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Endothelium-dependent relaxation and hyperpolarization in guinea-pig coronary artery: role of epoxyeicosatrienoic acid

[2]D.M. Eckman, N. Hopkins, C. McBride & [1]K.D. Keef

Department of Physiology & Cell Biology/352, University of Nevada School of Medicine, Reno, Nevada 89557, U.S.A.

1 Acetylcholine (ACh) elicits an endothelium-dependent relaxation and hyperpolarization in the absence of nitric oxide (NO) and prostaglandin synthesis in the guinea-pig coronary artery (GPCA). This response has been attributed to a factor termed endothelial-derived hyperpolarizing factor (EDHF). Recently it has been suggested that EDHF may be a cytochrome P450 product of arachidonic acid (AA) i.e., an epoxyeicosatrienoic acid (EET). The present study investigated whether this pathway could account for the response to ACh observed in the GPCA in the presence of 100 μM N^{ω}-nitro-L-arginine and 10 μM indomethacin.

2 ACh, AA and 11,12-EET each produced concentration-dependent relaxations in arteries contracted with the H_1-receptor agonist AEP (2,2-aminoethylpyridine). The AA-induced relaxation was significantly enhanced in the presence of the cyclo-oxygenase/lipoxygenase inhibitor, eicosatetranynoic acid (30 μM).

3 The cytochrome P450 inhibitors proadifen (10 μM) and clotrimazole (10 μM) inhibited ACh, lemakalim (LEM) and AA-induced relaxation, whereas 17-octadecynoic acid (100 μM) and 7-ethoxyresorufin (10 μM) were without effect on all three vasodilators. Proadifen and clotrimazole also inhibited ACh (1 μM) and LEM (1 μM)-induced hyperpolarization.

4 The ability of various potassium channel blockers to inhibit relaxation responses elicited with ACh, AA and 11,12-EET was also determined. Iberiotoxin (IBTX; 100 nM) was without effect on responses to ACh but significantly reduced responses to both AA and 11,12-EET. In contrast, 4-aminopyridine (4-AP; 5 mM) significantly reduced response to ACh but not responses to AA and 11,12-EET. Combined IBTX plus (4-AP) inhibited the ACh-induced relaxation to a greater extent than 4-AP alone. Apamin (1 μM), glibenclamide (10 μM) and $BaCl_2$ (50 μM) had no significant effect on responses to ACh, AA and 11,12-EET.

5 IBTX (100 nM) significantly reduced both 11,12-EET (33 μM) and AA (30 μM) hyperpolarization without affecting the ACh (1 μM)-induced hyperpolarization. In contrast, 4-AP significantly reduced the ACh-induced hyperpolarization without affecting either AA or 11,12-EET-induced hyperpolarizations.

6 In summary, our results suggest that the coronary endothelium releases a factor upon application of AA which hyperpolarizes the smooth muscle. The similarity of pharmacology between AA and 11,12-EET suggests that this factor is an EET. However, the disparity of pharmacology between responses to ACh versus responses to 11,12-EET do not support the hypothesis that EETs represent the predominant factor which ACh releases from the endothelium that leads to NO- and prostaglandin-independent hyperpolarization and relaxation in the GPCA.

Keywords: Acetylcholine; arachidonic acid; epoxyeicosatrienoic acid; hyperpolarization; membrane potential; potassium channels; EDHF; cytochrome P450; endothelium; coronary artery

Introduction

Acetylcholine (ACh) produces relaxation and hyperpolarization in the guinea-pig coronary artery which is due to the release of multiple vasoactive factors from the endothelium. Two of these factors are nitric oxide (NO) and the prostaglandin prostacyclin (see review by Thiemermann, 1991). However, endothelium-dependent relaxation and hyperpolarization can still be elicited following blockade of NO, prostaglandin and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation (Chen *et al*., 1988; Parkington *et al*., 1993; Eckman *et al*., 1994) suggesting that a third factor or group of factors is also released from the endothelium. The NO- and prostaglandin-independent response has been attributed to a factor which activates potassium channels in the smooth muscle membrane leading to hyperpolarization and relaxation of vascular smooth muscle. This factor has been termed endothelium-derived hyperpolarizing factor (EDHF; Chen *et al*., 1988). The chemical nature of EDHF is currently a subject of intense investigation.

Recent studies have suggested that in some blood vessels EDHF could be a cytochrome P450 metabolite of the arachidonic acid (AA) cascade (Hecker *et al*., 1994; Bauersachs *et al*., 1994; Campbell *et al*., 1996). The metabolism of AA by cytochrome P450 mono-oxygenase results in the formation of epoxyeicosatrienoic acids (EETs; Fitzpatrick & Murphy, 1989). There are four regioisomers of EETs: 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids. Exogenous application of EETs produces dilatation of pial arteries (Ellis *et al*., 1990; Gebremedhin *et al*., 1992) and coronary arteries of the cow, pig and dog (Rosolowsky *et al*., 1990; Rosolowsky & Campbell, 1993; Campbell *et al*., 1996; Graier *et al*., 1996) at concentrations ranging from 0.1 to 100 μM. In addition to relaxation, EETs also have been associated with an increase in K^+ channel activity in cat cerebral (Gebremedhin *et al*., 1992), rabbit portal vein, rat caudal artery, guinea-pig aorta, porcine coronary (Hu & Kim, 1993) and more recently bovine coronary arteries (Campbell *et al*., 1996). Finally, EETs have been shown to elicit membrane hyperpolarization of intact

[1] Author for correspondence.
[2] Present address: Department of Pharmacology, Medical Research Facility, University of Vermont, Burlington, Vermont 05405-0068, U.S.A.

arteries (bovine coronary, Campbell *et al*., 1996). These data taken together suggest that EETs represent a viable candidate for EDHF.

In contrast, other studies have suggested that the predominant EDHF is unlikely to be an EET because: (1) some cytochrome P450 blockers do not inhibit EDHF-induced responses (Corriu *et al*., 1996a; Zygmunt *et al*., 1996; Fukao *et al*., 1997; Ohlmann *et al*., 1997), (2) EETs do not relax some blood vessels (Zygmunt *et al*., 1996) and (3) EET and EDHF-induced responses are blocked by different potassium channel blockers (Fukao *et al*., 1997). Further complicating this situation is evidence that many of the cytochrome P450 blockers also inhibit responses to potassium channel openers (Graier *et al*., 1996; Edwards *et al*., 1996; Zygmunt *et al*., 1996; Fukao *et al*., 1997; Vanheel & Van de Voorde, 1997) making interpretations of results difficult.

The present study was undertaken to determine whether EETs can account for the NO- and prostaglandin-independent relaxation and hyperpolarization which occurs in the guinea-pig coronary artery in response to acetylcholine (ACh). Membrane potential and contractile activity were recorded from isolated ring segments of coronary artery treated with N^G-nitro-L-arginine L-NOARG and indomethacin to block production of NO and prostaglandins. Experiments were designed to determine whether 11,12-EET mimics the contractile and electrical effects of ACh and whether the precursor molecule AA, elicits responses that may be attributed to EET formation. Additional studies examined the effects of blockers of the cytochrome P450 pathway. Finally, the ability of various potassium channel blockers to inhibit responses to ACh, AA and 11,12-EET was determined. Our results suggest that application of AA to the coronary artery elicits a response which may be due to formation of EETs. However, they do not support the hypothesis that EETs represent the predominant EDHF released by ACh in the presence of NO and prostaglandin blockade.

Methods

Tissue preparation

Male albino guinea-pigs (350–550 g) were killed by CO_2 inhalation followed by exsanguination. The heart was immediately removed, placed in cold (4–6°C) oxygenated Krebs solution (in mM): NaCl 118.5, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11, $CaCl_2$ 2.5 and aerated with 95% O_2/5% CO_2. Segments (1–1.5 cm) of both the left descending and circumflex coronary arteries were dissected out and cleaned of all adhering fat and myocardial tissue. Ring segments 3 mm long and 200–300 μM in diameter were prepared for use in either contractile or intracellular recordings.

Tissue bath experiments

Tissues were mounted onto two triangular tungsten wires (89 μM diameter) and hung vertically in an isolated organ chamber (either 3 or 10 ml in volume). The bottom triangle was mounted to a stable hook while the top triangle was attached to a Grass (FT03c) strain gauge. The bath contained Krebs solution and was maintained at 37°C.

A resting force of 0.3 g was applied to the guinea-pig coronary artery. In preliminary experiments this was found to stretch vessels to near the optimal length for tension development (i.e. Lo). Vessels were initially equilibrated for 1–2 h with alternating 4 min exposures every 15 min to the histamine H_1-receptor agonist 2-(2-aminoethyl)-pyridine (AEP, 0.1–1 mM, Durant *et al*., 1975) and 60 mM KCl.

For experiments which tested the effects of cytochrome P450 or K^+ channel inhibitors on ACh; AA-, lemakalim- or 11,12-EET-induced vasodilatation each tissue served as its own control. Two sequential concentration-relaxation response relationships were obtained for a particular vasodilator before an experiment was begun. If reproducible responses were not obtained the tissue was discarded. In preliminary experiments we found that tissue which had the same concentration-relaxation response relationship with two sequential applications of vasodilator also had the same relationship for the third application of vasodilator. Following control responses the tissue was exposed to either a cytochrome P450 or K^+-channel blocker for 20–30 min. A third concentration-response curve was then obtained in the presence of blocker. Only one blocker was tested per tissue. Tissues were bathed in Krebs solution containing 100 μM L-NOARG and 10 μM indomethacin throughout the experiment.

Intracellular measurements

For experiments in which membrane potential was measured, a 5 mm segment of left circumflex or left descending coronary artery was employed. Vessel rings were mounted onto two parallel wires and a resting force of 0.3 g was applied to mimic the conditions present in the tissue bath experiments. All intracellular measurements were made through the adventitial surface with microelectrodes filled with 3 M KCl and having resistances between 70–100 MΩ. Impalements were judged on the basis of a rapid drop in potential upon entering the cell, a low noise level and minimal change in the electrode resistance and zero potential before and after implement. Signals were viewed on a digital oscilloscope (Hitachi) and stored on tape with a Vetter PCM Recording Adapter attached to a video cassette recorder.

Statistics

Statistical significance was determined by the two-tailed paired or unpaired *t* test. The changes were considered significant at $P<0.05$. The response to vasodilators was determined as % reduction of AEP-induced contraction (i.e., 100% relaxation was equivalent to complete reversal of the AEP-induced contraction). Vasodilators never reduced tone below the pre-contracted level of 0.3 g. Data are expressed as mean ± s.e.mean, *n* values reflect the number of animals studied. Because we were unable to obtain complete concentration-response relationships in the presence of some cytochrome P450- or K^+-channel-inhibitors, these responses were analysed by use of a repeated measures ANOVA (SASS). This technique allowed us to compare the whole curve rather than a specific point such as the traditional EC_{50} calculation produces.

Drugs used

Acetylcholine HCl (ACh), iberiotoxin, (IBTX), proadifen (SKF-525A), clotrimazole (Clot), 17-octadecynoic acid (17-ODYA), 7-ethoxyresorufin (7-ER), N^G-nitro-L-arginine (L-NOARG), U46619, indomethacin (Indo), eicosatetranynoic acid (ETYA), 4-aminopyridine (4-AP) arachidonic acid (AA, dissolved in bicarbonate buffer) and glibenclamide were purchased from Sigma (St. Louis, MO). 2-(2-aminoethyl)pyridine (AEP, Aldrich, Milwaukee, WI). 11,12-Expoxyeicosa-

trienoic acid (11,12-EET) was supplied in 100% ETOH at a concentration of 100 mM (Cayman Chemical, Ann Arbor, MI). Lemakalim was a generous gift from Smith Kline Beecham. Haemolysate was made according to the method of Bowman and Gillespie (1982) and was stored at 2°C for up to 3 days. Since the active component of haemolysate is oxyhaemoglobin we have referred to haemolysate as oxyhaemoglobin (HbO) throughout the text. Three volume % of haemolysate solution was added to the bath to attain a final concentration of approximatley 20 μM HbO. All compounds were dissolved in distilled water (unless otherwise stated) and stored on ice throughout the experiment.

Results

Comparison of the contractile and electrical properties of vasodilators

Effect of ACh, AA and 11,12-EET on contractile responses The effects of cumulative additions of ACh (0.01–10 μM), AA (0.1–100 μM) and 11,12-EET (1–40 μM) were measured in guinea-pig coronary arteries (GPCA, Figure 1) contracted with AEP (1 mM). Both ACh (Figure 1a) and 11,12-EET (Figure 1c) elicited concentration-dependent relaxation. In contrast, the response to AA was much smaller, i.e. the highest concentration of AA tested (100 μM) produced only a 30% relaxation of the GPCA (Figure 1b). The inability of AA to relax fully these vessels could be related to the simultaneous formation and release of both a relaxing and a contracting factor. To address this possibility we studied the effect of the dual cyclo-oxygenase and lipoxygenase inhibitor, ETYA (30 μM). ETYA significantly increased the amplitude of relaxation induced with AA and shifted the concentration-response relationship to the left. In contrast, there was no significant difference in the response to ACh or 11,12-EET in the presence or absence of ETYA (Figure 1). Because of the marked increase in the AA-induced response, ETYA (30 μM) was included in the remaining contractile experiments with AA in the GPCA.

Effect of ACh, AA and 11,12-EET on membrane potential EETs have been reported to hyperpolarize bovine coronary and rat mesenteric arteries (Campbell *et al*., 1996; Fukao *et al*., 1997). To determine whether this is the case in the GPCA, membrane potential was measured before and during application of ACh (1 μM) or 11,12-EET (33 μM). The 11,12-EET precursor, AA (30 μM) was also tested. These concentrations produced greater than 70% relaxation in tissue bath experiments. For these experiments a resting tension of 0.3 g was applied (i.e., equivalent to the tissue bath experiments). Membrane potential in stretched vessels (i.e., −42 mV) was more depolarized than in previous studies in which membrane potential was recorded in unstretched GPCA segments (e.g., −58 mV; Keef & Bowen, 1989). In some cases a small degree of myogenic tone also developed. Previous studies have shown that stretch can lead to both depolarization and myogenic tone (see D'Angelo & Meininger, 1994). After a stable impalement had been obtained, vessels were exposed to ACh, AA or 11,12-EET for 2.5–3.0 min. Each of these vasodilators elicited membrane hyperpolarization although the amplitude differed somewhat. ACh hyperpolarized cells by 26.4±1.5 mV (n=12) compared to 10.7±1.2 mV (n=10) for AA and 15.6±1.2 mV (n=10) for 11,12-EET (typical recording shown in Figure 5, data summarized in Table 1).

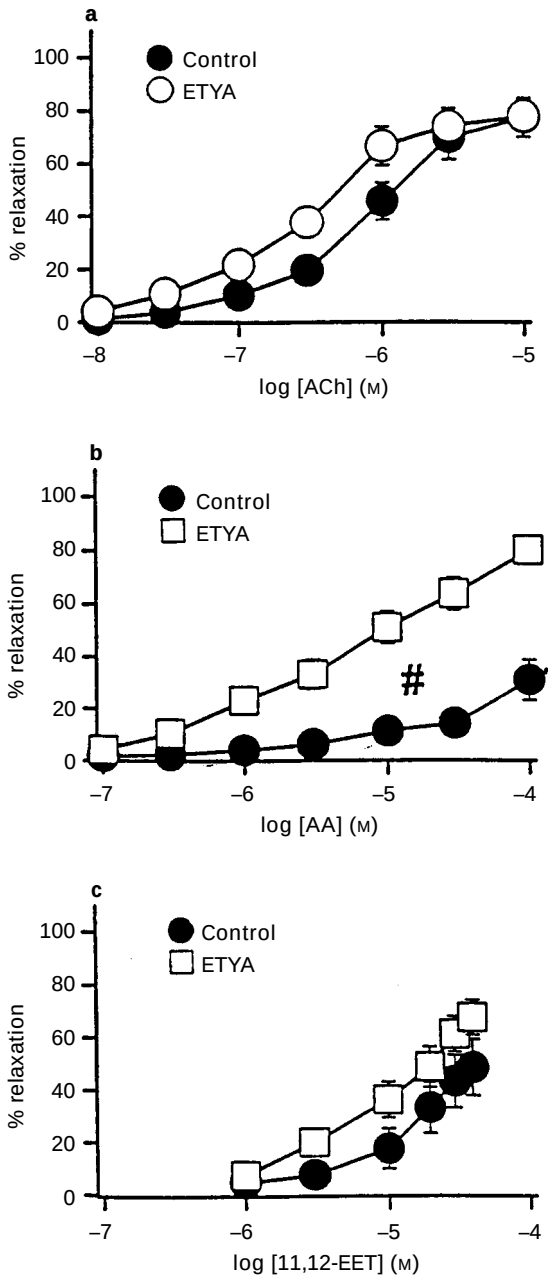

Figure 1 Comparison of acetylcholine (ACh; a), arachidonic acid (AA; b) and 11,12-epoxyeicosatrienoic acid (11,12-EET; c) induced relaxations in the absence (control) and presence of ETYA (10–30 μM) in the guinea-pig coronary artery. L-NOARG (100 μM) and indomethacin (10 μM) were present throughout. Shown are mean values with vertical lines indicating s.e.mean (n=9–15). # Indicates curves significantly different from one another $F \leqslant 0.05$.

Effect of cytochrome p450 inhibitors on vasodilators

Effect of cytochrome p450 inhibitors on ACh, AA and 11,12-EET-induced relaxation The most direct means to investigate the role of the cytochrome P450 pathway in the actions of ACh is to block cytochrome P450. However, as indicated in the introduction, problems have been obtained for these blockers. For this reason the effects of the cytochrome P450 blockers (i.e., proadifen or SKF 525A, 10 μM; clotrimazole 10 μM; 17-ODYA, 100 μM and 7-ER, 3–10 μM) were examined on responses elicited with the K_{ATP} channel opener lemakalim (Lem 0.1–10 μM) as well as on responses elicited with ACh (0.01–10 μM) and AA (0.3–100 μM). Of all the cytochrome P450 blockers tested, only those which inhibited lemakalim-

Table 1 Effect of the K^+-channel inhibitors on ACh, AA and 11, 12-EET-induced hyperpolarizations in the guinea-pig coronary artery

Experimental condition	*Control MP*	*Control Hyperpol*	*MP plus blocker*	*Hyperpol plus blocker*
4-AP (5 mM)				
ACh (1 μM, $n=7$)	-42.1 ± 0.8	25.3 ± 2.1	-42.5 ± 1.9	2.5 ± 1.5**
AA (30 μM, $n=4$)	-42.8 ± 1.6	11.5 ± 2.7	-36.1 ± 2.6	11.8 ± 3.5
11, 12-EET (33 μM, $n=5$)	-42.1 ± 1.0	16.3 ± 1.2	-40.0 ± 1.8	12.2 ± 2.1
IBTX (100 nM)				
ACh (1 μM, $n=3$)	-43.0 ± 2.5	27.0 ± 2.3	-37.1 ± 0.5	31.3 ± 2.9
AA (30 μM, $n=3$)	-42.3 ± 1.4	12.0 ± 1.5	-38.6 ± 2.0	6.3 ± 1.9*
11, 12-EET (33 μM, $n=3$)	-38.0 ± 4.0	12.3 ± 2.4	-37.0 ± 2.0	3.6 ± 1.9*

Resting membrane potential and hyperpolarization measurements are expressed as mean$\pm$s.e.mean. MP: membrane potential (mV); plus blocker: in the presence of K^+ channel blocker; Hyperpol: hyperpolarization; 4-AP: 4-aminopyridine; IBTX: iberiotoxin; ACh: acetylcholine; AA: arachidonic acid; 11, 12-EET: 11, 12-epoxyeicosatrienoic acid. Significantly different from control, * $P<0.05$; ** $P<0.001$.

induced relaxations also significantly inhibited ACh- and AA-induced relaxations (i.e., pradifen, clotrimazole and miconazole; see Figure 2 for example; all curves were found to be significantly shifted from the control responses at $F<0.05$) suggesting that these blockers have non-specific effects. In contrast, those blockers which were without effect on lemakalim responses were also without effect on responses to ACh and AA (i.e., 7-ER and 17-ODYA; see Figure 2 for example).

Effect of cytochrome p450 blockers on ACh and lemakalim-induced hyperpolarization The ability of proadifen (10 μM) to inhibit ACh-induced hyperpolarization was tested and compared to its effect on lemakalim-induced hyperpolarization. ACh (1 μM) gave rise to a 20.2 ± 1.2 mV hyperpolarization which was reduced to 1.0 ± 1.2 mV ($n=4$) in the presence of proadifen. Likewise proadifen reduced the lemakalim-induced hyperpolarization from 25 ± 5.1 to 5.5 ± 1.2 mV ($n=3$). In two experiments clotrimazole reduced the ACh-induced hyperpolarization from a mean of 27 mV to 18 mV and the lemakalim-induced hyperpolarization from 30 mV to 10 V. In one vessel miconazole reduced the ACh-induced hyperpolarization from 20 nV to 16 mV and the lemakalim-induced hyperpolarization from 29 mV to 21 mV, respectively. These results are in agreement with the previous extensive intracellular studies in rat mesenteric arteries (Fukao *et al.*, 1997).

Effect of potassium channel blockers on vasodilators

Effect of K^+-channel blockers on ACh, AA and 11,12-EET induced relaxations The next series of experiments tested a variety of different K^+ channel blockers, to determine whether a similar potency sequence was observed for effects on 11,12-EET,AA and ACh-induced relaxation. Concentration-response relationships were determined for ACh (0.01–100 μM), AA (0.3–300 μM) and 11,12-EET (0.1–40 μM) in the presence and absence of iberiotoxin (IBTX 100 nM), 4-aminopyridine (4-AP; 5 mM), apamin (1 μM), glibenclamide (10 μM) and $BaCl_2$ (50 μM). Combined IBTX plus 4-AP was also tested, as well as a combination of all 5 blockers (K-block).

IBTX had no significant effect on ACh-induced relaxations whereas 4-AP significantly shifted this relationship to the right (Figure 3A). In contrast, responses to AA and 11,12-EET were significantly shifted to the right by IBTX while 4-AP had no effect (Figure 3B, C). Apamin, glibenclamide and $BaCl_2$ were all without effect on all three vasodilators (Figure 4).

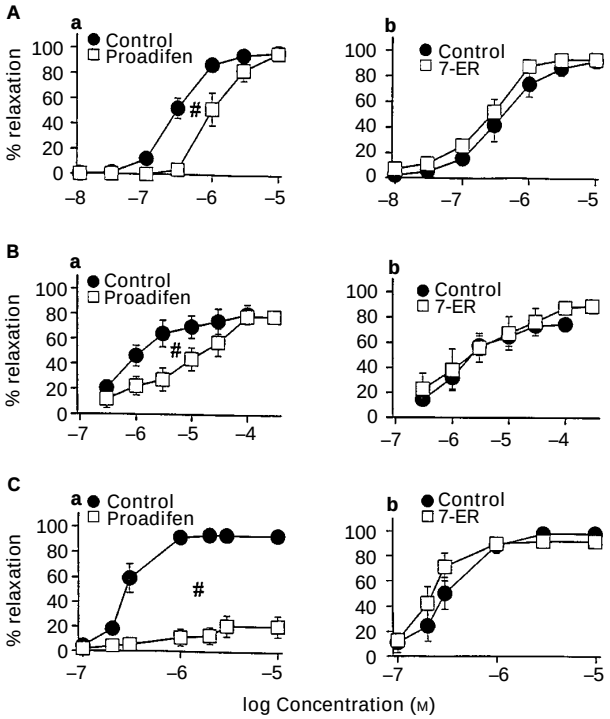

Figure 2 Effect of cytochrome p450 blockers on acetylcholine (A), arachidonic acid (B) and lemakalim (C) concentration-relaxation response relationships in the guinea-pig coronary artery. Responses to vasodilators were obtained in the absence (control) and presence of (a) proadifen (10 μM) and (b) 7-ethoxyresorufin (7-ER 10 μM). L-NOARG (100 μM) and indomethacin (10 μM) were present throughout. Shown are mean values and vertical lines indicate s.e.mean ($n=4-7$). # Indicates curves significantly different from one another $F\leqslant0.05$.

Combined IBTX and 4-AP produced a significantly greater inhibition of the ACh concentration-relaxation relationship than 4-AP alone whereas inclusion of 4-AP with IBTX had no greater effect on AA and 11,12-EET than IBTX alone (Figure 3). Finally, the shift in responses to ACh, AA and 11,12-EET in K-block solution ($n=6-9$) was the same as that observed with IBTX plus 4-AP (data not shown). Thus a significant relaxation can be obtained with high concentrations of ACh in the presence of K-block. To ensure that the remaining relaxation was not due to residual release of NO, additional experiments were undertaken in which oxyhaemoglobin (HbO 3%) was included in the bathing medium. The relaxation responses obtained in the presence of HbO plus K-block were

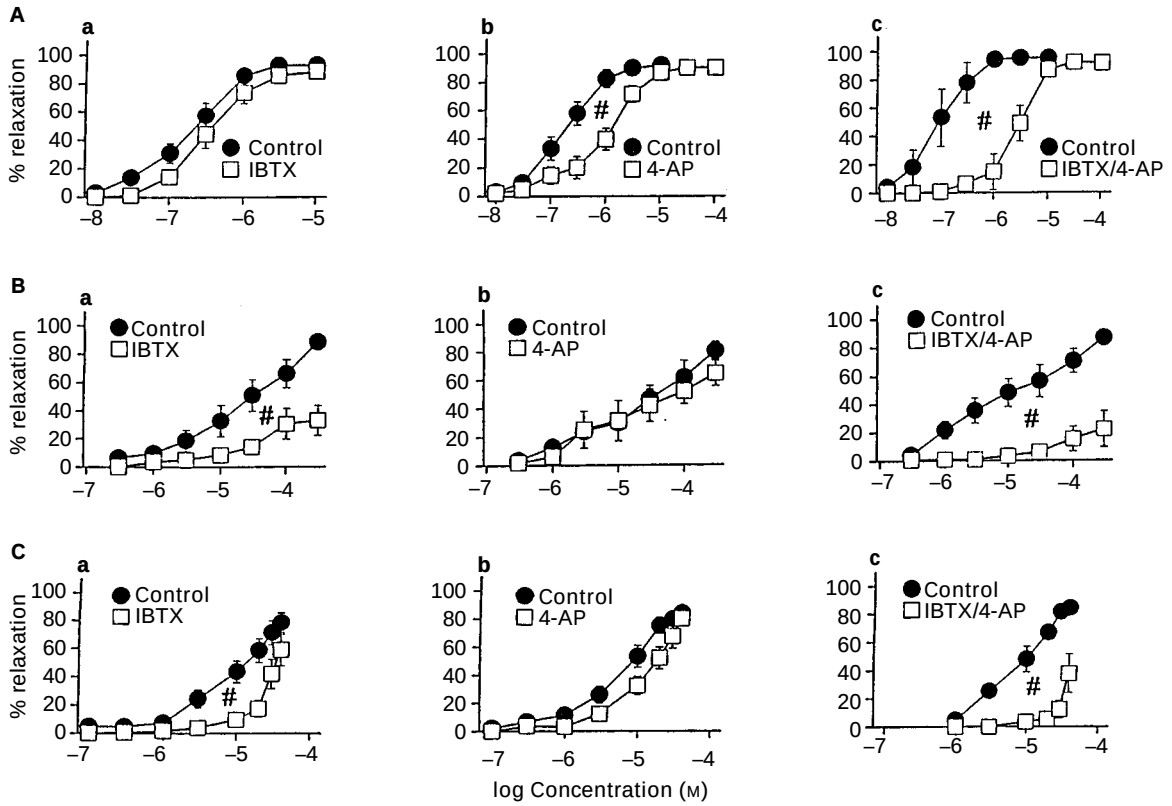

Figure 3 Effect of various K^+ channel blockers on acetylcholine (A), arachidonic acid (B) and 11,12-epoxyeicosatrienoic acid (C) concentration-relaxation response relationships in the guinea-pig coronary artery. Responses to vasodilators were obtained in the absence (control) and presence of iberiotoxin (IBTX, 100 nM; a), 4-aminopyridine (4-AP, 5 mM; b) and combined IBTX and 4-AP (c). L-NOARG (100 μM) and indomethacin (10 μM) were present throughout. Shown are mean values with vertical lines indicating s.e.mean ($n=3-11$). #Indicates curves significantly different from one another $F \leqslant 0.05$.

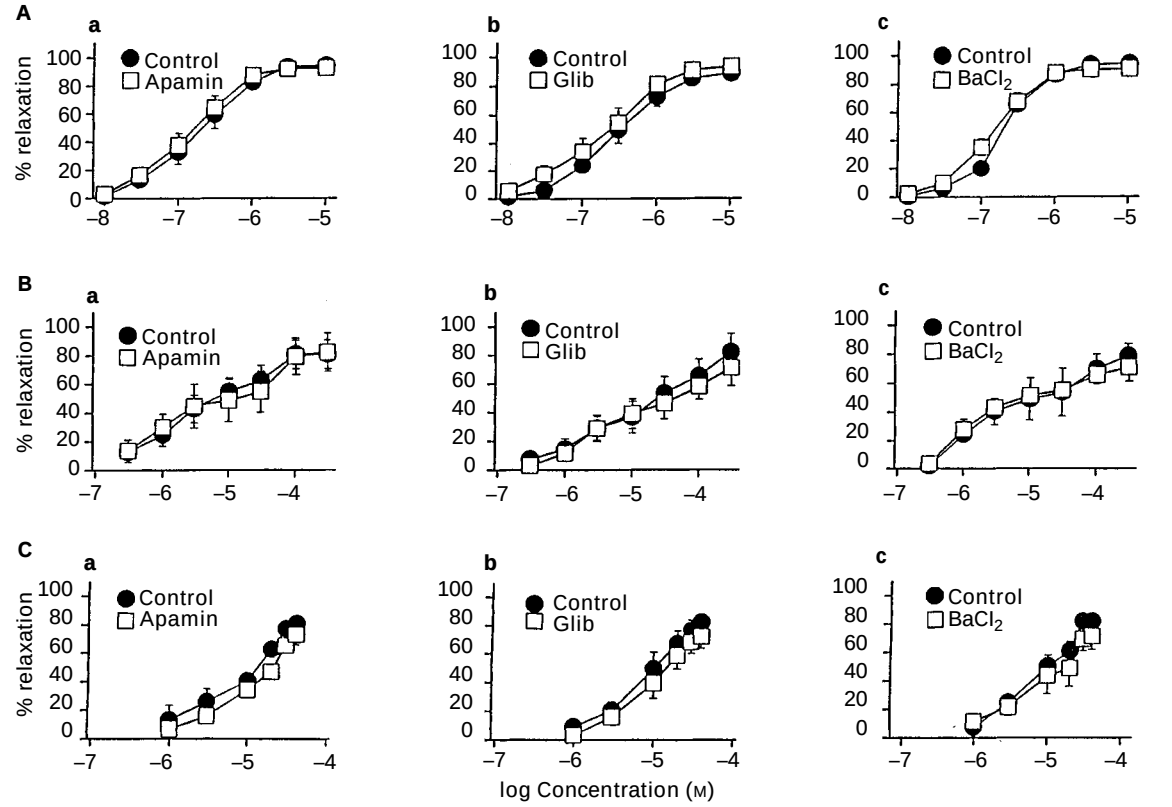

Figure 4 Lack of effect of various K^+ channel blockers on the acetylcholine (A), arachidonic acid (B) and 11,12-epoxyeicosatrienoic acid (C) concentration-relaxation resposne relationships in guinea-pig coronary artery. Responses to vasodilators were compared in the absence (control) or presence of apamin (1 μM, a), glibenclamide (5–10 μM, b) and $BaCl_2$ (50 μM, c). L-NOARG (100 μM) and indomethacin (10 μM) were present throughout. None of these K^+ channel blockers produced a significant shift of concentration-relaxation response relationships. Shown are mean values with vertical lines indicating s.e.mean ($n=3-14$).

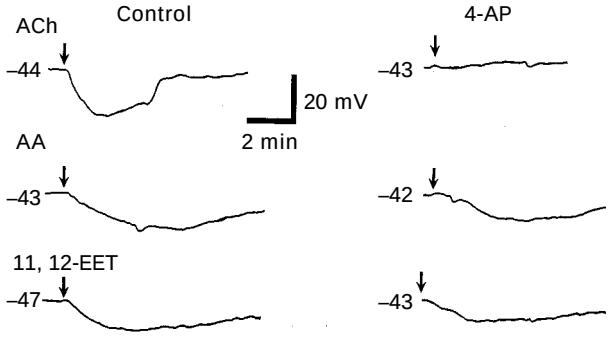

Figure 5 Comparison of acetylcholine (ACh, 1 μM), arachidonic acid (AA, 30 μM) and 11,12-epoxyeicosatrienoic acid (11,12-EET, 33 μM)-induced hyperpolarizations in the absence (control) or presence of 4-aminopyridine (4-AP, 5 mM) in the guinea-pig coronary artery. ACh, AA and 11,12 EET were each applied for 3 min. L-NOARG (100 μM) and indomethacin (10 μM) were present throughout. 4-AP reduced the ACh-induced hyperpolarization while leaving the responses to AA and 11,12 EET intact (see Table 1 for statistics).

the same as those observed in the absence of HbO with K-block ($n=3$).

Effect of K-channel inhibitors on ACh, AA and 11,12-EET induced hyperpolarizations Intracellular measurements were undertaken to determine the effect of 4-AP and IBTX on ACh (1 μM), AA (30 μM) and 11,12-EET (33 μM)-induced hyperpolarization (Figure 5). The results are tabulated in Table 1. The Ca^{2+}-activated K^+ blocker IBTX (100 nM) did not significantly depolarize resting membrane potential (-41.1 ± 1.6 mV vs -38.56 ± 1.5 mV respectively $n=9$, Table 1) but reduced the hyperpolarizations elicited with either 11,12-EET or AA. In contrast, the ACh-induced hyperpolarization was not significantly reduced by IBTX. The delayed rectifier K^+ channel blocker 4-AP (5 mM) had no significant effect on resting membrane potential (i.e., -42.1 ± 0.5 versus -40.1 ± 1.3 mV, $n=16$, Table 1). However, it significantly reduced the ACh-induced hyperpolarization, while having no significant effect on the AA- and 11,12-EET-induced hyperpolarizations. These results are in agreement with the contractile measurements which indicate that the potency of various K^+ channel blockers on ACh differ from their effect on AA and 11,12-EET.

Discussion

In recent years it has been suggested that the endothelium-dependent hyperpolarization which is observed in some blood vessels in response to agonists such as acetylcholine and bradykinin is due to a cytochrome P450 metabolite of the AA pathway (Hecker *et al*., 1994; Bauersachs *et al*., 1994; Campbell *et al*., 1996). The present study investigated whether this pathway could account for the ACh-induced NO- and prostaglandin-independent response observed in the guinea-pig coronary artery. Our results suggest that EET formation alone cannot explain much of the action of ACh in this vessel.

The response to 11,12-EET mimicked that of ACh in several ways. Of particular relevance, we found that 11,12-EET hyperpolarized and relaxed vessels. A number of studies have described the vasodilating characteristics of EETs (Ellis *et al*., 1990; Pfister *et al*., 1991; Gebremedhin *et al*., 1992; Rosolowsky & Campbell, 1993; Hecker *et al*., 1994; Campbell *et al*., 1996) and a few recent studies have measured EET-induced hyperpolarization (Campbell *et al*., 1996; Fukao *et al*., 1997). We also observed that AA, the putative precursor molecule for EET formation, relaxed and hyperpolarized the GPCA, further supporting the proposed role of EET as an EDHF. Although a number of studies have described the vasodilating characteristic of AA (Rosolowsky & Campbell, 1993; Lonigro *et al*., 1994; Campbell *et al*., 1996), few studies have measured its effect on membrane potential. In the rat mesenteric artery, 11,12-EET (10 μM) hyperpolarized cells by 7 mV while AA (100 μM) was without effect on membrane potential (Fukao *et al*., 1997). Four micromolar AA has previously been shown to hyperpolarize the GPCA in an indomethacin-sensitive manner (Parkington *et al*., 1993). In the present study we found that a higher concentration of AA (30 μM) hyperpolarized cells in the presence of indomethacin. Taken together these results suggest that AA addition may lead to formation of both cyclo-oxygenase as well as epoxygenase products which can hyperpolarize the smooth muscle. The contractile and electrical characteristics of 11,12-EET and AA in the GPCA are thus in keeping with the proposed role of EET as an EDHF. Likewise, the previously observed endothelial-dependence of AA-induced relaxation in coronary artery and endothelial independence of EET (Rosolowsky *et al*., 1990; Campbell *et al*., 1996) are in keeping with the proposed role of EET as an EDHF. We have observed similar effects in the monkey coronary artery, i.e., the AA relaxation was endothelium-dependent while the 11,12 EET-induced response was endothelium-independent ($n=3$, unpublished data). Although 11,12-EET mimicked the electrical and contractile behaviour of EDHF in the GPCA, the potency of 11,12-EET (i.e., 3–100 μM range) was less than that found in either the bovine or canine coronary artery (i.e., 0.03–30 μM range; Rosolowsky *et al*., 1990; Campbell *et al*., 1996), suggesting that EETs may play a greater role in canine and bovine coronary arteries than in the GPCA.

An interesting aspect of this study was the marked enhancement of AA-induced relaxations which occurred when ETYA was included in the bathing solutin. ETYA blocks cyclo-oxygenase (IC_{50} 8 μM) and lipoxygenase (IC_{50} 0.2–10 μM); Tobias & Hamilton, 1979; Bokoch & Reed, 1981; Salari *et al*., 1984; Moncada *et al*., 1985). Higher concentrations also appear to inhibit EET production (IC_{50} 40–100 μM; Capdevila *et al*., 1988; Revtyak *et al*., 1988). Our results suggest that the predominant effect of ETYA was to block the lipoxygenase pathway, since ETYA increased rather than decreased the relaxation response to AA while having no effect on either ACh or 11,12 EET-induced relaxation (indomethacin and L-NOARG present throughout). Other studies have also shown that ETYA was without effect on ACh-induced responses in blood vessels (Oyekan *et al*., 1994; Corriu *et al*., 1996a; Fukao *et al*., 1997). The lipoxygenase pathway metabolizes AA to at least three vasoconstricting compounds; leukotrienes (Badr *et al*., 1987), 12-HETE (Ma *et al*., 1991; Saito *et al*., 1992) and 15-HETE (Van Diest *et al*., 1991). Simultaneous release of one or more of these contractile substances along with EETs would be predicted to limit the relaxing action of the EETs.

Significant problems were encountered in this study when attempts were made to block cytochrome P450. The cytochrome P450 blockers tested either inhibited ACh as well as lemakalim (i.e., proadifen, clotrimazole and miconazole) or were without effect on the ACh-induced responses (i.e., 17-octadecynoic acid and 7-ethoxyresorufin). Indeed, proadifen not only blocked Ach and lemakalim-induced responses (present study) it also blocked EET-induced

relaxation and ACh-induced contraction (unpublished observations), making it a particularly unsuitable candidate to use to investigate the role of the cytochrome P450 pathway in intact tissues. Our results are very similar to those from a recent study by Fukao *et al.* (1997), which compared the effects of 10 different cytochrome P450 blockers on the hyperpolarization elicited with ACh and pinacidil in the rat mesenteric artery. This study showed that all 10 cytochrome P450 blockers tested were either without effect on the ACh-induced hyperpolarization (i.e., diethyldithiocarbamate, 17-octadecynoic acid, α-naphtoflavone, metyrapone, eicosatetraynoic acid, troleandomycin) or blocked the hyperpolarization elicited with both ACh and pinacidil (i.e,. ketoconazole, clotrimazole, miconazole, proadifen). Thus, the present study as well as that of Fukao *et al.* (1997) and others (Graier *et al.*, 1996; Edwards *et al.*, 1996; Zygmunt *et al.*, 1996; Corriu *et al.*, 1996a; Vanheel & Van de Voorde, 1997; Ohlmann *et al.*, 1997) all suggest that many cytochrome P450 blockers have either non-specific effects or are without effect on EDHF-induced responses.

Both EETs (Hecker *et al.*, 1994; Hu & Kim, 1994) and EDHF (for review see Brayden, 1993) lead to activation of K^+ channels in vascular smooth muscle. If EETs represent the primary EDHF then K^+ channel blockers should have a similar effect on the relaxation and hyperpolarization elicited with ACh and 11,12-EET. Likewise, if AA primarily leads to EET formation then K^+ channel blockers should have a similar effect on 11,12-EET and AA. We found that K^+ blockers had similar effects on AA and 11,12-EET providing additional evidence for the proposed endothelial conversion of AA to EETs (Rosolowsky *et al.*, 1990; Gebremedhin *et al.*, 1992; Campbell *et al.*, 1996). However, since our experiments were performed in the presence of indomethacin the observed effects may overestimate the activity of the epoxygenase pathway, i.e., indomethacin will shift AA metabolism away from the cyclo-oxygenase pathway and toward the epoxygenase pathway.

In contrast to the similarities observed between AA and 11,12-EET there were substantial differences in the potency of K^+ channel blockers on ACh versus 11,12-EET and AA. Iberiotoxin (IBTX) which blocks large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) produced a significant reduction of the 11,12-EET and AA-induced relaxation and hyperpolarization, while IBTX alone had no effect on ACh-induced responses. In contrast, 4-AP which blocks delayed rectifier type K^+ channels (Kv) significantly reduced relaxation and hyperpolarization to ACh without reducing responses to 11,12-EET and AA. These data are at odds with the hypothesis that EETs represent the predominant compounds released from the endothelium which hyperpolarize the smooth muscle. However they agree with previous studies of the bovine coronary artery which suggest that EETs enhance the activity of BK_{Ca} but not Kv (Hu & Kim, 1993; Campbell *et al.*, 1996; Li *et al.*, 1997). Since AA and 11,12 EET responses were greatly diminished by IBTX but unaffected by 4-AP, this suggests that the concentration of 4-AP used in this study (i.e., 5 mM) is unlikely to have a pronounced effect on BK_{Ca}.

In some blood vessels, such as the rat and rabbit mesenteric artery, the NO- and prostaglandin-independent response elicited with ACh is blocked by apamin, suggesting that EDHF activates small conductance Ca-activated K^+ channels (SK_{Ca}; Adeagbo & Triggle, 1993; Murphy & Brayden, 1995). However, in the GPCA apamin had no effect on all three vasodilators tested making this channel an unlikely target. Likewise, although there is increasing evidence that inwardly rectifying K^+ channels play a significant role in modulation of resting membrane potential in coronary artery (Knot *et al.*, 1996; Robertson *et al.*, 1996), responses to ACh, 11,12-EET and AA were unaffectd by $BaCl_2$ which blocks these channels (Robertson *et al.*, 1996).

The most interesting result to arise from experiments with K^+ channel blockers was the marked inhibition of ACh-induced relaxation and hyperpolarization produced by 4-AP. This blocker produced greater than 90% reduction of ACh (1 μM)-induced hyperpolarization and 50% reduction of the relaxation, suggesting that the response to ACh may be dependent upon Kv. The greater effect of 4-AP on hyperpolarization versus relaxation may be related to the conditions present during these experiments i.e., membrane potential was measured in the absence of agonist while relaxation was initiated after the tissue had been contracted with AEP. ACh may exert effects in the contracted tissue which are not apparent in the resting tissue. Differences in experimental conditions are also likely to explain why IBTX was without effect on ACh-induced hyperpolarization (Figure 5), while it shifted the dose-relaxation response relationship to the right when added in the presence of 4-AP (Figure 3).

Previous studies have shown little or no inhibition of EDHF-induced responses when low concentrations of 4-AP (i.e., 1 mM or less) were used (e.g., Petersson *et al.*, 1997). However, in a recent study in rabbit isolated coronary artery cells we found that 3 mM 4-AP was required for complete inhibition of Kv currents (Ishikawa *et al.*, 1997). Hence, 1 mM 4-AP may not be sufficient to block entirely Kv in intact tissues. In the guinea-pig basilar artery the EDHF-induced relaxation was reduced by the Kv blocker ciclazindol and abolished when ciclazindol was combined with apamin (Petersson *et al.*, 1997). Partial inhibition of relaxation was also observed when 4-AP (1 mM) was combined with apamin. The authors concluded that either two channels are involved in the actions of EDHF or, alternatively, that a single channel, structurally related to Kv and allosterically regulated by apamin is a target for EDHF (Petersson *et al.*, 1997). A similar study by this group in rat hepatic artery suggested that the target K-channel for EDHF was neither Kv nor BK_{Ca} but rather was structurally related to both (Zygmunt *et al.*, 1997). Thus, in both of these studies a 'Kv-like' conductance may be activated by EDHF. It is possible that a similar conductance is activated by EDHF in the GPCA.

In some blood vessels the NO- and prostaglandin-independent response can be blocked by a combination of charybdotoxin plus apamin while addition of either blocker alone is ineffective (Corriu *et al.*, 1996b; Zygmunt & Hogestatt, 1996; Petersson *et al.*, 1997; Zygmunt *et al.*, 1997). Interestingly, iberiotoxin plus apamin does not produce the same effect as charybdotoxin plus apamin (Petersson *et al.*, 1997; Zygmunt *et al.*, 1997). Since Kv channels are also inhibited by charybdotoxin (Chandy & Gutman, 1995), it is possible that the actions of charybdotoxin involve inhibition of Kv. In preliminary studies of the GPCA we also found that the actions of charybdotoxin and iberiotoxin differed, i.e., 100 nM charybdotoxin shifted the ACh concentration-relaxation response relationship to the same extent as 5 mM 4-AP ($n=4$, unpublished observation), while iberiotoxin was without effect (Figure 3). Since iberiotoxin is the more selective blocker of BK_{Ca} all subsequent experiments in the present study were carried out with iberiotoxin. However, this observation again suggests that the EDHF-induced response in the GPCA has similarities to the responses observed in the rat hepatic artery and guinea-pig basilar artery (Petersson *et al.*, 1997; Zygmunt *et al.*, 1997).

Although the results with 4-AP are intriguing it should be noted that 4-AP-sensitive channels are present on both the

smooth muscle (Volk & Shibata, 1993) and the endothelium (Chen & Cheung, 1992). Because of this complication it is not possible to identify definitively the site of action of 4-AP. This question can only be adequately addressed with donor-recipient style experiments in which 4-AP is applied exclusively to the recipient smooth muscle. However, inspite of the uncertainties regarding 4-AP, the marked difference in sensitivity of ACh versus 11,12-EET to IBTX argues that the predominant factor released by ACh which hyperpolarizes the smooth muscle is not an EET. Since IBTX produced some inhibition of the ACh-induced relaxation when combined with 4-AP, the cytochrome P450 pathway cannot be entirely excluded as a possible source of EDHFs, but our results clearly suggest that another factor(s) plays a more important role.

In the rat mesenteric artery it was also concluded that EETs are unlikely to be the predominant EDHF released in response to ACh, but the conclusion was based on somewhat different results, i.e., in this tissue glibenclamide (30 μM) blocked the 11,12-EET-induced hyperpolarization but had no effect on the ACh-induced hyperpolarization. In contrast, in the GPCA, we found that 11,12-EET and AA-induced relaxation were both unchanged in the presence of glibenclamide (present study) at a concentration (i.e., 10 μM) which blocks the lemakalim-induced relaxation in this vessel (Eckman *et al*., 1992).

In summary, our results suggest that the coronary endothelium releases a factor upon application of AA which hyperpolarizes the smooth muscle. The similarity of pharmacology between AA and 11,12-EET suggests that this factor is an EET. However, the disparity of pharmacology between responses to ACh versus responses to 11,12-EET do not support the hypothesis that an EET is the predominant factor released by ACh in the presence of NO- and prostaglandin-synthesis blockade.

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