small, and dye coupling between endothelial and smooth muscle cells also appears to be weak (Bény & Pacicca, 1994). We addressed the possibility of a transmission of an electrical signal between endothelial and smooth muscle cells via myoendothelial junctions by using octanol as an uncoupling agent (White *et al.* 1985). The finding that octanol had no effect on the NO/PGI_2-independent relaxant response to bradykinin supports the concept that the release of a humoral endothelium-derived factor is linked to the activation of K^+ channels in the smooth muscle cell membrane.

Both in the porcine and bovine coronary artery preparation, TBA, a non-selective inhibitor of K^+_{Ca} channels, but not the K^+_{ATP} channel inhibitor glibenclamide, almost abolished the NO/PGI_2-independent dilator response to bradykinin, suggesting the involvement of K^+_{Ca} channels in the EDHF-mediated relaxation (Cowan & Cohen, 1991; Holzmann *et al.* 1994). Apamin, a selective inhibitor of small conductance (6–14 pS) K^+_{Ca} channels (Blatz & Magleby, 1986), but not charybdotoxin, which blocks large conductance (35–250 pS) K^+_{Ca} channels (Miller, Moczydlowski, Latorre & Phillips, 1985), strongly inhibited the NO/PGI_2-independent dilator response to bradykinin, indicating that the EDHF-mediated relaxation in these vascular beds is due to the opening of small conductance K^+_{Ca} channels.

The relaxant response to bradykinin in isolated porcine coronary arteries is mediated by the release of at least two endothelium-derived vasodilator substances, NO and EDHF (Kauser & Rubanyi, 1992; Holzmann *et al.* 1994). While the mechanism by which NO formation occurs in endothelial cells is well established, little is known about the signal transduction cascade leading to the release of EDHF. Preconstriction with potassium chloride in the absence of L-NNA produced a shift to the right of the bradykinin concentration–response curve similar to that caused by L-NNA in segments preconstricted with U46619. Moreover, the amplitude of the dilator response to bradykinin was also significantly reduced, presumably due to the fact that high K^+ concentrations also attenuate the dilator response to NO. Blockade of the bradykinin (B_2) receptor by HOE 140 completely abrogated the dilator effect of bradykinin in the absence of L-NNA at low K^+ concentrations, suggesting (i) that the release of NO and EDHF following bradykinin administration is simultaneously triggered by activation of the bradykinin (B_2) receptor and the subsequent increase in intracellular Ca^{2+}, and (ii) that both autacoids contribute to the resulting relaxation to a similar extent, possibly in an additive manner (Holzmann *et al.* 1994).

The phospholipase A_2 inhibitor quinacrine significantly attenuated the NO/PGI_2-independent dilator response to bradykinin, suggesting that the release of arachidonic acid itself or that of a metabolite is involved therein. Since arachidonic acid *per se* did not influence the tone of endothelium-denuded porcine coronary arteries, the EDHF-type relaxation is likely to be mediated by a metabolite of this fatty acid. Neither cyclo-oxygenase inhibition with diclofenac nor lipoxygenases blockade with NDGA affected the EDHF-mediated relaxant response to bradykinin, leaving a cytochrome P450-derived arachidonic acid metabolite as the most likely candidate. A lack of effect has also been reported for other cyclooxygenase and lipoxygenase inhibitors in coronary arteries from various species (Pacicca, von der Weid & Bény, 1992; Nakashima, Mombouli, Taylor & Vanhoutte, 1993; Holzmann *et al.* 1994). Moreover, even though most coronary arteries are capable of synthesizing PGI_2 (Rosolowsky & Campbell, 1993), some respond only weakly to the prostanoid (Pinto, Abraham & Mullane, 1987), or not at all, such as the porcine coronary artery preparation employed in this study (Pacicca *et al.* 1992).

Further support for the notion that the NO/PGI_2-independent dilator response to bradykinin is mediated by a cytochrome P450-derived arachidonic acid metabolite comes from the finding that these relaxations were markedly reduced in the presence of the cytochrome P450 inhibitors clotrimazole and SKF525a. In contrast, the cytochrome P450 inhibitor, 7-ethoxyresorufin, which blocks the dilator effect of bradykinin in the rat kidney (Fulton, McGiff & Quilley, 1992), had no effect, suggesting that different cytochrome P450 isoenzymes may be involved in the metabolism of arachidonic acid in different vascular beds.

The possibility that this cytochrome P450-derived

arachidonic acid metabolite is an epoxide or a mixture thereof is substantiated by the following findings. Endothelium-intact canine (Pinto *et al.* 1987) and bovine (Rosolowsky & Campbell, 1993) coronary artery segments relax in response to exogenous arachidonic acid, and these relaxations can be partially blocked by both cyclo-oxygenase and cytochrome P450 inhibitors, whereas they are abolished by the combined treatment with these agents. These coronary arteries are also capable of metabolizing arachidonic acid to the four regiospecific epoxides at position 5, 8, 11 and 14, and the synthesis of these epoxides is abolished after cytochrome P450 inhibition. One cytochrome P450 inhibitor, SKF525a, also attenuated the bradykinin-induced relaxation in canine coronary arteries (Pinto *et al.* 1987), and the four epoxyeicosatetraenoic acids were shown to be potent coronary vasodilators irrespective of the presence of an intact endothelium (Rosolowsky & Campbell, 1993).

Our finding that 11,12-EET relaxes endothelium-denuded porcine coronary artery segments preconstricted with U46619 confirms these observations. Although we do not provide direct experimental evidence for a hyperpolarization of the coronary smooth muscle by the epoxide, i.e. by monitoring changes in membrane potential, this can be inferred from the lack of effect of 11,12-EET following preconstriction of the segments with depolarizing concentrations of K^+. Moreover, the equally strong inhibitory effect of TBA on the dilator response to 11,12-EET implies an involvement of K^+_{Ca} channels in this response. Indeed, all four epoxides have been shown *per se* to activate these channels in cultured coronary smooth muscle cells (Hu & Kim, 1993).

Taken together these findings suggest that in the coronary endothelium, bradykinin stimulates the release of arachidonic acid from phospholipids which in turn is metabolized to one or several epoxides via the cytochrome P450 pathway. These epoxides diffuse either freely or via gap junctions not susceptible to uncoupling by octanol to the underlying smooth muscle cells where they enhance K^+_{Ca} channel activity with subsequent vasodilation. According to our sandwich bioassay experiments, the release of these epoxides appears to be strictly abluminal, possibly due to the compartmentalization of the enzymes involved. Moreover, due to their hydrophobic and labile nature, these epoxides may not diffuse beyond the first layer of smooth muscle cells. In view of the tight electrical coupling of smooth cells, however, hyperpolarization of the first smooth muscle cell layer would be sufficient to cause a hyperpolarization and hence relaxation of the entire vascular wall. This may also explain why EDHF, unlike the endothelium-derived relaxing factor (NO), cannot be monitored by conventional bioassay techniques (Kauser & Rubanyi, 1992).

When compared with NO, the physiological importance of EDHF released in certain vascular beds may have been underestimated. In isolated coronary arteries, for example, approximately 60% of the dilator response to bradykinin is resistant to inhibition of either NO synthase or cyclo-oxygenase (Holzmann *et al.* 1994). In the coronary microcirculation of the rat heart, only the duration, but not the amplitude, of the dilator response to bradykinin is affected by the combined treatment with an inhibitor of NO synthase and cyclo-oxygenase (Baydoun & Woodward, 1991). Similarly, the duration, but not the amplitude, of the systemic hypotensive response to acetylcholine, ATP or bradykinin in the anaesthetized rat *in vivo* is reduced following application of an NO synthase inhibitor (O'Shaughnessy, Newman & Warren, 1992).

These findings imply that the release of EDHF from the vascular endothelium in the coronary circulation may play an important role in the control of local vascular tone under physiological conditions as well as in conditions where NO synthesis is impaired, i.e. in atherosclerosis, hypertension or ischaemia. Moreover, therapeutics which have been shown to restore impaired endothelium-dependent vasodilations, such as angiotensin I-converting enzyme (ACE) inhibitors, enhance both the NO-dependent and -independent dilator response to bradykinin in isolated coronary arteries (Mombouli, Illiano, Nagao, Scott-Burden & Vanhoutte, 1992), indicating that part of their beneficial cardiovascular effects may also be related to an enhanced release of EDHF.

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