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Multiple pathways underlying endothelium-dependent relaxation in the rabbit isolated femoral artery

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1 In isolated segments of the rabbit femoral artery stimulated with noradrenaline, both acetylcholine (1nM–10 μM) and the calcium ionophore A23187 (1 nM–100 μM) evoked endothelium-dependent smooth muscle relaxation and hyperpolarization while bradykinin (0.01–100 nM) had no effect.

2 The nitric oxide synthase inhibitors, N^G-nitro-L-arginine (L-NOARG; 100 μM; 20 min) or N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM; 20 min) each abolished the hyperpolarization and the majority of the relaxation to acetylcholine (maximal response reduced from 96.8 ± 2.3% to 2.0 ± 1.4%).

3 The potassium channel blocker, glibenclamide (10 μM; 10 min) also abolished the change in membrane potential to acetylcholine but did not modify the smooth muscle relaxation.

4 In contrast, neither L-NAME nor glibenclamide modified the comparable responses of the femoral artery to A23187, which were also unaffected by the cyclo-oxygenase inhibitor, indomethacin (10 μM).

5 In artery segments stimulated with potassium chloride (25 mM), the maximal change in tension and membrane potential evoked by A23187 (100 μM) was significantly reduced from 95.0 ± 4.5% and 23.0 ± 2.0 mV to 69.0 ± 10.1% and 12.0 ± 1.5 mV, respectively. Under these conditions L-NAME further reduced the relaxation but not the accompanying hyperpolarization to A23187.

6 Endothelium-denuded arterial segments sandwiched with endothelium-intact 'donor' segments gave qualitatively similar relaxant responses to those described above for acetylcholine and A23187.

7 Exogenous nitric oxide (0.5–10 μM) stimulated a transient relaxation in pre-contracted artery segments, which at concentrations above 5 μM was accompanied by smooth muscle hyperpolarization (maximum 8.5 ± 3.2 mV; $n = 4$). The hyperpolarization but not the relaxation to nitric oxide was abolished by either glibenclamide or 25 mM potassium.

8 These data indicate that in the femoral artery, acetylcholine-induced relaxation can be attributed solely to the release of nitric oxide from the endothelium, which then stimulates relaxation independently of a change in smooth muscle membrane potential. In contrast, both the relaxation and hyperpolarization evoked by A23187 appear to be mediated predominantly by nitric oxide-independent pathways which appear to involve a diffusible factor released from the endothelium. The results suggest that this diffusible hyperpolarizing factor can be released from endothelial cells in the femoral artery by A23187 but not by acetylcholine.

Keywords: Endothelium-dependent hyperpolarization, relaxation; rabbit isolated femoral artery; A23187; acetylcholine; nitric oxide

Introduction

Endothelium-derived relaxing factor (EDRF) has now been identified as nitric oxide, or a closely related molecule, which is synthesized from L-arginine by nitric oxide synthase (Palmer *et al.*, 1987). However, in many vessels endothelium-dependent relaxations, particularly to agents such as bradykinin and the calcium ionophore, A23187, are resistant to inhibitors of the nitric oxide transduction pathway. This indicates that other endothelium-derived factors may contribute to the local regulation of vascular smooth muscle tone (Chen & Suzuki, 1989; Cowan & Cohen, 1991; Nagao & Vanhoutte, 1992). Furthermore, direct measurements of smooth muscle guanosine 3':5'-cyclic monophosphate (cyclic GMP) have shown in isolated preparations such as the rat kidney, canine femoral vein and porcine coronary artery, that endothelium-dependent relaxations are unaffected when cyclic GMP formation is inhibited (Cacofeiro & Nasjletti, 1991; Cowan & Cohen, 1991; Vidal *et al.*, 1991).

Endothelium-dependent relaxation is usually accompanied by hyperpolarization of the vascular smooth muscle cell membrane and, although application of exogenous nitric oxide can elicit membrane hyperpolarization under certain conditions in some vessels, it has been suggested that the hyperpolarization may be mediated by a factor (EDHF) which is distinct from nitric oxide (Taylor *et al.*, 1988; Tare *et al.*, 1990). The release of EDHF may explain the resistance of some endothelium-dependent responses to inhibitors of nitric oxide synthase (Garland & McPherson, 1992; Nagao & Vanhoutte, 1992).

Preliminary experiments in the rabbit isolated femoral artery indicated that acetylcholine-evoked relaxations could be inhibited by L-NAME, but that relaxations elicited by the calcium ionophore A23187 were unaffected (Plane *et al.*, 1992). This suggested that acetylcholine and A23187 might have a differential influence on the release of nitric oxide and EDHF in this large artery. We have now investigated the contribution of smooth muscle hyperpolarization to endothelium-dependent relaxation in the femoral artery by making simultaneous measurements of changes in smooth muscle membrane potential and tension. In addition, we have also investigated the action of bradykinin, which may stimulate relaxation in arterial smooth muscle predominantly via the release of EDHF (Nagao & Vanhoutte, 1992; Nakashima *et al.*, 1993).

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Methods

New Zealand white rabbits (2–3 kg) of either sex were anaesthetized with an intravenous injection of sodium pentobarbitone (60 mg kg^{-1}, i.v.) and killed by rapid exsanguination. The femoral arteries were carefully removed, cleaned of adhering fat and connective tissue and cut into cylindrical segments 2–3 mm in length. Segments were then mounted in a two-channel Mulvany-Halpern myograph (model 400A; J.P. Trading, Denmark) for simultaneous recording of changes in smooth muscle membrane potential and tension, as previously described (Garland, 1987). Briefly, two tungsten wires (each 40 μm diameter) were passed through the lumen of the segment and each wire attached to a metal foot in the myograph. The tissue segments were stretched between the two wires to a previously determined optimal preload of 1g, and superfused at 7–8 ml min^{-1} with Krebs buffer which had been bubbled with 95% O_2/5% CO_2. In some experiments, the endothelial cell layer was removed by gently rubbing the intimal surface with a human hair.

Drugs were equilibrated with the perfusate before it entered the tissue chamber. Nitric oxide solutions were injected from a gas-tight syringe close to the arterial segment, in volumes of not greater than 200 μl.

Electrophysiology

Measurement of smooth muscle membrane potential was made with a glass microelectrode advanced through the adventitial surface of the arterial segment. The electrodes were filled with 2 M KCl and had resistances of 60–120 MΩ. Membrane electrical events were recorded through a high impedance d.c. preamplifier (Neurolog 102G) and, together with data from the isometric force transducer, stored on disc (CVMS, McPherson Scientific).

Sandwich preparations

Endothelium-denuded segments of the femoral artery were mounted between stainless steel hooks in 10 ml organ baths under a resting tension of 1 g for isometric recording of tension changes. The tissues were maintained at 37°C in Krebs buffer bubbled with 95%O_2/5%CO_2. Removal of the endothelium was confirmed by the absence of any relaxation to acetylcholine or A23187 following pre-constriction with phenylephrine. 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 a detector tissue denuded of endothelium. The tissues were then rechallenged with acetylcholine or A23187 in the absence and presence of L-NAME (100 μM).

Solutions and drugs

All experiments were carried out in Krebs buffer with the following composition (mM): NaCl 122, $NaHCO_3$ 25.5, KCl 5.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, disodium EDTA 0.027, ascorbate 0.114, and glucose 9.4. K^+ 25 mM Krebs solution was prepared by direct replacement of NaCl with KCl.

Drugs used were acetylcholine chloride (BDH), noradrenaline bitartrate (arterenol, Sigma), calcium ionophore A23187 (Sigma), phenylephrine (Sigma), N^G-nitro-L-arginine methyl ester (Sigma), N^G-nitro-L-arginine (Sigma) and glibenclamide (gift from Hoechst).

Preparation of nitric oxide solutions

Nitric oxide gas (research grade, BDH) was injected into Krebs solution which had been bubbled with helium (BOC) for 45–60 min. Nitric oxide solutions were injected from a gas-tight syringe close to the artery segments, in volumes of not greater than 200 μl. Control injections of helium-gassed Krebs solution were made to assess the extent of any potential injection artifacts.

Analysis of data

Relaxations are expressed as the percentage decrease in the tone induced by either noradrenaline (0.1–1.0 μM) or potassium chloride (25 mM). Data are expressed as mean ± s.e. mean. The significance of differences between mean values was calculated with the Wilcoxon test for paired samples.

Results

Membrane and tension responses to acetylcholine

Smooth muscle cells in the femoral artery were electrically quiescent and had a mean resting membrane potential of −64.3 ± 4.8 mV (51 cells from 29 preparations). The application of acetylcholine (1 nM–10 μM) to cells pre-contracted and depolarized with noradrenaline (0.1–1 μM; mean background contraction and depolarization of 14.9 ± 4.6 mN and 19.0 ± 2.3 mV, $n = 12$) evoked concentration-dependent smooth muscle repolarization and relaxation, both of which were endothelium-dependent. The maximal relaxation evoked by acetylcholine (10 μM) was 96.8 ± 2.3% ($n = 4$), and was accompanied by a maximal increase in membrane potential of 9.0 ± 2.7 mV ($n = 4$). The EC_{50} values for the relaxation and repolarization to acetylcholine were not significantly different (0.25 ± 0.05 μM and 0.29 ± 0.06 μM; $n = 4$; $P > 0.05$), although the concentration required to initiate membrane hyperpolarization was ten fold higher than for relaxation. A representative trace showing simultaneous measurements of changes in membrane potential and tension to acetylcholine is shown in Figure 1a.

Pre-incubation of femoral artery segments with either L-NOARG or L-NAME (100 μM; 20 min) caused an endothelium-dependent increase in basal tone of 4.41 ± 0.87 mN ($n = 12$) but had no significant effect on the resting membrane potential of the smooth muscle cells (mean resting membrane potential in the presence of L-NOARG or L-NAME was −63.0 ± 5.0 mV; $n = 5$; $P > 0.05$). After exposure to either of these inhibitors, acetylcholine-evoked repolarization was abolished and relaxation was dramatically reduced to only 2.0 ± 1.4% ($n = 4$; $P < 0.05$) in cells pre-stimulated with noradrenaline. Concentration-response curves for acetylcholine-evoked relaxation and hyperpolarization in the presence and absence of L-NAME are shown in Figure 2.

The application of the potassium channel blocker glibenclamide (10 μM; 10 min) did not affect either the basal tone or the resting membrane potential in smooth muscle of the femoral artery (mean resting membrane potential in the presence of glibenclamide was −64.5 ± 5.0 mV ($n = 3$)). However, after exposure to glibenclamide acetylcholine-evoked hyperpolarization was abolished although relaxation was not significantly altered (maximal response in the presence of glibenclamide; 94.6 ± 6.0%; $n = 4$; $P > 0.05$). Potassium chloride (25 mM) induced a similar contraction to noradrenaline (11.7 ± 4.7 mN; $n = 8$; $P > 0.05$), although the extent of smooth muscle depolarization was greater (29.5 ± 2.3 mV; $n = 4$; $P < 0.05$). In the presence of 25 mM potassium chloride, acetylcholine-evoked hyperpolarization was abolished, but as with glibenclamide, relaxation was not significantly altered (maximal response 89.9 ± 9.6%; $n = 4$; $P > 0.05$). Following exposure to L-NAME (100 μM), the relaxation to acetylcholine in segments contracted by potassium chloride was inhibited to a similar extent as in the segments contracted with noradrenaline. The maximal relaxation was reduced to only 10.3 ± 1.3% ($n = 4$; $P < 0.05$).

Indomethacin (10 μM) did not alter the relaxation to ace-

tylcholine in either noradrenaline- or potassium chloride-contracted tissues. In the presence of indomethacin, the maximal relaxation evoked by acetylcholine in noradrenaline- and potassium chloride-contracted arterial segments was 95.3 ± 4.0% ($n = 3$) and 90.5 ± 7.9% ($n = 3$), respectively.

Membrane and tension responses to A23187

The calcium ionophore, A23187 (1 nM–100 μM) evoked a concentration-dependent smooth muscle relaxation and repolarization in arterial segments stimulated with

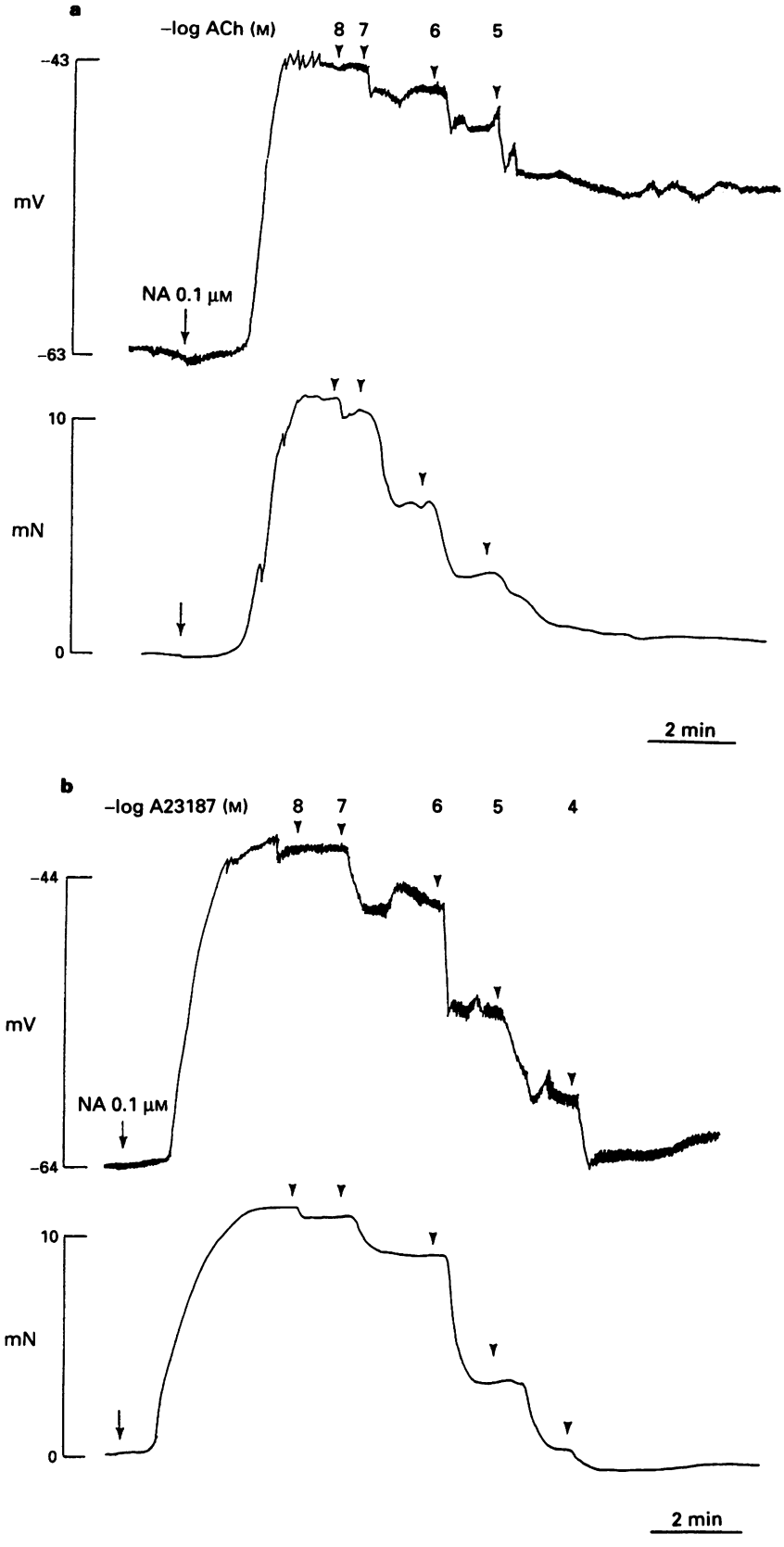

Figure 1 Representative traces showing simultaneous records of changes in membrane potential and tension elicited by acetylcholine (0.01–10 μM) and A23187 (0.01–100 μM) in noradrenaline (1.0 μM)-stimulated segments of the rabbit femoral artery: (**a**) acetylcholine (**b**) A23187.

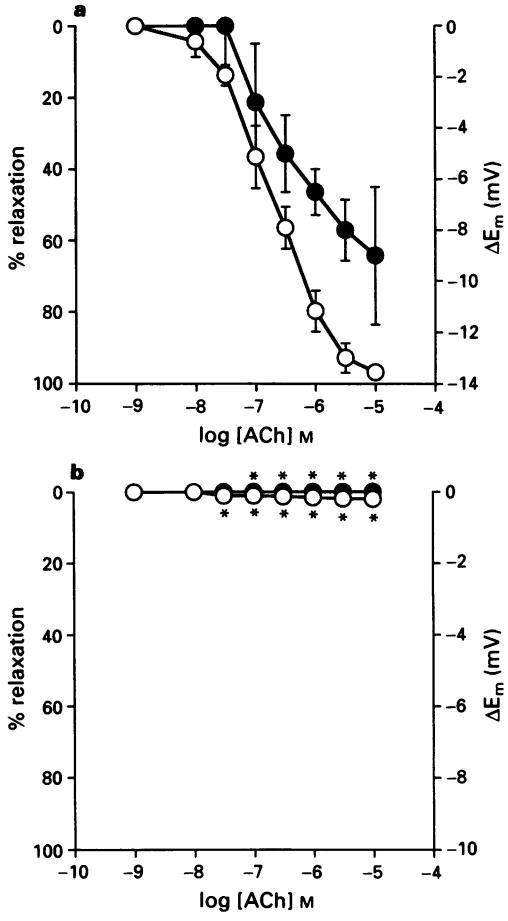

Figure 2 Mean concentration-response curves for acetylcholine in the rabbit femoral artery pre-contracted with noradrenaline (0.1–1.0 μM), in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM; $n = 4$). Points show relaxation (○) and repolarization (●) and are the mean ± s.e. mean from 4 separate experiments. (a) Control responses; (b) in the presence of L-NAME. *$P < 0.05$.

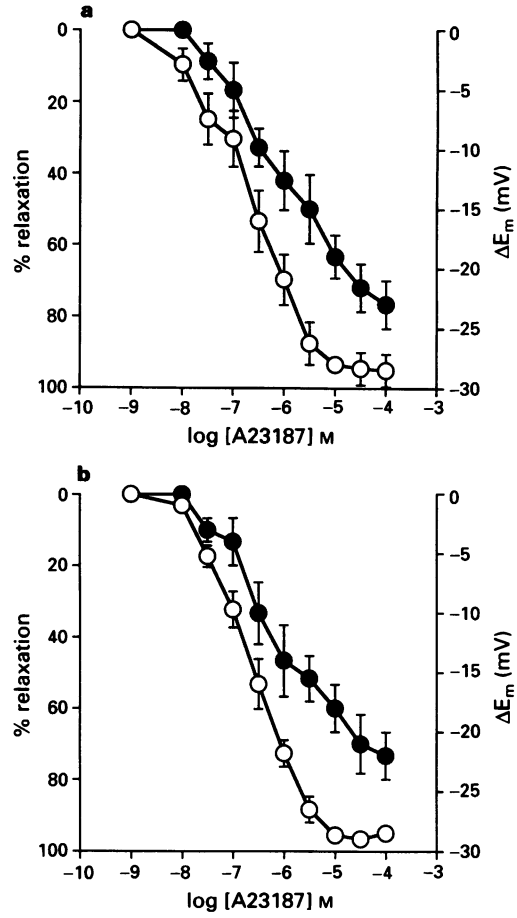

Figure 3 Mean concentration-response curves for A23187 in the rabbit femoral artery pre-contracted with noradrenaline (0.1–1.0 μM), in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM; $n = 4$). Points show relaxation (○) and repolarization (●) and are the mean ± s.e. mean from 4 separate experiments. (a) Control responses; (b) in the presence of L-NAME. *$P < 0.05$.

noradrenaline (0.1–1 μM; mean background contraction and depolarization of 13.5 ± 3.0 mN and 17.5 ± 2.5 mV; $n = 12$). The maximal change in membrane potential and tension to A23187 (100 μM) was 23.0 ± 2.0 mV and 95.7 ± 2.5% ($n = 8$), respectively. As with acetylcholine, the concentration required to initiate repolarization was higher than for relaxation (0.01 μM and 0.03 μM, respectively). A representative trace of changes in membrane and tension to A23187 is shown in Figure 1b. Both the changes in membrane potential and tension were abolished by removal of the endothelium.

Unlike acetylcholine, both the relaxation and repolarization evoked by A23187 was unchanged by prior exposure to either L-NOARG or L-NAME (100 μM; 20 min) or to the potassium channel inhibitor glibenclamide (10 μM; 10 min). The maximal relaxation and change in membrane potential to A23187 in the presence of L-NAME was 95.5 ± 2.4% and 22.1 ± 3.5 mV, and in the presence of glibenclamide, 98.0 ± 1.5% and 21.5 ± 3.0 mV ($n = 4$; $P > 0.05$). Figure 3 shows the concentration-response curves for A23187-evoked relaxation and repolarization in noradrenaline-contracted tissues in the presence and absence of L-NAME.

In arterial segments pre-contracted with potassium chloride (mean background contraction and depolarization of 15.6 ± 3.5 mN and 26.8 ± 5.1 mV; $n = 6$), both the relaxation and repolarization to A23187 were significantly reduced compared to responses in noradrenaline-stimulated tissues. The maximal change in tension and membrane potential to A23187 was reduced to 69.0 ± 10.1% and 12.0 ± 1.5 mV ($n = 4$; $P < 0.05$), respectively. Subsequent exposure to L-NAME (100 μM) further attenuated the A23187-evoked relaxation in potassium chloride-contracted tissues, reducing the maximal relaxation to 42.0 ± 5.6% ($n = 4$) while the accompanying hyperpolarization was unaltered. Glibenclamide (10 μM) reduced the maximum relaxation in the presence of potassium to 57.7 ± 5.3% ($n = 4$; $P < 0.05$), and the repolarization to 6.1 ± 1.5 mV ($n = 4$; $P < 0.05$). Addition of L-NAME and glibenclamide together did not further depress relaxation. Figure 4 shows the concentration-response curves for A23187-evoked relaxation in potassium-contracted tissues in the presence and absence of L-NAME.

The cyclo-oxygenase inhibitor, indomethacin, did not affect relaxation to A23187 in either noradrenaline or potassium chloride-constricted artery segments. The maximal relaxation to A23187 in noradrenaline and potassium chloride contracted tissues in the presence of indomethacin was 97.3 ± 2.5% ($n = 3$) and 68 ± 8.3% ($n = 3$), respectively.

Transfer of tension changes in sandwich preparations

The use of sandwich preparations of femoral artery showed that the tension changes to acetylcholine and to A23187 could be transferred from one strip to another ($n = 8$). As shown in Figure 5 the application of either acetylcholine or A23187 to segments of femoral artery without an endothelium induced relaxation only if a donor tissue with a functional endothelium was wrapped round the denuded segment.

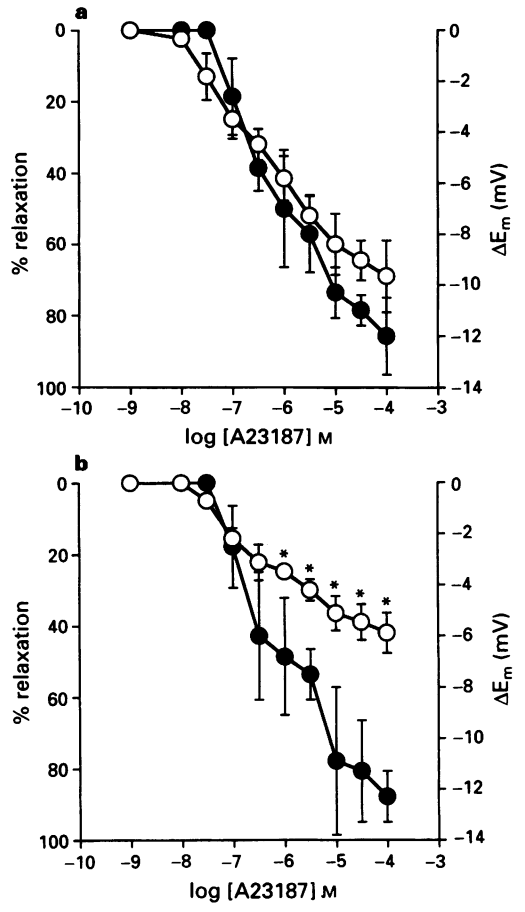

Figure 4 Mean concentration-response curves for A23187 in the rabbit femoral artery pre-contracted with potassium chloride (25 mM), in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM; $n = 4$). Points show relaxation (○) and repolarization (●) and are the mean ± s.e. mean from 4 separate experiments. (a) Control responses; (b) in the presence of L-NAME. *$P < 0.05$.

The concentration-dependent relaxation to acetylcholine was almost totally abolished in the presence of L-NAME (100 μM; Figure 5c), whereas similar responses to A23187 were largely unaffected (Figure 5e). However, the relaxation to A23187 was reduced in the presence of L-NAME if the strips were initially contracted with 25 mM potassium, rather than phenylephrine (1 μM; Figure 5f).

Membrane and tension responses to bradykinin

The application of bradykinin (0.01–100 μM) to either resting or noradrenaline-stimulated tissues failed to evoke any change in smooth muscle tone or membrane potential ($n = 4$).

Membrane and tension responses to nitric oxide

Bolus doses of exogenous nitric oxide (0.5–10 μM), applied close to the arterial segment, initiated transient, concentration-dependent relaxation in tissues pre-contracted with noradrenaline (0.1–1 μM). At concentrations above 5 μM, smooth muscle relaxation was accompanied by a small, transient increase in membrane potential. The maximal change in membrane potential and tension evoked by 10 μM nitic oxide was 8.5 ± 3.2 mV and 86.5 ± 6.6% ($n = 4$), respectively. In the presence of glibenclamide (10 μM) or in tissues pre-contracted with 25 mM potassium chloride, the change in membrane potential to nitric oxide was abolished ($n = 4$), but the relaxation was unaffected. The maximal relaxation evoked by nitric oxide in the presence of either 25 mM potassium chloride or glibenclamide was 80.6 ± 7.9% ($n = 4$; $P > 0.05$) and 85.9 ± 7.4% ($n = 4$; $P > 0.05$), respectively.

Discussion

These data indicate that in the rabbit femoral artery, in common with other vessels, mechanisms which are both nitric oxide-dependent and -independent can contribute to endothelium-dependent smooth muscle relaxation. However, the relative importance of these mechanisms appears to vary, with nitric oxide-independent mechanisms apparently making a larger contribution to the responses evoked by A23187 than other agonists. Of particular interest is the finding that the endothelium-dependent hyperpolarization and relaxation to acetylcholine can be explained solely in terms of nitric oxide release, in spite of the fact that mechanisms independent of nitric oxide, but also capable of providing an important drive to relaxation, are functional in the same vessel. Simple contractile measurements in a variety of isolated arteries from the rat, have led to the suggestion that the relaxation to acetylcholine is mediated by nitric oxide in large, conducting vessels, while in smaller arteries voltage-sensitive mechanisms may predominate viz. EDHF (Nagao *et al.*, 1992). This suggestion was based mainly on the marked relaxation to acetylcholine which persisted in the presence of nitro-arginine in the smaller arteries, while a similar manoeuvre abolished the equivalent responses in larger vessels. Some studies are consistent with this suggestion, showing hyperpolarization in smooth muscle cells of large arteries which appears not to be of primary importance for relaxation and which, in some cases, is sensitive to glibenclamide (Chen *et al.*, 1988; Huang *et al.*, 1988; Brayden, 1990; Rand & Garland, 1992). However there are exceptions, as in large coronary arteries from the guinea-pig the smooth muscle cells developed an endothelium-dependent hyperpolarization to acetylcholine which was resistant to both L-NOARG and glibenclamide (Chen *et al.*, 1991).

The occurrence of more than one endothelium-dependent mechanism which can mediate relaxation in the same vessel has been demonstrated previously in a number of arteries. In the rabbit and bovine pulmonary arteries, bradykinin-evoked relaxation was partially antagonized by either methylene blue or indomethacin, and abolished by these two agents in combination (Chand *et al.*, 1987; Ignarro *et al.*, 1987). Furthermore, in rabbit coronary arteries prostacyclin appears also to contribute to bradykinin-evoked dilatation recorded in the presence of L-NOARG (Jackson *et al.*, 1993). However, in this as in other studies, a role for prostanoids in the nitric oxide-independent response to A23187 is unlikely, because indomethacin was without effect (Cowan & Cohen, 1991; Garland & McPherson, 1992; Adeagbo & Triggle, 1993).

Acetylcholine-evoked relaxation of the femoral artery was accompanied by repolarization of the smooth muscle cell membrane with both of the responses being inhibited by the nitric oxide synthase inhibitors, L-NAME or L-NOARG. This indicates that, as in the rabbit basilar and guinea-pig uterine arteries, the release of endothelium-derived nitric oxide has an important role to play in both the change in membrane potential and in tension to acetylcholine (Tare *et al.*, 1990; Rand & Garland, 1992). This proposal is supported by the finding that relaxation to exogenous nitric oxide was also accompanied by an increase in the membrane potential of the smooth muscle cells, with a maximum response of similar magnitude to that elicited by acetylcholine. In addition, both the acetylcholine and nitric oxide-evoked changes in membrane potential were totally blocked by the sulphonylurea compound, glibenclamide, and by an increased extracellular potassium concentration. The latter observations indicate that glibenclamide-sensitive potassium channels most probably underlie these membrane responses. Similar findings

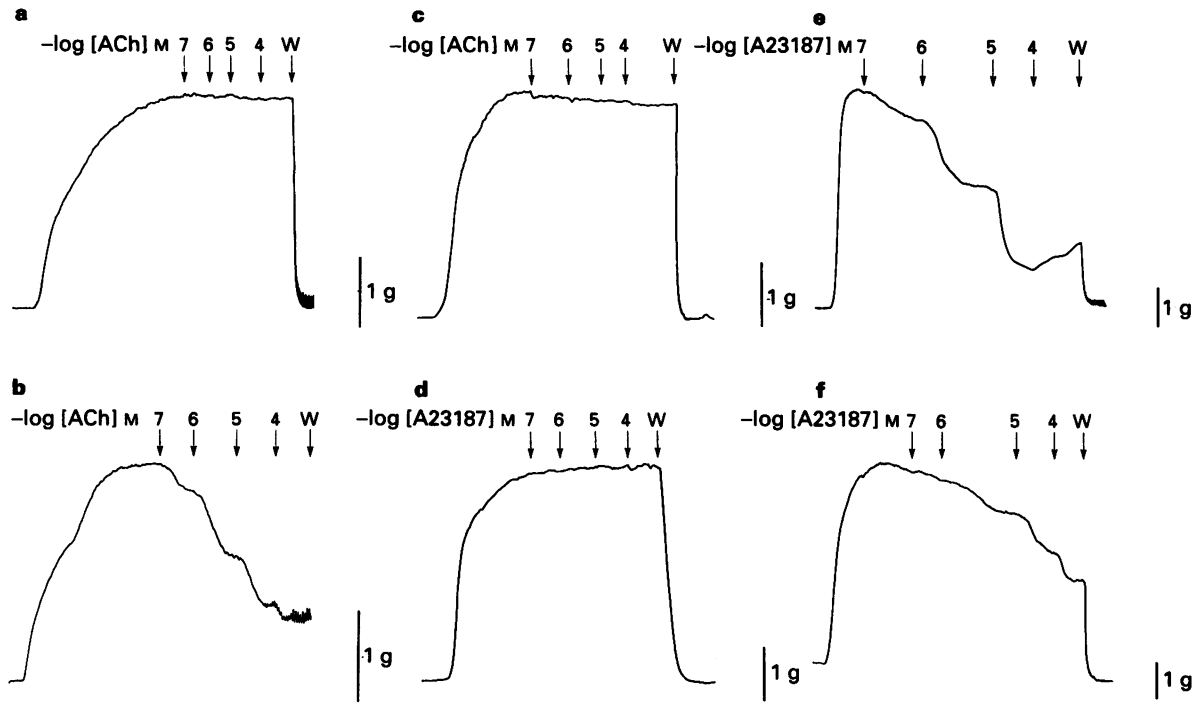

Figure 5 Representative traces, from eight experiments, showing tension change in the isolated femoral artery to either acetylcholine or A23187. Apart from (**f**), segments were precontracted by phenylephrine 1 μM. The artery segment was denuded of endothelium and exposed to acetylcholine in the absence (**a**) or presence of a donor segment containing functional endothelium (**b**) or a donor segment in the additional presence of N^G-nitro-L-arginine methyl ester (L-NAME) (**c**). Similar experiments using A23187 rather than acetylcholine are illustrated in (**d**). A23187 alone; (**e**) A23187 plus donor segment and L-NAME; (**f**) A23187 in an artery segment precontracted by 25 mM potassium.

have also been reported in other vessels, such as the rabbit middle cerebral artery, where nitric oxide- and acetylcholine-evoked changes in membrane potential were also inhibited by glibenclamide or raised extracellular potassium (Brayden, 1990). Our observations are in agreement with the studies of Huang *et al.* (1988) who suggested that the relaxation to acetylcholine in the femoral artery was mediated by nitric oxide. However, we were additionally able to block hyperpolarization to acetylcholine using specific nitric oxide-synthase inhibitors.

Inhibition of both the acetylcholine- and nitric oxide-induced hyperpolarization did not influence smooth muscle relaxation to either agent, indicating that the change in membrane potential is not normally important for reductions in tone. This contrasts with the rat mesenteric artery, where both nitric oxide, acting via cyclic GMP, and a nitric oxide-independent hyperpolarization stimulate significant relaxation in the response to acetylcholine, and where the nitric oxide-independent relaxations are markedly reduced under conditions which inhibit repolarization (Garland & McPherson, 1992; Waldron *et al.*, 1993). The potassium channel opener, levcromakalim, completely reversed the contraction to either noradrenaline or raised potassium in the femoral artery, so a link between an increase in membrane potential and smooth muscle relaxation is functional in these cells (Thirstrup & Nielson-Kidsk, 1992; Plane & Thomas, unpublished observations). Failure to reduce smooth muscle relaxation by blocking hyperpolarization presumably reflects a supramaximal action of nitric oxide via cyclic GMP.

The calcium inophore, A23187, also elicited both endothelium-dependent relaxation and repolarization in noradrenaline-stimulated segments of the rabbit femoral artery. However, in contrast to the acetylcholine-evoked responses, both the relaxation and the repolarization to A23187 were unaffected by pre-incubation with either of the nitric oxide synthase inhibitors L-NAME and L-NOARG, indicating that nitric oxide release does not make a significant contribution to these responses. This is consistent with an earlier study, where methylene blue, at a concentration which inhibited the relaxation evoked by sodium nitroprusside, did not modify A23187-evoked relaxation (Plane *et al.*, 1992). In addition to inhibiting the enzyme soluble guanylate cyclase, methylene blue also inactivates nitric oxide via the extracellular generation of superoxide anion. Therefore this observation suggests that the mediator of A23187-evoked responses, unlike nitric oxide, is not susceptable to oxygen-derived free radicals (Wolin *et al.*, 1990).

Although endothelium-dependent hyperpolarization is mediated by the opening of potassium channels, the type of channel involved has not been defined and may even vary between different vessels. Variation may also, at least in part, reflect the predominance of either EDHF or nitric oxide in the control of vessel tone. For example, in the rabbit basilar and middle cerebral arteries, endothelium-dependent hyperpolarization to acetylcholine is mediated by glibenclamide-sensitive potassium channels, whereas in the rat small mesenteric artery glibenclamide does not modify the increase in membrane potential to acetylcholine, even though glibenclamide-sensitive potassium channels are present in this vessel (Brayden, 1990; McPherson & Angus, 1990; Plane & Garland, 1993). In the rat perfused mesenteric bed, the component of smooth muscle relaxation to acetylcholine which is presumably mediated by this change in membrane potential, is in fact sensitive to the blocking action of apamin (Adeagbo & Triggle, 1993). Endothelium-dependent relaxation which is not mediated by nitric oxide has also been reported to be insensitive to glibenclamide in the porcine and guinea-pig coronary arteries (Chen *et al.*, 1991; Cowan & Cohen, 1992).

In the present study, nitric oxide- and acetylcholine-evoked hyperpolarization were each blocked by glibenclamide, while the A23187-evoked changes in smooth muscle membrane potential were not modified, except in the presence of raised extracellular potassium. This observation again indicates that A23187 operates through a different mechanism from acetyl-

choline, a mechanism which presumably involves potassium channels as both the repolarization and relaxation to A23187 were significantly reduced by raised extracellular potassium chloride concentrations. The glibenclamide sensitive component was only revealed once the predominant hyperpolarizing response to A23187 had been markedly reduced by lowering the potassium gradient. The glibenclamide-sensitive component may well reflect the release of nitric oxide, as under these conditions a similar sensitivity to L-NAME was also apparent. A direct effect of 25 mM potassium on the production or release of nitric oxide is unlikely, as in this and other studies, L-NAME-sensitive relaxations to acetylcholine were not significantly different in noradrenaline- or potassium chloride-contracted tissues (Parsons *et al.*, 1991; Plane & Garland, 1993). As both the amplitude of the repolarization and the nitric oxide-independent component of relaxation to A23187 were reduced by a similar amount, a casual link between the two events is indicated, supporting the possibility that smooth muscle hyperpolarization is responsible for nitric oxide-independent relaxation. Similar observations have recently been made with other agonists in vessels such as the porcine and canine coronary arteries and rat mesenteric arteries (Cowan & Cohen, 1991; 1992; Garland & McPherson, 1992; Nagao & Vanhoutte, 1992).

The fact that smooth muscle relaxation to acetylcholine and A23187 was recorded in the experiments with sandwich preparations of the femoral artery, which had a qualitatively similar sensitivity to L-NAME and raised extracellular potassium as in the individual segments, provides strong evidence that both nitric oxide and a transferable hyperpolarizing factor can be released by endothelial cells in this artery. Although it has been suggested that electrotonic spread of hyperpolarization from endothelial cells could underlie smooth muscle relaxation, the balance of available evidence favours the release of a diffusible hyperpolarizing factor. For example, acetylcholine induced smooth muscle hyperpolarization in smooth muscle cells of guinea-pig coronary artery denuded of endothelium, but only if sandwiched with a carotid artery with an intact endothelium. The hyperpolarization induced in this way was not sensitive to either nitroarginine or glibenclamide, but was blocked in the presence of TEA (Chen *et al.*, 1991). In the present study, relaxation was obtained in sandwiched preparations under conditions which would favour nitric oxide-independent hyperpolarization and associated smooth muscle relaxation, strongly supporting the suggestion that a diffusible hyperpolarizing factor is released from endothelial cells by A23187.

In contrast to acetylcholine, nitric oxide and A23187, bradykinin failed to evoke any measurable change in either smooth muscle membrane potential or tone in the femoral artery. Bradykinin has been shown to cause endothelium-dependent relaxation predominately by the release of EDHF in other vessels (Nagao & Vanhoutte, 1992). A similar effect of bradykinin has also been reported in human coronary arteries, an effect which is mimicked by A23187 (Nakashima *et al.*, 1993). As A23187 appeared to stimulate endothelium-dependent relaxation in the femoral artery predominately via EDHF, the lack of any response to bradykinin suggests an absence of receptors for this tachykinin on endothelial cells in the rabbit femoral artery.

In conclusion, these data indicate that in the rabbit femoral artery, which is a large condduit vessel, acetylcholine-evoked relaxations can be explained by the release of nitric oxide which then acts to stimulate relaxation by a voltage-independent mechanism. In contrast, in the femoral as in other arteries, A23187-evoked relaxations appear to be mediated predominantly by mechanisms independent of the release of nitric oxide and the activation of guanylate cyclase. A23187-evoked relaxations are mediated by a diffusible factor and are reduced together with accompanying smooth muscle hyperpolarization, indicating that the change in membrane potential provides a significant drive to relaxation.

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