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Evidence against a role of cytochrome P450-derived arachidonic acid metabolites in endothelium-dependent hyperpolarization by acetylcholine in rat isolated mesenteric artery

[1]Mitsuhiro Fukao, Yuichi Hattori, Morio Kanno, *Ichiro Sakuma & *Akira Kitabatake

Departments of Pharmacology and *Cardiovascular Medicine, Hokkaido University School of Medicine, Sapporo 060, Japan

1 In rat mesenteric artery, acetylcholine (ACh) causes endothelium-dependent hyperpolarization by releasing endothelium-derived hyerpolarizing factor (EDHF). Recent evidence suggests that EDHF may be a cytochrome P450-derived arachidonic acid metabolite. The aim of the present study was to investigate whether such a metabolite is indeed contributing to ACh-induced hyperpolarization observed in rat mesenteric artery.

2 The phospholipase A_2 inhibitor quinacrine (30 μM) nearly completely eliminated ACh-induced hyperpolarization. However, the hyperpolarizing effect of pinacidil was also abolished in the presence of quinacrine.

3 The imidazole antimycotic agents ketoconazole (50 μM), clotrimazole (30 μM) and miconazole (10 μM), which bind to the heme moiety of cytochrome P450, eliminated not only ACh-induced hyperpolarizations but also those induced by pinacidil. SKF525A (30 μM), a prototype inhibitor of the enzyme, also abolished the hyperpolarizing responses to both agents. In contrast, neither 17-octadecynoic acid (10 μM), a mechanism-based inhibitor of cytochrome P450 metabolism of fatty acids, nor eicosatetraynoic acid (20 μM), an inhibitor of all arachidonic acid metabolic pathways, altered ACh-induced hyperpolarization. Furthermore, the hyperpolarization was unaffected by the preferential inhibitors of specific cytochrome P450 isozymes, α-naphtoflavone (1 μM), diedthyldithiocarbamate (50 μM), metyrapone (20 μM) and troleandomycin (10 μM).

4 Pretreatment of rats with lipopolysaccharide (2 mg kg^{-1}) and exposure to nitroprusside (10 μM), both of which are expected to inhibit cytochrome P450 activity due to nitric oxide overproduction, were without effect on ACh-induced hyperpolarization. Pretreatment of rats for 3 days with pentobarbitone (80 mg kg^{-1} day^{-1}), a cytochrome P450 inducer, also did not affect the hyperpolarizing response to ACh.

5 Arachidonic acid in concentrations up to 100 μM had no detectable effect on smooth muscle membrane potential. 11,12-Epoxyeicosatrienoic acid (EET, 10 μM), one of cytochrome P450-derived epoxygenase metabolites of arachidonic acid, elicited a small endothelium-independent membrane hyperpolarization. The hyperpolarizing response to EET was blocked by glibenclamide (30 μM), in contrast to the response to ACh.

6 These results suggest that the contribution of a cytochrome P450-derived metabolite of arachidonic acid to ACh-induced hyperpolarization via EDHF release is minimal or absent in rat mesenteric artery.

Keywords: Endothelium-derived hyperpolarizing factor; acetylcholine; hyperpolarization; arachidonic acid; cytochrome P450; epoxyeicosatrienoic acid; rat mesenteric artery

Introduction

The vascular endothelium plays an essential role in the regulation of vascular smooth muscle tone by the release of endothelium-derived relaxing factors (EDRF) and contracting factors (Furchgott & Vanhoutte, 1989). EDRF is now considered to be nitric oxide (NO) (Palmer *et al*., 1987) or a related nitroso compound (Myers *et al*., 1990). Endothelial cells also mediate hyperpolarization of smooth muscle cells in response to vasodilators such as acetylcholine (ACh) (Chen *et al*., 1988; Feletou & Vanhoutte, 1988), adenosine 5′-diphosphate (ADP; Brayden & Willman, 1989), histamine (Chen & Suzuki, 1989) and substance P (Bolton *et al*., 1986). This endothelium-dependent hyperpolarization is likely to be due to a diffusible substance released from the endothelium rather than an electrical signal transmitted from endothelial cells to smooth muscle cells (Chen *et al*., 1991; Bény & Pacicca, 1994). Recent evidence mostly indicates that endothelium-dependent hyperpolarization is not mediated by NO (Bény & Brunet, 1988; Garland & McPherson, 1992), but presumably by an as yet unidentified endothelium-derived hyperpolarizing factor (EDHF) (Bény & Brunet, 1988; Chen *et al*., 1988; Feletou & Vanhoutte, 1988; Taylor & Weston, 1988).

Arachidonic acid is oxidatively metabolized by three distinct enzyme systems; cyclo-oxygenase, lipoxygenase and cytochrome P450-dependent epoxygenase (Fitzpatrick & Murphy, 1989). Numerous tissues produce biologically-active metabolites of arachidonic acid via the cytochrome P450 pathway (Fitzpatrick & Murphy, 1989), and in the vasculature, this co-enzyme is predominantly localized in the endothelium (Abraham *et al*., 1985). It has been demonstrated that the EDHF-mediated vasodilator response to bradykinin is attenuated by the cytochrome P450 inhibitors, SKF525A and clotrimazole, in bovine, porcine and rat coronary arteries (Hecker *et al*., 1994; Lischke *et al*., 1995), suggesting that cytochrome P450-derived arachidonic acid metabolites may serve as EDHF. However, these inhibitors of arachidonic acid metabolism and cytochrome P450 systems possess a variety of ancillary actions that create problems for interpreting accurately the experimental results obtained in their presence. Indeed, early studies with these inhibitors proposed that EDRF might be a metabolite of arachidonic acid produced via lipoxygenase or cytochrome P450 (Singer & Peach, 1983a,b).

[1] Author for correspondence.

SKF525A, which is frequently used to inhibit cytochrome P450-dependent mechanisms, has numerous additional actions that may lead to misinterpretation of experimental results (Kalsner *et al*., 1970; McMurtry *et al*., 1976; Neubig *et al*., 1979). Thus, even if chemical agents known as cytochrome P450 inhibitors have interfered with the expression of EDHF-mediated vasodilator response to bradykinin, such actions might be due to effects unrelated to inhibition of cytochrome P450. Evidence in favour of this possibility has recently been obtained (Zygmunt *et al*., 1996).

The present study was designed to determine whether a cytochrome P450-derived arachidonic acid metabolite is a potential candidate for EDHF. With this aim, we observed effects of various kinds of cytochrome P450 inhibitors on ACh-induced endothelium-dependent hyperpolarization in rat mesenteric artery and compared these results with their effects on EDHF-unrelated hyperpolarization induced by pinacidil. Our results demonstrate that the EDHF-mediated response in rat mesenteric artery is not linked to the generation of cytochrome P450-derived arachidonic acid metabolites.

Methods

Male Wistar rats, between 10 and 17 weeks old and weighing 210–340 g, were anaesthetized with diethyl ether. The main branch of the superior mesenteric arteries was carefully excised from the animals under ether anaesthesia and placed on a plate containing oxygenated physiological salt solution (PSS) at room temperature. The arteries were dissected free from surrounding connective tissues, cut into rings 3 mm in length, and opened longitudinally. Care was taken to ensure that the endothelial layer was not damaged during the processing of the tissue preparation. Where indicated, the endothelial cells were removed by gently rubbing the intimal surface of the vessel with a moistened cotton ball. The lack of a response to ACh was taken as evidence for the complete removal of the endothelium. The tissue was pinned down, intimal side upward, on the bottom of an organ chamber (capacity 3 ml), and superfused at a constant flow rate of 7 ml min^{-1} with PSS aerated with 95% O_2 and 5% CO_2. The temperature of perfusate was kept constant at 37°C. PSS contained the following (mM): NaCI 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. After the preparations had equilibrated for at least 60 min, glass microelectrodes filled with 3 M KCl (tip resistance 40–80 MΩ) were inserted into the smooth muscle cells from the intimal side. Electrical signals were monitored continuously on an oscilloscope (Nihon Kohden, VC-10, Tokyo, Japan) and recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan). Following stable membrane potentials for at least 2 min, the hyperpolarizing response to ACh was determined by continuous recordings of membrane potential from a single cell. Two min after application of ACh, the preparation was washed with drug free PSS, until the membrane potential reverted to the resting level (usually 5 min). The test drugs were then applied and 20–30 min later, ACh was again administered. We also examined the effect of these test drugs on hyperpolarization induced by pinacidil, an ATP-sensitive K^+ channel opener. Cytochrome P450 inhibitors were used in concentrations shown to reduce cytochrome P450 activity by 70–100% in vascular tissues or other tissues (Pinto *et al*., 1986; Oyekan *et al*., 1991; Zou *et al*., 1994; Newton *et al*., 1995).

In a series of experiments carried out to examine the influence of NO synthase on ACh-induced hyperpolarization, a bolus dose of lipopolysaccharide (2 mg kg^{-1}, i.p.) was administered 3 days before the mesenteric artery was isolated. In some experiments, in order to evaluate the effect of the induction of cytochrome P450 enzymes on ACh-induced hyperpolarization, phenobarbitone (80 mg kg^{-1}, i.p.) was given for 3 days.

The compounds used were as follows: ACh chloride, tetraethylammonium chloride and tetra-*n*-butylammonium bromide (Wako, Osaka, Japan); pinacidil (Shionogi, Osaka, Japan); indomethacin, N^G-nitro-L-arginine (L-NOARG), quinacrine, clotrimazole, miconazole, 17-octadecynoic acid (17-ODYA), α-naphtoflavone, diethyldithiocarbamate, metyrapone, troleandomycin, sodium nitroprusside and lipopolysaccharide (Sigma, St. Louis, MO, U.S.A); SKF525A (N,N-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) and ketoconazole (BIOMOL Research, Plymouth Meeting, PA, U.S.A.); eicosatetraynoic acid (ETYA) and 11,12-epoxyeicosatrienoic acid (11,12-EET) (Cascade Biochem Ltd., Berkshire U.K.) and phenobarbitone sodium (Tanabe, Osaka, Japan).

All values are presented in terms of the means ± s.e. mean. Analysis by Student's *t* test was performed for paired and unpaired observations as appropriate. *P* values less than 0.05 were considered significant.

Results

Hyperpolarizations induced by ACh and pinacidil

The resting membrane potentials of vascular smooth muscle cells in rat mesenteric artery were -51.7 ± 0.3 mV ($n=79$). As previously found (Fukao *et al*., 1995), ACh (1 μM) caused membrane hyperpolarization by -16.3 ± 0.4 mV($n=55$), an effect which was not observed in tissues without endothelium. Consistent with earlier observations (Garland & McPherson, 1992), neither indomethacin (10 μM) nor L-NOARG (100 μM) affected the hyperpolarizing response to ACh. However, this was eliminated by high K^+ medium (25 mM), tetraethylammonium (10 mM) and tetrabutylammonium (500 μM), but was not changed by glibenclamide (30 μM). These findings collectively suggest that membrane hyperpolarization produced by ACh in rat mesenteric artery is mediated by EDHF. On the other hand, pinacidil (1 μM), a K^+-channel opener, hyperpolarized the membrane potential regardless of whether the endothelium was present or not. The hyperpolarization induced by pinacidil was inhibited by glibenclamide.

Effect of phospholipase A_2 inhibitor

Arachidonic acid is released from membrane phospholipids via the enzyme phospholipase A_2, which can be inhibited by quinacrine. In the presence of quinacrine (30 μM), there was a significant depolarization of the membrane (7.5 ± 1.5 mV, $n=4$) and a marked suppression of hyperpolarization produced by subsequently-applied ACh (1 μM) (Figure 1a). The peak amplitudes of ACh-induced hyperpolarizations were -15.4 ± 0.5 and -1.5 ± 0.3 mV ($n=4$) before and after the addition of quinacrine, respectively. However, the hyperpolarizing response to pinacidil (1 μM) was also eliminated by quinacrine (Figure 1b).

Effect of cytochrome P450 inhibitors

The imidazole antimycotic agents, ketoconazole (50 μM), clotrimazole (30 μM) and miconazole (10 μM) significantly depolarized the membrane by 3.0 ± 1.4 ($n=5$), 7.7 ± 1.0 ($n=7$), 8.6 ± 0.8 mV ($n=5$), respectively. In their presence, the hyperpolarizing response to ACh (1 μM) was almost completely abolished (Figure 2a–c and Table 1). Miconazole also inhibited the hyperpolarizing response to pinacidil (Figure 2d). Other derivatives also caused an inhibition of pinacidil-induced hyperpolarization (data not shown).

SKF525A is metabolized by cytochrome P450 to an intermediate that inhibits cytochrome P450-dependent mixed-function oxidases via type I binding (Mannering, 1971). Like the antimycotic agents, SKF525A (30 μM) also depolarized the membrane by 7.8 ± 2.3 mV ($n=4$) and abolished the hyperpolarizing effects of ACh (1 μM) (Figure 3a and Table 1). SKF525A also suppressed pinacidil-induced hyperpolarizations (Figure 3b).

17-ODYA is a suicide-substrate inhibitor which selectively inhibits cytochrome P450 isoenzymes based on substrate specificity (Muerhoff *et al*., 1989). 17-ODYA (10 μM) produced no detectable change in the resting membrane potential and hardly modified ACh-induced hyperpolarization (Figure 4a and Table 1). Another suicide-substrate inhibitor ETYA (20 μM), which is a competitive analogue of arachidonic acid and is able to block all pathways of arachidonic acid metabolism (McGiff, 1991), also had no detectable effect on the resting membrane potential and ACh-induced hyperpolarization (Figure 4a and Table 1).

'Cytochrome P450' is a complex of co-factors and a member of the hemoprotein superfamily, of which cytochrome P450 subtypes 1A1, 1A2, 2E1 and 3A might be present in human umbilical vein endothelial cells based on the presence of the relevant mRNA (Farin *et al*., 1994). α-Naphtoflavon inhibits cytochrome P450 1A1, 1A2, 2C7, 2C8 (Chang *et al*., 1994). Diethyldithiocarbamate inhibits cytochrome P450 2A6, 2B6, 2E1 (Chang *et al*., 1994). Metyrapone inhibits cytochrome P450 11B1 (Denner *et al*., 1995) and 2B subfamily (Knickle & Bend, 1994). None of these inhibitors affected the resting membrane potential or the hyperpolarizing response to ACh (Table 1).

Effect of procedures modifying cytochrome P450

Cytochrome P450 activity is inhibited by NO (Wink *et al*., 1993; Khatsenko *et al*., 1993), and lipopolysaccharide and certain cytokines can induce the synthesis of NO in vascular endothelium (Radomski *et al*., 1990) and smooth muscle (Rees *et al*., 1990; Busse & Mulsch, 1990; Fleming *et al*., 1991). In mesenteric arteries taken from rats treated with lipopolysaccharide, the resting membrane potentials averaged -51.5 ± 0.7 mV ($n = 24$), a value which was comparable to

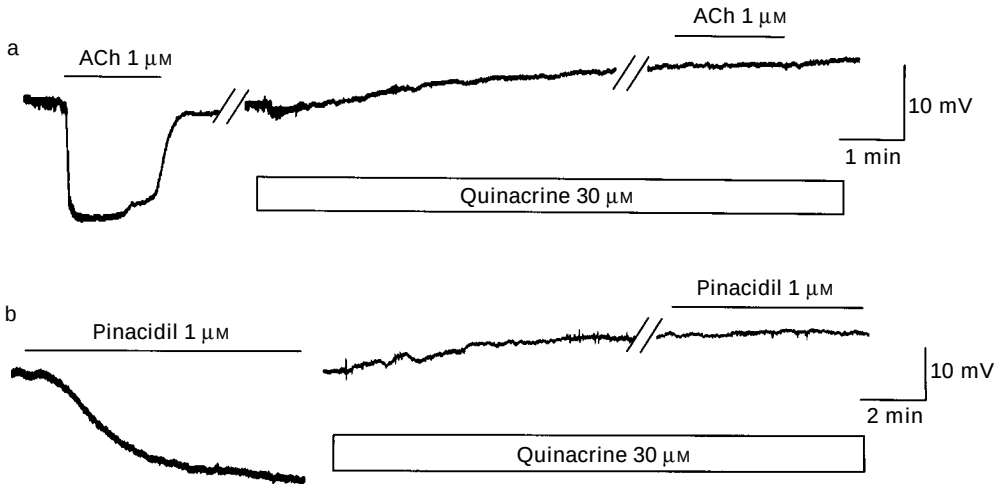

Figure 1 Effect of quinacrine on the hyperpolarizing responses to ACh (a) and pinacidil (b). ACh and pinacidil were present for the periods indicated by the horizontal bars. Quinacrine was added to the bath 20–30 min before the second application of ACh or pinacidil.

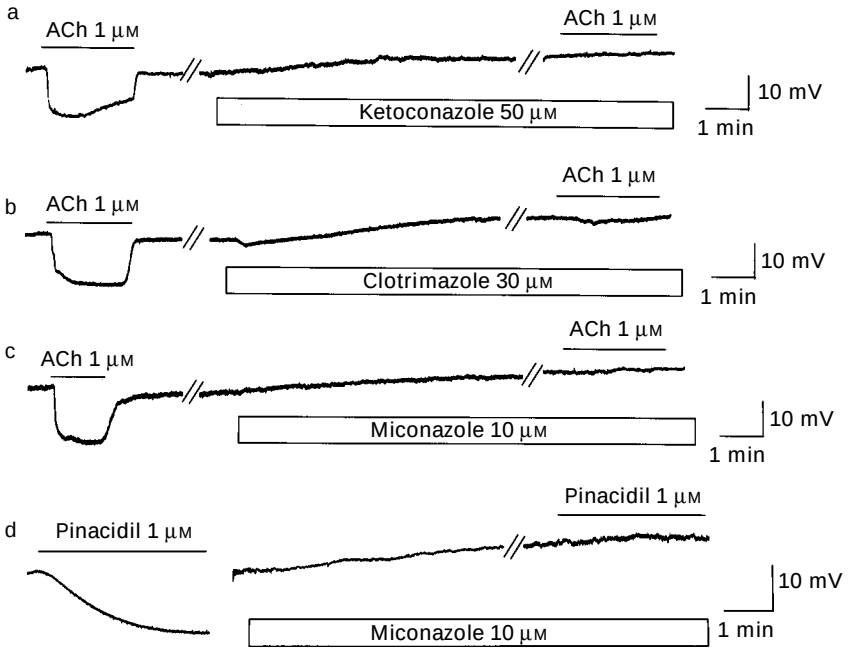

Figure 2 Effect of ketoconazole (a), clotrimazole (b) and miconazole (c, d) on the hyperpolarizing responses to ACh (a–c) and pinacidil (d). ACh and pinacidil were present for the periods indicated by the horizontal bars. The imidazole agents were added to the bath 20–30 min before the second application ACh or pinacidil.

Table 1 Effects of cytochrome P450 inhibitors on the hyperpolarizing response to 1 μM ACh

Inhibitors	*Concentration* (μM)	*Hyperpolarization* (mV) *Untreated*	*Treated*	*% inhibition*	n
Ketoconazole	30	−15.0±0.7	−3.8±0.8*	75	4
	50	−16.3±0.8	0*	100	4
Clotrimazole	30	−14.5±1.1	−2.0±1.0*	86	4
Miconazole	10	−16.2±0.5	−3.8±1.7*	77	6
SKF 525A	30	−16.0±1.1	−2.2±0.2*	86	5
Eicosatetraynoic acid	20	−17.8±0.9	−17.3±1.4	3	4
17-Octadecynoic acid	10	−18.0±1.2	−17.7±1.4	2	3
α-Naphtoflavone	1	−18.3±1.0	−17.8±1.0	3	4
Metyrapone	20	−15.8±1.4	−14.0±0.6	11	4
Troleandomycin	10	−16.0±0.6	−16.0±1.1	0	4
Diethyldithiocarbamate	50	−15.8±0.2	−15.0±1.0	5	4

*$P<0.001$ vs. untreated.

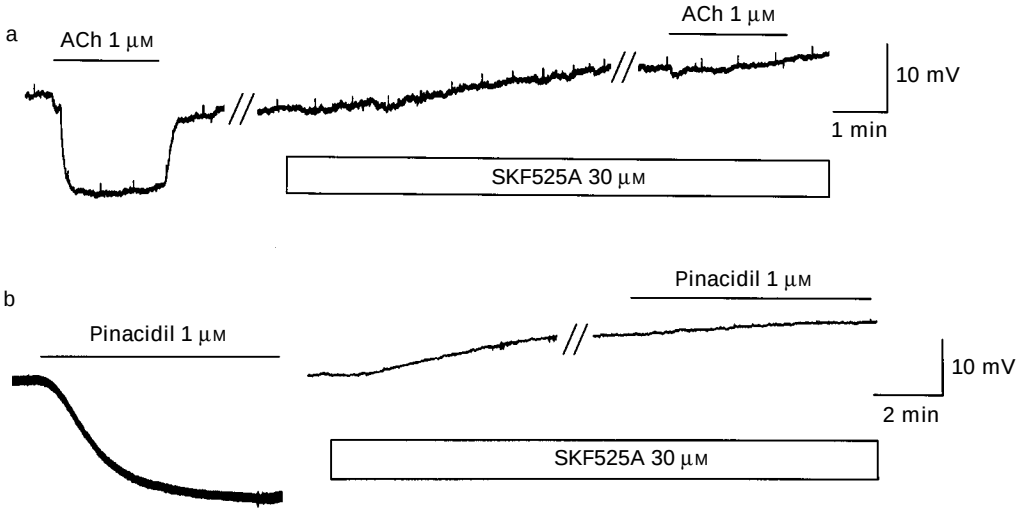

Figure 3 Effect of SKF525A on the hyperpolarizing responses to ACh (a) and pinacidil (b). ACh and pinacidil were present for the periods indicated by the horizontal bars. SKF525A was added to the bath 20–30 min before the second application ACh or pinacidil.

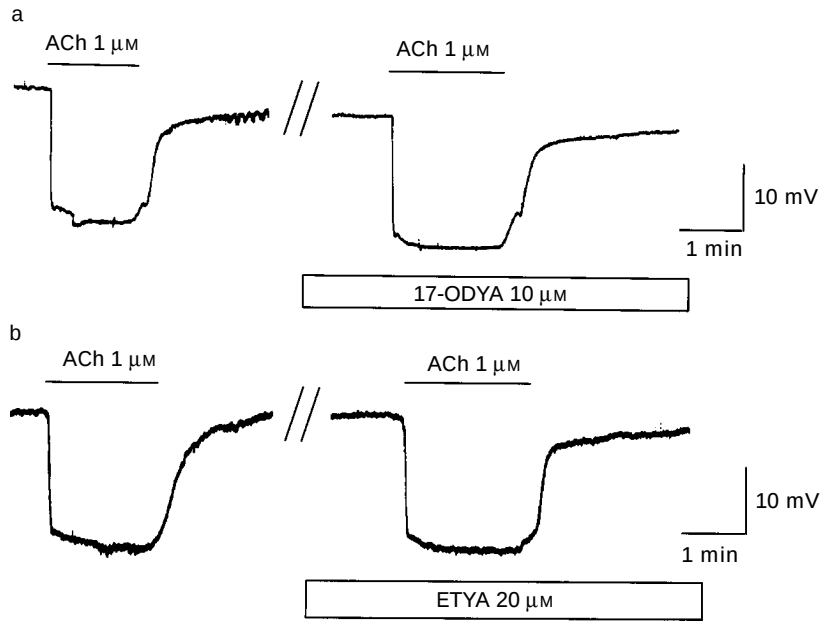

Figure 4 Effect of 17-octadecynoic acid (17-ODYA) (a) and eicosatetraynoic acid (ETYA) (b) on the hyperpolarizing responses to ACh. ACh was present for the periods indicated by the horizontal bars. 17-ODYA and ETYA were added to the bath 20–30 min before the second application of ACh.

Figure 5 The hyperpolarizing response to ACh in an artery taken from a rat pretreated with phenobarbitone (a) and in the presence of nitroprusside (b). ACh was present for the periods indicated by the horizontal bars. Nitroprusside (SNP) was added to the bath 5 min before the second application of ACh.

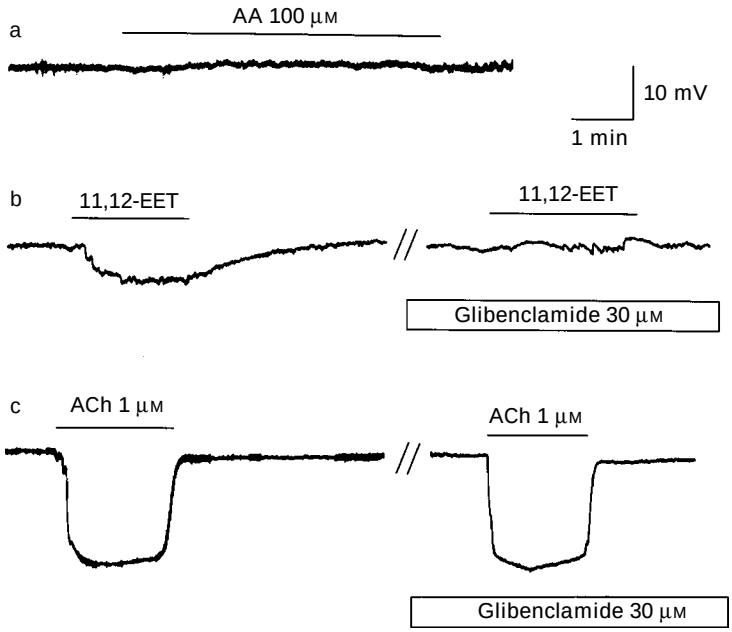

Figure 6 Changes in membrane potentials elicited by arachidonic acid (a), 11,12-epoxyeicosatrienoic acid (11,12-EET) (b) and ACh (c). Arachidonic acid (AA), 11,12-EET and ACh were present for the periods indicated by the horizontal bars. Glibenclamide was added to the bath 20 min before second application of 11,12-EET or ACh.

that obtained in untreated rat mesenteric arteries. The hyperpolarizing response to ACh in arteries from rats that received lipopolysaccharide was the same as that in untreated arteries (Figure 5a); ACh (1 μM) hyperpolarized by 16.5 ± 0.6 mV, 6 pretreated preparations. The effect of nitroprusside (10 μM), an NO donor, was also examined. Nitroprusside elicited a small hyperpolarization (-3.0 ± 0.5 mV $n = 5$), but did not substantially alter ACh-induced hyperpolarizations (Figure 5b). In the presence of nitroprusside, the hyperpolarization produced by 1 μM ACh was -15.4 ± 0.9 mV ($n = 5$).

Phenobarbitone is a well-known inducer of hepatic cytochrome P450. In mesenteric arteries harvested from rats treated with phenobarbitone, the resting membrane potentials were -54.3 ± 1.5 mV ($n = 4$). The response to 1 μM ACh was unchanged by pretreatment with phenobarbitone (-14.3 ± 0.5 mV, $n = 4$).

Hyperpolarization produced by 11,12-EET

The metabolism of arachidonic acid by the cytochrome P450 system results in the formation of EETs, dihydroxyeicosatrienoic acids and monohydroxyeicosatetraenoic acids in various tissues (Capdevila *et al*., 1981; Fitzpatrick *et al*., 1986). EETs are known to possess potent vasodilating activity (Carroll *et al*., 1987; Proctor *et al*., 1987). In tissues with endothelium, the membrane potential of smooth muscle cells was not changed by arachidonic acid in concentrations up to 100 μM (Figure 6a). However, as shown in Figure 6b, 11,12-EET (10 μM) produced membrane hyperpolarization, independent of the presence or absence of the endothelium. The onset and development of the hyperpolarization were much slower in the case of 11,12-EET than in the case of ACh. The peak amplitude of the hyperpolarizing response to 11,12-EET was -6.7 ± 0.2 mV ($n = 6$). In contrast to the response to ACh, the hyperpolarization elicited by 11,12-EET was nearly completely eliminated by glibenclamide (30 μM) (Figure 6).

Discussion

Recent experimental evidence suggests that EDHF is one of the cytochrome P450-derived arachidonic acid metabolites, presumably an epoxide or a mixture thereof. First, following inhibition of NO and prostacyclin formation with L-NOARG and indomethacin or diclofenac, the endothelium-dependent vasorelaxant responses to ACh and bradykinin are depressed by the inhibitors of phospholipase A_2 or cytochrome P450 (Hecker *et al*., 1994; Fulton *et al*., 1995; Lischke *et al*., 1995). Second, pretreatment of rats with phenobarbitone, a potent inducer of hepatic cytochrome P450, amplifies the vasodilator responses to ACh and bradykinin (Randall & Hiley, 1988; Oyekan, 1995). Finally, EETs, cytochrome P450-derived arachidonic acid metabolites, are vasorelaxant (Proctor *et al*.,

1987; Carroll *et al*., 1987; Ellis *et al*., 1990; Hecker *et al*., 1994) and activate Ca^{2+}- activated K^+ channels in vascular smooth muscle cells (Hu & Kim, 1993).

In the present study, the contribution of a cytochrome P450-dependent mechanism via metabolism of arachidonic acid to the EDHF-mediated response to ACh in rat mesenteric artery was assessed by monitoring changes in the membrane potential of smooth muscle cells. As demonstrated in our previous study (Fukao *et al*., 1995), ACh causes endothelium-dependent hyperpolarization in rat mesenteric artery. ACh-induced hyperpolarization was not affected by L-NOARG or indomethacin, which is consistent with earlier suggestions that NO or prostanoids are not involved in this response (Garland & McPherson, 1992). ACh failed to produce hyperpolarization in high K^+ medium. Moreover, tetraethylammonium and tetrabutylammonium, but not glibenclamide, eliminated ACh-induced hyperpolarization. These properties of the hyperpolarization are in good agreement with the concept that EDHF hyperpolarizes the membrane of smooth muscle cells by opening a K^+ channel other than the ATP-sensitive K^+ channel (Chen *et al*., 1991). Thus, it is reasonable to conclude that ACh-induced hyperpolarization in rat mesenteric artery is caused by EDHF released from the endothelium.

In this study, the phospholipase A_2 inhibitor quinacrine almost completely abolished ACh-induced hyperpolarization. Hecker *et al*. (1994) have also demonstrated that quinacrine significantly attenuates the EDHF-mediated relaxant response to bradykinin in porcine coronary artery. However, this does not imply that the release of arachidonic acid itself or that of a metabolite is involved in the EDHF-mediated responses. We found that the hyperpolarizing response to pinacidil, an ATP-sensitive K^+ channel opener, could be suppresed by quinacrine. In addition, quinacrine by itself caused membrane depolarization. These findings suggest that quinacrine may interfere with several types of K^+ channels in vascular smooth muscle cells in a nonspecific manner. Thus, quinacrine appears to be an inappropriate agent to address the role of arachidonic acid or its metabolite in the EDHF-mediated responses.

Evidence which has been claimed to provide strong support for the view that EDHF is a cytochrome P450-derived arachidonic acid metabolite was apparently provided by the findings that the cytochrome P450 inhibitors SKF525A and clotrimazole markedly reduce the EDHF-mediated relaxation of vascular tissues (Hecker *et al*., 1994; Lischke *et al*., 1995). However, the present study showed that SKF525A and imidazole antimycotic agents including clotrimazole eliminated not only ACh-induced hyperpolarizations but also those induced by pinacidil. Furthermore, these cytochrome P450 inhibitors *per se* depolarized the smooth muscle membrane. Thus, it is most likely that SKF525A and imidazole derivatives suppressed the EDHF-mediated responses due to their nonspecific actions on K^+ channels which appear to be unrelated to inhibition of cytochrome P450. Evidence in favour of this review has recently been independently obtained (Edwards *et al*., 1996; Zygmunt *et al*., 1996). Contrary to these findings, Corriu *et al*. (1996) have shown that the ACh-induced hyperpolarization in guinea-pig carotid artery is not affected by SKF525A and clotrimazole. The reason for this discrepancy is not clear, but may be related to the differences in the incubation time with these inhibitors.

In contrast, 17-ODYA, a mechanism-based inhibitor which inhibits the cytochrome P450-dependent process of arachidonic acid metabolism at concentrations below 5 μM (Zou *et al*., 1994), did not affect ACh-induced hyperpolarization even at a concentration of 10 μM. ETYA, which inhibits all pathways of arachidonic acid metabolsim (McGiff, 1991), also had no effect on ACh-induced hyperpolarization. At present, we do not have any definite knowledge of the isozyme pattern of P450 in the endothelial cells. Farin *et al*. (1994) provided evidence of the presence of several isozymes in the umbilical vein endothelial cells. In the presence of diethyldithiocarbamate, metyrapone or troleandomycin, which have been shown to inhibit several P450 isozymes selectively, the hyperpolarizing effect produced by ACh was no less than the control response to ACh. Finally, based on the present results with a variety of cytochrome P450 inhibitors, it can be concluded that a cytochrome P450-derived arachidonic acid metabolite does not substantially contribute to the hyperpolarization produced by ACh in rat mesenteric artery.

Further investigation revealed that ACh-induced hyperpolarization was well preserved under the condition of NO overproduction due to pretreatment of the animals with lipopolysaccharide or exposure of isolated vessels to nitroprusside. It has been demonstrated that NO and NO-releasing compounds inhibit cytochrome P450 activities (Wink *et al*., 1993; Khatsenko *et al*., 1993). Thus, the present data suggest that cytochrome P450 activities suppressed by NO generated from lipopolysaccharide and nitroprusside are not involved in ACh-induced hyperpolarization. In addition, we found that the cytochrome P450-inducing agent phenobarbitone did not augment ACh-induced hyperpolarization. This is inconsistent with data from previous studies which showed that pretreatment of rats with phenobarbitone enhances vasodilatations produced by ACh in mesenteric arterial bed (Randall & Hiley, 1988) and by bradykinin in kidney (Oyekan, 1995). The reason for this discrepancy is not clear, but may be related to the multicomponent nature of the *in vivo* vasodilator response, unlike *in vitro* hyperpolarizations. Hence, this study demonstrates that modulation of cytochrome P450 by the agents which inhibit or induce the enzyme did not affect the hyperpolarization elicited by ACh, providing further evidence against the suggestion that the hyperpolarizing response to ACh is mediated by a cytochrome P450-derived arachidonic acid metabolite.

Hecker *et al*. (1994) have shown that EDHF may be an epoxy metabolite of arachidonic acid formed via the cytochrome P450 mono-oxygenase pathway. Indeed, EETs are vasodilators in some tissues (Carroll *et al*., 1987; Proctor *et al*., 1987). If this hypothesis were true, then arachidonic acid and EETs should cause hyperpolarization.

However, we found that arachidonic acid failed to hyperpolarize the smooth muscle membrane in concentrations up to 100 μM. On the other hand, 11,12-EET produced a small, but definite hyperpolarization. However, in contrast to the actions of ACh, the hyperpolarizing effect of 11,12-EET was almost completely abolished by glibenclamide, indicating that 11,12-EET may activate the ATP-sensitive K^+ channel. Hu & Kim (1993) have shown that EETs activate the Ca^{2+}-activated K^+ channel in vascular smooth muscle cells, but this K^+ channel activation is not blocked by glibenclamide. Therefore, the ionic mechanism responsible for membrane hyperpolarization of EETs appear to be different from that of ACh.

Taken together the present data demonstrate that cytochrome P450 does not play a major role in endothelium-dependent hyperpolarization produced by ACh, at least in the rat isolated mesenteric artery studied here. Our results suggest that EDHF released by ACh is not a cytochrome P450-derived arachidonic acid metabolite, a conclusion also reached following similar studies in rat hepatic artery (Zygmunt *et al*., 1996). Indeed, our data are consistent with an action of the cytochrome P450 inhibitors on the K^+-channel opened by EDHF (Edwards *et al*., 1996). However, the possibility that a cytochrome P450-derived arachidonic acid may act as 'EDHF' in other blood vessels such as those in the kidney, cannot be ruled out.

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