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Interactions between endothelium-derived relaxing factors in the rat hepatic artery: focus on regulation of EDHF

Peter M. Zygmunt, [1]Frances Plane, Marie Paulsson, [1]Christopher J. Garland & [2]Edward D. Högestätt

Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University, Lund, Sweden and [1]Department of Pharmacology, University of Bristol, Bristol, UK

1 In rat isolated hepatic arteries contracted with phenylephrine, acetylcholine and the calcium ionophore A23187 each elicit endothelium-dependent relaxations, which involve both nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). However, the contribution of prostanoids to these responses, and the potential interaction between EDHF and other endothelium-derived relaxing factors have not been examined.

2 In the presence of the NO synthase inhibitor N^G-nitro-L-arginine (L-NOARG, 0.3 mM) and a mixture of charybdotoxin (0.3 μM) and apamin (0.3 μM), inhibitors of the target potassium (K) channel(s) for EDHF, acetylcholine and A23187 each induced a concentration-dependent and almost complete relaxation, which was abolished in the additional presence of indomethacin (10 μM). Thus, in addition to EDHF and NO, a relaxing factor(s) generated by cyclo-oxygenase (COX) contributes to endothelium-dependent relaxation in the rat hepatic artery.

3 The resting membrane potentials of endothelium-intact and endothelium-denuded vascular segments were -57 mV and -52 mV, respectively ($P>0.05$). In intact arteries, the resting membrane potential was not affected by L-NOARG plus indomethacin, but reduced to -47 mV in the presence of charybdotoxin plus apamin. Acetylcholine and A23187 (10 μM each) elicited a hyperpolarization of 13 mV and 15 mV, respectively. The hyperpolarization induced by these agents was not affected by L-NOARG plus indomethacin (12 mV and 14 mV, respectively), but reduced in the presence of charybdotoxin plus apamin (7 mV and 10 mV, respectively), and abolished in the combined presence of charybdotoxin, apamin and indomethacin.

4 The NO donor 3-morpholino-sydnonimine (SIN-1) induced a concentration-dependent relaxation, which was unaffected by charybdotoxin plus apamin, but abolished by the selective soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM). SIN-1 (10 μM) did not alter the resting membrane potential in endothelium-denuded vascular segments.

5 The COX-dependent relaxation induced by acetylcholine was abolished following exposure to 30 mM KCl, but unaffected by glibenclamide (10 μM). The prostacyclin analogue iloprost induced a concentration-dependent relaxation, which was also abolished in 30 mM KCl and unaffected by the combined treatment with glibenclamide, charybdotoxin and apamin. Iloprost (10 μM) induced a glibenclamide-resistant hyperpolarization (8 mV with and 9 mV without glibenclamide) in endothelium-denuded vascular segments.

6 Exposure to SIN-1 or iloprost did not affect the EDHF-mediated relaxation induced by acetylcholine (i.e. in the presence of L-NOARG and indomethacin). Replacement of L-NOARG with the NO scavenger oxyhaemoglobin (10 μM) or the soluble guanylate cyclase inhibitor ODQ (10 μM) or methylene blue (10 μM), which all significantly inhibited responses to endothelium-derived NO, did not affect the acetylcholine-induced relaxation in the presence of indomethacin, indicating that endogenous NO also does not suppress EDHF-mediated responses.

7 These results show that, in addition to EDHF and NO, an endothelium-derived hyperpolarizing factor(s) generated by COX contributes significantly to endothelium-dependent relaxation in the rat heptic artery. Neither this factor nor NO seems to regulate EDHF-mediated responses. Thus, EDHF does not serve simply as a 'back-up' system for NO and prostacyclin in this artery. However, whether EDHF modulates the NO and COX pathways remains to be determined.

Keywords: Endothelium-derived hyperpolarizing factor; endothelium-derived relaxing factors; hyperpolarization; iloprost; membrane potential; nitric oxide; potassium channels; prostacyclin; vascular endothelium

Introduction

In addition to nitric oxide (NO) and prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) appears to be an important mediator of vasodilatation in various vascular beds (see Cohen & Vanhoutte, 1995; Garland *et al.*, 1995), including the rat hepatic artery (Zygmunt *et al.*, 1994c). Prostacyclin and NO induce vascular smooth muscle relaxation via stimulation of adenylate and guanylate cyclase, respectively (Vane *et al.*, 1982; Moncada & Higgs, 1993). In contrast, EDHF is thought to stimulate potassium (K) channels without the involvement of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) or guanosine 3′:5′-cyclic monophosphate (cyclic GMP), although the identity of the target K channel(s) has not been definitively established (Zygmunt *et al.*, 1994a,c; Cohen & Vanhoutte, 1995; Garland *et al.*, 1995; Zygmunt & Högestätt, 1996; Zygmunt *et al.*, 1997). However, part of the relaxation induced by NO and prostacyclin may also involve activation

[2] Author for correspondence at: Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University Hospital, S-221 85 Lund, Sweden.

of K channels either indirectly via cyclic nucleotide-dependent protein phosphorylation or, as recently suggested for NO, via a direct interaction with the channel protein (Archer *et al*., 1994; Bolotina *et al*., 1994; Murphy & Brayden, 1995a,b).

Although EDHF, NO and prostacyclin operate mainly through different effector or second messenger systems, there is still the potential of cross-talk between these factors. Nitric oxide has been shown to inhibit cyclo-oxygenase (COX) and cytochrome P450 as well as its own synthesis via feed-back inhibition of NO synthase (Khatsenko *et al*., 1993; Wink *et al*., 1993; Ravichandran *et al*., 1995; Ziyaat *et al*., 1996). It has been suggested that EDHF is formed by a cytochrome P450-dependent mono-oxygenase (Campbell *et al*., 1996; Chen & Cheung, 1996; Popp *et al*., 1996). Thus, NO may regulate the synthesis of EDHF by inhibiting this enzyme. Interactions through cross-talk between second messenger systems or by activation of K channels in the endothelium or smooth muscle are also feasible. For example, hyperpolarization of the endothelial cell membrane by one mediator could increase the electrical driving force for calcium and, hence, promote calcium-dependent release of other relaxing factors (Lückhoff & Busse, 1990).

In some blood vessels, NO seems to be the primary mediator of endothelium-dependent relaxation, whereas EDHF and prostanoids contribute to a limited extent (Cohen & Vanhoutte, 1995; Garland *et al*., 1995; Zygmunt *et al*., 1995). Recent studies have shown that NO and cyclic GMP can impair endothelium-dependent relaxation mediated by EDHF (Olmos *et al*., 1995; Bauersachs *et al*., 1996; McCulloch *et al*., 1997), suggesting that EDHF may function as a 'back-up' system, which is up-regulated when NO synthesis is failing (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996; Kemp *et al*., 1995).

We have challenged the validity of this hypothesis in the present study by examining the effects of the NO donor 3-morpholino-sydnonimine (SIN-1) and inhibitors acting at different levels of the NO pathway on relaxations mediated by EDHF in the rat hepatic artery. Furthermore, EDHF-mediated relaxations were recorded in the presence and absence of the prostacyclin mimetic iloprost. The K channel(s) activated by EDHF in this artery is effectively inhibited by a combination of charybdotoxin and apamin (Zygmunt & Högestätt, 1996), which has allowed us to disclose the different pathways contributing to endothelium-dependent relaxation in this blood vessel.

Methods

Experimental procedure

Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was removed and divided into ring segments, 1–2 mm long, which were suspended between two metal pins in organ baths (2.5 ml), containing warmed (37°C) physiological salt solution (PSS) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_2 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and (+)-glucose 6.0. The PSS was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, resulting in a pH of 7.4. During an equilibration period of about one hour, the resting wall tension was adjusted to approximately 2 mN mm^{-1} vessel length. 'Isometric' tension was measured by a force-displacement transducer (Grass Instruments FT03C, USA), connected to a polygraph (for details, see Högestätt *et al*., 1983). In order to assess the contractile capacity, each vessel segment was contracted first by an isosmolar 60 mM potassium solution (prepared as the PSS except for an equimolar substitution of NaCl with KCl) and then by 10 μM phenylephrine.

Relaxations induced by acetylcholine, A23187, SIN-1 and iloprost were studied in vessels contracted by phenylephrine. The concentration of phenylephrine was titrated for each vascular segment to give a contraction equivalent to 70–90% of the initial response to 10 μM phenylephrine (Zygmunt *et al*., 1994a). When stable contractions were obtained, the vasodilators were added cumulatively to determine concentration-response relationships. In some experiments, SIN-1 or iloprost was added on top of phenylephrine-induced contractions. The phenylephrine concentration was then increased to regain the initial level of tension before cumulative addition of acetylcholine. The incubation time with indomethacin, L-NOARG, charybdotoxin, apamin and glibenclamide was at least 20 min. Each vessel segment was exposed to only one treatment.

Electrophysiology

Recording of smooth muscle membrane potential was made on ring segments of the rat hepatic artery suspended in a myograph at 37°C as previously described (Waldron & Garland, 1994). Briefly, glass microelectrodes filled with 2 M KCl and of a tip resistance between 80–120 MΩ were advanced from the adventitial side of the artery at resting tension.

Calculations and statistics

Relaxations are expressed as the percentage reversal of the phenylephrine-induced contraction immediately before addition of the vasodilators. The maximal relaxation induced by each concentration of vasodilator was determined and used in subsequent calculations. The negative logarithm of the drug concentration eliciting half maximal relaxation (pEC_{50}) was determined by linear regression analysis, using the values immediately above and below half maximal response. E_{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 of pEC_{50} and E_{max} values was performed by using Student's t-test (two-tailed) or multiple analysis of variance (MANOVA) followed by Bonferroni Dunn's *post hoc* test (Statview 4.12). Statistical significance was accepted when $P<0.05$.

Drugs

Acetylcholine chloride (Aldrich); apamin (Alomone labs); A23187, glibenclamide, methylene blue, N^G-nitro-L-arginine, L-phenylephrine hydrochloride and human haemoglobin (Sigma); synthetic charybdotoxin (Latoxan); indomethacin (Confortid, Dumex); levcromakalim (SmithKline Beecham); 3-morpholino-sydnonimine hydrochloride (Cassella AG); iloprost (Ilomedin, Schering AG). A23187 and glibenclamide were each dissolved in absolute ethanol while levcromaklim was dissolved in 70% ethanol. Apamin and charybdotoxin were dissolved in saline. All other drugs were dissolved in distilled water. Oxyhaemoglobin was prepared as described previously (Zygmunt *et al*., 1994b). Stock solutions of the substances were stored at −70°C.

Results

Endothelium-derived relaxing factors

Acetylcholine and A23187 each induced concentration-dependent relaxations in phenylephrine-contracted rat hepatic arteries. Neither the COX inhibitor indomethacin (10 μM) nor the NO synthase inhibitor N^G-nitro-L-arginine (L-NOARG, 0.3 mM) or treatment with a mixture of the K channel inhibitors charybdotoxin (0.3 μM) and apamin (0.3 μM), which prevent the action of EDHF (Zygmunt & Högestätt 1996), significantly affected the relaxations induced by acetylcholine or A23187 (Table 1). The same was found when either L-NOARG or charybdotoxin plus apamin was combined with indomethacin (Table 1). However, treatment with L-NOARG, charybdotoxin and apamin together attenuated the relaxations induced by acetylcholine, but not those elicited by A23187 (Table 1). When the arterial segments were incubated with all inhibitors together (L-NOARG, charybdotoxin, apamin and indomethacin), the relaxations induced by acetylcholine and A23187 were converted into concentration-dependent contractions in the majority of preparations (Figure 1, Table 1).

Thus, after appropriate pharmacological treatment, at least three distinct endothelium-derived relaxing factors could be distinguished. Apart from relaxations mediated by EDHF (in the presence of L-NOARG and indomethacin) and NO (in the presence of indomethacin, charybdotoxin and apamin), a COX-dependent relaxation was revealed in the presence of L-NOARG, charybdotoxin and apamin (Figure 1). Endothelium-derived NO, EDHF and the relaxing factor(s) generated by COX each almost completely relaxed the phenylephrine-induced contraction (Table 1). Whereas acetylcholine was a less effective activator of the COX pathway than of the EDHF and NO pathways, A23187 was an equally potent activator of all three pathways (Table 1).

Endothelium-derived hyperpolarizing factors

The resting membrane potential of endothelium-intact arterial segments was -57 ± 1.5 mV ($n=30$ cells from 16 preparations). This was not significantly different from the resting membrane potential recorded in endothelium-denuded preparations (-52 ± 2.5 mV, $n=10$ cells from 6 preparations). L-NOARG (0.3 mM) plus indomethacin (10 μM) also did not significantly affect the membrane potential of the smooth muscle cells; the resting membrane potential in the presence of these inhibitors was -54 ± 2.9 mV ($n=9$ cells from 8 preparations). In contrast, application of charybdotoxin plus apamin (0.3 μM each) caused a small but significant depolarization; the resting membrane potential was -47 ± 1.0 mV ($n=10$ cells from 8 preparations) in the presence of these inhibitors ($P<0.01$).

Acetylcholine and A23187 (both 10 μM) each caused a similar degree of hyperpolarization with mean values of 13 ± 1.0 mV ($n=17$ cells from 10 preparations) and 15 ± 1.3 mV ($n=9$ cells from 8 preparations), respectively (Figure 2). The amplitude of the hyperpolarization to both acetylcholine and A23187 was unaffected by L-NOARG and indomethacin either alone (not shown) or in combination (Figure 2). The changes in membrane potential elicited by acetylcholine and A23187 in the presence of the combination of these inhibitors were 12 ± 1.2 mV and 14 ± 1.5 mV ($n=8$ cells from 8 preparations in each case), respectively. However, the hyperpolarization to both acetylcholine and A23187 was significantly reduced in the presence of charybdotoxin and apamin together (Figure 2). The changes in membrane potential elicited by acetylcholine and A23187 in the presence of these inhibitors were 7.0 ± 1.0 mV and 10 ± 1.0 mV ($n=5$ cells from 5 preparations in each case), respectively. In the presence of indomethacin, charybdotoxin and apamin together, the hyperpolarization to both acetylcholine and A23187 was abolished ($n=4$ cells from 4 preparations in each case, Figure 2).

Effects of K channel inhibitors

To test whether the relaxations mediated by NO involved activation of K channels sensitive to apamin and charybdotoxin, the effect of this toxin combination (0.3 μM each) was studied on relaxations induced by the NO donor SIN-1 in the presence of indomethacin (10 μM). The SIN-1-induced relaxation was unaffected by this treatment; the pEC_{50} and E_{max} values were 6.5 ± 0.4 and 96 ± 2% in the presence and 6.3 ± 0.2 and 99 ± 1 % in the absence of charybdotoxin plus

Table 1 Effects of inhibitors of COX (indomethacin), NO synthase (L-NOARG) and the target K channel(s) for EDHF (charybdotoxin plus apamin) on the endothelium-dependent relaxation induced by acetylcholine and A23187 in rat hepatic arteries contracted by phenylephrine

Inhibitors	n	*Acetylcholine* pEC_{50}	E_{max} (%)	n	*A23187* pEC_{50}	E_{max} (%)
Control	10	7.6±0.2	99±1	7	6.3±0.1	92±3
Indomethacin 10 μM	8	7.7±0.3	94±1	6	5.8±0.1	98±1
L-NOARG 0.3 mM	7	7.7±0.1	97±2		–	–
Charybdotoxin 0.3 μM Apamin 0.3 μM	4	7.5±0.1	100±1		–	–
Indomethacin 10 μM L-NOARG 0.3 mM	11	7.6±0.1	95±2	11	6.0±0.1	89±2
Indomethacin 10 μM Charybdotoxin 0.3 μM Apamin 0.3 μM	15	7.8±0.1	98±1	10	6.0±0.1	98±1
L-NOARG 0.3 mM Charybdotoxin 0.3 μM Apamin 0.3 μM	11	6.3±0.2*	91±1	9	6.2±0.1	76±8
Indomethacin 10 μM L-NOARG 0.3 mM Charybdotoxin 0.3 μM Apamin 0.3 μM	12	–	−22±11* (contraction)	6	–	−44±11* (contraction)

Data are presented as mean±s.e.mean. *Significantly different from controls ($P<0.05$).

apamin ($n=4$). The toxin mixture was also without effect on SIN-1-induced relaxations in the additional presence of 0.3 mM L-NOARG ($n=7$, data not shown). Similarly, glibenclamide (10 μM), an inhibitor of ATP-sensitive potassium channels, had no effect on relaxations induced by SIN-1 in the presence of L-NOARG, indomethacin, charybdotoxin and apamin; the pEC_{50} and E_{max} values for SIN-1 were 6.2 ± 0.2 and 94 ± 6% in the presence and 6.5 ± 0.2 and 96 ± 4 % in the absence of glibenclamide ($n=6$). SIN-1 (10 μM) also did not alter the resting membrane potential of the smooth muscle cells of endothelium-denuded arterial segments (Figure 3); the membrane potential before and after exposure to SIN-1 was -56 ± 2.0 mV and -57 ± 2.0 mV ($n=5-6$ cells from 3 preparations in each case), respectively .

The effect of apamin plus charybdotoxin was also tested on relaxations elicited by the stable prostacyclin mimetic iloprost in the presence of L-NOARG. Again, this toxin combination was found to have no effect; the pEC_{50} and E_{max} values for iloprost were 7.0 ± 0.2 and 89 ± 8% in the presence and 7.0 ± 0.2 and 86 ± 6 % in the absence of charybdotoxin and apamin ($n=4$). Similar results were obtained when the experiments were performed in the additional presence of indomethacin ($n=5$, data not shown). In endothelium-denuded arterial segments, iloprost (10 μM) caused a hyperpolarization of 9.0 ± 1.5 mV ($n=5$ cells from 3 preparations, Figure 3), which was unaffected by glibenclamide (8.5 ± 1.0 mV, $n=3$ cells from 2 preparations).

Treatment with glibenclamide (10 μM) was also without effect on the COX-dependent relaxation induced by acetylcholine or the relaxation evoked by iloprost in the combined presence of L-NOARG, charybdotoxin and apamin; the pEC_{50} and E_{max} values for acetylcholine were 5.8 ± 0.1 and 84 ± 4% in the presence and 5.8 ± 0.2 and 84 ± 4% in the absence of glibenclamide ($n=7$). The corresponding values for iloprost were 7.6 ± 0.2 and 94 ± 3% in the presence and $7.6+0.1$ and 99 ± 1% in the absence of glibenclamide ($n=5$).

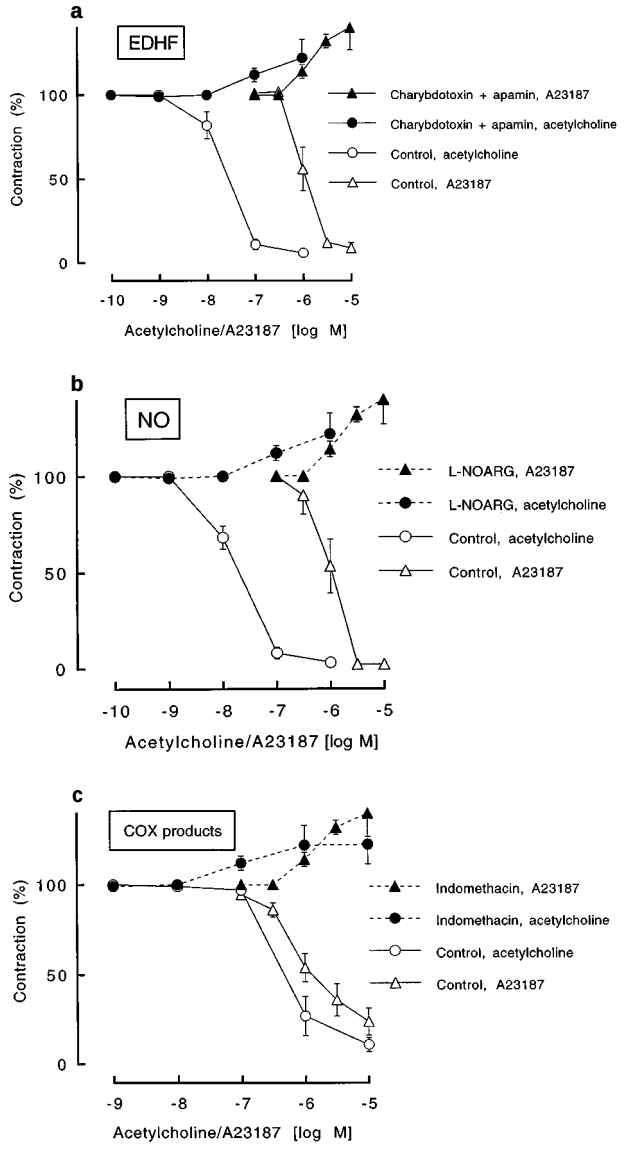

Figure 1 Concentration-relaxation curves for the endothelium-dependent relaxation induced by acetylcholine (circles) and A23187 (triangles) in phenylephrine-contracted hepatic arteries. The relaxation mediated by EDHF (a; $n=11$), NO (b; $n=15$ and 10 for acetylcholine and A23187, respectively) and COX products (c; $n=11$ and 9 for acetylcholine and A23187, respectively) were disclosed in the presence of 10 μM indomethacin plus 0.3 mM L-NOARG (EDHF), 10 μM indomethacin, 0.3 μM charybdotoxin plus 0.3 μM apamin (NO) and 0.3 mM L-NOARG, 0.3 μM charybdotoxin plus 0.3 μM apamin (COX). Solid symbols in (a) indicate responses in the presence of all these inhibitors (the same data are presented in (b) and (c), broken lines, for comparision). One hundred percent on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of acetylcholine or A23187. Data are presented as means and vertical lines show s.e.mean.

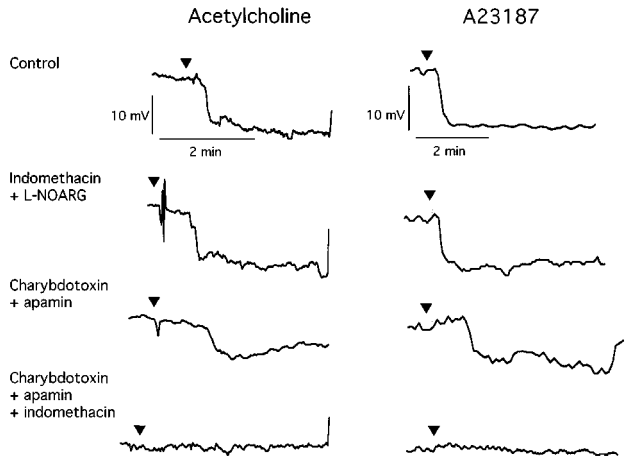

Figure 2 Traces showing the effect of acetylcholine (10 μM) and the calcium ionophore A23187 (10 μM) on the membrane potential in endothelium-intact ring segments of hepatic artery at resting tension. EDHF-mediated hyperpolarizations were obtained in the presence of indomethacin (10 μM) plus L-NOARG (0.3 mM). COX-dependent hyperpolarizations were revealed in the presence of charybdotoxin (0.3 μM) plus apamin (0.3 μM). However, no hyperpolarization occurred in the presence of charybdotoxin, apamin and indomethacin together.

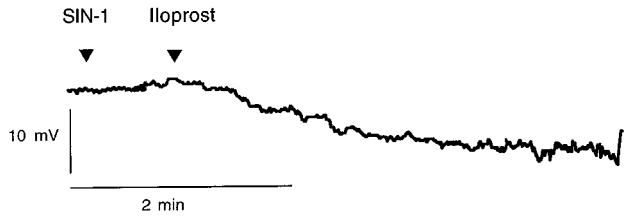

Figure 3 Trace showing the effects of the NO donor SIN-1 (10 μM) and the prostacyclin analogue iloprost (10 μM) on the membrane potential in an endothelium-denuded ring segment of hepatic artery at resting tension.

Effect of 30 mM KCl

As shown previously in the rat hepatic artery, both hyperpolarization and relaxation mediated by EDHF were prevented by 30 mM KCl (Zygmunt *et al*., 1994c). Similarly, 30 mM KCl abolished the COX-dependent relaxation induced by acetylcholine and the relaxation elicited by iloprost in the presence of L-NOARG, charybdotoxin and apamin together (Figure 4). However, acetylcholine evoked a pronounced NO-mediated relaxation in the presence of 30 mM KCl and indomethacin; the pEC_{50} and E_{max} values for acetylcholine were 7.5 ± 0.1 and $87 \pm 4\%$ ($n=6$). These responses were almost identical with acetylcholine-induced relaxations mediated by NO in normal PSS, containing indomethacin, charybdotoxin and apamin together (see Table 1).

Effects of SIN-1 and iloprost on EDHF-mediated relaxations

To address the possibility that NO as well as COX-dependent autocoids such as prostacyclin inhibit the EDHF pathway, the effects of SIN-1 and iloprost were studied on EDHF-mediated relaxations induced by acetylcholine as follows. When stable contractions by phenylephrine were obtained in the presence of L-NOARG (0.3 mM) and indomethacin (10 μM), either SIN-1 (100 – 1000 nM; mean concentration = 583 ± 189 nM, $n=6$) or iloprost (80 – 300 nM; mean concentration = 154 ± 42 nM, $n=5$) was added to the organ bath until the contraction was reduced by $82 \pm 7\%$ (range = 52 – 100%) for SIN-1 ($n=6$) and $82 \pm 6\%$ (range = 61 – 97%) for iloprost ($n=5$). The phenylephrine concentration was then increased to regain the initial level of tension before addition of acetylcholine (Figure 5). Under these conditions, the acetylcholine-induced relaxation was unaffected by SIN-1 and iloprost (Figure 5). Both SIN-1 and iloprost caused sustained relaxations throughout these experiments (Figure 5). As shown in separate experiments, the relaxation induced by SIN-1 was abolished by the selective soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM, Figure 6), indicating that the effect of SIN-1 was indeed produced by activation of this enzyme.

Effect of endothelium-derived NO on EDHF-mediated relaxation

To examine whether an on-going NO synthesis could influence the formation of EDHF, acetylcholine-induced relaxation recorded in the presence of L-NOARG was compared with relaxations obtained in the presence of the NO scavenger oxyhaemoglobin (10 μM) or the soluble guanylate cyclase inhibitor ODQ (10 μM) or methylene blue (10 μM). Replacement of L-NOARG with either oxyhaemoglobin, ODQ or methylene blue, all of which significantly inhibited the action of endogenous NO, had no effect on the acetylcholine-induced relaxation (Figure 7).

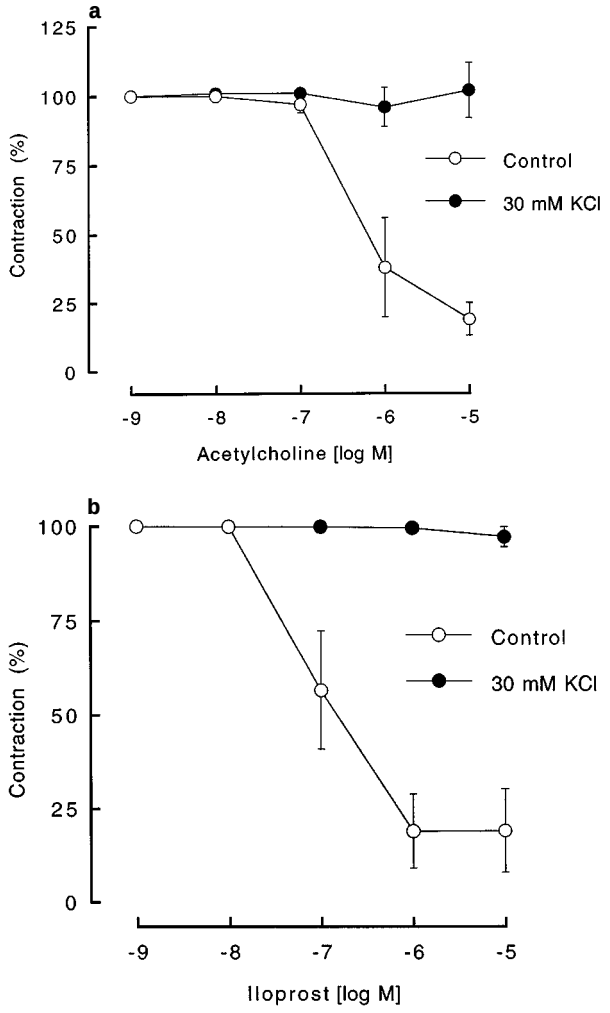

Figure 4 Effects of 30 mM KCl on (a) the COX-dependent relaxation induced by acetylcholine and (b) the relaxation induced by iloprost in hepatic arteries contracted by phenylephrine. L-NOARG (0.3 mM), charybdotoxin (0.3 μM) and apamin (0.3 μM) were present in all experiments. Control experiments were performed in normal PSS (4.6 mM KCl). One hundred percent on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of acetylcholine or iloprost. Data are presented as means and vertical lines show s.e.mean of five to seven experiments.

Discussion

The present study provides evidence that at least three different relaxing factors contribute to the endothelium-dependent relaxation induced by acetylcholine and A23187 in the rat hepatic artery. Responses mediated by NO and EDHF have been described previously in this artery (Zygmunt *et al*., 1994a,c), but the observation of a COX-dependent hyperpolarizing factor, which contributed significantly to the endothelium-dependent relaxation, is a novel finding. Indeed, this factor was able to relax almost fully phenylephrine-contracted arteries.

In the presence of L-NOARG and indomethacin, acetylcholine and A23187 have been shown to cause endothelium-dependent relaxations, which are sensitive to charybdotoxin plus apamin, in the rat hepatic artery (Zygmunt & Högestätt, 1996). The present study confirms that such EDHF-mediated relaxations are antagonized by charybdotoxin plus apamin, and also shows that the smooth muscle hyperpolarization triggered by acetylcholine and A23187 is prevented by these K channel inhibitors. These findings further support a causal relationship between the hyperpolarization and relaxation mediated by EDHF in the rat hepatic artery (Zygmunt *et al*., 1994c).

A striking observation was the lack of effect of indomethacin either alone or when combined with the NO synthase inhibitor L-NOARG or charybdotoxin plus apamin, inhibitors of the target K channel(s) for EDHF (Zygmunt & Högestätt, 1996). This clearly illustrates that lack of effect of indomethacin does not provide solid proof to discard a role for prostanoids in endothelium-dependent relaxation unless all

additional inhibitory pathways have been suppressed, and raises concern that this factor may have been overlooked before (see also Murphy & Brayden, 1995b; Corriu *et al*., 1996).

Prostacyclin is the main product of COX in the vascular endothelium, affecting both smooth muscle tone and platelet function (Vane *et al*., 1982). In the present study, the stable prostacyclin analogue iloprost could mimic the action of the COX-dependent relaxing factor(s). Both factors elicited a hyperpolarization and relaxation, which were of similar amplitude and unaffected by glibenclamide. Furthermore, neither factor could evoke a relaxation when the extracellular K concentration was raised to 30 mM, suggesting that the relaxations were indeed mediated by membrane hyperpolarization. An indomethacin-sensitive smooth muscle hyperpolarization in response to acetylcholine has also been demonstrated in rabbit mesenteric and guinea-pig coronary and carotid arteries (Parkington *et al*., 1993; Murphy & Brayden, 1995b; Corriu *et al*., 1996). In contrast to the present study, the COX-dependent hyperpolarization as well as the hyperpolarization induced by iloprost were considered to be of no importance for the relaxation in the guinea-pig coronary artery (Parkington *et al*., 1993; 1995), whereas this aspect was not studied in rabbit mesenteric and guinea-pig carotid arteries (Murphy & Brayden, 1995b; Corriu *et al*., 1996). In rabbit mesenteric artery, both the endogenous hyperpolarization and that produced by iloprost were blocked by glibenclamide, an inhibitor of ATP-sensitive K channels (Murphy & Brayden, 1995b). However, the COX-dependent relaxation and the hyperpolarization and relaxation elicited by iloprost in the rat hepatic artery were unaffected by glibenclamide at a concentration of 10 μM, which completely prevents the hyperpolarization and relaxation induced by the K-channel opener levcromakalim in this artery (Zygmunt *et al*., 1994c). Furthermore, K channels sensitive to charybdotoxin and apamin also do not seem to be involved, since these toxins did not affect the iloprost-induced relaxation. Thus, the mechansims behind the COX-dependent and iloprost-induced

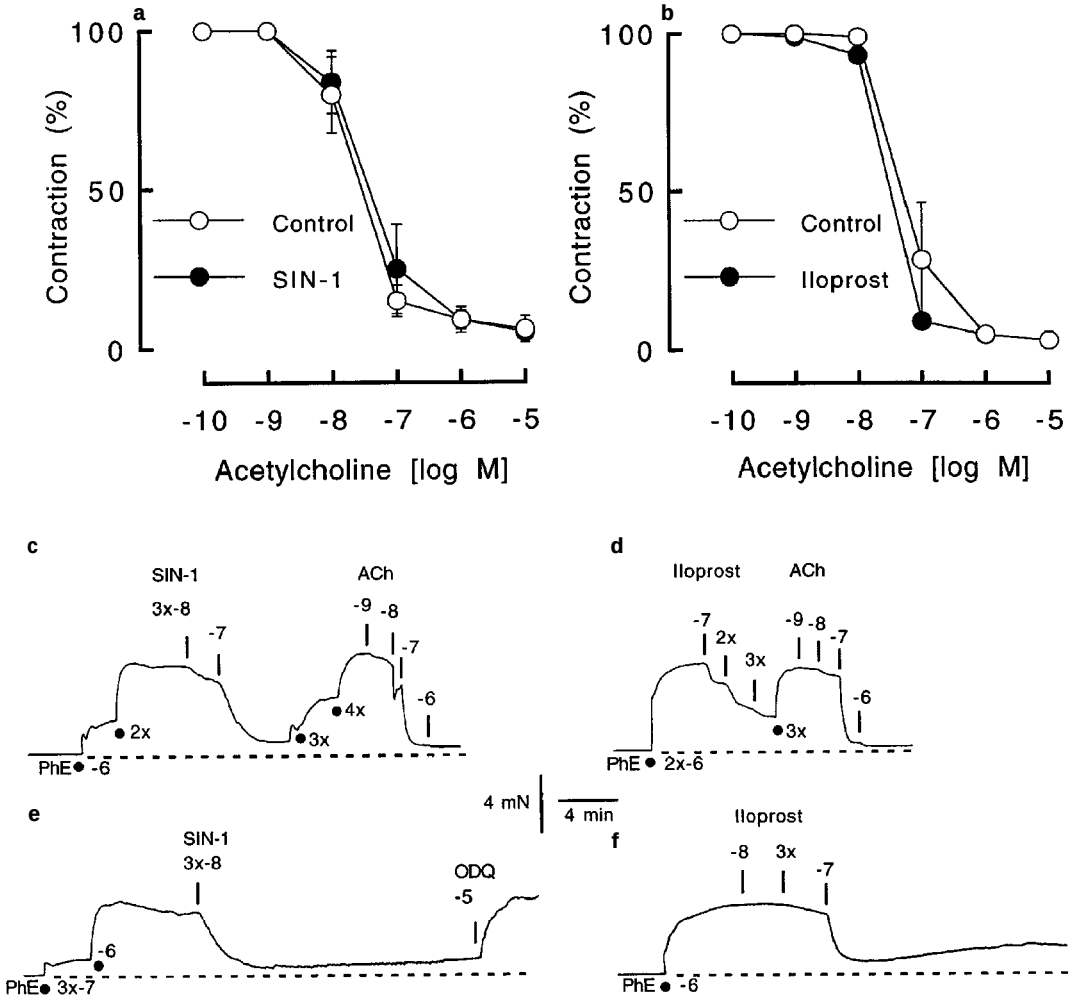

Figure 5 Effects of SIN-1 (a) and iloprost (b) on EDHF-mediated relaxations induced by acetylcholine in phenylephrine-contracted hepatic arteries. SIN-1 or iloprost was given at a concentration which relaxed the phenylephrine-induced contraction by approximately 80% (see Results). The phenylephrine concentration was then increased to regain the initial level of tension. All experiments were perfomed in the presence of L-NOARG (0.3 mM) and indomethacin (10 μM). One hundred percent on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of acetylcholine. Data are presented as means and vertical lines show s.e.mean of five to six experiments. Traces showing the effects of SIN-1 (c) and iloprost (d) on EDHF-mediated relaxations evoked by acetylcholine (ACh) in phenylephrine (PhE)-contracted arteries in the presence of L-NOARG and indomethacin. As shown by lower traces, the relaxations induced by SIN-1 (e) and iloprost (f) persisted throughout the experimental period. Addition of the soluble guanylate cyclase inhibitor ODQ after 20 min reversed the SIN-1-induced relaxation, whereas ODQ itself had no contractile effect in the absence of SIN-1 (not shown). Drug concentrations are shown as log molar concentrations. Dashed lines indicate the basal tension level before addition of phenylephrine.

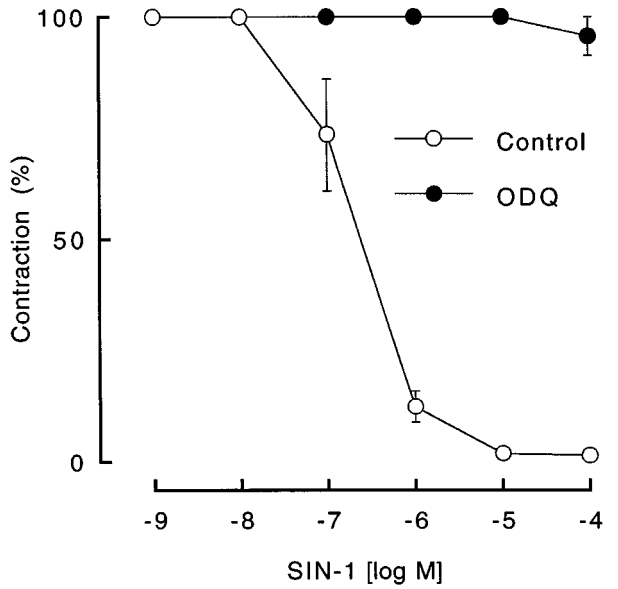

Figure 6 Effect of SIN-1 in the absence (control) and presence of the guanylate cyclase inhibitor ODQ (10 μM) in hepatic arteries contracted by phenylephrine. The experiments were perfomed in the presence of L-NOARG (0.3 mM) and indomethacin (10 μM). One hundred percent on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of SIN-1. Data are presented as means and vertical lines show s.e.mean of five experiments.

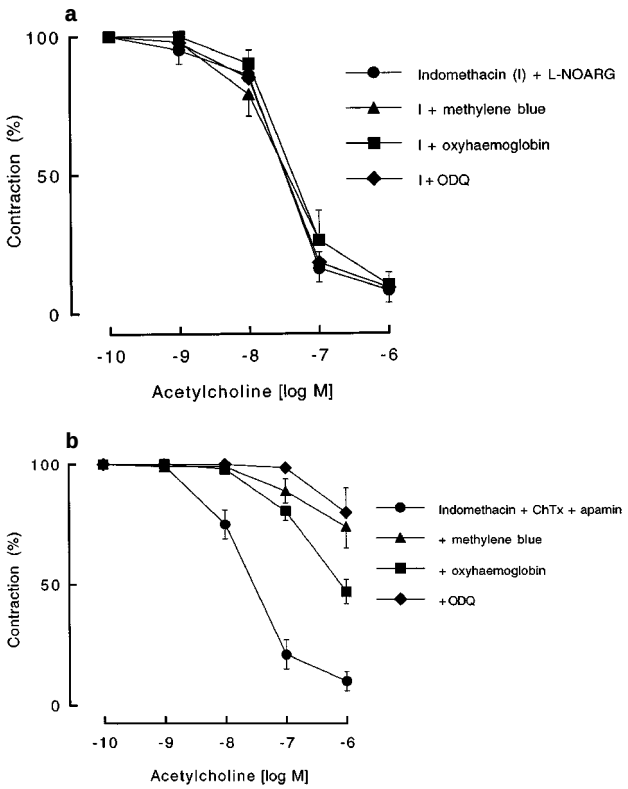

Figure 7 (a) Acetylcholine-induced relaxations in phenylephrine-contracted hepatic arteries recorded in the presence of indomethacin (10 μM) combined with either L-NOARG (0.3 mM, $n=9$), oxyhaemoglobin (10 μM, $n=9$), ODQ (10 μM, $n=6$) or methylene blue (10 μM, $n=6$). (b) Effects of oxyhaemoglobin (10 μM, $n=7$), ODQ (10 μM, $n=6$) and methylene blue (10 μM, $n=6$) on acetylcholine-induced relaxations mediated by endothelium-derived NO, i.e in the presence of indomethacin (10 μM), charybdotoxin (ChTx; 0.3 μM) and apamin (0.3 μM). Controls ($n=9$) were recorded in the absence of oxyhaemoglobin, ODQ and methylene blue. One hundred percent on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of acetylcholine. Data are presented as means and vertical lines show s.e.mean.

hyperpolarization and relaxation are presently unclear and are the subject of an on-going study.

In contrast to A23187, acetylcholine was a less potent stimulator of the COX pathway than of the NO and EDHF pathways. Whether this means that the COX pathway on the one hand and the NO and EDHF pathways on the other hand are regulated in part by different second messenger systems remains to be established. An increase in the endothelial calcium concentration seems to be crucial for activation of all three pathways (Lückhoff *et al*., 1988; Parsaee *et al*., 1992; Busse *et al*., 1993; Higuchi *et al*., 1996; Fukao *et al*., 1997a). However, the production of NO and prostacyclin in bovine aortic endothelial cells differ with regard to calcium sensitivity, the latter being less sensitive (Parsaee *et al*., 1992). Whether such a difference in calcium sensitivity could explain the present findings is unclear, since the calcium ionophore A23187 was equally potent in activating all three pathways.

It has been proposed that EDHF serves as a 'back-up' system, which is turned on when the NO synthesis is compromised or inhibited by L-arginine analogues (see Introduction). For example, NO may suppress the release of EDHF by inhibiting cytochrome P450 mono-oxygenase (Olmos *et al*., 1995), the enzyme claimed to generate EDHF (Campbell *et al*., 1996; Chen & Cheung, 1996; Popp *et al*., 1996). Different approaches were used in the present study to examine whether NO regulates responses mediated by EDHF. Firstly, exposure to the NO donor SIN-1 did not affect the EDHF-mediated relaxation, an observation also made in porcine coronary arteries (Nagao & Vanhoutte, 1992). SIN-1 also failed to affect the EDHF-mediated hyperpolarization in rabbit mesenteric arteries (Murphy & Brayden, 1995a). However, SIN-1 or the cyclic GMP analogue 8-Br cyclic GMP inhibited relaxations mediated by EDHF in canine coronary arteries and the rat perfused mesenteric arterial bed, possibly by affecting the target K channel(s) for EDHF (Olmos *et al*., 1995; McCulloch *et al*., 1997), indicating that the activity of EDHF may be down-regulated by NO in certain vascular beds. Secondly, the acetylcholine-induced relaxation was unaltered when L-NOARG was replaced by the NO scavenger oxyhaemoglobin or the soluble guanylate cyclase inhibitors ODQ and methylene blue, all of which inhibit the NO pathway mainly at sites distal to the endothelium. These findings suggest that the vasodilatory effects of EDHF are not suppressed by NO or mediated by products of NO synthase formed only in the presence of N^G-substituted L-arginine analogues in the rat hepatic artery. EDHF also seems to act independently of the COX pathway, since iloprost was unable to affect the EDHF-mediated relaxation. However, the results do not exclude the possibility that EDHF may down-regulate the activity of COX and NO synthase. Selective inhibitors of the EDHF synthesis would be needed to answer this question definitively.

Nitric oxide has been shown to activate charybdotoxin-sensitive K channels in vascular smooth muscle (Archer *et al*., 1994; Bolotina *et al*., 1994). In rat small mesenteric arteries, the relaxation induced by the NO donor SIN-1 is inhibited by charybdotoxin (Plane *et al*., 1996). In other arteries, glibenclamide inhibits NO-mediated hyperpolarization, implicating ATP-sensitive K channels in the action of NO (Parkington *et al*., 1995; Murphy & Brayden, 1995a,b;

Corriu *et al*., 1996). However, in the present study, glibenclamide and charybdotoxin, either alone (unpublished observations) or together with apamin, did not affect the SIN-1-induced relaxation. Furthermore, neither endogenous NO nor SIN-1 elicited a smooth muscle hyperpolarization, and exposure to 30 mM KCl had no effect on the acetylcholine-induced relaxation mediated by endogenous NO, indicating that K channel activation is not crucial for the action of NO in this artery. This strongly suggests that NO does not interact with large-conductance calcium-activated or ATP-sensitive K channels, or the target K channel(s) for EDHF in the rat hepatic artery. The findings also suggest that the treatment used to prevent EDHF-mediated relaxations (i.e. charybdotoxin plus apamin) did not affect the response to endogenous NO. Thus, the relaxation induced by acetylcholine and A23187 in the presence of indomethacin, charybdotoxin and apamin should reflect the 'true' NO-mediated relaxation.

We have previously examined the possibility that EDHF is an arachidonic acid metabolite formed by cytochrome P450 mono-oxygenase in the rat hepatic artery (Zygmunt *et al*., 1996). As already mentioned, NO has been shown to inhibit cytochrome P450-dependent enzymes (Wink *et al*., 1993; Khatsenko *et al*., 1993). The fact that the NO-donor SIN-1 did not affect the EDHF-mediated relaxation in the present study further support our previous conclusion that EDHF is not produced by this enzyme system in the rat hepatic artery (Zygmunt *et al*., 1996). Similarly, the NO donor nitroprusside was without effect on the hyperpolarization mediated by EDHF in rat small mesenteric arteries (Fukao *et al*., 1997b).

The results of the present study show that, in addition to EDHF and NO, the vascular endothelium produces a COX-dependent hyperpolarizing factor(s), which makes an important contribution to the endothelium-dependent relaxation induced by acetylcholine and A23187 in the rat heptic artery. Neither this factor nor NO seems to regulate the synthesis or effect of EDHF in this artery. Thus, EDHF apparently acts independently of NO and COX, and therefore may not be regarded simply as a 'back-up' system for NO and prostacyclin in this vessel. However, whether EDHF modulates the NO and COX pathways remains to be established and will probably await the discovery of selective inhibitors of the synthesis of EDHF.

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