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The relative importance of nitric oxide and nitric oxide-independent mechanisms in acetylcholine-evoked dilatation of the rat mesenteric bed

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1 The relative contribution of nitric oxide (NO) to acetylcholine-induced smooth muscle relaxation was investigated in the rat perfused mesenteric vasculature and in isolated segments of second, third and fourth order arterial branches.

2 The EC_{50} values and maximal relaxation to acetylcholine were not significantly different in the sequential arterial branches, being approximately 0.05 μM and 85%, respectively.

3 The NO synthase inhibitor L-N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) reduced acetylcholine-evoked endothelium-dependent dilatation and relaxation in the perfused mesenteric bed and in isolated arterial segments. The maximum response to acetylcholine in both preparations was reduced by between 35% to 40% while the EC_{50} values were increased by 5–6 fold. L-NAME had no effect on basal smooth muscle tone in either case.

4 In contrast, endothelium-dependent dilatation of the perfused mesenteric bed evoked by A23187 (0.002–20 nmol), was unaffected by exposure to L-NAME. The EC_{50} values and maximal responses elicited by A23187 (20 nmol) before and after exposure to L-NAME were 0.96 ± 0.5 nmol and $67.0 \pm 7.0\%$ ($n = 4$), and 0.7 ± 0.4 nmol and $70.0 \pm 5.0\%$ ($n = 4$; $P > 0.01$), respectively.

5 Perfusion of the isolated mesenteric bed with raised K^+-Krebs buffer (25 mM) had no effect on basal tone, but reduced the amplitude of both acetylcholine- and A23187-evoked dilatation. The maximum responses to acetylcholine (2 μmol) and A23187 (20 nmol) were reduced from $67.5 \pm 7.3\%$ and $65.4 \pm 8.2\%$ to $18.9 \pm 11.0\%$ ($n = 5$; $P < 0.01$) and $13.5 \pm 12.0\%$ ($n = 4$; $P < 0.01$), respectively.

6 Exposure of the mesenteric bed to L-NAME in the presence of raised K^+-Krebs further reduced the maximal response elicited by acetylcholine to only $8.9 \pm 2.8\%$ ($n = 4$; $P < 0.01$).

7 These results indicate that acetylcholine-evoked vasodilatation of the rat mesenteric vasculature is mediated by both NO-dependent and -independent mechanisms. The relative contribution made by these mechanisms does not appear to differ in sequential branches of the mesenteric artery. In contrast, A23187-evoked vasodilatation appears to be mediated predominantly by a NO-independent mechanism which is sensitive to increases in the extracellular potassium concentration and may reflect the action of endothelium-derived hyperpolarizing factor (EDHF).

Keywords: Acetylcholine; nitric oxide; rat mesenteric bed; endothelium; EDHF; N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

Endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide (NO), or a closely related molecule, which is synthesized from L-arginine by the enzyme NO synthase (reviewed by Moncada *et al.*, 1991). Structural analogues of L-arginine such as N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) have been used to block this pathway and inhibit relaxation mediated by the release of endothelium-derived NO (Rees *et al.*, 1989). However, in several blood vessels, such as the porcine coronary artery and rabbit femoral artery, endothelium-dependent relaxations are not inhibited by NO synthase inhibitors, indicating that other endothelium-derived factors, distinct from NO, may contribute to the local regulation of smooth muscle tone (Chen *et al.*, 1988; Taylor & Weston, 1988; Nagao & Vanhoutte, 1992; Plane *et al.*, 1992).

Endothelium-dependent relaxation evoked by agents such as acetylcholine is accompanied by smooth muscle hyperpolarization. Although exogenous NO can evoke smooth muscle hyperpolarization in some vessels under certain conditions, there is now considerable evidence that a separate endothelium-derived hyperpolarizing factor (EDHF) is also released and contributes to reductions in smooth muscle tone, accounting for the relaxation not mediated by NO (Chen *et al.*, 1988; Tare *et al.*, 1990; Garland & McPherson, 1992; Nagao & Vanhoutte, 1992). Furthermore, when endothelium-dependent hyperpolarization is abolished by increased extracellular potassium concentration, the remaining relaxation is completely blocked by inhibitors of NO synthase (Nagao & Vanhoutte, 1992; Waldron *et al.*, 1993).

The relative contribution of NO and EDHF to endothelium-dependent relaxation appears to vary with both different vessels and agonists (Palmer *et al.*, 1988; Rees *et al.*, 1989; Plane *et al.*, 1992). A comparison of various isolated arteries from the rat indicates that the NO-independent component of relaxation may be more prominent in small arteries. In contrast, NO is probably the predominant factor released from the endothelium in large conduit arteries (Nagao *et al.*, 1992). Several investigations in the microcirculation also suggest a heterogeneous distribution of endothelial cell function, with the contribution made by NO varying along the vascular network (Tang & Joyner, 1992; Hester *et al.*, 1993).

In the perfused rat mesenteric bed, acetylcholine-evoked relaxations have been reported to be completely inhibited by L-arginine analogues such as L-NOARG and L-NAME, suggesting that the reductions in tone are mediated solely by

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endothelium-derived NO (Moore et al., 1990). However, this contrasts with studies in isolated arterial segments from this vascular bed, where acetylcholine-evoked relaxations were found to be largely resistant to NO synthase inhibitors (Garland & McPherson, 1992; Bennett et al., 1992; Waldron et al., 1993). In third order arterial branches from Wistar-Kyoto rats, both the relaxation and hyperpolarization induced by acetylcholine were found to be insensitive to L-arginine analogues, suggesting a major contribution from EDHF (Garland & McPherson, 1992; Waldron et al., 1993). The membrane potential change evoked by acetylcholine, and a substantial component of the relaxation, are inhibited by 25 mM potassium, a concentration which has no effect on relaxation to exogenous or endothelium-derived NO (Parsons et al., 1991; Plane & Garland, 1993; Waldron et al., 1993). In the absence of a change in membrane potential, the remaining component of the relaxation to acetylcholine can be blocked by L-NAME. These observations show that increasing the external potassium concentration to 25 mM in the mesenteric circulation can be used to inhibit the tension change to hyperpolarization, and presumably to differentiate between the relative contribution made by each of these mechanisms.

In the present study, we have attempted to resolve the apparently conflicting observations made in the perfused mesenteric preparation and in isolated arterial segments. We have investigated the relative importance of NO-dependent and -independent mechanisms in endothelium-dependent dilatation of the isolated, perfused mesenteric bed of the rat to acetylcholine and the calcium ionophore A23187. In addition, the possibility that the relative contribution from NO and NO-independent mechanisms varied between second, third and fourth order branch arteries from this vascular bed was also examined. Some of these results have been presented in preliminary form to the British Pharmacological Society (Parsons et al., 1993).

Methods

Rat isolated mesenteric arteries

Endothelium-dependent relaxations of isolated segments of rat mesenteric arteries were studied as previously described (Garland & McPherson, 1992). Briefly, arterial segments (2 mm in length) from either second, third or fourth order branches from the superior mesenteric artery were mounted in a small vessel myograph (model 400A, J.P. Trading, Denmark) and tension changes displayed via a Kipp & Zonen pen recorder. Vessels were allowed to equilibrate under zero force for 30 min before the construction of a passive diameter-tension curve (Mulvany & Halpern, 1977). From this curve the effective transmural pressure was calculated. The vessels were set at tensions equivalent to those generated at 0.9 times the diameter of the vessel at 100 mmHg. Non-linear curve fitting of the passive diameter-tension curve was achieved using a custom written programme for the IBM PC (NORMALIZE, G.A. McPherson). Vessel diameters at an equivalent transmural pressure of 100 mmHg (D_{100}) are given in the text.

After an initial equilibration period of 60 min, tissues were pre-contracted with a submaximal concentration of noradrenaline (1–10 μM) and relaxed to cumulative concentrations of acetylcholine (1 nM–30 μM). Following washout, NO synthase inhibitors were pre-incubated in the organ bath for 30–60 min before the contraction-relaxation cycle was repeated.

Rat isolated perfused mesentery

Rat mesentery preparations were isolated as described by Moore et al. (1990). Briefly, the mesenteric vascular bed was cannulated via the superior mesenteric artery, separated from the intestine, and perfused at 5 ml min^{-1} with warmed (37°C) Krebs buffer which had been bubbled with 95% O_2/CO_2. Perfusion pressure was monitored continuously by a Bell & Howell pressure transducer connected to a pen recorder.

To induce tone, noradrenaline (10–50 μM) was added to the Krebs reservoir at a concentration sufficient to increase perfusion pressure by 80–90 mmHg, representing approximately 70–80% of the maximum response. Bolus doses of acetylcholine (0.02 nmol–2 μmol), A23187 (0.002–20 nmol) or sodium nitroprusside (0.002 nmol–2 μmol) were injected in a small volume (100 μl) to prevent injection artifacts. In some experiments, preparations were perfused for 1 min with 3-(3-cholamidopropyl) 1-propanesulphonate (CHAPS; 4.7 mg ml^{-1}) to remove the endothelial cell layer. Following exposure to CHAPS, responses to acetylcholine were abolished, whereas dilatation evoked by sodium nitroprusside was unaltered. Inhibitors of NO biosynthesis were added to the Krebs reservoir and allowed to perfuse the tissues for at least 30 min before pre-constriction.

Solutions and drugs

Tissues were maintained in Krebs buffer of the following composition (mM): NaCl 119, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.18, disodium EDTA 0.027, and glucose 11.0. Raised K^+-Krebs solution (25 mM KCl) was prepared by direct replacement of NaCl with KCl.

Drugs used were A23187 (Sigma), acetylcholine chloride (BDH), L-NAME (Sigma), L-NOARG (Sigma), noradrenaline bitartrate (arterenol, Sigma) and sodium nitroprusside (Sigma). All drugs were dissolved in Krebs buffer except A23187, the stock solution of which was dissolved in dimethylsulphoxide (DMSO).

Analysis of data

In all experiments, relaxations were expressed as a percentage decrease in the induced level of tone. All data are expressed as mean ± s.e.mean. The significance between mean values was calculated by Student's t test, with rejection of the null hypothesis at the 5% level ($P<0.05$). In some experiments, analysis of variance (ANOVA) was used to compare the effects of L-NAME and raised K^+ when used together.

In experiments on isolated arterial segments, EC_{50} values are expressed as $-\log$ M whereas in the perfused preparation, where bolus doses of drugs were added, EC_{50} values are quoted in μmol.

Results

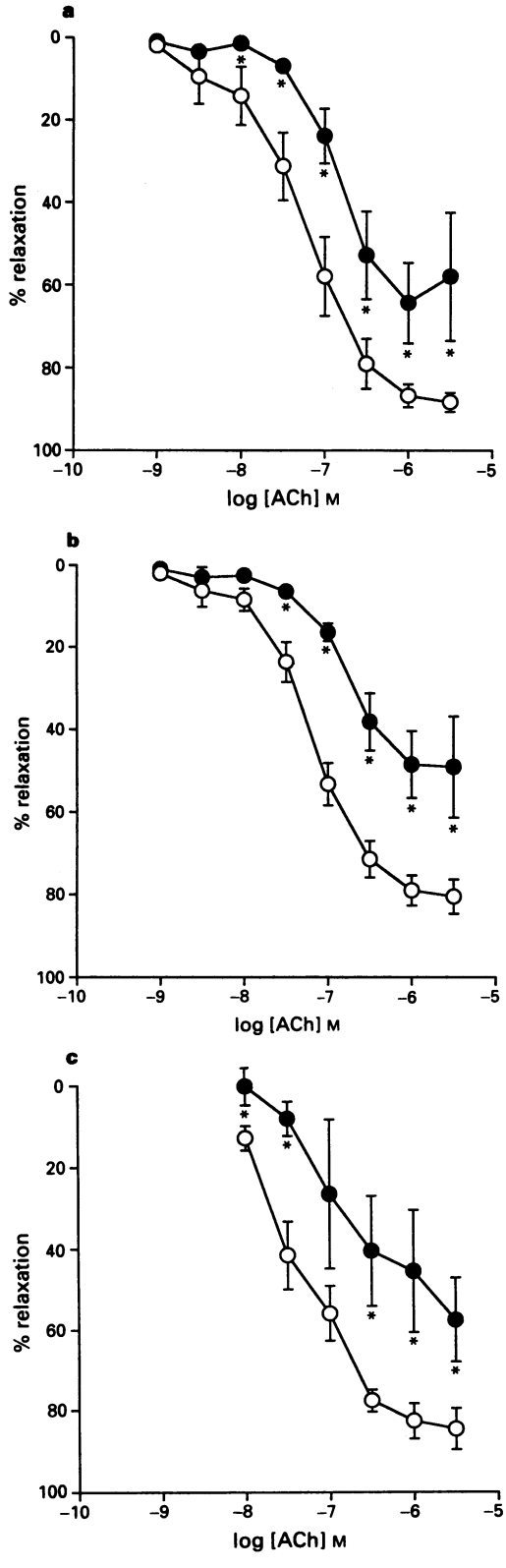
Acetylcholine-evoked relaxation of isolated segments from the mesenteric bed

Acetylcholine (1 nM–10 μM) evoked dose-dependent relaxation in noradrenaline-contracted segments of second (D_{100} = 454 ± 15 μm; n = 7), third (D_{100} = 349 ± 18 μm; n = 14) and fourth (D_{100} = 316 ± 12 μm; n = 6) order branches of rat mesenteric arteries. The EC_{50} values and maximal relaxation evoked by acetylcholine were not significantly different between the arterial segments from the different branches (Table 1). Concentration-response curves for acetylcholine-evoked relaxations in the different arterial branches are shown in Figure 1 (a, b and c).

Pre-incubation of tissues with L-NAME (100 μM; 30 min) did not alter the resting tension of any of the segments from the different arterial branches. However, following exposure to L-NAME, acetylcholine-evoked relaxations were depressed to a similar extent in each arterial branch (Figure 1). In each case there was a 5 to 6 fold increase in the EC_{50} concentration and the maximal response was reduced by approximately 40% (Table 1). Increasing the incubation time with

Table 1 Comparison of % maximal relaxation of induced tone (R_{max}) and EC_{50} values (−log M) for acetylcholine in isolated arterial segments in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM)

Branch	D_{100} (μm)	*Control* R_{max}	*Control* EC_{50}	*L-NAME* (100 μM) R_{max}	*L-NAME* (100 μM) EC_{50}
2nd	454 ± 15	88.3 ± 2.3	7.15 (7.34–6.96)	58.0 ± 15.5	6.72 (6.86–6.57)
3rd	349 ± 18	80.6 ± 4.2	7.25 (7.57–6.97)	49.2 ± 12.0	6.74 (6.98–6.49)
4th	316 ± 12	84.7 ± 5.0	7.44 (7.76 ± 7.11)	57.7 ± 10.4	6.83 (7.25–6.41)



L-NAME to 60 min did not further depress the acetylcholine-evoked responses (data not shown).

Indomethacin (10 μM) was without effect on acetylcholine-evoked relaxations in the presence or absence of L-NAME ($n = 3$).

Effect of L-NAME on endothelium-dependent dilatation of the isolated perfused mesenteric bed

Bolus injections of acetylcholine (0.2 nmol–2 μmol), evoked transient, dose-dependent decreases in perfusion pressure of tissues pre-constricted with noradrenaline (10–50 μM). These responses were abolished following removal of the endothelium with CHAPS. The maximal response evoked by acetylcholine (2 μmol) was 77.2 ± 7.6% ($n = 12$) relaxation of induced tone and the EC_{50} value was 32.7 ± 13.1 nmol ($n = 12$).

When added to tissues pre-constricted with noradrenaline, L-NAME (100 μM) and L-NOARG (100 μM) caused a small, rapid, endothelium-dependent increase in perfusion pressure of 11.8 ± 2.4 mmHg ($n = 4$), which then declined to the previous level within 8–10 min. In the absence of induced tone, infusion of the L-arginine analogues for up to 30 min had no effect on basal perfusion pressure, and did not enhance the contractions evoked by noradrenaline. Acetylcholine-evoked responses were significant depressed in 80% of preparations following exposure to the NO synthase inhibitors. In the remainder, relaxation was not modified. In the tissues in which NO synthase inhibitors reduced relaxation, the maximum response to acetylcholine (2 μmol) was reduced by 35.4 ± 3.0% ($n = 6$; $P < 0.01$). A similar reduction was observed in the presence of L-NOARG. Dose-response curves for acetylcholine-induced dilatation in the presence and absence of L-NAME are shown in Figure 2. Removal of L-NAME from the Krebs buffer did not restore acetylcholine-evoked dilatation over the 45 min recovery period of the experiments, although responses were partially restored by the infusion of 100 μM L-arginine (71% of maximal response; $n = 2$).

Bolus injections of A23187 (0.002–20 nmol) also evoked transient, dose-dependent reductions in the perfusion pressure of noradrenaline-constricted preparations which were abolished by removal of the endothelium. The maximal response evoked by A23187 (0.02 μmol) was 65.44 ± 8.2% ($n = 4$) relaxation of induced tone and the EC_{50} value was 0.96 ± 0.48 nmol ($n = 4$). However, in contrast to acetylcholine, A23187-evoked responses were not depressed and in some cases were increased, by prior exposure to L-NAME (100 μM) for up to 60 min. The maximal response evoked by A23187 in the presence of L-NAME was 68.14 ± 5.1% ($n = 4$; $P > 0.05$) and the EC_{50} value was 0.7 ± 0.4 nmol

Figure 1 Mean concentration-response curves for acetylcholine-evoked relaxation in isolated segments of second (a), third (b) and fourth (c) order branches of the rat mesenteric bed, in the presence (●) and absence (○) of N^G-nitro-L-arginine methyl ester (100 μM). Points are the mean with s.e.mean from 3 or 4 separate experiments, *$P < 0.01$.

($n = 4$; $P > 0.05$). Figure 3 shows dose-response curves for A23187-induced relaxation of noradrenaline-constricted tissues in the presence and absence of L-NAME.

Indomethacin (10 μM) was without effect on responses to either acetylcholine or A23187, in either the presence or the absence of L-NAME (data not shown).

The nitrovasodilator agent, sodium nitroprusside (0.002–20 nmol), evoked dose-dependent reductions in noradrenaline-induced tone, and these relaxations were unaffected by removal of the endothelium or exposure to L-NAME (100 μM; 30 min). The maximal response evoked by sodium nitroprusside in the presence and absence of L-NAME was 77.5 ± 3.9% and 79.0 ± 5.0% ($n = 3$; $P > 0.01$).

Effect of raised K^+ Krebs buffer on endothelium-dependent dilatation of the isolated, perfused mesenteric bed

Perfusion of isolated mesenteric preparations with raised K^+-Krebs buffer had no effect on basal perfusion pressure and did not enhance noradrenaline-evoked constriction. However, in the presence of raised K^+-Krebs buffer, the dilatation of noradrenaline-induced tone evoked by acetylcholine or A23187 was significantly reduced.

In the presence of raised K^+-Krebs buffer, the responses to low doses of acetylcholine were not significantly inhibited, but the maximal response was reduced from 77.2 ± 9.2 to 18.9 ± 11.1% ($n = 5$; $P < 0.01$) compared to control. Subsequent exposure to L-NAME (100 μM), in the continued presence of raised K^+-Krebs buffer, did not further depress the acetylcholine-evoked dilatation (maximal response 8.9 ± 2.8%; $n = 4$; ANOVA). Dose-response curves for acetylcholine-evoked responses in the presence of raised K^+-Krebs buffer, alone or together with L-NAME, are shown in Figure 4.

In the presence of raised K^+-Krebs buffer, the responses to all doses of A23187 (0.002–20 nmol) were significantly inhibited by 80–90%, compared to control responses in normal Krebs buffer. The maximal response to A23187 (20 nmol) in the presence of raised K^+-Krebs buffer was

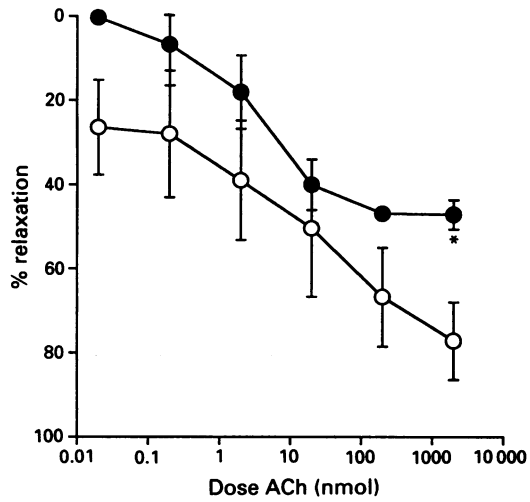


Figure 2 Mean dose-response curves for acetylcholine-evoked dilatation of the rat isolated perfused mesenteric bed pre-constricted with noradrenaline (1–10 μM). Points show dilatation in the presence (●) and absence (○) of N^G-nitro-L-arginine methyl ester (100 μM), and are the mean with s.e.mean from 6 separate experiments.

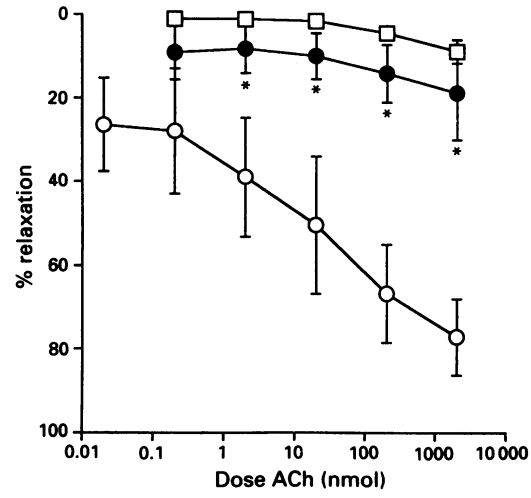


Figure 4 Mean dose-response curves for acetylcholine-evoked dilatation of the rat isolated perfused mesenteric bed pre-constricted with noradrenaline (1–10 μM). Points show dilatation in the absence (○) and presence of 25 mM K^+-Krebs buffer (●), or 25 mM K^+-Krebs buffer and N^G-nitro-L-arginine methyl ester (100 μM; □). Points are the mean with s.e.mean from 4 separate experiments, $*P < 0.01$.

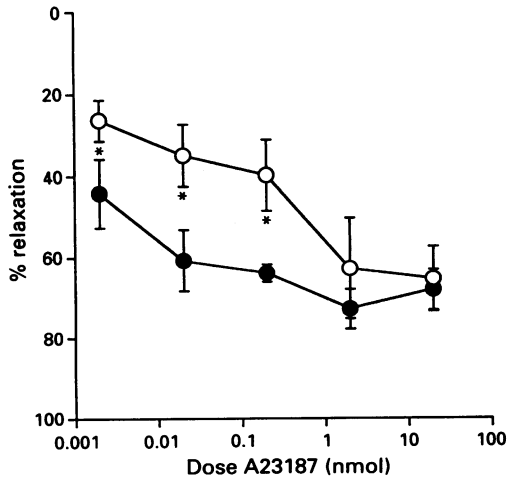


Figure 3 Mean dose-response curves for A23187-evoked dilatation of the rat isolated perfused mesenteric bed pre-constricted with noradrenaline (1–10 μM). Points show dilatation in the presence (●) and absence (○) of N^G-nitro-L-arginine methyl ester (100 μM), and are the mean with s.e.mean from 4 separate experiments, $*P < 0.01$.

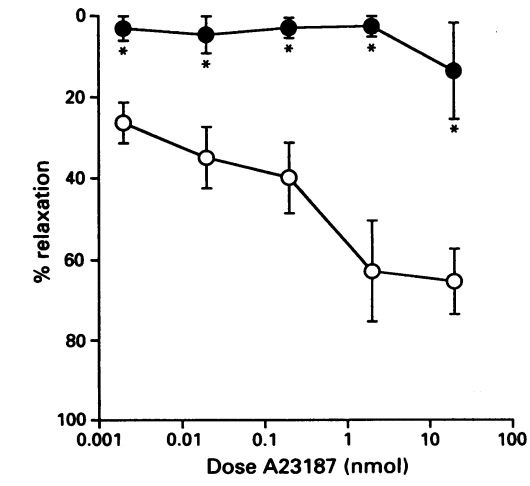


Figure 5 Mean dose-response curves for A23187-evoked dilatation of the rat isolated perfused mesenteric bed pre-constricted with noradrenaline (1–10 μM). Points show dilatation in the absence (○) and presence of 25 mM K^+-Krebs buffer (●), and are the mean with s.e.mean from 4 separate experiments, $*P < 0.01$.

13.5 ± 11.9% ($n = 4$; $P < 0.01$). Dose-response curves for A23187 in normal and raised K^+-Krebs buffer are shown in Figure 5. Relaxations evoked by the nitrovasodilator, sodium nitroprusside, were unaltered in the presence of high K^+-Krebs buffer (results not shown), and the cyclo-oxygenase inhibitor, indomethacin (10 μM) did not alter responses to either acetylcholine or A23187.

Discussion

These data indicate that endothelium-dependent dilatation of the rat mesenteric vasculature is mediated by both NO-dependent and -independent mechanisms. The relative contribution made by these separate mechanisms appears to vary with acetylcholine or A23187, and as in studies of other arteries, the NO-independent mechanisms appear to make a larger contribution in the responses evoked by A23187 (Nagao & Vanhoutte, 1992; Plane *et al.*, 1992).

In the rat perfused mesenteric vasculature, L-NAME and L-NOARG attenuated the reversal of noradrenaline-induced tone evoked by the muscarinic agonist, acetylcholine, reducing the maximum response by around 35% and shifting the concentration-response curve to the right. However, in the presence of raised K^+-Krebs buffer, the maximum response to acetylcholine was reduced by 72% and, subsequent exposure to L-NAME almost abolished the relaxation to acetylcholine. These results are in agreement with previous observations on isolated third order branches of the mesenteric bed, which indicated that acetylcholine-evoked relaxation was mediated by the release of NO and, at higher concentrations, a hyperpolarizing factor distinct from NO and inhibited by 25 mM potassium (Waldron *et al.*, 1993). As in the isolated segments, the potassium-sensitive pathway appears to make a very significant contribution to acetylcholine-evoked smooth muscle relaxation.

In contrast, A23187-evoked dilatation was not inhibited by exposure to the L-arginine analogue L-NAME, indicating that NO is probably not important in smooth muscle relaxation to this agent in the rat mesenteric vasculature. Interestingly, at some doses of A23187 the dilatation was increased following exposure to L-NAME. We do not have an explanation for this observation and it was not investigated further.

However, as with acetylcholine, the relaxation to A23187 was significantly reduced in the presence of a raised concentration of potassium, suggesting that A23187 may induce relaxation by a mechanism which is dependent on increased potassium conductance.

Previous studies have also demonstrated that factors distinct from NO contribute to endothelium-dependent relaxation in this vascular bed. For example, methylene blue and oxyhaemoglobin are much less effective in blocking acetylcholine-evoked dilatation in the perfused mesentery, in concentrations which completely inhibit equivalent responses in other arteries such as the rabbit aorta (Khan *et al.*, 1992). In addition, oxyhaemoglobin and methylene blue were found to inhibit completely relaxation to low concentrations of acetylcholine in the rat mesenteric bed, but were less effective against higher concentrations, when up to 58% relaxation persisted (Khan *et al.*, 1992). This profile was very similar to the responses to acetylcholine obtained in the presence of L-NAME in the present study, and imply that relaxation to low concentrations of acetylcholine predominantly reflects the release of NO, while at higher concentrations, an additional factor, probably EDHF, makes a major contribution.

The identity of the mediator of NO-independent relaxation is unclear although, in some vessels, such as the rabbit and bovine pulmonary artery, endothelium-dependent relaxation is mediated by the release of both NO and products of arachidonic acid metabolism (Chand *et al.*, 1987; Ignarro *et al.*, 1987). In the rabbit coronary artery, bradykinin-evoked relaxation which persists in the presence of L-NOARG is mediated by prostacyclin acting through glibenclamide-sensitive potassium channels (Jackson *et al.*, 1993). A role for prostanoids in the NO-independent responses to acetylcholine and A23187 in the rat mesentery is unlikely, as indomethacin was without effect in this and a previous study (Garland & McPherson, 1992).

L-NAME and L-NOARG did not affect the resting tone in either the perfused mesenteric preparation or in isolated arteries, indicating that the basal release of NO is low in the mesenteric bed. A transient endothelium-dependent contraction was observed when L-NAME was added to perfused preparations stimulated with noradrenaline, which may suggest that NO release is stimulated during arterial contraction. However, following exposure to the NO synthase inhibitor, noradrenaline-induced contractions were not enhanced indicating that NO release does not have an important modulatory role in this vascular bed. Another recent study has also reported that L-NAME increased the basal perfusion pressure in this bed, but only in the presence of increased extracellular potassium (Adeagbo & Triggle, 1993). These workers suggested that EDHF rather than NO is the principle regulator of basal perfusion pressure, the contribution of NO only becoming apparent after the action of EDHF had been abolished (Adeagbo & Triggle, 1993).

In the rabbit aorta, elevated potassium concentrations have been shown to inhibit the relaxation to both acetylcholine and sodium nitroprusside, by inhibiting the formation and action of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the vascular smooth muscle (Collins *et al.*, 1988). As vasodilatation of the perfused mesenteric bed evoked by the nitrovasodilator, sodium nitroprusside, was unaltered in the presence of 25 mM potassium in the mesenteric bed, it is unlikely that this potassium concentration has a significant inhibitory action on the formation or the cellular actions of cyclic GMP. This is in agreement with other studies which have also found that this concentration of potassium does not inhibit either the release or actions of endothelium-derived NO or the smooth muscle responses to exogenous NO (Parsons *et al.*, 1991; Plane & Garland, 1993; Waldron *et al.*, 1993).

In other studies, higher concentrations of potassium (up to 60 mM) had no effect on the size of relaxation to endothelium-derived NO, indicating that inhibition of either the release or the actions of NO are unlikely to account for the reduced relaxation to acetylcholine and A23187 observed in the presence of 25 mM K^+-Krebs buffer (Parsons *et al.*, 1991; Plane *et al.*, 1992; Plane & Garland, 1993). In fact, in the perfused preparation, the presence of 25 mM K^+ had little effect on the relaxation to the lowest dose of acetylcholine, but significantly depressed vasodilatation stimulated by higher doses of either acetylcholine or A23187. This supports the idea that the release of an additional factor, which is sensitive to increases in the extracellular potassium concentration, depends on the level of intracellular calcium within the endothelium. If this is the case, then it appears that EDHF production is more sensitive to calcium influx than is the synthesis of NO.

In a previous study on the perfused mesenteric bed, L-NOARG (200 μM) was found to abolish acetylcholine-evoked dilatation (Moore *et al.*, 1990). In contrast, in our experiments L-NAME (100 μM) and L-NOARG (100 μM) reduced the maximum response to acetylcholine in both the perfused preparation and isolated arterial segments by only 35%. This inhibition was not improved by increasing the concentration of the NO synthase inhibitors to 200 μM, or by increasing the exposure time to 60 min. Furthermore, in 20% of the perfused preparations, acetylcholine-evoked responses were totally resistant to the blocking action of NO synthase inhibitors. The reasons for the discrepancy between these studies is unclear, but in the light of our observations, it does not appear to be due to a variation in the relative contribution of NO to relaxation down the vascular bed, as L-NAME reduced acetylcholine-evoked relaxation to a similar extent in

each of the second, third and fourth order branches of the mesenteric artery.

In conclusion, these data indicate that in the rat mesenteric vasculature, endothelium-dependent vasodilatation is mediated by the release of both NO and an additional factor which is probably EDHF. The relative contribution made by these two mechanisms to the overall relaxation appears to vary with different agonists. Relaxation to low doses of acetylcholine appears to be mediated by NO, whereas higher concentrations activate an additional mechanism. Relaxation to A23187 appears to be independent of NO production, and mediated by a potassium-sensitive mechanism alone, implying a major role for EDHF.

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