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Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery

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1 The possibility that the endothelium-derived hyperpolarising factor (EDHF) in the rat hepatic artery is a cytochrome P450 mono-oxygenase metabolite of arachidonic acid was examined in the present study. In this preparation, acetylcholine elicits EDHF-mediated relaxations in the presence of the nitric oxide (NO) synthase and cyclo-oxygenase inhibitors N^{ω}-nitro-L-arginine (L-NOARG) and indomethacin, respectively.

2 17-Octadecynoic acid (17-ODYA, 50 μM), a suicide-substrate inhibitor of the cytochrome P450 mono-oxygenases responsible for the production of 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs), had no effect on acetylcholine-induced relaxations in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM). Furthermore, 5,6-, 8,9-, 11,12- and 14,15- EETs failed to relax arteries without endothelium in the presence of L-NOARG plus indomethacin.

3 Proadifen and clotrimazole, which are inhibitors of several isoforms of cytochrome P450 mono-oxygenases, inhibited acetylcholine-induced relaxations in the presence of L-NOARG plus indomethacin. The concentration of acetylcholine which caused half-maximal relaxation was about 3 and 30 times higher in the presence than in the absence of clotrimazole (3 μM) and proadifen (10 μM), respectively. The maximal relaxation was reduced by proadifen but not by clotrimazole. Proadifen (10 μM) also inhibited acetylcholine-induced hyperpolarization in the presence of L-NOARG plus indomethacin.

4 In the presence of 30 mM K^+ plus indomethacin (10 μM), acetylcholine induced an L-NOARG-sensitive relaxation mediated via release of NO. Under these conditions, proadifen (10 μM) shifted the acetylcholine concentration-response curve 6 fold to the right without affecting the maximal relaxation. Clotrimazole (3 μM) was without effect on these responses. The relaxant actions of the NO donor, 3-morpholino-sydnonimine, were unaffected by proadifen (10 μM).

5 The relaxant effects of the opener of ATP-sensitive potassium channels, levcromakalim, were abolished by proadifen (10 μM) and strongly attenuated by clotrimazole (3 μM). Proadifen (10 μM) also abolished the hyperpolarization induced by levcromakalim (1 μM).

6 The lack of effect of 17-ODYA on relaxations mediated by EDHF, together with the failure of extracellularly-applied EETs to produce relaxation, collectively suggest that EDHF is not an EET in the rat hepatic artery. It seems likely that inhibition of ion channels in the smooth muscle rather than reduced EDHF formation in the endothelium offers a better explanation for the actions of the cytochrome P450 inhibitors proadifen and clotrimazole.

Keywords: Arachidonic acid; cytochrome P450; vascular endothelium; epoxyeicosatrienoic acid; hyperpolarization; membrane potential; nitric oxide; potassium channels

Introduction

There is now much evidence that endothelium-dependent smooth muscle hyperpolarization is mediated by an endogenous agent commonly referred to as EDHF (endothelium-derived hyperpolarizing factor). EDHF is distinct from nitric oxide (NO) and prostanoids and contributes significantly to vasodilatation in several vascular regions in different species including man (Zygmunt *et al.*, 1994a,b; Garland *et al.*, 1995; Petersson *et al.*, 1995). Although it is believed to be a diffusible factor, the identity of EDHF has not yet been established.

EDHF may be a labile metabolite of arachidonic acid formed through cytochrome P450-dependent mono-oxygenase activity (Komori & Vanhoutte, 1990). Certain metabolites of arachidonic acid such as 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs) can relax blood vessels (Pfister *et al.*, 1991; Gebremedhin *et al.*, 1992; Rosolowsky & Campbell, 1993; Hecker *et al.*, 1994) and activate potassium (K) channels in vascular smooth muscle cells (Gebremedhin *et al.*, 1992; Hu & Kim, 1993). The mono-oxygenases themselves seem to be located primarily within the endothelium (Abraham *et al.*, 1985) and endothelial cells are capable of synthesizing EETs (see Harder *et al.*, 1995). These findings, together with the ability of cytochrome P450 inhibitors, such as proadifen and clotrimazole, to attenuate EDHF-mediated vasodilatation, support the view that EDHF is an arachidonic acid metabolite derived via a cytochrome P450-dependent mono-oxygenase (Fulton *et al.*, 1992; 1994; 1995; Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Lischke *et al.*, 1995).

The aim of the present study was to examine how endothelium-dependent and -independent responses of the rat hepatic artery are modified by three mechanistically-distinct inhibitors of cytochrome P450 mono-oxygenase. Two of these, proadifen and clotrimazole, are inhibitors of a wide range of cytochrome P450-dependent enzymes. In contrast, 17-octadecynoic acid (17-ODYA) is a selective inhibitor of cytochrome P450 mono-oxygenases involved in the formation of EETs and hydroxyeicosatrienoic acids (Zou *et al.*, 1994a,b). Some of these results have been presented to the British Pharmacological Society (Zygmunt *et al.*, 1996).

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Methods

Female Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was removed and cut into ring segments, 1–2 mm long, and suspended between two metal pins in organ baths containing physiological salt solution (PSS) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and glucose 11. The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 (carbogen) at a temperature of 37°C, resulting in a pH of 7.4. During an equilibration period of about 1 h, the vessels were repeatedly stretched until a stable resting tension of approximately 2 $mNmm^{-1}$ was obtained. Isometric tension was measured as previously described (Högestätt *et al.*, 1983). In some preparations, the endothelium was removed by insufflating carbogen (0.2 l min^{-1}) through the vessel lumen for 4 min. Lack of relaxation in response to 10 μM acetylcholine was used as a criterion of a successful removal of the endothelium.

Relaxations were studied in preparations contracted by phenylephrine. The concentrations of phenylephrine was titrated for each vascular segment to give a contraction amounting to 50–70% of an initial response to 10 μM phenylephrine (Zygmunt *et al.*, 1994a). When experiments were performed in a 30 mM K^+ PSS (KCl was added to the PSS without correcting for changes in osmolarity) in order to prevent the contribution of EDHF to endothelium-mediated relaxations, the final 50–70% level of contraction was achieved by addition of phenylephrine (Zygmunt *et al.*, 1994b). When stable contractions were obtained, the vasodilators were added cumulatively to determine concentration-response relationships. The incubation time with proadifen, clotrimazole, 17-ODYA, L-NOARG and indomethacin was 1 h. Control experiments with vehicle were performed in the same manner.

Electrophysiological recordings were made as previously described (Zygmunt *et al.*, 1994b). Briefly, glass microelectrodes filled with 0.5 M KCl (tip resistance 80–150 MΩ) were advanced from the adventitial side of the artery at resting tension. A successful impalement was characterized by a sudden negative shift in voltage followed by a stable negative potential for 5 min.

Calculations and statistics

The negative logarithm of the drug concentration eliciting 50% of the maximal relaxation (pRC_{50}) was determined by linear regression analysis by use of the data points immediately above and below the half-maximal response. R_{max} refers to the maximal relaxation achieved (100% denotes a complete reversal of the phenylephrine-induced contraction). Values are presented as mean ± s.e.mean, and *n* indicates the number of vascular segments (animals) examined. Statistical analysis was performed by using Student's *t* test (two-tailed) or multiple analysis of variance (MANOVA). Statistical significance was accepted when $P<0.05$.

Drugs

The following drugs were used: acetylcholine chloride (ACh), A23187, (−)-phenylephrine hydrochloride, N^{ω}-nitro-L-arginine, 3-morpholino-sydnonimine hydrochloride and clotrimazole (all from Sigma, St Louis, MO, U.S.A.); indomethacin (Confortid, Dumex, Copenhagen, Denmark); levcromakalim and proadifen (SKF 525A) (SmithKline Beecham, Brentford, UK); apamin (Alomone labs, Jerusalem, Israel); synthetic charybdotoxin (Latoxan, Rosans, France). 5,6-, 8,9-, 11,12- and 14,15-EETs (Biomol, U.S.A.). EETs were obtained as an oil dissolved in ethanol; the volume of ethanol was reduced under nitrogen to obtain a stock solution of 10 mM. 17-ODYA was synthesized as previously described (Shak *et al.*, 1985). A23187, clotrimazole and 17-ODYA were each dissolved in absolute ethanol while levcromakalim was dissolved in 70% ethanol. Apamin and charybdotoxin were each dissolved in saline. All other drugs were dissolved in distilled water. Stock solutions of the substances were stored at −70°C.

Results

Effects of 17-ODYA and EETs

The studies showing that EETs are capable of hyperpolarizing and relaxing vascular smooth muscle (see Introduction) prompted us to examine whether EDHF in rat hepatic artery might be an EET. Initially, endothelium-intact segments of hepatic artery were exposed to 50 μM 17-ODYA, an agent which acts as a suicide-substrate inhibitor of the epoxygenase pathway responsible for the generation of EETs (Zou *et al.*, 1994a,b). In the presence of L-NOARG (0.3 μM) plus indomethacin (10 μM), conditions which eliminate relaxations to endothelium-derived NO (EDNO) (Zygmunt *et al.*, 1994a,b), 17-ODYA had no effect on acetylcholine-induced relaxations. The pRC_{50} and R_{max} values for acetylcholine were 7.6 ± 0.1 and 96 ± 1% in the absence, and 7.5 ± 0.1 and 96 ± 3% in the presence of 17-ODYA, respectively (Figure 1, $n=5$).

To investigate further any possible relaxant actions of EETs, we examined the effects of 5,6-, 8,9-, 11,12- and 14,15-EETs on contractions induced by phenylephrine in segments of rat hepatic artery from which the endothelium had been removed. As shown in Figure 2, 8,9- and 11,12-EETs at a concentration of 10 μM (higher concentrations could not be tested because of solvent effects) were unable to relax the tissues ($n=4$). Similar results were obtained with 5,6- and 14,15-EETs ($n=4$, data not shown). In contrast, levcromakalim applied in the continuing presence of the EETs produced a full inhibition of phenylephrine-induced contractions, indicating that the tissues were capable of relaxing in response to a hyperpolarizing stimulus (Figure 2).

Effects of proadifen and clotrimazole

Modulation of responses to EDHF With L-NOARG (0.3 mM) plus indomethacin (10 μM) in the Krebs solution, the relaxant potency (pRC_{50}) of acetylcholine was significantly lower in the presence than in the absence of 10 μM proadifen (test, 6.3 ± 0.2;

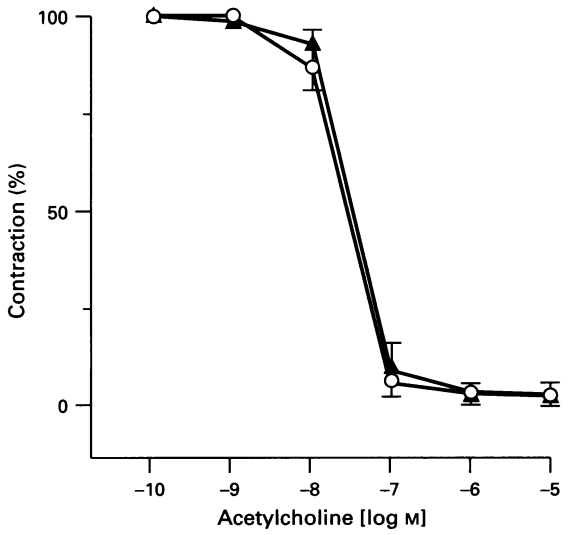

Figure 1 Relaxation induced by acetylcholine in the absence (○) and presence (▲) of 17-octadecynoic acid (17-ODYA, 50 μM) in arteries contracted by phenylephrine. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM). Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as mean ± s.e.mean of five experiments.

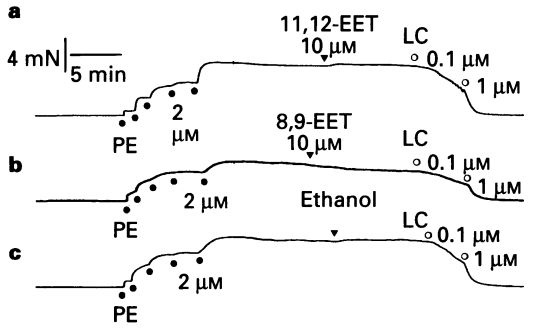

Figure 2 Traces showing the effect of (a) 11,12-epoxyeicosatrienoic acid (11,12-EET), (b) 8,9-EET and (c) solvent (0.1% ethanol) in endothelium-denuded arteries contracted by phenylephrine (PE). The relaxant effects of levcromakalim (LC) are also shown. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM).

control, 7.8±0.3, $n=6$, $P<0.01$) and 3 μM clotrimazole (test, 7.0±0.1; control, 7.5±0.1, $n=7$, $P<0.05$) (Figure 3). Although the maximal relaxation was not significantly reduced in the presence of 3 μM clotrimazole (test, 73±11%; control, 94±1%, $n=7$, $P=0.08$), there was a significant reduction when 10 μM proadifen was present (test, 89±4%; control, 98±1%, $n=6$, $P<0.01$) (Figure 3). Any effects of higher concentrations of proadifen and clotrimazole could not be examined due to inhibition of phenylephrine-induced contractions.

To determine whether the inhibitory effects of proadifen were mediated by inhibition of muscarinic receptors on the endothelial cells, the calcium (Ca)-ionophore A23187 (0.1–3 μM) was used to elicit receptor-independent release of EDHF (see Zygmunt & Högestätt, 1996). In the presence of L-NOARG (0.3 μM) plus indomethacin (10 μM), 3 μM A23187 relaxed the phenylephrine-induced contraction by 85±2% in the absence and 35±9% in the presence of 10 μM proadifen, respectively ($n=5$, $P<0.001$).

When cells of the hepatic artery were impaled with microelectrodes in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM), the membrane potential of preparations exposed to 10 μM proadifen was less negative compared to controls (test, −45±1 mV; control, −55 mV, $n=3$, $P<0.01$). Under control conditions, 1 μM acetylcholine produced a maximal hyperpolarization of 14±1 mV ($n=3$), a value which was reduced to 3±2 mV ($n=3$, $P<0.01$) in the presence of 10 μM proadifen (Figure 4).

Modulation of responses to EDNO and NO-donors These studies were carried out to determine whether the inhibition of EDHF by proadifen and clotrimazole extended to mechano-inhibitory pathways involving other mechanisms.

We have previously shown (Zygmunt *et al.*, 1994b; Zygmunt & Högestätt, 1996) that the relaxant responses to EDHF in the rat hepatic artery can be abolished either in the presence of a PSS containing 30 mM K^+ or in normal PSS to which charybdotoxin plus apamin (each 300 nM) have been added. Under similar conditions, but in the absence of L-NOARG, acetylcholine-induced relaxations are produced by EDNO. In 30 mM K^+PSS containing indomethacin (10 μM), 10 μM proadifen caused a rightward shift of the concentration-response curve to acetylcholine without affecting the maximal relaxation (Figure 5). The pRC_{50} values for acetylcholine were 7.5±0.1 in the absence and 6.7±0.1 in the presence of proadifen ($n=6$, $P<0.001$). In contrast to proadifen, 3 μM clotrimazole had no effect on EDNO-mediated relaxations (Figure 5).

In PSS containing indomethacin (10 μM) together with charybdotoxin plus apamin (each 300 nM), the EDNO-mediated relaxations generated on exposure to A23187 were unaffected by proadifen (10 μM). The pRC_{50} and R_{max} values for A23187 were 6.0±0.1 and 97±2% in the absence, and

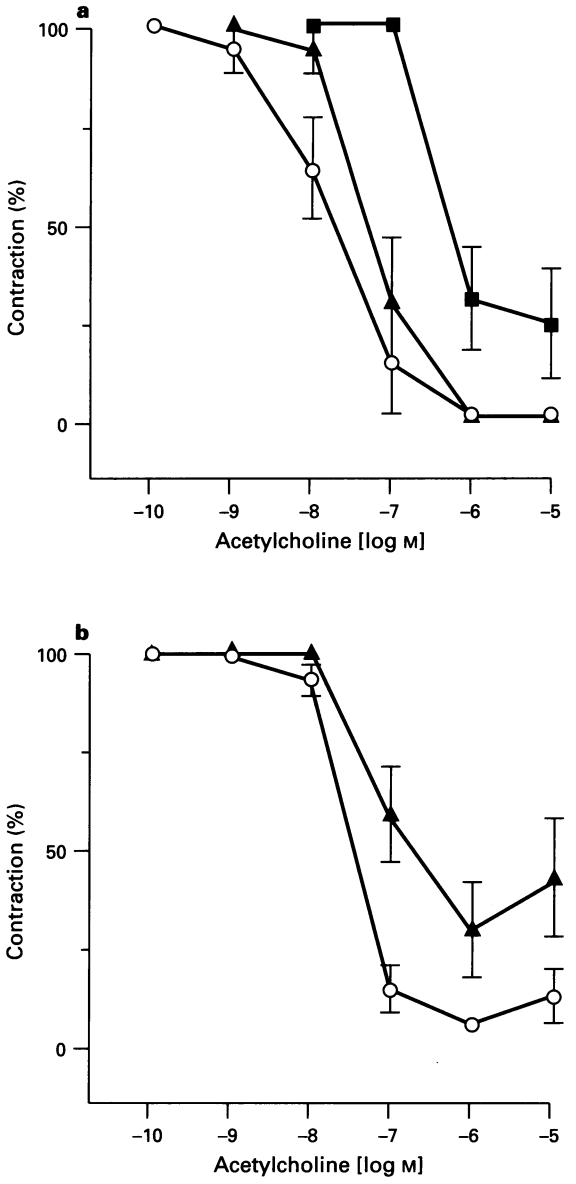

Figure 3 Relaxation induced by acetylcholine in the absence (○) and presence of (a) 1 μM (▲) and 10 μM (■) proadifen and (b) 3 μM clotrimazole (▲) in arteries contracted by phenylephrine. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM). Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as mean±s.e.mean of six to seven experiments.

6.2±0.1 and 91±2% in the presence of proadifen, respectively ($n=4$). Similarly, relaxations induced by the NO-donor 3-morpholino-sydnonimine (SIN-1) in normal PSS were unaffected by proadifen (10 μM). The pRC_{50} and R_{max} values for SIN-1 were 7.1±0.2 and 100±1% in the absence, and 7.0±0.1 and 99±1% in the presence of proadifen, respectively ($n=5$).

Modulation of responses to levcromakalim Relaxations of smooth muscle induced by levcromakalim are believed to involve the opening of plasmalemmal ATP-sensitive potassium channels (K_{ATP}; Edwards & Weston, 1993). The effects of this agent were therefore assessed in the presence of proadifen or clotrimazole to indicate whether K-channel inhibition may play some part in the ability of these cytochrome P450 inhibitors to antagonize EDHF. The relaxant effects of levcromakalim were inhibited by 1 μM proadifen or 3 μM clotrimazole and abolished by 10 μM proadifen (Figure 6). By use of microelectrodes, it was shown that levcromakalim produces a marked membrane hyperpolarization of 23 mV and 25 mV in cells from two different rat hepatic arteries, an

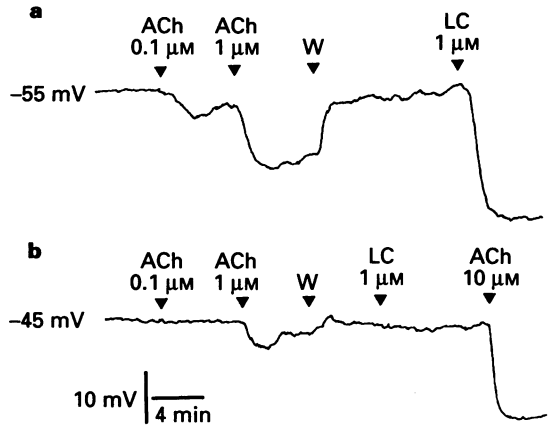

Figure 4 Traces showing the hyperpolarization induced by acetylcholine (ACh) and levcromakalim (LC) in the absence (a) and presence (b) of 10 μM proadifen in arteries at resting tension. W denotes washout. The recordings in (a) and (b) are from separate experiments. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM).

effect which was abolished in the presence of 10 μM proadifen (Figure 4).

Discussion

Is EDHF an EET?

It has been suggested that the endogenous K-channel opener, EDHF, might be an EET generated via a cytochrome P450-dependent mono-oxygenase located in the endothelial cells (see Hecker *et al.*, 1994; Harder *et al.*, 1995). The ability of EETs to relax smooth muscle and to open K-channels (Pfister *et al.*, 1991; Gebremedhin *et al.*, 1992; Hu & Kim, 1993; Rosolowsky & Campbell, 1993; Hecker *et al.*, 1994), together with the finding that inhibitors of this cytochrome can antagonize EDHF (Fulton *et al.*, 1992; 1994; 1995; Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Lischke *et al.*, 1995), collectively suggest that EDHF could indeed be an EET.

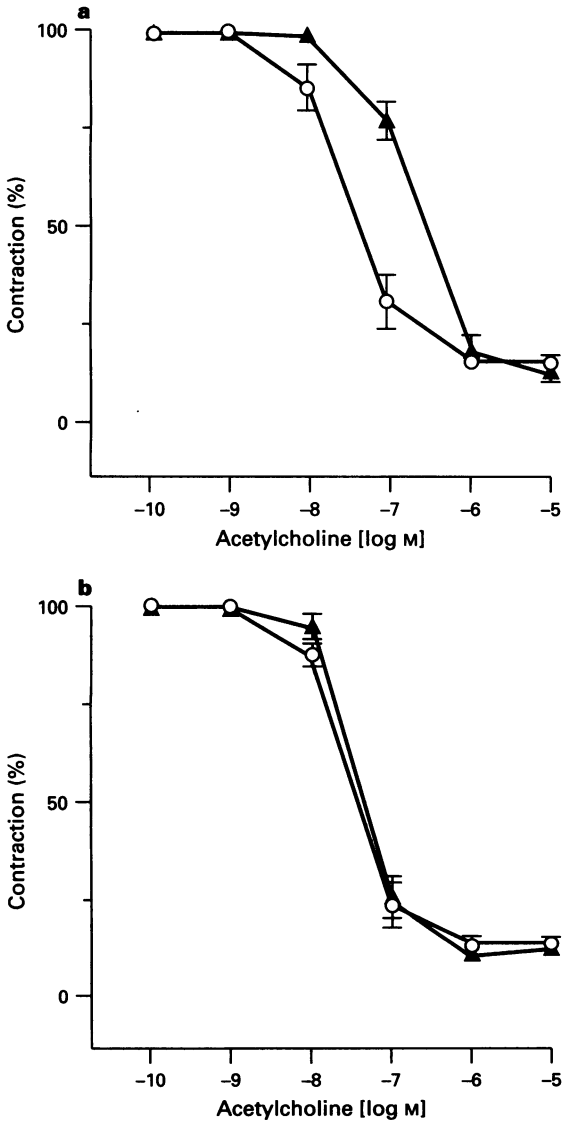

Figure 5 Relaxation induced by acetylcholine in the absence (○) and presence (▲) of (a) 10 μM proadifen and (b) 3 μM clotrimazole in arteries contracted by phenylephrine in 30 mM K^+ PSS. The experiments were performed in the presence of indomethacin (10 μM). Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as mean ± s.e.mean of six experiments.

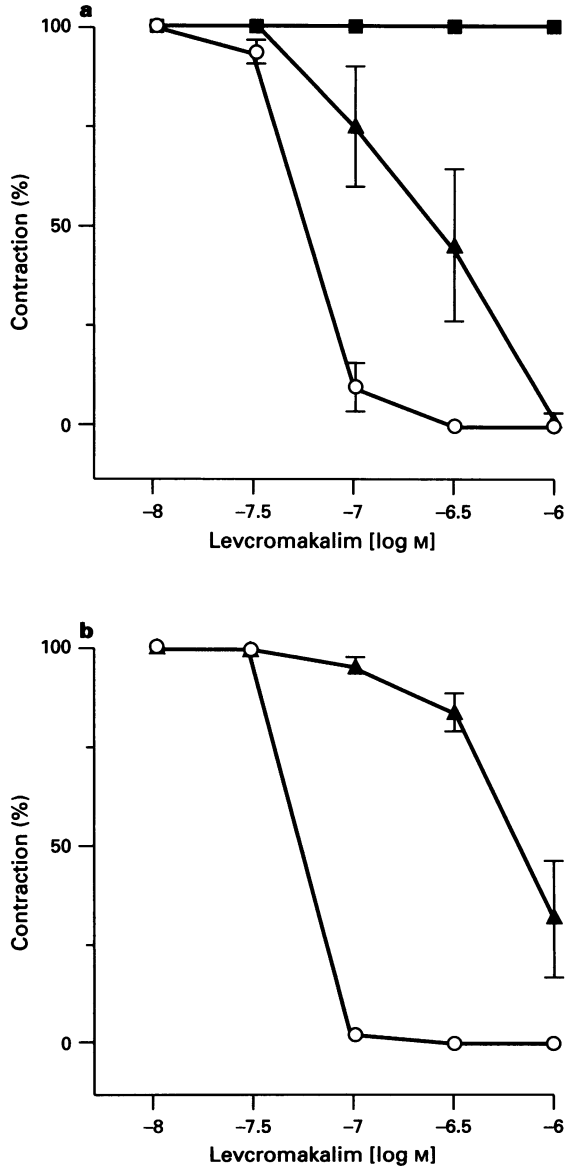

Figure 6 Relaxation induced by levcromakalim in the absence (○) and presence of (a) 1 μM (▲) and 10 μM (■) proadifen and (b) 3 μM clotrimazole (▲) in arteries contracted by phenylephrine. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM). Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as mean ± s.e.mean of six to seven experiments.

In the present study, two approaches were adopted to test this proposition. Firstly, tissues were exposed to the suicide-substrate inhibitor 17-ODYA, which is a potent inhibitor of the cytochrome P450-dependent epoxygenase responsible for the formation of EETs (Zou *et al.*, 1994a,b). No effect on EDHF-mediated relaxations was observed even though the concentration of 17-ODYA employed (50 μM) was at least ten times higher than that which almost completely inhibits the formation of EETs in renal microvessels and cortical microsomes of rats (Zou *et al.*, 1994a,b). Additionally, no relaxations were generated when segments of artery denuded of endothelium were exposed to either 5,6-, 8,9-, 11,12- or 14,15-EETs in the presence of L-NOARG plus indomethacin.

The lack of effect of 17-ODYA and of the EETs in the present study is a strong indication that EDHF is unlikely to be an EET in rat hepatic artery. This finding contrasts, however, with observations made in other test systems. For example, in the rat isolated perfused heart, 17-ODYA (at a concentration of only 2 μM) inhibited the bradykinin-induced vasodilator responses, which were possibly mediated by EDHF (Fulton *et al.*, 1995). Furthermore, EETs are reported to relax blood vessels from several vascular regions (Pfister *et al.*, 1991; Gebremedhin *et al.*, 1992; Rosolowsky & Campbell, 1993; Hecker *et al.*, 1994). The reasons for the discrepancy between these findings and those of the present study are not certain, but the differences could indicate that 'EDHF' is not the same in all tissues. Alternatively, the presence of indomethacin in the present study and its absence in earlier investigations (Pfister *et al.*, 1991; Gebremedhin *et al.*, 1992; Rosolowsky & Campbell, 1993) may be relevant, since indomethacin inhibits the relaxant effect of 5,6-EET (Carroll *et al.*, 1990; Ellis *et al.*, 1990). EETs may thus be metabolised to relaxant substances via cyclo-oxygenase giving rise to the false conclusion that EDHF is an EET.

Is EDHF the product of a cytochrome P450-dependent pathway?

To obtain more information about the involvement of a cytochrome P450-dependent pathway, experiments were also conducted in the presence of proadifen and clotrimazole, two mechanistically-different inhibitors of a large number of cytochrome P450-dependent systems (see Murray & Reidy, 1990). The present study clearly showed that proadifen and clotrimazole each inhibited endothelium-dependent relaxations mediated by EDHF in the rat hepatic artery. Furthermore, as demonstrated in microelectrode experiments, the hyperpolarization in response to EDHF was also inhibited by proadifen, which is consistent with a reduced formation of this factor.

A substantial part of the EDHF-induced relaxation remained in the presence of proadifen and clotrimazole, but higher concentrations could not be used without affecting the contractile response to phenylephrine. In spite of this limitation, the present results could be consistent with the view that EDHF is a metabolite of arachidonic acid, distinct from EETs, generated by a cytochrome P450-dependent pathway within the endothelial cells.

Are proadifen and clotrimazole selective inhibitors of cytochrome P450?

To consolidate the view that EDHF might be a cytochrome P450-dependent metabolite, it was necessary to establish whether proadifen and clotrimazole were *selective* inhibitors of this cytochrome and two approaches were used to investigate this question. One of these involved the use of relaxations generated either by EDNO or by a synthetic NO-donor, while the second strategy utilised responses to the K_{ATP} opener levcromakalim. Although EDHF is not thought to open K_{ATP} (see Garland *et al.*, 1995; Zygmunt & Högestätt, 1996), this agent provided a convenient method of generating smooth muscle hyperpolarization, the modulation of which by proadifen and clotrimazole would provide a preliminary indication of an interaction between these agents and relaxations caused by an increase in membrane potential.

We have previously used either a 30 mM K^+ PSS or a combination of apamin plus charybdotoxin to prevent the contribution of EDHF to relaxations induced by acetylcholine and A23187, thereby disclosing relaxations mediated by EDNO in the rat hepatic artery (Zygmunt *et al.*, 1994b; Zygmunt & Högestätt, 1996). Under these conditions, proadifen but not clotrimazole inhibited the EDNO-mediated relaxation in response to acetylcholine (present study). The relaxations induced by the NO-donor SIN-1 were, however, unaffected by proadifen, suggesting that the inhibition of EDNO by this agent was exerted on the endothelium rather than on the NO-effector pathway in smooth muscle. Since A23187-induced relaxations mediated by EDNO were unaffected, it seems unlikely that proadifen reduces the formation of EDNO by acting on endothelial NO-synthase. The inhibitory effects of proadifen may be related to its anti-muscarinic properties (Taylor *et al.*, 1980) or to an interaction with other processes involved in muscarinic receptor-response coupling, eventually leading to increased Ca^{2+} levels in endothelial cells. Such properties could also contribute to the ability of proadifen to inhibit EDHF-mediated responses, since an increase in endothelial Ca^{2+} concentration seems to be important not only for the synthesis of NO, but also for the formation of EDHF (Suzuki & Chen, 1990). However, proadifen also inhibited the receptor-independent relaxation produced by A23187 in the presence of L-NOARG plus indomethacin.

The hyperpolarization generated by EDHF (and by other K-channel openers like levcromakalim) is believed to relax smooth muscle indirectly through closure of voltage-sensitive Ca-channels (Edwards & Weston, 1993). Thus, when such channels are closed by nimodipine in the rat hepatic artery, the subsequent relaxations to EDHF and levcromakalim are reduced (Zygmunt 1994b). Since cytochrome P450 inhibitors, including clotrimazole and proadifen, are also inhibitors of voltage-sensitive Ca-channels (Kalsner *et al.*, 1970; Triggle *et al.*, 1979; Villalobos *et al.*, 1992), this action may thus contribute to their effect on EDHF- mediated relaxations.

Fulton *et al.* (1994) showed that clotrimazole had no effect on the vasodilatation produced by bolus injections of cromakalim into rat coronary arteries. In addition, Corriu *et al.* (1996) failed to detect any inhibitory effects of proadifen and clotrimazole on EDHF-mediated hyperpolarization in guinea-pig carotid artery. In that investigation the effect of only one concentration of acetylcholine was examined. It is therefore possible that the effects of the cytochrome P450 inhibitors on the acetylcholine-induced hyperpolarization were underestimated (c.f. Figures 3 and 4 in the present study). In contrast, we found that both proadifen and clotrimazole inhibited the relaxations mediated by either EDHF or levcromakalim in the rat hepatic artery. Furthermore, in those experiments in which membrane potential was measured, proadifen also inhibited EDHF- and levcromakalim-induced hyperpolarizations. Moreover, other workers have shown that cytochrome P450 inhibitors including clotrimazole and proadifen inhibit Ca-activated K-channels and displace radioactivity-labelled charybdotoxin from binding sites in erythrocytes (Alvarez *et al.*, 1992; Brugnara *et al.*, 1993). Clotrimazole and proadifen also inhibit the delayed rectifier current in rat vasculature (Zygmunt *et al.*, 1996). Collectively, these data show that cytochrome P450 inhibitors interact with several types of K-channel. Since the identity of the K-channel(s) activated by EDHF in the rat hepatic artery is unknown (Zygmunt & Högestätt, 1996), any interaction by these inhibitors with K-channels has to be considered when evaluating their effects on EDHF-mediated responses in this tissue.

Conclusions

The lack of effect of 17-ODYA on relaxations mediated by EDHF together with the failure of extracellularly-applied EETs to produce relaxation, collectively suggest that EDHF is

not an EET in the rat hepatic artery. The modulation of K-channels by proadifen and clotrimazole together with their Ca-antagonistic properties suggests that inhibition of K- and Ca-channels in smooth muscle rather than reduced EDHF formation in the endothelium offers a better explanation for the actions of the cytochrome P450 inhibitors. Further studies to characterize the effects of proadifen, clotrimazole and 17-ODYA on smooth muscle ion channels are in progress.

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