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NO/PGI_2-independent vasorelaxation and the cytochrome P450 pathway in rabbit carotid artery

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1 The nature and cellular mechanisms that are responsible for endothelium-dependent relaxations resistant to indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME) were investigated in phenylephrine (PE) precontracted isolated carotid arteries from the rabbit.

2 In the presence of the cyclo-oxygenase inhibitor, indomethacin (10 μM), acetylcholine (ACh) induced a concentration- and endothelium-dependent relaxation of PE-induced tone which was more potent than the calcium ionophore A23187 with pD_2 values of 7.03 ± 0.12 ($n=8$) and 6.37 ± 0.12 ($n=6$), respectively. The ACh-induced response was abolished by removal of the endothelium, but was not altered when indomethacin was omitted (pD_2 value 7.00 ± 0.10 and maximal relaxation $99 \pm 3\%$, $n=6$). Bradykinin and histamine (0.01–100 μM) had no effect either upon resting or PE-induced tone ($n=5$).

3 In the presence of indomethacin plus the NO synthase inhibitor, L-NAME (30 μM), the response to A23187 was abolished. However, the response to ACh was not abolished, although it was significantly inhibited with the pD_2 value and the maximal relaxation decreasing to 6.48 ± 0.10 and $67 \pm 3\%$, respectively (for both $P<0.01$, $n=8$). The L-NAME/indomethacin insensitive vasorelaxation to ACh was completely abolished by preconstriction of the tissues with potassium chloride (40 mM, $n=8$).

4 The Ca^{2+}-activated K^+ (K_{Ca}) channel blockers, tetrabutylammonium (TBA, 1 mM, $n=5$) and charybdotoxin (CTX, 0.1 μM, $n=5$), completely inhibited the nitric oxide (NO) and prostacyclin (PGI_2)-independent relaxation response to ACh. However, iberiotoxin (ITX, 0.1 μM, $n=8$) or apamin (1–3 μM, $n=6$) only partially inhibited the relaxation.

5 Inhibitors of the cytochrome P450 mono-oxygenase, SKF-525A (1–10 μM, $n=6$), clotrimazole (1 μM, $n=5$) and 17-octadecynoic acid (17-ODYA, 3 μM, $n=7$) also reduced the NO/PGI_2-independent relaxation response to ACh.

6 In endothelium-denuded rings of rabbit carotid arteries, the relaxation response to exogenous NO was not altered by either K_{Ca} channel blockade with apamin (1 μM, $n=5$) or CTX (0.1 μM, $n=5$), or by the cytochrome P450 mono-oxygenase blockers SKF-525A (10 μM, $n=4$) and clotrimazole (10 μM, $n=5$). However, the NO-induced response was shifted to the right by LY83583 (10 μM, $n=4$), a guanylyl cyclase inhibitor, with the pD_2 value decreasing from 6.95 ± 0.14 to 6.04 ± 0.09 ($P<0.01$).

7 ACh (0.01–100 μM) induced a concentration-dependent relaxation of PE-induced tone in endothelium-denuded arterial segments sandwiched with endothelium-intact donor segments. This relaxation to ACh was largely unaffected by indomathacin (10 μM) plus L-NAME (30 μM), but abolished by the combination of indomethacin, L-NAME and TBA (1 mM, $n=5$).

8 These data suggest that in the rabbit carotid artery: (a) ACh can induce the release of both NO and EDHF, whereas A23187 only evokes the release of NO from the endothelium, (b) the diffusible EDHF released by ACh may be a cytochrome P450-derived arachidonic acid metabolite, and (c) EDHF-induced relaxation involves the opening of at least two types of K_{Ca} channels, whereas NO mediates vasorelaxation via a guanosine 3′: 5′-cyclic monophosphate (cyclic GMP)-mediated pathway, in which a cytochrome P450 pathway and K_{Ca} channels do not seem to be involved.

Keywords: Acetylcholine; NO; endothelium-derived hyperpolarizing factor (EDHF); Ca^{2+}-activated K^+ channels; cytochrome P450; vasorelaxation; carotid artery

Introduction

The vascular endothelium regulates the tone of the underlying smooth muscle through the release of potent vasoactive agents. Since the discovery of endothelium-dependent vascular relaxation (Furchgott & Zawadzki, 1980), an intensive research effort has been directed to the identification of an endothelium-derived relaxing factor (EDRF). EDRF is likley to be nitric oxide (Ignarro *et al*., 1987; Palmer *et al*., 1987; 1988) or a related compound (Myers *et al*., 1990). In addition to EDRF, considerable evidence shows that several receptor-dependent agonists, such as acetylcholine (ACh), bradykinin, histamine and substance P, release a factor that causes vascular smooth muscle hyperpolarization: the so-called endothelium-derived hyperpolarizing factor (EDHF) which has yet to be identified (Chen *et al*., 1991; Cowan & Cohen, 1991; Suzuki *et al*., 1992; Holzmann *et al*., 1994). Several studies have demonstrated that the electrophysiological and pharmacological properties of EDHF differ from EDRF in many respects (see reviews by Garland *et al*., 1995; Waldron *et al*., 1996). For instance, EDHF-mediated relaxation and membrane hyperpolarization are resistant to inhibitors of the L-arginine-nitric oxide pathway, such as oxyhaemoglobin, methylene blue or N^G-nitro-L-arginine methyl ester (L-NAME; Chen & Suzuki, 1990; Nagao & Vanhoutte, 1992; Holzmann *et al*., 1994).

The contribution of EDHF to endothelium-dependent relaxations appears rather variable depending upon species, tissue and agonist (Nagao & Vanhoutte, 1993). In addition, it is generally considered that EDHF may be greater importance in

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resistance than in large conduit arteries and may play a significant role in the determination of the peripheral vascular resistance (Nagao & Vanhoutte, 1993; Garland *et al*., 1995). However, in the rabbit carotid artery, which is a large conduit artery, ACh has been shown to generate an endothelium-dependent hyperpolarization (Chen & Suzuki, 1990) and relaxation (Cowan *et al*., 1993). ACh-induced relaxation of rabbit carotid artery was blocked only partially by L-NAME, whilst the L-NAME resistant response was nearly abolished by elevated external K^+ or potassium channel blockers, such as charybdotoxin (Cowan *et al*., 1993). These studies demonstrated that the endothelial cells of rabbit carotid artery may release EDHF and hyperpolarize the underlying vascular smooth muscle cells by opening potassium channels. However, the identity of EDHF remains unknown and the validity of this hypothesis still remains to be established.

The aims of the present study were to determine (a) whether a diffusible EDHF is released, by use of 'sandwich-tissue' experiments wherein an endothelium-intact and an endothelium-denuded tissue are brought together, (b) the contribution of L-NAME/indomethacin insensitive relaxation in the rabbit carotid artery segments precontracted by phenylephrine and (c) the effects of K^+ channel blockers and cytochrome P450 inhibitors on the ACh-induced relaxation in order to characterize the nature and mechanism of action of EDHF.

Methods

Vasorelaxation experiments

New Zealand white rabbits (1–2 kg) of either sex were killed by exsanguination after anaesthesia with an intravenous injection of sodium pentobarbitone (240 mg kg^{-1}). The common carotid artery was quickly excised and placed in cold physiological salt solution (PSS) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and dextrose 11.1 The pH of the solution after saturation with 95% O_2 + 5% CO_2 gas mixture was 7.4. Solutions of high K^+ PSS were made by equimolar replacement of NaCl with KCl. Adherent fat and connective tissue were cleaned carefully from the artery which was then cut into 5 mm rings. In some experiments, the endothelial cell layer was removed by gently rubbing the intimal surface of the rings with a piece of PE 90 tubing. Confirmation that the endothelium was functionally absent was obtained by lack of a relaxation response to ACh (10 μM). Each ring was suspended between platinum hooks and mounted in a 10 ml organ bath containing PSS maintained at 37°C and was bubbled continuously with 95% O_2 + 5% CO_2. The rings were stretched in a stepwise fashion to a tension of 7 g (Cowan *et al*., 1993; Najibi *et al*., 1994). The tissues were equilibrated for 2 h, during which time the bath solution was changed every 20 min. Isometric tension was recorded with a force displacement transducer (Grass FT 03) coupled to a Grass polygraph model 7E. All vessels were treated with indomethacin (10 μM) to block prostanoid production by cyclo-oxygenase. Rings were contracted with either phenylephrine (1 μM) or high K^+ PSS (40 mM) and were relaxed by half-log increases in the concentration of ACh and A23187 (0.01 to 30 μM). The tone developed by phenylephrine or high K^+ PSS was similar (27.5 ± 4.9 mN vs 26.0 ± 5.9 mN, $P > 0.05$, $n = 8$). The tissues were only exposed to two concentration-response curves, either the first curve in the absence of any blocker and the second in the presence of L-NAME or the first curve in the presence of L-NAME and the second in the presence of L-NAME in combination with a Ca^{2+} -activated K^+ (K_{Ca}) channel blocker or cytochrome P450 blocker. The second concentration-response curves were performed after 1 h of washing with PSS and blockers were added to the tissues 30 min before the commencement of the second curve. Control curves to ACh were performed either in the absence or presence of L-NAME to determine the effects of time and repeated exposure to ACh on the relaxation. There was no significant difference between the first and second concentration-response curves to ACh ($n = 6$, data not shown).

In some experiments, rings of carotid artery without endothelium were contracted to the same degree with phenylephrine (1 μM) and were relaxed by half-long increases in the concentration of nitric oxide (0.01 to 30 μM). After 1 h of washing with PSS, the tissues were pretreated with K_{Ca} channel blockers, cytochrome P450 blockers or a guanylyl cyclase inhibitor for 30 min before repeated exposure to nitric oxide.

Sandwich experiments

Sandwich preparations were made similar to those described by Plane *et al*. (1995). Briefly, the endothelial layer in a long segment of the carotid artery was removed by gently rubbing the intimal surface of the rings with a piece of PE 90 tubing. The endothelium-denuded segment was then cut into several segments, one of which was used to confirm removal of the endothelium by the absence of any relaxation to acetylcholine (10 μM) following the pre-constriction with phenylephrine (1 μM). To examine the transferable nature of the nitric oxide-independent relaxations, donor segments with an intact endothelium were wrapped round and attached with a small pin to the other endothelium-denuded segments. The sandwich preparations were mounted between stainless steel hooks in 10 ml organ baths containing PSS with indomethacin (10 μM), maintained at 37°C and bubbled continuously with 95% O_2 + 5% CO_2, under a resting tension of 7 g for isometric recording of tension changes. After equilibration for 2 h, the preparations were contracted with phenylephrine (1 μM) and then challenged with ACh (10 nM – 100 μM) in the absence and presence of L-NAME (30 μM).

Drugs

The following drugs were used: (−)-phenylephrine hydrochloride, acetylcholine bromide, calcium ionophore A23187, histamine dihydrochloride, bradykinin, apamin, tetrabutylammonium hydrogen sulphate (TBA), clotrimazole, 17-octadecynoic acid (17-ODYA), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and indomethacin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Charybdotoxin (CTX), iberiotoxin (ITX) and SKF525A (N,N-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.) and LY83583 (6-anilino-5,8-quinolinedione) was from Lilly Research Laboratories (Indianapolis, IN, U.S.A.).

Stock solutions of clotrimazole and 17-ODYA were prepared in ethanol; the stock solution of indomethacin was made in 4% (w/v) $NaHCO_3$; A23187 was dissolved in dimethyl sulphoxide; all other compounds dissolved freely in distilled water. The final concentrations of all solvents used (<0.1%) had no effects in preliminary experiments. Nitric oxide was prepared by bubbling gaseous nitric oxide (Liquid Carbonic Inc.) for 20 min through a gas tight vial containing ice-cold distilled water which had been bubbled with research grade argon (Union Carbide) for 20 min, producing a saturated solution of exogenous nitric oxide of ~1 mM. Nitric oxide in solution was serially diluted immediately before use in gas tight vials with cold degassed distilled water and then injected into the organ bath in volumes of 10–30 μl with a gas tight syringe.

Statistical analysis

Vasorelaxant responses were measured as percentage inhibition of phenylephrine- or KCl-induced contraction. pD_2 values were determined as the negative log molar concentration of vasorelaxant which caused 50% of the maximal effect. All data are expressed as means ± s.e.mean and differences between means determined by Student's *t* test for paired data. A *P* value less than 0.05 was considered significant. In all experiments, *n* equals the number of animals from which rings were taken.

Results

Characterization of the relaxant response to ACh

In endothelium-intact rings of rabbit carotid arteries precontracted with phenylephrine (1 μM), ACh induced a concentration-dependent relaxant response with a pD_2 value of 7.03 ± 0.12 and maximal relaxation reaching $99 \pm 1\%$ of the phenylephrine-induced contraction (Figure 1a). The ACh-induced response was abolished by removal of the endothelium, but was not altered when the cyclo-oxygenase inhibitor indomethacin (10 μM) was omitted from PSS (pD_2 value 7.00 ± 0.10 and maximal relaxation $99 \pm 3\%$). In the presence of indomethacin plus the NO synthase inhibitor, L-NAME (30 μM), the concentration-response curve of ACh was significantly shifted to the right with the pD_2 value decreasing to 6.48 ± 0.10 ($P<0.01$). The maximal relaxation to ACh was also significantly decreased to $67 \pm 3\%$ ($P<0.01$, Figure 1a).

In the presence of high K^+PSS (40 mM), carotid artery preparations contracted to a similar degree as phenylephrine (1 μM)-contracted preparations. ACh also induced a concentration-dependent relaxant response with a pD_2 value of 6.20 ± 0.10 and maximal relaxation reaching $63 \pm 5\%$ of the high K^+ PSS-induced contraction (Figure 1b). The ACh-induced concentration-dependent relaxant response was not affected by indomethacin 10 μM (pD_2 6.18 ± 0.15 and maximal relaxation $65 \pm 6\%$). However, the relaxation to ACh was blocked completely by indomethacin plus L-NAME (30 μM; Figure 1b).

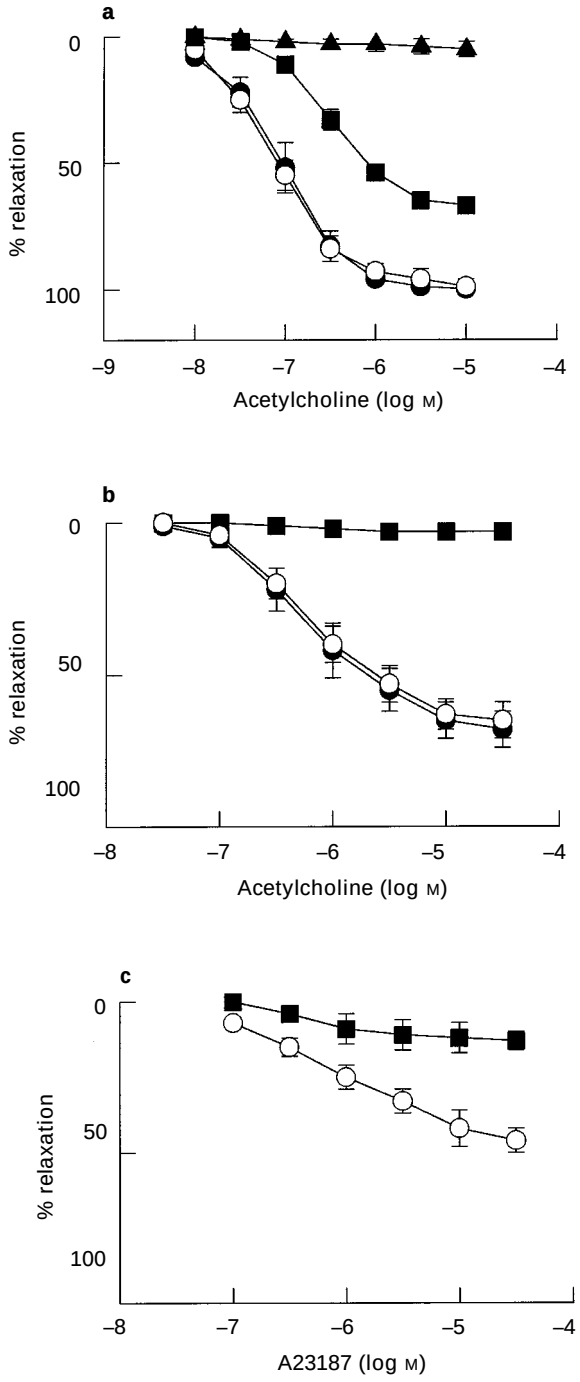

Figure 1 Mean concentration-response curves for acetylcholine and A23187 in the rabbit carotid artery precontracted with phenylephrine (1 μM; a, c) and high K^+ PSS (40 mM, b) in the absence (○) and the presence of indomethacin (10 μM, ●) or indomethacin plus N^G-nitro-L-arginine methyl ester hydrochloride (30 μM, ■). (▲) Endothelium was removed. Points are the mean and vertical lines show s.e.mean from 6–8 separate experiments.

Response to A23187, bradykinin and histamine

In endothelium-intact rings of rabbit carotid arteries precontracted with phenylephrine (1 μM), A23187 induced a concentration-dependent relaxant response, though its potency was much less than that of ACh. The pD_2 value of A23187 was 6.37 ± 0.12 and the maximal relaxation was $46 \pm 4\%$ of the phenylephrine-induced contraction. The A23187-induced vasorelaxation response was significantly inhibited ($13 \pm 3\%$, $P<0.01$, $n=6$) by indomethacin (10 μM) plus L-NAME (30 μM; Figure 1c). The application of bradykinin or histamine (0.01–100 μM) to either resting or phenylephrine-stimulated rings failed to evoke any change in smooth muscle tone ($n=5$, data not shown).

Effects of K^+ channel blockers on the relaxant response to ACh

Pretreatment with apamin (1 or 3 μM), which did not affect either basal tone or phenylephrine-induced contraction, partially inhibited the L-NAME/indomethacin insensitive relaxation to ACh. In the presence of apamin (1 or 3 μM), the pD_2 for ACh decreased from 6.56 ± 0.10 to 6.24 ± 0.14 and 6.20 ± 0.08, respectively ($P<0.05$ for both). The maximal relaxation to ACh was also significantly decreased from $71 \pm 4\%$ to $45 \pm 6\%$ and $38 \pm 5\%$ ($P<0.01$ for both), respectively (Figure 2a). ITX (0.1 μM) partially inhibited the L-NAME/indomethacin insensitive relaxation to ACh. In the presence of ITX, the pD_2 for ACh decreased from 6.39 ± 0.06 to 6.08 ± 0.14 ($P<0.05$). The maximal relaxation to ACh was also significantly decreased from $70 \pm 7\%$ to $37 \pm 6\%$ ($P<0.01$; Figure 2b). However, CTX (0.1 µM) and TBA (1 mM) completely inhibited the L-NAME/indomethacin insensitive response to ACh (Figure 2c and d).

Effects of cytochrome P450 inhibitors on relaxant response to ACh

Pretreatment with SKF-525A (1 or 10 μM) did not affect either basal tone or phenylephrine-induced contraction. However, under these conditions the L-NAME/indomethacin insensitive response to ACh was inhibited in a dose-dependent manner (Figure 3a). In the presence of SKF-525A (1 μM), the pD_2 value for ACh decreased from 6.44 ± 0.07 to 6.10 ± 0.06 ($P<0.01$). The maximal relaxation to ACh was also significantly decreased from $68 \pm 6\%$ to $35 \pm 7\%$ ($P<0.01$). SKF-525A (10 μM) further inhibited the L-NAME/indomethacin insensitive response to ACh with the maximal relaxation significantly decreasing from $68 \pm 6\%$ to $14 \pm 5\%$ ($P<0.01$). Both 17-ODYA (3 μM) and clotrimazole (1 μM) also significantly inhibited the L-NAME/indomethacin insensitive response to ACh (Figure 3b and c). In the presence of 17-ODYA (3 μM) or clotrimazole (1 μM), the pD_2 values for ACh decreased from 6.56 ± 0.08 to 6.30 ± 0.11 ($P<0.05$) and from 6.50 ± 0.14 to

6.43±015 ($P>0.05$), respectively, and the maximal relaxations to ACh were significantly decreased from 65±3% to 25±7% ($P<0.01$) and from 64±5% to 22±3% ($P<0.01$), respectively.

Characterization of the relaxant response to NO

In endothelium-denuded rings of rabbit carotid arteries precontracted with phenylephrine (1 μM), exogenous NO induced a concentration-dependent relaxant response. This response was not altered by either the K_{Ca} channel blockers (apamin 1 μM or CTX 0.1 μM, Figure 4a and b) or the cytochrome P450 mono-oxygenase blockers (SKF-525A 10 μM or clotrimazole 10 μM; Figure 4c and d). However, the NO-induced response was shifted to the right by a guanylyl cyclase inhibitor LY83583 (10 μM) with the pD_2 value decreasing from 6.95±0.14 to 6.04±0.09 ($P<0.01$). The maximal relaxation to NO was not changed by LY83583 pretreatment (Figure 4e).

Sandwich experiments

The maximal relaxation to ACh (100 μM) in endothelium-denuded arterial segments was only 8±1%, thus confirming full removal of the endothelial cells on the detector tissue (Figure 5a). However, the ACh-evoked maximal relaxations in the denuded detector tissue sandwiched with an intact endothelium donor tissue reached 70±3% in the presence of indomethacin (10 μM) (Figure 5b). In the presence of indomethacin plus L-NAME (30 μM), the relaxation to ACh was significantly reduced but not abolished (51±2%, Figure 5c). The L-NAME/indomethacin insensitive relaxation to ACh was further abolished by TBA (1 mM). Only 14±1% of the maximal relaxation to ACh remained (Figure 5d).

Discussion

The findings described above indicate that in the rabbit carotid artery, as with other vessels such as the rabbit femoral artery (Plane *et al*., 1995) and rat aorta (Chen *et al*., 1988; Hatake *et al*., 1995), both nitric oxide-dependent and -independent endothelium-dependent mechanisms exist which evoke smooth muscle relaxation. It has previously been shown that A23187, bradykinin and histamine cause relaxation in arterial smooth muscle predominantly via the release of EDHF, especially in porcine coronary artery (Nagao & Vanhoutte, 1992; Holzmann *et al*., 1994), rabbit femoral artery (Plane *et al*., 1995) and the rat mesenteric arterial bed (Adeagbo & Triggle, 1993; Waldron & Garland, 1994). For this reason we have investigated the action of these agonists in the rabbit carotid artery. Of particular interest is the finding that endothelium-dependent relaxation to A23187, which appeared to relax fe-

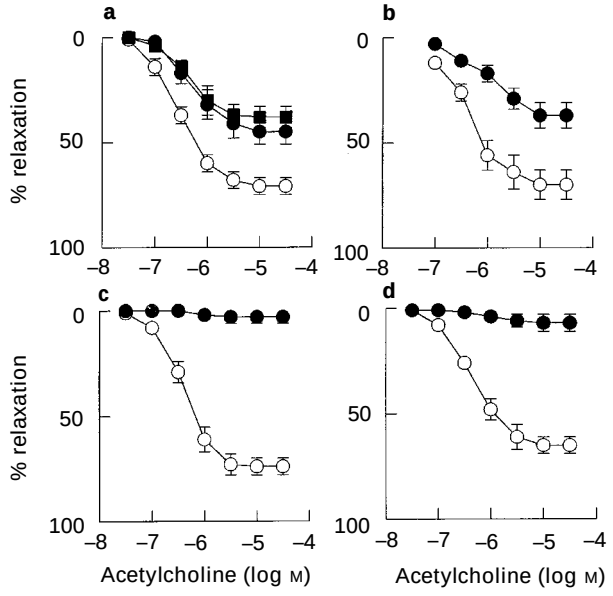

Figure 2 Mean concentration-response curves for acetylcholine in the rabbit carotid artery pretreated with indomethacin (10 μM) plus N^G-nitro-L-arginine methyl ester hydrochloride (30 μM) in the absence (○) and the presence of the K^+ channel blockers (●): (a) apamin (1 μM, ●; 3 μM, ■; $n=6$), (b) iberiotoxin (0.1 μM, $n=8$), (c) charybdotoxin (0.1 μM, $n=5$) and (d) tetrabutylammonium (1mM, $n=5$). Points are the mean and vertical lines show s.e.mean.

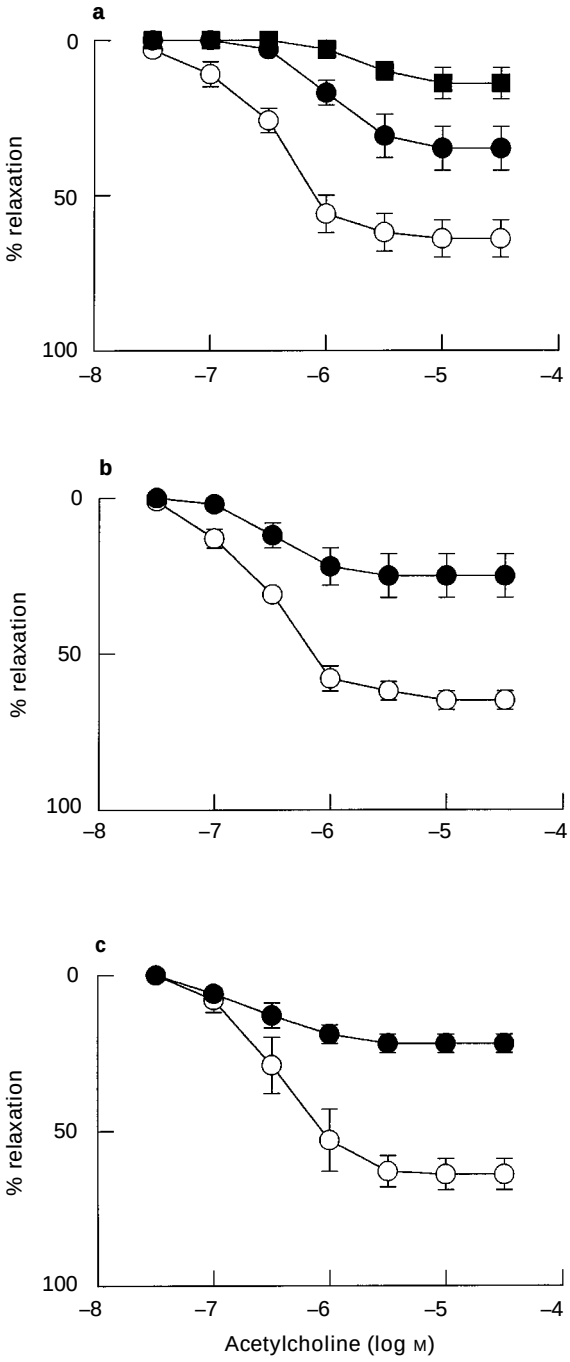

Figure 3 Mean concentration-response curves for acetylcholine in the rabbit carotid artery pretreated with indomethacin (10 μM) plus N^G-nitro-L-arginine methyl ester hydrochloride (30 μM) in the absence (○) and the presence of cytochrome P450 inhibitors (●): (a) SKF-525A (1 μM, ●; 10 μM, ■; $n=6$), (b) 17-octadecynoic acid (3 μM, $n=7$) and (c) clotrimazole (1 μM, $n=5$). Points are the mean and vertical lines show s.e.mean.

moral artery preparations predominantly via EDHF (Plane *et al*., 1995), can be explained solely in terms of nitric oxide release in the rabbit carotid artery. In contrast, endothelium-dependent relaxation to ACh, which has been explained solely in terms of nitric oxide release in the femoral artery (Plane *et al*., 1995), appears to involve both nitric oxide and EDHF in the rabbit carotid artery. The lack of any response to bradykinin and histamine, which have been shown to cause endothelium-dependent relaxation predominantly by the release of EDHF in other vessels, suggests an absence of receptors for these agonists on endothelial cells in the rabbit carotid artery.

In the present study, ACh induced a dose-dependent vasorelaxation, which was completely abolished by removal of the endothelium and this relaxation was not altered when the cyclo-oxygenase inhibitor indomethacin was omitted from the PSS. These results suggest that ACh-induced relaxation of rabbit carotid artery is endothelium-dependent and rules out an involvement of relaxant prostanoids, including prostacyclin (PGI_2) in the relaxation response to ACh. In the presence of the NO synthase inhibitor, L-NAME (30 μM), ACh-induced vasorelaxation was significantly inhibited (approximately 33%), but not abolished. This L-NAME/indomethacin insensitive relaxation response was abolished when carotid artery segments were preconstricted with high K^+ PSS suggesting that it was mediated by endothelium-dependent hyperpolarization.

In the present study, sandwich-tissue experiments further substantiate the conclusion that ACh stimulates endothelial cells of the rabbit carotid artery to release two different kinds of diffusible relaxing factors: one of which is L-NAME-sensitive, presumably NO, and the other, which is L-NAME-insensitive, has the characteristics of an EDHF. Although we did not provide direct experimental evidence for a hyperpolarization of the carotid smooth muscle by ACh, i.e. by simultaneously monitoring changes in membrane potential, this can be inferred from previous studies. Thus, Chen & Suzuki (1990) showed that in smooth muscle cells of the rabbit carotid artery, ACh (0.01 – 10 μM) generated concentration-dependent hyperpolarization to a maximum of approximately 13 mV only in the tissues with an intact endothelium. Our results are also consistent with Cowan *et al*. (1993) who demonstrated that relaxation of rabbit carotid artery is mediated by two different mechanisms, one dependent upon NO and subsequent cyclic GMP accumulation, and another independent of NO. The latter mechanism is likely to be hyperpolarization mediated by EDHF.

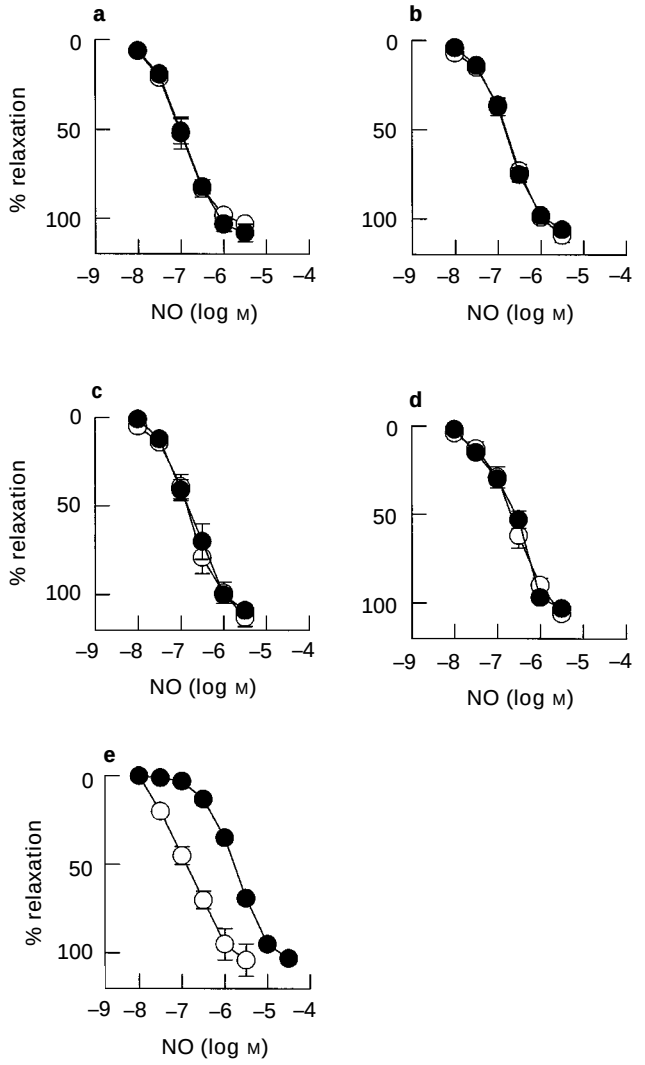

Figure 4 Mean concentration-response curves for exogenous nitric oxide (NO) in the rabbit, endothelium-denuded carotid artery in the absence (○) and the presence of K_{Ca} channel blockers, cytochrome P450 mono-oxygenase blockers or guanylyl cyclase inhibitor (●): (a) apamin (1 μM, $n=5$), (b) charybdotoxin (0.1 μM, $n=5$), (c) SKF-525A (10 μM, $n=4$), (d) clotrimazole (10 μM, $n=5$) and (e) LY83583 (10 μM, $n=4$). Points are the mean and vertical lines show s.e. mean.

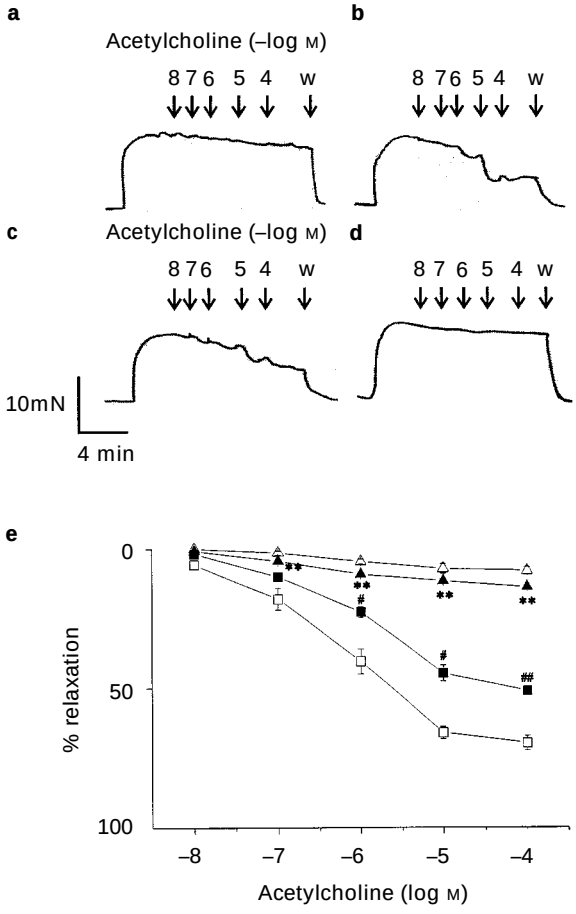

Figure 5 Traces: representative recordings of a sandwich experiment in the isolated carotid artery. (a) Endothelium-denuded tissue alone. (b) Sandwich preparation of an endothelium-denuded ring in the presence of an endothelium-intact vessel. (c) Sandwich preparation in the presence of indomethacin (10 μM) plus N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, 30 μM). (d) Sandwich preparation in the presence of indomethacin, L-NAME and tetrabutylammonium (1 mM). (e) Mean concentration-response curves for acetylcholine in endothelium-denuded tissues (△), sandwiched vessels (□), sandwiched vessels in the presence of indomathacin (10 μM) plus L-NAME (30 μM) (■), and in the additional presence of tetrabutylammonium (1mM). (▲). Points are the mean and vertical lines s.e.mean from 5 separate experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs sandwiched vessels; $^{**}P<0.01$ vs sandwiched vessels in the presence of indomethacin plus L-NAME.

These findings also demonstrate that the relaxant response to ACh in rabbit isolated carotid artery is mediated by at least two endothelium-derived vasodilator substances, most probably NO and EDHF. Despite extensive investigation, the nature and cellular mode of action of EDHF have not yet been elucidated (Garland *et al*., 1995; Waldron *et al*., 1996). Three kinds of K_{Ca} channels have been described: large conductance (BK_{Ca}), intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}; Cook, 1990). ITX is a highly selective and potent blocker of BK_{Ca} channels and apamin is a selective blocker of SK_{Ca} channel (Cook, 1990; Galvez *et al*., 1990; Nelson & Quayle, 1995). Previous studies in our laboratory with the rat isolated, perfused mesenteric artery preparation indicated that an L-NAME/indomethacin-insensitive vasorelaxant factor is released by ACh and histamine (Adeagbo & Triggle, 1993). Furthermore, this relaxation was inhibited by high K^+ and by apamin. In the present study, four K^+ channels blockers with different selectivity for subtypes of K_{Ca} channels were used to determine which subtype(s) of K_{Ca} channel plays a functional role in the relaxation response to ACh in the rabbit carotid artery. Although CTX has been employed as a specific blocker of BK_{Ca}, it is known to inhibit other K_{Ca} channels, for example IK_{Ca}, as well as other voltage-dependent K^+ channels (Cook, 1990; Nelson & Quayle, 1995). For this reason, we also employed ITX and apamin in the present study to analyse in more detail which kind(s) of K_{Ca} channel may be involved in the action of EDHF. TBA and CTX were used as nonselective blockers of K_{Ca} channels (Cook, 1990) and in the present study, both agents abolished L-NAME/indomethacin-insensitive ACh-induced relaxation, suggesting that K_{Ca} channels play a role in the NO/PGI_2-independent relaxation. Although apamin (1 μM) significantly inhibited the L-NAME/indomethacin-insensitive ACh-induced relaxation, increasing the concentration of apamin to 3 μM did not abolish the relaxation to ACh. ITX (0.1 μM) also significantly inhibited the NO/PGI_2-independent ACh-induced relaxation, but did not completely abolish the relaxation to ACh. Taken together these findings suggest that, in the rabbit carotid artery, both BK_{Ca} and SK_{Ca} are involved in mediating the L-NAME/indomethacin-insensitive relaxation to ACh.

Although the nature of EDHF has not yet been elucidated, recent studies indicate that EDHF may be a cytochrome P450 product (Hecker *et al*., 1994; Fulton *et al*., 1995; Garland *et al*., 1995; Lischke *et al*., 1995; Campbell *et al*., 1996). In the present study, we provide evidence that the cytochrome P450 inhibitors, SKF-525A, clotrimazole and 17-ODYA modulate NO/PGI_2-independent relaxation to ACh in the rabbit carotid artery. Our results are in general agreement with those of Hecker *et al*. (1994), Fulton *et al*. (1995) and Lischke *et al*. (1995), in which cytochrome P450 inhibitors significantly inhibited the NO/PGI_2-independent relaxation to bradykinin in porcine coronary artery, in the rat heart and in the rabbit carotid artery, respectively. In our studies, pretreatment of the artery with SKF-525A (1–10 μM) inhibited the L-NAME/indomethacin insensitive relaxation to ACh in a dose-dependent manner. In light of the fact that SKF-525A, an agent that is metabolized by cytochrome P450 to an intermediate that inhibits the enzyme, is a nonspecific cytochrome P450 inhibitor (Oyekan *et al*., 1994), we selected two other more specific cytochrome P450 inhibitors: clotrimazole, an imidazole that binds to the heme moiety of the enzyme and 17-ODYA, an irreversible inhibitor of long chain fatty acid metabolizing cytochrome P450 enzyme (Harder *et al*., 1995). These agents were applied at concentrations previously shown to cause selective inhibition of cytochrome P450 (Oyekan *et al*., 1994; Zou *et al*., 1994). Both clotrimazole and 17-ODYA markedly inhibited the L-NAME/indomethacin-insensitive response to ACh in the rabbit carotid artery. Our findings provide strong evidence in support of the hypothesis that the L-NAME/indomethacin-insensitive response to ACh in rabbit carotid artery involves a cytochrome P450-derived arachidonic acid metabolite. However, it should be noted that our results are inconsistent with those of Corriu *et al*. (1996) and Zygmunt *et al*. (1996), in which studies cytochrome P450 inhibitors did not alter endothelium-dependent hyperpolarization in the guinea-pig isolated carotid artery and 17-ODYA did not inhibit the NO/PGI_2-independent relaxation to ACh in the rat hepatic artery. The reasons for these discrepancies are not certain but may be explained by species and/or tissue differences or could indicate that the nature and/or cellular mechanisms of EDHF are not the same in all tissues.

Recently, Lishcke *et al*. (1995) demonstrated an inhibitory action of clotrimazole on the NO/PGI_2-independent relaxation response to ACh in the rabbit carotid artery and concluded that EDHF may be a product of cytochrome P450. Unlike the present study, they neither compared the effects of ACh and A23187 on NO versus EDHF release nor investigated the 'transferability' of the putative EDHF. It has been shown that SKF-525A and analogues have non-selective inhibitory effects on Ca^{2+}-dependent non-vascular smooth muscle contraction (Triggle *et al*., 1979) and that SKF-525A and clotrimazole inhibit the relaxation induced by levcromakalim, a K_{ATP} channel activator (Zygmunt *et al*., 1996). Thus some caution is required concerning the data obtained from using some cytochrome P450 inhibitors. However, in the present study the cytochrome P450 inhibitors had no effect either on basal or phenylephrine-induced tone or on exogenous NO-induced relaxation of endothelium-denuded carotid artery. This suggests that the inhibitory effects of cytochrome P450 inhibitors, at the concentrations used in the present study, are not due to a direct action on the membrane channels or inhibition of NO mediated relaxation. In support of our hypothesis, Campbell *et al*. (1996) have found that neither SKF-525A nor miconazole (10 μM) affected the open-state probability of K^+ channels or nitric oxide synthase activity.

The differential abilities of A23187 and ACh to release NO or EDHF in the rabbit femoral (Plane *et al*., 1995) versus the rabbit carotid arteries may infer that there may be more than one EDHF. It is clear that the contribution of EDHF to endothelium-dependent relaxation appears rather variable depending upon the tissue source and agonist employed (Nagao & Vanhoutte, 1993). However, the relative physiological importance of NO versus EDHF in the carotid artery is unknown. Najibi *et al*., (1994) found that in hypercholesterolemia, the contribution of NO in carotid artery was reduced, but a non-NO factor, putatively EDHF, maintained a normal ACh-induced relaxation. This suggests that EDHF released from the endothelial cells of carotid artery may play an important role in pathological conditions where NO synthesis is impaired. Further studies will be necessary to determine the contribution of EDHF released in large conduit arteries in physiological and pathological conditions.

In the present study, endothelium-denuded rings of the rabbit carotid artery preconstricted with phenylephrine relaxed to exogenously applied NO. This relaxation response was not altered by either K_{Ca} channel blockers or cytochrome P450 mono-oxygenase blockers but was significantly inhibited by the guanylyl cyclase inhibitor, LY83583 (Kontos & Wei, 1993). These results are in general agreement with those of Bialecki & Carol (1995), in which K_{Ca} channel blockers did not inhibit the NO donor S-nitroso-N-acetylpenicillamine (SNAP)-mediated relaxation of guinea-pig aorta and carotid artery. Our findings provide further evidence for the different nature and cellular mechanisms of action of EDHF versus NO. The former, possibly a cytochrome P450 metabolite, mediates vasorelaxation via activation of K_{Ca} channels and the latter via the guanylyl cyclase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway.

In conclusion, we have demonstrated that in the rabbit carotid artery, A23187-evoked relaxation of phenylephrine-induced contractions appears to be mediated by the release of NO. In contrast, ACh-evoked relaxations are mediated by the release of both NO and EDHF. The diffusible EDHF released by ACh may be a cytochrome P450-derived arachidonic acid metabolite, which probably causes hyperpolarization of the underlying smooth muscle cells by a mechanism of action in-

volving both BK_{Ca} and SK_{Ca} channels. However, electrophysiological measurement of whole cell and individual K_{Ca} channel currents from the carotid artery are required for definitive confirmation of our findings, most particularly with respect to the identification of the subtype of K_{Ca} channel involved in mediating the effects of EDHF.

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