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Characterization and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed

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1 We have used the isolated, buffer-perfused, mesenteric arterial bed of the rat (preconstricted with methoxamine or 60 mM K^+) to characterize nitric oxide (NO)-independent vasorelaxation which is thought to be mediated by the endothelium-derived hyperpolarizing factor (EDHF).

2 The muscarinic agonists carbachol, acetylcholine (ACh) and methacholine caused dose-related relaxations in preconstricted preparations with ED_{50} values of 0.18 ± 0.04 nmol ($n=8$), 0.05 ± 0.02 nmol ($n=6$) and 0.26 ± 0.16 nmol ($n=5$), respectively. In the same preparations N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) significantly ($P<0.05$) decreased the potency of all the agents (ED_{50} values in the presence of L-NAME: carbachol, 0.66 ± 0.11 nmol; ACh, 0.28 ± 0.10 nmol; methacholine, 1.97 ± 1.01 nmol). The maximal relaxation to ACh was also significantly ($P<0.05$) reduced (from 85.3 ± 0.9 to 73.2 ± 3.7%) in the presence of L-NAME. The vasorelaxant effects of carbachol were not significantly altered by the cyclo-oxygenase inhibitor indomethacin (10 μM; $n=4$).

3 The K^+ channel blocker, tetraethylammonium (TEA, 10 mM) also significantly ($P<0.001$) reduced both the potency of carbachol ($ED_{50} = 1.97 \pm 0.14$ nmol in presence of TEA) and the maximum relaxation ($R_{max} = 74.6 \pm 3.2$% in presence of TEA, $P<0.05$, $n=3$). When TEA was added in the presence of L-NAME ($n=4$), there was a further significant ($P<0.001$) decrease in the potency of carbachol ($ED_{50} = 22.4 \pm 13.5$ nmol) relative to that in the presence of L-NAME alone, and R_{max} was also significantly ($P<0.05$) reduced (74.6 ± 4.2%). The ATP-sensitive K^+ channel inhibitor, glibenclamide (10 μM), had no effect on carbachol-induced relaxation ($n=9$).

4 High extracellular K^+ (60 mM) significantly ($P<0.01$) reduced the potency of carbachol ($n=5$) by 5 fold (ED_{50}: control, 0.16 ± 0.04 nmol; high K^+, 0.88 ± 0.25 nmol) and the R_{max} was also significantly ($P<0.01$) reduced (control, 83.4 ± 2.7%; high K^+, 40.3 ± 9.2%). The residual vasorelaxation to carbachol in the presence of high K^+ was abolished by L-NAME (100 μM; $n=5$). In preparations preconstricted with high K^+, the potency of sodium nitroprusside was not significantly different from that in preparations precontracted with methoxamine, though the maximal response was reduced (62.4 ± 3.4% high K^+, $n=7$; 83.1 ± 3.1% control, $n=7$).

5 In the presence of the cytochrome P450 inhibitor, clotrimazole (1 μM, $n=5$ and 10 μM, $n=4$), the dose-response curve to carbachol was significantly shifted to the right 2 fold ($P<0.05$) and 4 fold ($P<0.001$) respectively, an effect which was further enhanced in the presence of L-NAME. R_{max} was significantly ($P<0.01$) reduced by the presence of 10 μM clotrimazole alone, being 86.9 ± 2.5% in its absence and 61.8 ± 7.8% in its presence ($n=6$).

6 In the presence of the cell permeable analogue of cyclic GMP, 8-bromo cyclic GMP (6 μM), the inhibitory effects of L-NAME on carbachol-induced relaxation were substantially enhanced (ED_{50}: L-NAME alone, 0.52 ± 0.11 nmol, $n=5$; L-NAME + 8-bromo cyclic GMP, 1.42 ± 0.28 nmol, $n=7$. R_{max}: L-NAME alone, 82.2 ± 2.4%; L-NAME + 8-bromo cyclic GMP, 59.1 ± 1.8%. $P<0.001$). These results suggest that the magnitude of the NO-independent component of vasorelaxation is reduced when functional cyclic GMP levels are maintained, suggesting that basal NO (via cyclic GMP) may modulate EDHF activity and, therefore, on loss of basal NO production the EDHF component of endothelium-dependent relaxations becomes functionally greater.

7 The present investigation demonstrates that muscaranic receptor-induced vasorelaxation in the rat mesenteric arterial bed is mediated by both NO-dependent and independent mechanisms. The L-NAME-insensitive mechanism, most probably occurs via activation of a K^+ conductance and shows the characteristics of EDHF-mediated responses. Finally, the results demonstrate that EDHF activity may become upregulated on inhibition of NO production and this may compensate for the loss of NO.

Keywords: Mesenteric arterial bed; N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide; endothelium; carbachol; endothelium-derived hyperpolarizing factor (EDHF); potassium channels; tetraethylammonium (TEA); clotrimazole; cyclic GMP

Introduction

Vasorelaxation to muscarinic agonists is endothelium-dependent (Furchgott & Zawadzki, 1980) and is largely mediated by the endothelium-derived relaxing factor (EDRF) which has been identified as nitric oxide (NO) (Palmer *et al.*, 1987). However, the endothelium-dependent relaxation induced by muscarinic agonists has been associated with hyperpolarization of the vascular smooth muscle (Bolton *et al.*, 1984; Bolton & Clapp, 1986; Komori & Suzuki, 1987; Taylor *et al.*, 1988; Chen *et al.*, 1988) which does not appear to be due to NO. Furthermore, in a variety of vascular beds there are substantial proportions of the endothelium-dependent relaxations which are insensitive to NO synthase inhibiti on (Adeagbo & Triggle, 1993; Parsons *et al.*, 1994; for review see Garland *et al.*, 1995). Accordingly, the

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existence of a further endothelial factor, termed endothelium-derived hyperpolarizing factor (EDHF) has been hypothesized (Feletou & Vanhoutte, 1988; Taylor & Weston, 1988; Chen *et al*., 1988; Chen & Suzuki, 1990; McPherson & Angus, 1991) and this is thought to increase potassium conductance and contribute to endothelium-dependent relaxation (Chen & Suzuki, 1989; Cowan *et al*., 1993).

In support of there being two distinct mediators of endothelium-dependent relaxations, Chen *et al*. (1988) observed that inhibitors of NO activity, such as haemoglobin and methylene blue inhibited relaxation but not hyperpolarization to acetylcholine (ACh) in the rat thoracic aorta and pulmonary artery. Furthermore, the $^{86}Rb^+$ efflux stimulated by ACh was unaffected by either inhibitor, whereas the ACh-induced increases in cyclic GMP were abolished. Garland & McPherson (1992) studied relaxation and hyperpolarization to NO and ACh in rat isolated small mesenteric arterial vessels and showed that preincubation of arterial segments with haemoglobin had no effect on the responses to ACh, whereas it abolished the hyperpolarization and relaxation induced by NO. This supports the observation of Tare *et al*. (1990) that NO can indeed induce hyperpolarization, but also lends support to the notion that this NO-induced hyperpolarization is not involved in mediating the hyperpolarization evoked by ACh (Garland & McPherson, 1992). In 1993, Adeagbo & Triggle showed that ACh-induced vasorelaxation in the rat mesenteric arterial bed was partially inhibited by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and that increasing the extracellular K^+ concentration reduced the residual L-NAME-insensitive component of vasorelaxation to ACh. Similarly in rat isolated small mesenteric arterial segments, Waldron & Garland (1994) demonstrated that both L-NAME and 25 mM extracellular K^+ individually reduced relaxation and hyperpolarization to ACh and that 25 mM K^+ in the presence of L-NAME abolished the L-NAME-insensitive component. Cowan *et al*. (1993) showed that in the rabbit thoracic aorta, L-NAME abolished ACh-induced vasorelaxation. In contrast, ACh-induced relaxation in the abdominal aorta, and the carotid and iliac arteries was only partially reduced by L-NAME, but was inhibited by the presence of either the K^+ channel blocker tetraethylammonium (TEA) or charybdotoxin (Ca^{2+}-dependent K^+ channel blocker). This suggests that there are tissue specific differences in the contribution of NO and EDHF to vasorelaxation and hyperpolarization; indeed it appears that EDHF activity becomes of greater importance in resistance vessels (Garland *et al*., 1995).

The identity of EDHF has remained elusive, although evidence suggests that it might be derived from arachidonic acid via the cytochrome P450 pathway (Campbell *et al*., 1996). Furthermore, NO synthase inhibitor-insensitive vasorelaxation is attenuated by cytochrome P450 inhibitors (Bauersachs *et al*., 1994; Fulton *et al*., 1995; Hecker *et al*., 1994; Campbell *et al*., 1996). However, the identification of EDHF as a cytochrome P450 product has recently been challenged as not all inhibitors of this system inhibit EDHF activity (Corriu *et al*., 1996), while some of the inhibitors act as K^+ channel inhibitors (Zygmunt *et al*., 1996), and could therefore block EDHF activity at the site of action rather than synthesis. Furthermore, we have recently shown that EDHF activity is selectively antagonized by the highly selective cannabinoid antagonist, SR 141716A, and that the endogenous cannabinoid anandamide, which is derived from arachidonic acid, mimics the actions of EDHF (Randall *et al*., 1996). We have now, therefore, proposed that anandamide, or related cannabinoid substance, represents EDHF (Randall *et al*., 1996).

The purpose of the present investigation was to determine the contribution of NO and EDHF relaxations in the isolated perfused mesenteric arterial bed of the rat, to attempt to characterize the EDHF-mediated vasorelaxation and to examine whether there is any interaction between the two systems.

Preliminary accounts of this work have been presented to the British Pharmacological Society (McCulloch & Randall, 1996a,b).

Methods

Preparation of the isolated buffer-perfused superior mesenteric arterial bed

Male Wistar rats (200–350 g; Bantin & Kingman, Hull, Humberside) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p., Sagatal, Rhône Mérieux, Harlow, Essex). A midline incision was made, and the superior mesenteric artery was cannulated. The vascular bed was flushed with Krebs-Henseleit solution before the arterial vasculature was dissected away from the intestines and transferred to a jacketed organ bath (37°C) as described previously by Randall & Hiley (1988). The tissue was perfused at 5 ml min^{-1} with gassed (95% O_2/5% CO_2), Krebs-Henseleit solution at 37°C (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10), by means of a peristaltic pump (Watson Marlow 504S). In the case of experiments involving high K^+, 60 mM K^+ isotonic Krebs-Henseleit buffer was prepared by substituting equimolar concentrations of NaCl with KCl.

The perfusion pressure in superior mesenteric arterial bed was continuously monitored by means of a pressure transduer placed close to the inflow cannula, coupled to a Maclab 4e recording system (AD Instruments, New South Wales, Australia). Flow was kept constant (5 ml min^{-1}) and therefore changes in perfusion pressure represented alterations in vascular resistance. At the end of each experiment, the pressure drop across the cannula was measured and subtracted from the recorded basal persfusion pressure in order to determine the actual pressure across the bed.

Experimental protocol

Following a 30 min equilibration period, perfusion pressure was raised by addition of methoxamine (10–60 μM) to the perfusion fluid. The vasorelaxant effects of carbachol, acetylcholine and methacholine were assessed in the absence and presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM). Vasorelaxants were administered close-arterially as bolus doses in random order. In view of the augmented vasoconstrictor responses in the presence of L-NAME, consistent with the blockade of basal NO synthase activity, the concentration of methoxamine used in these experiments was reduced (to 1–4 μM) to induce an equivalent level of tone.

The vasorelaxant effects of carbachol were assessed in the absence and presence of the K^+ channel blocker TEA (10 mM) and then in the presence of 100 μM L-NAME in separate experiments. In order to investigate further the contribution of EDHF to the vasorelaxation in response to carbachol, the K^+ concentration of the physiological buffer was increased to 60 mM by isotonic replacement of NaCl with KCl.

The involvement of ATP-sensitive K^+ channels in vasorelaxation to carbachol was assessed by constructing dose-response curves in the absence and presence of 10 μM glibenclamide (Randall & McCulloch, 1995). The NO synthase inhibitor, L-NAME (100 μM) was also used in the presence of 10 μM glibenclamide. Additionally, the effects of the cytochrome P450 inhibitor clotrimazole (1 μM–10 μM) on the relaxant effects of carbachol were assessed in the absence and presence of L-NAME (100 μM).

The potential involvement of the second messenger guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in the modulation of K^+ channel activity was assessed by using the cell permeable analogue, 8-bromo-cyclic GMP at a concentration of 6 μM. This concentration had been established in preliminary experiments as the EC_{50} for the relaxation of established tone. Dose-response curves to carbachol were obtained in the absence and presence of 8-bromo cyclic GMP alone and in the combined presence of both 8-bromo cyclic GMP and 100 μM L-NAME. Due to the relaxant effects of 8-bromo cyclic GMP, control curves were constructed at reduced tone (given by 5–10 μM methoxamine), such that the level of tone was comparable to that in the presence of 8-bromo cyclic GMP.

Data and statistical analysis

The data are presented as mean $\pm$ s.e.mean and were compared by analysis of variance (ANOVA) with significant differences between groups being located by Bonferroni's *post-hoc* test. ED_{50} values for vasorelaxant responses were obtained from individual dose-response curves as the dose at which the half-maximal relaxant response occurred. The ED_{50} was determined by fitting the data to the logistic equation:

$$R_{max} = \frac{R_{max} \cdot A^{n_H}}{ED_{50}{}^{n_H} + A^{n_H}}$$

where R is the reduction in tone, A the dose of the vasorelaxant, R_{max} the maximum reduction of established tone, n_H the slope function and ED_{50} the dose of vasorelaxant giving half the maximal relaxation. The curve fitting was carried out by use of KaleidaGraph software (Synergy, Reading, PA, U.S.A.) running on a Macintosh computer. The ED_{50} values were converted to logarithmic values for statistical analysis.

Drugs

All drugs were prepared on the day of the experiment. Methoxamine hydrochloride, carbachol chloride, N^G-nitro-L-arginine methyl ester hydrochloride, acetylcholine chloride, methacholine chloride, sodium nitroprusside and tetraethylammonium acetate (all from Sigma Chemical Company, Poole, Dorset) were dissolved and diluted in Krebs-Henseleit solution. Clotrimazole (Sigma Chemical Company) was dissolved in ethanol. 8-bromo cyclic GMP (Sigma Chemical Company) was dissolved in 0.1 M NaOH as a stock solution of 10 mM. Glibenclamide (Hoechst UK Ltd., Hounslow, Middlesex) was dissolved in dimethylsulphoxide (DMSO), to give a stock solution of 0.2 M; the final concentration of DMSO in the Krebs-Henseleit buffer solution was <0.005% (v/v).

Results

Basal perfusion pressures and established tone

In the 116 preparations used, basal perfusion pressure was 21.4 $\pm$ 0.7 mmHg. In the 57 preparations to which 10–60 μM methoxamine was added, tone was increased by 89.4 $\pm$ 3.2 mmHg above basal. The presence of the NO synthase inhibitor L-NAME (100 μM) alone in the perfusion fluid did not significantly influence basal perfusion pressure, but after addition of methoxamine (1–4 μM), the perfusion pressure was increased by 85.9 $\pm$ 4.3 mmHg ($n=34$) above the basal pressure. In experiments carried out at low tone, in the absence of L-NAME, methoxamine (1–4 μM) increased perfusion pressure by 53.9 $\pm$ 4.4 mmHg ($n=8$) above the baseline and, in the presence of L-NAME, methoxamine (0.5–0.75 μM) raised perfusion pressure by 51.6 $\pm$ 5.4 mmHg ($n=5$). In the absence of L-NAME, 8-bromo cyclic GMP (6 μM) reduced the methoxamine-induced tone by 42.3 $\pm$ 4.1% ($n=3$) such that it was 60.8 $\pm$ 12.5 mmHg above basal whereas in the presence of L-NAME, 8-bromo cyclic GMP reduced tone by 42.7 $\pm$ 4.6% ($n=4$), to 59.2 $\pm$ 9.2 mmHg above basal. Use of Krebs-Henseleit containing 60 mM K^+ raised the perfusion pressure by 78.6 $\pm$ 7.2 mmHg ($n=5$) above basal.

Effects of L-NAME on vasorelaxant responses to muscarinic agonists

Carbachol caused dose-related relaxations of methoxamine-induced tone and the dose-response curve had an $ED_{50} = 0.18 \pm 0.04$ nmol and an $R_{max} = 84.9 \pm 3.2\%$ ($n=8$). Following the addition of L-NAME (100 μM), there was a significant ($P<0.05$), 3.7 fold, rightward shift in the dose-response curve ($ED_{50} = 0.66 \pm 0.11$ nmol), with no significant change in the maximum relaxation ($R_{max} = 79.1 \pm 2.8\%$; Figure

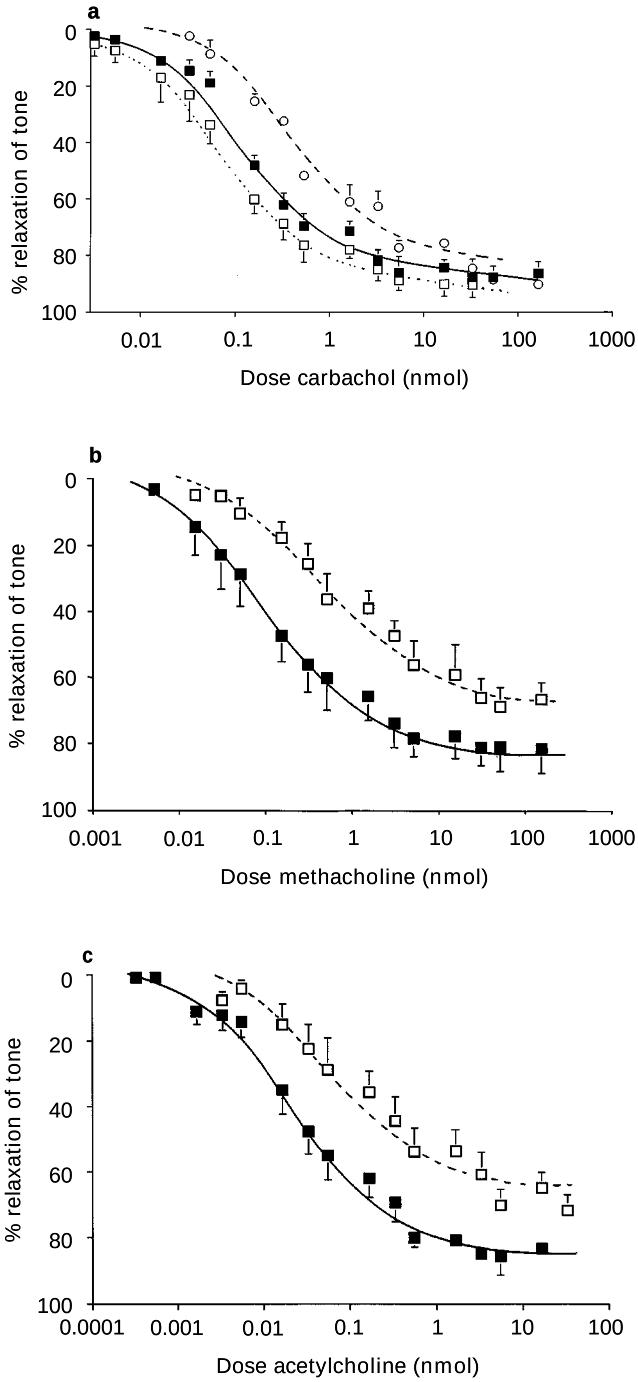

Figure 1 Log dose-response curves for relaxation of methoxamine-induced tone in the rat isolated perfused superior mesenteric arterial bed by (a) carbachol ($n=8$), (b) methacholine ($n=5$) and (c) acetylcholine ($n=6$) in the absence (solid symbol) and presence (open symbol) of L-NAME (100 μM). In (a) the relaxant responses to carbachol in the presence of 10 μM indomethacin ($n=4$) are also shown (□). Values are shown as mean and vertical lines indicate s.e.mean.

1a). In control preparations inclusion of 10 μM indomethacin had no significant effects on the dose-response curve to carbachol ($ED_{50} = 0.088 \pm 0.017$ nmol and $R_{max} = 89.9 \pm 2.6\%$; Figure 1a; $n=4$).

Methacholine also gave dose-related relaxations ($ED_{50} = 0.26 \pm 0.17$ nmol, $R_{max} = 80.8 \pm 3.5\%$ ($n=5$)). Addition of L-NAME significantly ($P<0.05$) decreased the potency of methacholine ($ED_{50} = 1.97 \pm 1.01$ nmol, $n=5$) by 7.6 fold, while R_{max} was unchanged ($75.1 \pm 5.8\%$; $n=5$; Figure 1b).

Acetylcholine produced dose-related relaxations of tone ($ED_{50} = 0.05 \pm 0.02$ nmol, $R_{max} = 85.3 \pm 0.9\%$), and the presence of L-NAME caused a significant ($P<0.01$), 5.6 fold, rightward shift in the dose-response curve ($ED_{50} = 0.28 \pm 0.10$ nmol, $n=6$), accompanied by a significant ($P<0.05$) reduction in the maximum response ($R_{max} = 73.2 \pm 3.7\%$; $n=6$; Figure 1c).

Effects of TEA on vasorelaxation to carbachol in the absence and presence of L-NAME

In the presence of 10 mM tetraethylammonium (TEA) the dose-response curve to carbachol was significantly ($P<0.001$) shifted 10 fold to the right ($ED_{50} = 0.18 \pm 0.04$ nmol in control, $n=7$, vs 1.97 ± 0.14 nmol in the presence of TEA, $n=3$) with a significant ($P<0.05$) reduction in the maximal relaxation ($88.2 \pm 3.2\%$ vs $74.6 \pm 3.2\%$; Figure 2).

When TEA was added in the presence of L-NAME, there was a further significant ($P<0.01$), 34 fold, rightward shift in the dose-response curve to carbachol ($ED_{50} = 22.4 \pm 13.5$ nmol) relative to L-NAME alone ($ED_{50} = 0.66 \pm 0.11$ nmol), with a significant ($P<0.05$) reduction in R_{max} ($88.9 \pm 2.4\%$ vs $74.6 \pm 4.2\%$; $n=4$; Figure 2). Therefore, the dose-response curve to carbachol was significantly ($P<0.05$) shifted to the right 11 fold compared with TEA alone, and the R_{max} was unchanged.

Sodium nitroprusside (10 pmol – 101 nmol) relaxed methoxamine-induced tone with an $ED_{50} = 0.19 \pm 0.06$ nmol and $R_{max} = 87.9 \pm 3.3\%$ ($n=3$) and these responses were unaffected by the presence of 10 mM TEA ($ED_{50} = 0.17 \pm 0.03$ nmol and $R_{max} = 88.9 \pm 4.0\%$; $n=3$).

Effects of high extracellular K^+ on vasorelaxation to carbachol in the absence and presence of L-NAME

In the presence of 60 mM extracellular K^+, there was a significant ($P<0.01$), 5 fold, decrease in the potency of carbachol relative to that for the relaxation against methoxamine-induced tone (ED_{50} against methoxamine, 0.16 ± 0.04 nmol; against 60 mM K^+ 0.88 ± 0.25 nmol). There was also a significant ($P<0.01$) reduction in the maximal relaxation which was $40.3 \pm 9.2\%$ ($n=5$; Figure 3a) as compared to $83.4 \pm 2.7\%$ against methoxamine ($n=5$). In the presence of both 60 mM K^+ and L-NAME, the vasorelaxant responses to carbachol were abolished (Figure 3a).

Sodium nitroprusside (10 pmol – 336 nmol) also relaxed high K^+-induced tone. In this respect maximum relaxation was depressed ($62.4 \pm 3.4\%$; $P<0.001$; $n=7$) relative to the maximum responses against methoxamine-induced tone ($83.1 \pm 3.1\%$; $n=7$) (Figure 3b). The ED_{50} values were 0.75 ± 0.19 nmol (against methoxamine) and 1.88 ± 0.64 nmol (against 60 mM K^+), which were not significantly different.

Effects of glibenclamide on vasorelaxant responses to carbachol in the absence and presence of L-NAME

The ATP-sensitive K^+ channel inhibitor, glibenclamide (10 μM), had no significant effect on vasorelaxation to carbachol ($ED_{50} = 0.24 \pm 0.04$ nmol in the absence of glibenclamide and 0.26 ± 0.07 nmol in its presence). R_{max} was also unaffected ($89.2 \pm 1.3\%$, control, versus $86.6 \pm 2.7\%$, glibenclamide; $n=9$; Figure 4).

In the presence of both glibenclamide and L-NAME, the dose-response curve for carbachol was not significantly affected compared with that in the presence of L-NAME alone

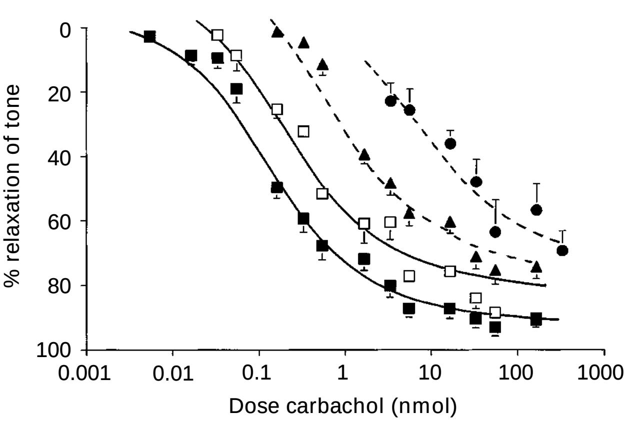

Figure 2 Log dose-response curves for relaxation of methoxamine-established tone by carbachol in the rat isolated superior mesenteric arterial bed in the absence (■, $n=7$) and presence (□, $n=7$) of L-NAME (100 μM), in the presence of TEA alone (10 mM, ▲, $n=3$), and in the presence (●, $n=4$) of both L-NAME (100 μM) and TEA (10 mM). Values are shown as mean and vertical lines indicate s.e.mean.

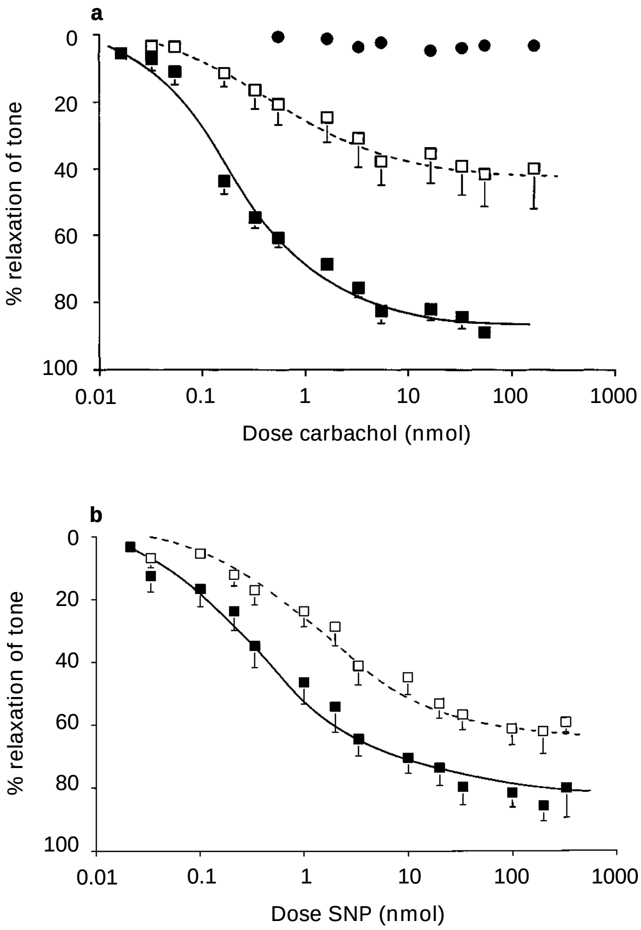

Figure 3 Log dose-response curves for relaxation of the rat isolated superior mesenteric arterial bed by (a) carbachol ($n=5$) and (b) sodium nitroprusside ($n=7$) when tone was increased by methoxamine (■) or 60 mM K^+ (□). In (a) the effects of 100 μM L-NAME on the relaxation of tone induced by 60 mM K^+ are also shown (●). Values are shown as mean and vertical lines indicate s.e.mean.

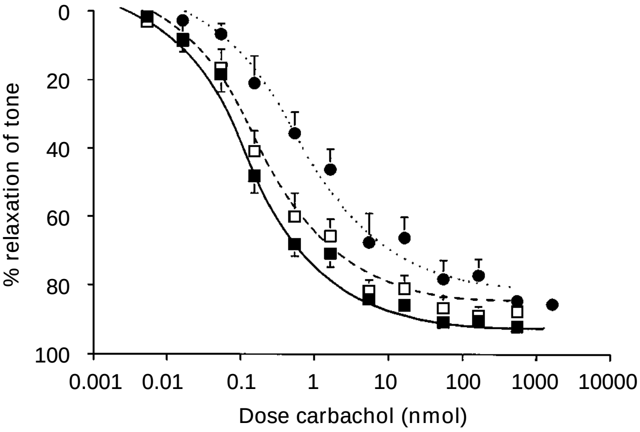

Figure 4 Log dose-response curves for relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed by carbachol in the absence (■, $n=10$) and presence (□, $n=9$) of glibenclamide (10 μM) and in the presence of both glibenclamide (10 μM) and L-NAME (100 μM, ●, $n=3$). Values are shown as mean and vertical lines indicate s.e.mean.

with an $ED_{50}=1.64\pm1.03$ nmol and $R_{max}=83.6\pm1.5\%$ in the presence of both agents ($n=3$), compared to $ED_{50}=0.83\pm0.16$ nmol and $R_{max}=79.1\pm2.8\%$ in the presence of L-NAME alone ($n=11$).

Effects of clotrimazole on vasorelaxation to carbachol in the absence and presence of L-NAME

Clotrimazole (1 μM) caused the dose-response curve to carbachol to be significantly ($P<0.05$) shifted to the right ($ED_{50}=0.18\pm0.03$ nmol, control, $n=10$, versus 0.35 ± 0.10 nmol in the presence of clotrimazole, $n=5$) with no change in maximal response relative to that obtained with carbachol alone ($86.9\pm2.5\%$ versus $81.6\pm5.7\%$; Figure 5a). Increasing the concentration of clotrimazole to 10 μM caused the dose-response curve to be significantly ($P<0.001$) shifted approximately 2 fold to the right ($ED_{50}=0.75\pm0.12$ nmol) and produced a significant ($P<0.01$) reduction in maximal relaxation ($R_{max}=61.8\pm7.8\%$) relative to that observed for carbachol alone (Figure 5a).

When clotrimazole (1 μM) was added in the presence of L-NAME the dose-response curve to carbachol was significantly ($P<0.001$) shifted to the right ($ED_{50}=44.9\pm41.7$ nmol) relative to carbachol alone and there was a significant ($P<0.05$) reduction in the maximum relaxation ($R_{max}=67.2\pm6.8\%$, $n=5$) relative to carbachol alone (Figure 5b). There was a further reduction in the maximum response in the presence of both 10 μM clotrimazole and 100 μM L-NAME, when the R_{max} was $12.1\pm1.6\%$ ($n=4$) and this was significantly ($P<0.001$) less than in the controls (data given above) and the potency ($ED_{50}=27.1\pm16.1$ nmol) was also significantly ($P<0.001$) reduced.

Sodium nitroprusside (20 pmol – 1 μmol) relaxed methoxamine-induced tone ($ED_{50}=1.74\pm0.39$ nmol and $R_{max}=83.4\pm2.6\%$; $n=11$) and these responses were not significantly affected by the presence of 10 μM clotrimazole ($ED_{50}=1.08\pm0.46$ nmol and $R_{max}=82.2\pm4.8\%$; $n=4$).

Effects of 8-bromo cyclic GMP on vasorelaxation to carbachol in absence and presence of L-NAME

In the presence of 6 μM 8-bromo cyclic GMP ($n=3$), the R_{max} for carbachol ($79.4\pm3.5\%$) was signficantly ($P<0.01$) reduced compared to control ($87.9\pm2.3\%$, $n=8$) but there was no change in the ED_{50} (0.11 ± 0.01 nmol in the absence of 8-bromo cyclic GMP 0.19 ± 0.07 nmol in the presence of 8-bromo cyclic GMP, Figure 6). The dose-response curve for carbachol obtained in the presence of L-NAME alone ($n=8$) was described by an ED_{50} of 0.52 ± 0.11 nmol and an R_{max} of $82.2\pm2.4\%$. In the presence of both L-NAME and 8-bromo cyclic GMP ($n=7$), there was a significant ($P<0.001$) rightward shift in the dose-response curve ($ED_{50}=1.42\pm0.28$

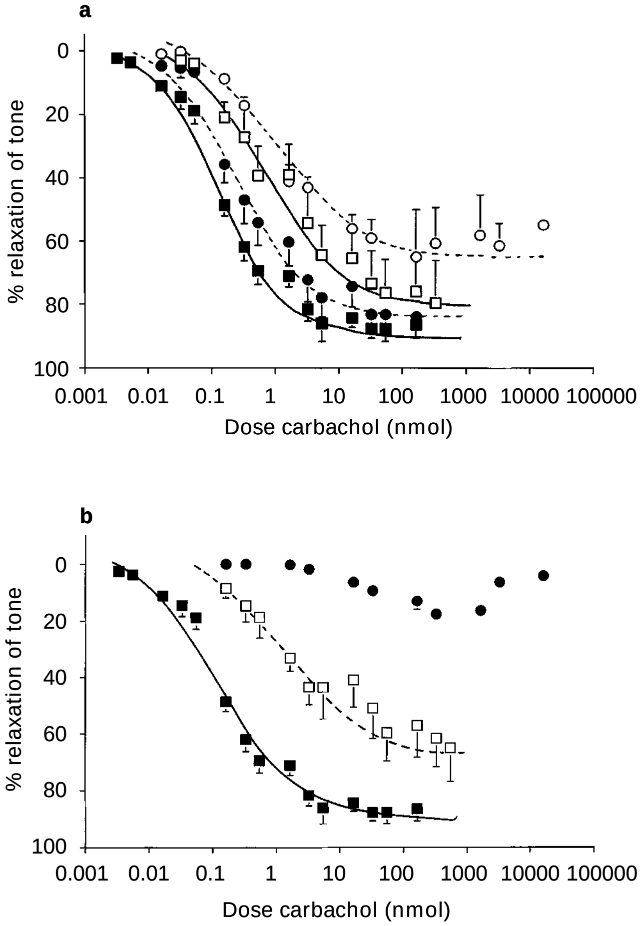

Figure 5 Log dose-response curves for relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed by carbachol (a) in the absence (■, $n=10$) and presence (□) of L-NAME (100 μM) or clotrimazole (1 μM, ●, $n=5$, or 10 μM, ○, $n=4$); and (b) in the presence of either clotrimazole 1 μM (□, $n=5$) or 10 μM (●, $n=6$) and L-NAME (100 μM). Values are given as mean and vertical lines show s.e.mean.

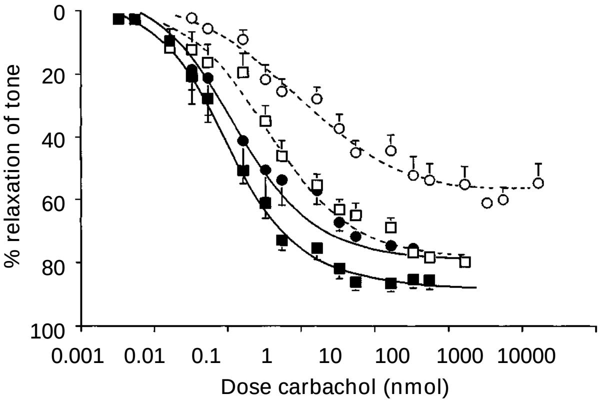

Figure 6 Log dose-response curves for relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed by carbachol in the absence (■, $n=8$) and presence (□, $n=5$) of L-NAME, in the presence of 8-bromo cyclic GMP (6 μM, ●, $n=3$) and in the presence of both L-NAME (100 μM) and 8-bromo cyclic GMP (6 μM, ○, $n=7$). Values are shown as mean and vertical lines indicate s.e.mean.

nmol), with a significant ($P<0.001$) reduction in R_{max} ($59.1 \pm 1.8\%$, Figure 6), such that the effects of L-NAME on carbachol-induced vasorelaxation were substantially enhanced by the presence of 8-bromo cyclic GMP.

Discussion

The results of the present study clearly demonstrate that, in the rat mesenteric arterial bed, vasorelaxation to muscarinic agonists has two components; one of which is L-NAME-sensitive (and presumably mediated via NO), while the other is sensitive to both TEA, a K^+ channel inhibitor, and clotrimazole, a cytochrome P450 inhibitor which is also thought to block K^+ channels. On the basis of these results it is likely that the second component of the relaxation is mediated via the putative EDHF and this is in agreement with the conclusions of Adeagbo & Triggle (1993) that both EDHF and NO are involved in the responses of the rat mesenteric bed to acetylcholine. The results of the present study further show that there appears to be interaction, or 'cross-talk', between NO and EDHF, such that EDHF activity is enhanced on loss of NO.

Inhibition of NO synthase with a high concentration of L-NAME (100 μM) decreased the potencies of carbachol, methacholine and acetylcholine by 3.7, 5.6 and 7.6 fold respectively, but only in the case of acetylcholine was there any reduction in maximal relaxation. These observations suggest that there is a mechanism, in addition to the NO pathway, which can mediate vasorelaxation to muscarinic agonist-induced vasorelaxation. A similar NO-independent component of vascular relaxation was observed in the human forearm by Chowienczyk *et al.* (1993). However, this varied between muscarinic agonists, in that responses to acetycholine were partly NO-independent and those to methacholine were entirely NO-independent. The phenomenon of NO-independent, but endothelium-dependent relaxations, also occurs in rat isolated small mesenteric arterial segments (Garland & McPherson, 1992); the magnitude of which was sufficient for these authors to conclude that NO was not involved in mediating vasorelaxation to acetylcholine. The L-NAME-insensitive component has generally been attributed to the existence of an EDHF (Feletou & Vanhoutte, 1988; Taylor & Weston, 1988; Chen *et al.*, 1988; Chen & Suzuki, 1990; McPherson & Angus, 1991) which acts by increasing membrane K^+ conductance, thus inducing hyperpolarization and relaxation.

In order to assess the contribution made by EDHF to carbachol-induced relaxation, K^+ channels were blocked by TEA. In the presence of TEA, both the potency and reactivity of carbachol were reduced, thereby implicating K^+ channels in muscarinic agonist-induced relaxation. In preliminary experiments, TEA was found to have no effect on the contractile responses of the rat ileum to carbachol, thereby ruling out TEA acting as an antimuscarinic agent (Amoah & Randall, unpublished observations). Furthermore, TEA did not oppose vasorelaxation induced by sodium nitroprusside. The present findings with TEA agree with similar observations made by Chen *et al.* (1991) and Parkington *et al.* (1995) in guinea-pig coronary artery and by Cowan *et al.* (1993) in rabbit abdominal aorta and carotid artery.

The addition of TEA in the presence of L-NAME further reduced the potency of carbachol, by about 34 fold relative to the potency seen in the presence of L-NAME alone, indicating that there are at least two mechanisms by which carbachol can induce vasorelaxation in the rat mesenteric arterial bed; one involving K^+ channels and the other involving the NO pathway. This contrasts with the findings of Garland & McPherson (1992) who concluded that release of NO was not involved in ACh-induced vasorelaxation in isolated small mesenteric arteries. This may perhaps be explained by the difference in the muscarinic agonists employed, such that acetylcholine and carbachol may act on different subtypes of muscarinic receptors which may be coupled to different effector systems (Rubanyi *et al.*, 1987; Jaiswal *et al.*, 1991) or by the size of the vessels being studied as the effects observed in the perfused bed occur in smaller vessels than those used by Garland & McPherson (1992). Carbachol was less potent as a vasorelaxant in the presence of TEA than in the presence of L-NAME alone, suggesting a greater contribution of EDHF than NO to carbachol-induced relaxation in the rat mesenteric arterial bed, a conclusion also made by Adeagbo & Triggle (1993).

The inhibition of K_{ATP} channels by glibenclamide had no effect on vasorelaxation to carbachol indicating that these channels are not involved in mediating the responses to EDHF. Though this observation has not been made before in the rat mesentery, this agrees with the results of Parkington *et al.* (1995) and Hecker *et al.* (1994) in porcine and bovine coronary arteries, and of Cowan *et al.* (1993) in rabbit thoracic aortae and carotid and iliac arteries.

Raising extracellular K^+ reduces the electrochemical gradient for K^+ efflux and depolarizes the cell membrane. Therefore, the substantial decrease in potency and maximal response in the presence of high K^+ would suggest that a K^+ conductance was important in mediating relaxation to carbachol and confirms the previous work by Adeagbo & Triggle (1993) and Waldron & Garland (1994). When L-NAME was also present, relaxation was completely abolished, suggesting that although both EDHF and NO mediate the relaxation, no other factor is involved.

Others have shown that NO itself may act via K^+ channel activation and hyperpolarization (Tare *et al.*, 1990). However, in the present study vasorelaxation to the nitric oxide donor sodium nitroprusside was much less affected by preconstricting the vessels with high K^+, and was not significantly affected by TEA, which would appear to rule out K^+ channel activation as a major action of endothelium-derived NO in this vascular bed. Further, in the present study both K^+ channel blockade and raised extracellular K^+ were effective at inhibiting endothelium-dependent relaxations following NO synthase inhibition, providing further evidence for the presence of both NO and EDHF components.

The identity of EDHF has remained elusive, but Furchgott & Zawadzki (1980) ruled out the possibility of cyclo-oxygenase involvement in relaxation to acetylcholine, as both indomethacin and aspirin had no effect on ACh-induced vasorelaxation, and this has since been confirmed by many other investigators (Randall & Hiley, 1988; Garland & McPherson, 1992; Bauersachs *et al.*, 1992; Hecker *et al.*, 1994; Hatake *et al.*, 1995). Furchgott & Zawadzki (1980) showed that eicosatetraynoic acid (an inhibitor of arachidonic acid metabolism) and mepacrine (an inhibitor of phospholipase A_2) reduced ACh-induced vasorelaxation, thus perhaps providing evidence of the involvement of an arachidonic acid metabolite in EDHF-mediated vasorelaxation.

In the present study, the cytochrome P450 inhibitor clotrimazole caused concentration-related inhibitions of carbachol-induced vasorelaxation, providing further evidence for the hypothesis that EDHF may be a cytochrome P450-derived arachidonic acid metabolite. Indeed, Hecker *et al.* (1994) have shown that 100 μM clotrimazole attenuated the endothelium-dependent vasodilator effect of bradykinin in the rat coronary vasculature. However, experiments involving clotrimazole should be viewed with caution as this agent may also inhibit K^+ channel activation (Zygmunt *et al.*, 1996). Nonetheless, regardless of the site of action of this agent, it would appear to be an effective EDHF antagonist.

Kilpatrick & Cocks (1994) showed in the porcine coronary artery that both NO and EDHF are responsible for endothelium-dependent relaxation *in vitro*, in accordance with our findings in the rat mesenteric arterial bed. They found that NO was able to mediate all the relaxation response, whether or not hyperpolarization occurred and that when this NO pathway was inhibited, residual relaxation occurred through the hyperpolarization pathway, such that 60–80% relaxation could be achieved. The fact that both TEA and clotrimazole had, in the present study, greater impact after NO synthase inhibition suggests that the influence of EDHF increases after loss of NO release. This introduces the possibility that basal NO production may modulate the activity of the EDHF pathway, supporting the findings of Kilpatrick & Cocks (1994). Indeed, this back-up mechanism may be of pathophysiological importance as Kemp *et al.* (1995) demonstrated that, in a sheep model of experimental pulmonary hypertension, endothelium-dependent relaxation is diminshed while K^+ conductance-mediated relaxation becomes more important.

We have previously shown that cyclic GMP may modulate K^+ channel activity, such that their activation is enhanced on reduction of basal cyclic GMP levels (McCulloch & Randall, 1996c). This led us to see if functionally replacing cyclic GMP, which is reduced on loss of basal NO production, with the cell permeable analogue 8-bromo cyclic GMP, could influence the effects of L-NAME. The presence of 8-bromo cyclic GMP alone had minimal effects on vasorelaxation to carbachol, whereas in its presence the inhibitory effects of L-NAME on carbachol-induced vasorelaxation were substantially enhanced. One possible explanation is that basal NO formation, through generating basal cyclic GMP levels, may modulate K^+ channel activation, such that when basal NO is removed K^+ channel activation by EDHF is enhanced. Thus, in the absence of basal NO production, the L-NAME-insensitive component of vasorelaxation is upregulated, as suggested by Kilpatrick & Cocks (1994). In the context of the present investigation, this may explain why L-NAME alone appeared to have only modest effects against carbachol-induced vasorelaxation because of the upregulation of the EDHF/K^+ channel pathway on loss of NO. This upregulation is suppressed by 8-bromo cyclic GMP and thus the effects of L-NAME are potentiated in its presence. We therefore provide evidence for interaction, or 'cross-talk', between NO and EDHF such that, when NO is inhibited, the modulatory effect of NO (via cyclic GMP) on the EDHF/K^+ channel pathway is removed. Thus the impact of EDHF is increased and this may compensate in whole or in part for the loss of NO activity. The precise mechanisms underlying this cross-talk are unclear and could involve cyclic GMP influencing either the release of EDHF or its activation of K^+ channels. Interaction with, or modulation of, K^+ channels would seem most likely as there is good evidence for cyclic GMP modulating the activity of Ca^{2+}-activated K^+ channels in vascular smooth muscle (Williams *et al.*, 1988). Potentially, loss of cyclic GMP control could allow EDHF more scope to activate these channels, thereby increasing the functional importance of EDHF. This back-up mechanism may be of considerable importance in disease states where NO activity is impaired.

In summary, the results of the present investigation demonstrate that endothelium-dependent vasorelaxation to carbachol in the rat mesenteric arterial bed is mediated by two pathways, one is L-NAME-sensitive and the other L-NAME-insensitive. There is evidence that the L-NAME-insensitive vasorelaxant effects of muscarinic agonists are mediated through an increase in K^+ conductance. Finally, the results strongly suggest that the NO pathway may modulate the activity of the EDHF pathway, through actions of cyclic GMP on K^+ channel activation, such that EDHF activity is upregulated on loss of NO.

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