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Endothelium-dependent relaxation to acetylcholine in the rabbit basilar artery: importance of membrane hyperpolarization

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1 Muscarinic stimulation of isolated, preconstricted segments of the basilar artery, with either acetylcholine or carbachol, was followed by endothelium-dependent smooth muscle relaxation and membrane hyperpolarization.

2 Smooth muscle relaxation to acetylcholine was stimulated in the presence of lower concentrations than the associated hyperpolarization (EC_{50} values 3.2 μM and 31.6 μM, respectively), and was sustained during agonist application, while the hyperpolarization was relatively transient.

3 Repeated exposure to acetylcholine was associated with loss of membrane hyperpolarization, while smooth muscle relaxation was unaltered. Following a second exposure to 100 μM acetylcholine, mean hyperpolarization was markedly depressed from 8.5 to 2 mV, and subsequent exposures failed to induce any hyperpolarization. Relaxations with a similar amplitude and rate of development, were recorded with each subsequent addition of acetylcholine.

4 The competitive substrate inhibitors for nitric oxide synthase, L-N^G-monomethyl arginine (100 μM L-NMMA) or L-N^G-nitro arginine methyl ester (100 μM L-NAME), modified the form and amplitude of both the relaxation and the hyperpolarization to acetylcholine. In the majority of experiments, both the hyperpolarization and the relaxation were almost totally abolished.

5 Neither nitric oxide, applied directly in physiological salt solution, nor sodium nitroprusside, produced smooth muscle hyperpolarization except in high concentrations. Reproducible, small amplitude (around 2 mV) hyperpolarization followed the application of either NO gas (15 μM) or sodium nitroprusside (100 μM), both of which induced almost maximal smooth muscle relaxation.

6 These data show that muscarinic stimulation of endothelial cells in the rabbit basilar artery is followed by both smooth muscle hyperpolarization and relaxation. They indicate that nitric oxide is involved in both of these responses, but that the smooth muscle hyperpolarization is not an essential component of the relaxation.

Keywords: Vascular smooth muscle; endothelial cells; EDHF; nitric oxide; sodium nitroprusside; L-N^G-nitro arginine methylester (L-NAME); L-N^G-monomethylarginine (L-NMMA); membrane potential

Introduction

As well as relaxing vascular smooth muscle in an endothelium-dependent fashion (by releasing endothelium-derived relaxing factor (EDRF)), acetylcholine and other muscarinic agonists also stimulate endothelium-dependent smooth muscle hyperpolarization (Bolton *et al.*, 1984; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Brayden, 1990; Garland & McPherson, 1991; McPherson & Angus, 1991). Smooth muscle hyperpolarization to acetylcholine, like the relaxation, is mediated, at least in part, by the release of a diffusible factor (Feletou & Vanhoutte, 1988; Chen *et al.*, 1991).

Whether or not the endothelium-dependent hyperpolarization to acetylcholine is mediated in any way by nitric oxide, which either is or is closely related to EDRF, is currently the subject of some controversy. In contrast to the relaxation induced by acetylcholine in the rat aorta and pulmonary artery, hyperpolarization was not blocked by either oxyhaemoglobin or methylene blue, leading to the suggestion that a separate hyperpolarizing factor (EDHF) was released simultaneously with EDRF (Chen *et al.*, 1988; Taylor & Weston, 1988). The concept of a separate hyperpolarizing factor was supported by the failure of exogenous nitric oxide to induce smooth muscle hyperpolarization in concentrations which induced marked relaxation in a number of vascular preparations (Komori *et al.*, 1988; Huang *et al.*, 1988; Brayden, 1990). Also, hyperpolarization to acetylcholine was not reduced by inhibitors of nitric oxide synthase in both the guinea-pig coronary artery and rat small mesenteric artery (Chen *et al.*, 1991; Garland & McPherson, 1992).

However, pronounced smooth muscle hyperpolarization and relaxation was demonstrated to exogenous nitric oxide in the guinea-pig uterine artery (Tare *et al.*, 1990a) and the rat small mesenteric artery (Garland & McPherson, 1992). In addition, Tare and co-workers were able to reduce both the hyperpolarization and relaxation to acetylcholine with the nitric oxide synthase inhibitor, L-N^G-monomethyl arginine (L-NMMA), indicating that nitric oxide may contribute to both the hyperpolarization and relaxation induced by this agonist.

The present study investigated the role of nitric oxide in the smooth muscle relaxation and hyperpolarization to muscarinic stimulation in the rabbit basilar artery. Some of these results have been communicated in a preliminary form (Rand & Garland, 1991a,b).

Methods

White rabbits of either sex (2–3 kg) were anaesthetized with i.v. sodium pentobarbitone (60 mg kg^{-1}) and killed by rapid exsanguination. The brain was removed and placed in physiological salt solution (PSS) at room temperature. The basilar artery was carefully removed and cut into cylindrical

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segments 1–2 mm in length. Segments were then mounted in a tissue chamber for recording changes in smooth muscle membrane potential and tension, as previously described (Garland, 1987). Briefly, two tungsten wires (each of 25 μm diameter) were passed through the segment's lumen and each wire attached to a small plastic foot. One foot was coupled to an isometric force transducer (Harvard Biosciences, 52-9529) and the other to a microdrive (Prior, code 71). The segment was stretched between the wires, under a previously determined optimal pre-load of 500 mg and superfused (at 1–3 ml min^{-1}) with PSS which had been gassed with 95% O_2 + 5% CO_2 and warmed to 36°C. Concentration-response curves were constructed from responses to single concentrations of agonist. Both agonists, antagonists and enzyme inhibitors were equilibrated with the superfusate before it entered the tissue chamber. In the case of nitric oxide gas, PSS containing the gas (usually 200 μl) was injected close to the artery segment from a gas-tight syringe. In some experiments, endothelial cells were destroyed by carefully passing a blunt syringe needle (25 gauge) through the artery lumen, and then cutting and mounting segments as described above. The success of this manoeuvre was confirmed by a loss of relaxation and, in some cases, hyperpolarization to acetylcholine. In addition, in a number of experiments, the loss of the endothelium was confirmed histologically by staining with haematoxylin and eosin.

Electrophysiology

Measurement of smooth muscle membrane potential was made with a glass microelectrode, advanced through the adventitial surface of the artery segment. The electrodes were filled with 2 M KCl and had resistances of 80–120 MΩ. Membrane electrical events were recorded through a high-input impedance d.c. preamplifier (Neurology 102G) and digitized, together with data from the isometric transducer, and stored on disc.

Solutions and drugs

Experiments were made in physiological salt solution (PSS) of 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. At the end of an experiment contraction was stimulated with 100 mM K-PSS. This solution was prepared by direct replacement of NaCl with KCl.

Drugs used were acetylcholine chloride (BDH); noradrenaline bitartrate (arterenol, Sigma); atropine sulphate (Sigma); carbamylcholine chloride (carbachol, Sigma); histamine acid phosphate (Sigma); D-monomethyl-L-arginine and N^G-monomethyl-L-arginine (gifts from Dr H. Hodson, Wellcome Foundation); N^G-nitro L-arginine methyl ester and sodium nitroprusside (both Sigma).

Preparation of nitric oxide solution

Nitric oxide (BDH) was injected, with a gas tight syringe, into PSS which had been bubbled with research grade helium (BOC) for 45–60 min. The PSS contained an appropriate concentration of noradrenaline, in order to prevent local dilution artifacts during injection. Nitric oxide solution was then injected into the tissue chamber in volumes of 100–200 μl, with a gas tight syringe. Control injections of helium gassed PSS, containing noradrenaline, were always performed to assess the extent of potential injection artifacts.

Analysis of data

Relaxations are expressed as a percentage decrease in the initial tone to noradrenaline, and in some cases histamine. 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.

Results

Endothelium-dependent responses to acetylcholine

Acetylcholine (0.01–100 μM) hyperpolarized smooth muscle cells in the basilar artery, when applied either in the presence or absence of noradrenaline to depolarize and contract the muscle cells. Figure 1 summarizes the relaxant and hyperpolarizing responses obtained in the presence of noradrenaline, which was applied in variable concentrations (between 10 and 100 μM) in order to stimulate similar-sized contraction and depolarization in each experiment. Acetylcholine was applied against a mean background contraction and depolarization of 2.5 ± 0.3 mN and 6 ± 0.5 mV ($n = 53$), respectively. Threshold relaxation was obtained with 0.1 μM acetylcholine, and relaxation increased with increasing concentrations of acetylcholine, until contraction was totally reversed in the presence of 1 mM acetylcholine. Hyperpolarization, in contrast, was only recorded with acetylcholine concentrations in excess of 1 μM, and increased to 10 ± 1.0 mV in the presence of 1 mM acetylcholine. Hyperpolarization was not observed in artery segments after the endothelium had been rubbed, which also markedly reduced acetylcholine-induced relaxation (> 70%).

In the continued presence of acetylcholine, smooth muscle relaxation was sustained, whereas the membrane hyperpolarization was relatively transient. Although smooth muscle hyperpolarization was always initiated (1–9 s) before relaxation, the membrane potential had returned to pre-stimulation levels within approximately 10–15 min (742 ± 106 s, mean ± s.e. mean; $n = 5$) Figure 2a.

In addition, smooth muscle hyperpolarization, but not relaxation, desensitized rapidly with repeated application of acetylcholine. Figure 2b illustrates this point. Exposure to 100 μM acetylcholine was associated with a mean reversal of noradrenaline-induced contraction by 93 ± 2% and a hyperpolarization of 8.5 ± 1.0 mV ($n = 5$ paired observations in each case). After 60 min wash-out, re-exposure to the same concentration of acetylcholine stimulated relaxation of a similar size and rate (90% reversal of contraction), but now the hyperpolarization was significantly reduced, 1.9 ± 0.42 mV ($P < 0.001$; $n = 9$). Subsequent exposure to acetylcholine every 60 min, over a period of 2–3 h, was followed by relaxation which again developed at the same rate and had the same amplitude as the first response. However, after the second exposure to acetylcholine, no hyperpolarization was detected, and hyperpolarization did not return during the period of the experiment.

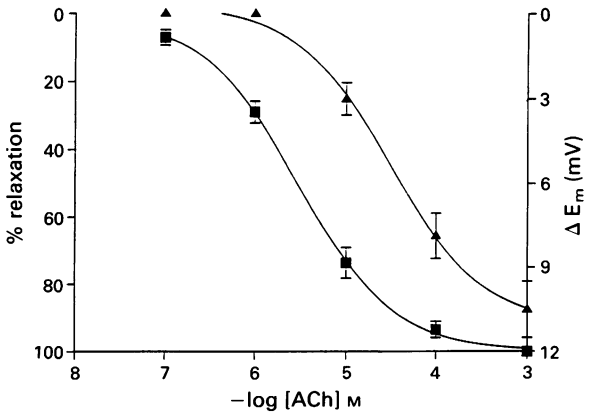

Figure 1 Mean concentration-response curves to acetylcholine in the rabbit basilar artery. Points show the mean hyperpolarization (▲) and relaxation (■) recorded from arteries preconstricted with noradrenaline. Points are the mean from at least five separate experiments; s.e. mean shown by vertical bars.

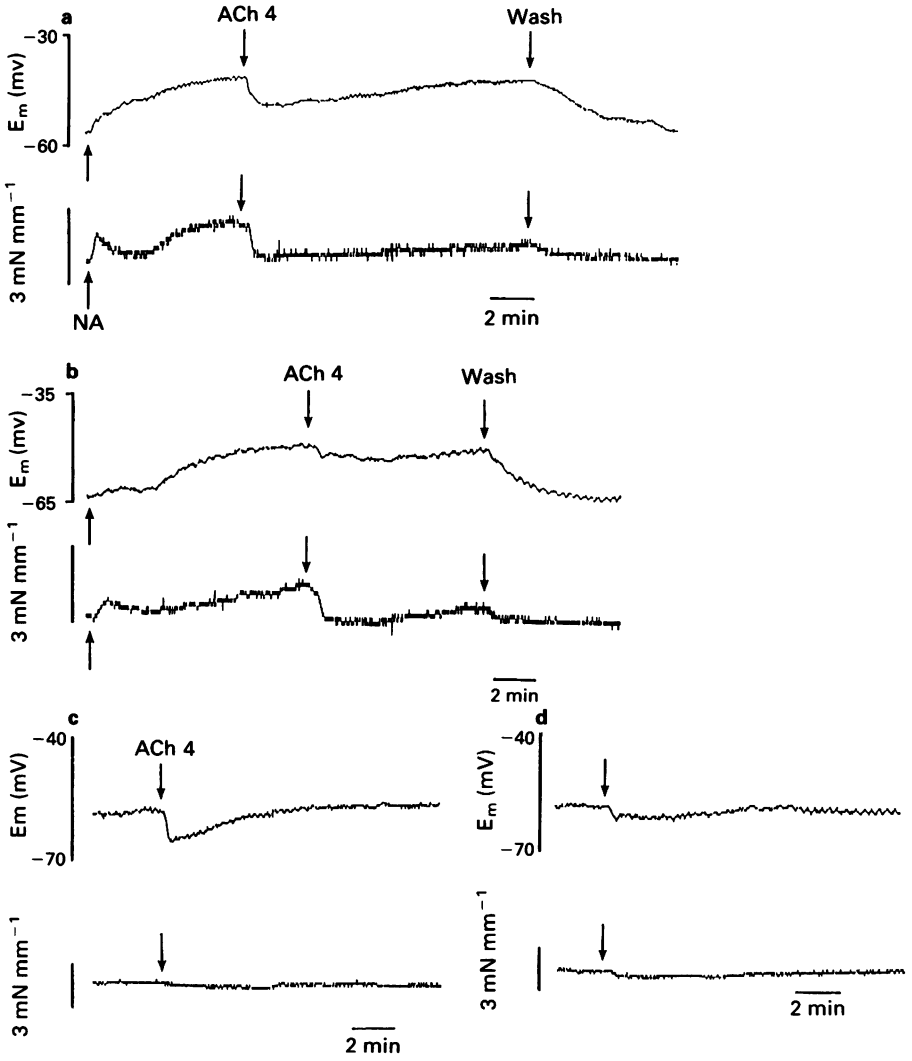

Figure 2 (a,b,c,d) Representative trace showing the response to acetylcholine (ACh) in the presence (a,b) and absence (c,d) of noradrenaline (NA) to induce contraction. Acetylcholine (-log M) hyperpolarized and relaxed smooth muscle in the basilar artery (a), when reapplied to the same artery 60 min later (b), the hyperpolarization, but not the relaxation, was markedly depressed. In the absence of contraction, acetylcholine hyperpolarized the smooth muscle cell (c), and this hyperpolarization was also depressed when acetylcholine was reapplied to the cell 60 min later (d).

Similar membrane responses were observed in the absence of prior smooth muscle stimulation. Acetylcholine (100 μM) stimulated hyperpolarization which had a similar amplitude range (7–8.5 mV in 2 separate experiments) and time course to the responses obtained in the presence of noradrenaline (Figure 2c). Again, desensitization of the hyperpolarization was marked after a single exposure to acetylcholine (Figure 2d).

Effect of carbachol and atropine

Carbachol (0.01–100 μM) produced endothelium-dependent smooth muscle hyperpolarization and relaxation which was very similar to the response obtained with acetylcholine. The hyperpolarization to 100 μM carbachol was of similar amplitude (6.3 ± 0.6 mV, $n = 5$) to that observed with an equivalent concentration of acetylcholine (8.5 ± 1.0 mV, $n = 5$). Hyperpolarization also preceded the onset of relaxation by a similar time (3 ± 0.7 vs 4 ± 1.0 s, $n = 5$), took a similar period to reach maximum (33.4 ± 9.0 vs 38.0 ± 3.0 s, $n = 5$ separate experiments in each case), and was reduced to a similar level after an initial exposure to carbachol (2.1 ± 1.0 vs 2.0 ± 0.4 mV, $n = 5$ and 9, respectively). However, in the continued presence of carbachol, the membrane potential returned to pre-stimulation levels more rapidly than was the case with acetylcholine (382.0 ± 42.0 s; range 292–514 s, $n = 5$). The relaxant potency of carbachol was slightly less than that of acetylcholine (EC_{50} values 14 μM and 2.7 μM, respectively), but both agents induced maximal relaxation.

Atropine (0.1 μM) reversibly blocked the smooth muscle relaxation to acetylcholine, the mechanical effects of 10 μM acetylcholine being reduced from 60% to 8%, while those to 100 μM acetylcholine were reduced from 86% to 27% (each $n = 5$). Hyperpolarization to 100 μM acetylcholine was totally abolished by the presence of atropine ($n = 5$).

Effects of L-NMMA and L-NAME

Competitive substrate inhibitors for nitric oxide synthase reduced both the hyperpolarization and the relaxation to acetylcholine. However, the extent of the observed inhibition was variable (Table 1). In some cases, only a minimal hyperpolarization and relaxation remained (group1, Table 1), while in other tissues the form of the hyperpolarization became biphasic, while relaxation was only minimally reduced (group 2, Table 1). These effects are illustrated in Figure 3. Apart from the extent of the inhibition of responses to acetylcholine, the only other apparent difference between the two groups of tissues was in the size of the additional contraction produced by the synthase inhibitors. Significant blockade of

Table 1 The effects of L-N^G-monomethyl arginine (L-NMMA) and L-N^G-nitro arginine methyl ester (L-NAME) on the endothelium-dependent hyperpolarization and relaxation of the rabbit basilar artery to acetylcholine

	Group I			*Group II*	
	L-NMMA		*L-NAME*	*L-NMMA*	
	10^{-5}M	10^{-4}M	10^{-4}M	10^{-5}M	10^{-4}M
	$n=6$	$n=5$	$n=4$	$n=3$	$n=4$
Resting membrane potential (mV)	59 ± 5	64 ± 5	56 ± 2	61 ± 4	54 ± 3
Contraction to noradrenaline (mN)	4.6 ± 2.0	4.1 ± 1.6	3.5 ± 1.3	3.4 ± 2.5	3.8 ± 2.5
Depolarization to noradrenaline (mV)	13 ± 5.0	17 ± 7.0	8 ± 1.0	12 ± 3.0	7 ± 1.0
Contraction:inhibitor + noradrenaline (mN)	9.4 ± 1.8	16.5 ± 3.1	15.0 ± 1.9	6.1 ± 3.8	8.8 ± 4.0
Additional depolarization (mV)	2 ± 1.0	3 ± 0.9	3.1 ± 1.3	2 ± 1.0	2 ± 0.8
% relaxation to 100 μM acetylcholine	38 ± 6	37 ± 5	32 ± 3	90 ± 10	91 ± 6
Hyperpolarization to 100 μM acetylcholine	3 ± 1.0	2.5 ± 0.5	3.1 ± 1.0	7.5 ± 1.5	9 ± 1.4
Contraction to 100 mM K^+ (mN)	27.1 ± 2.7	29.3 ± 3.1	29.8 ± 3.0	31 ± 2.0	28.3 ± 1.9

Each value is the mean derived from the stated number (n) of observations ± s.e. mean.

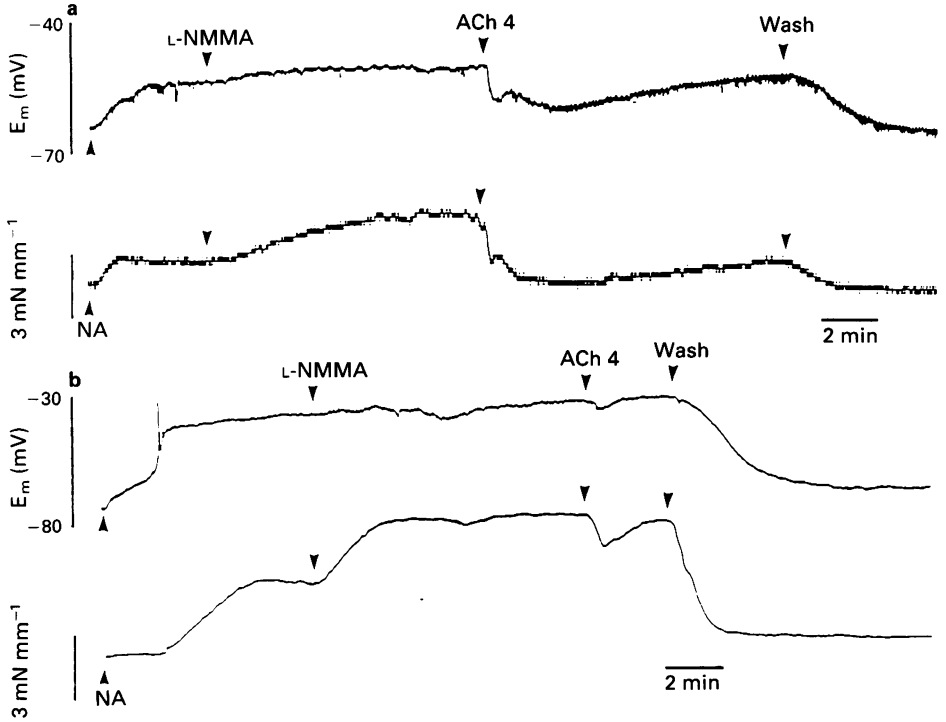

Figure 3 Representative traces showing the different responses obtained in the presence of the nitric oxide synthase inhibitor L-N^G-monomethyl arginine (L-NMMA, 100 μM) with smooth muscle depolarization and contraction induced with noradrenaline (NA). In a small number of arteries, the hyperpolarization and relaxation to acetylcholine (ACh, -log M) had a similar magnitude to control (Figure 2a), but both responses became biphasic (a). In the majority of arteries tested, both the hyperpolarization and the relaxation to acetylcholine were markedly reduced following the application of L-NMMA (b).

the responses to acetylcholine was associated with a large additional tissue contraction to either L-NMMA or L-NAME (group 1). This phenomenon is, however, unlikely to underlie the differences in the extent of the inhibition of responses to acetylcholine by the nitric oxide synthase inhibitors, for two reasons. First, both groups developed a similar contraction when subsequently stimulated with 100 mM K^+-PSS, indicating that the smaller responses to L-NMMA in group 2 did not reflect damaged tissue. Second, the larger contractions produced by L-NMMA and L-NAME in group 1 would

not in themselves reduce the subsequent responses to acetylcholine, as in control experiments acetylcholine produced >90% relaxation in artery segments contracted to between 40 and 60% of the response to 100 mM K^+-PSS, with either histamine ($n = 15$) or noradrenaline ($n = 6$).

D-NMMA (100 μM) did not alter the tension or the membrane potential in artery segments pre-contracted with noradrenaline, nor did it modify the subsequent relaxation and hyperpolarization to acetylcholine ($n = 3$).

Membrane and tension responses to nitric oxide

Nitric oxide, applied either as a gas in solution or via the application of sodium nitroprusside, stimulated endothelium-independent relaxation in segments of basilar artery contracted with noradrenaline. Over the concentration range 0.1 μM–100 μM, sodium nitroprusside produced sustained relaxation, which reversed the noradrenaline-induced contraction by 92.6 ± 1.4% ($n = 6$). Nitric oxide gas, injected close to the tissue, initiated a transient relaxation. At the highest concentration tested (15 μM), nitric oxide reversed the noradrenaline-induced contraction by 74.7 ± 6.1% ($n = 8$), a response which decayed within 20 s. The EC_{50} values for sodium nitroprusside and nitric oxide gas were not different (3 ± 0.9 vs 2.2 ± 0.8 μM; Figure 4a).

Membrane hyperpolarization was only recorded with high concentrations of nitric oxide (15 μM), concentrations which were associated with almost maximal tissue relaxation. Hyperpolarizations with an amplitude of 3 ± 0.3 mV were stimulated by 100 μM sodium nitroprusside applied to smooth muscle with a membrane potential of 43.5 ± 1.7 mV and contracted by 3.8 ± 1.0 mN in the presence of noradrenaline ($n = 5$). The hyperpolarizations were reproducible (up to 3 determinations), slow to peak and lagged behind the onset of smooth muscle relaxation by around 15 s. Larger hyperpolarizations could be induced with 10 mM sodium nitroprusside, which produced a slow, but reproducible hyperpolarization of 19 ± 2 mV and which totally relaxed the arterial segments ($n = 3$). No cross-desensitization of either hyperpolarization or relaxation was observed between acetylcholine and sodium nitroprusside. This was the case whether sodium nitroprusside was applied before or after acetylcholine ($n = 3$).

The highest concentration of nitric oxide gas tested (15 μM), produced only a weak hyperpolarization of 2.2 ± 0.4 mV ($n = 5$). As with sodium nitroprusside, the hyperpolarizations could be reproduced (at least three applications), and were associated with marked smooth muscle relaxation Figure 4b. If nitric oxide was applied to unstimulated smooth muscle cells (membrane potential of 61 ± 7 mV; $n = 4$), hyperpolarizations were recorded which had a similar size to those observed in the presence of noradrenaline.

Discussion

These data indicate very clearly that the membrane hyperpolarization which is induced by muscarinic stimulation in the rabbit basilar artery, does not make an important contribution to the concurrent smooth muscle relaxation. The most direct evidence for this statement comes from the experiments involving repeated application of acetylcholine. After three exposures to acetylcholine, membrane hyperpolarization was completely abolished, but the relaxation was unaltered, both in the amplitude attained and the rate at which it developed. A further indication that membrane hyperpolarization is not required for smooth muscle relaxation to acetylcholine in the basilar artery, comes from the absence of a correlation between the concentration of acetylcholine required for hyperpolarization and relaxation, and from the time course for both of these responses. In the latter, the membrane potential returned to prestimulation levels in the continued presence of either acetylcholine or carbachol, while relaxation was sustained.

Overall, these observations contrast with other arteries, in that they argue against an important role for smooth muscle hyperpolarization in relaxation. For example, in the aorta and the pulmonary and mesenteric arteries from the rat, there is a close correlation between acetylcholine-induced smooth muscle hyperpolarization and relaxation (Taylor *et al.*, 1988; Chen & Suzuki, 1989; Garland & McPherson, 1992). As marked smooth muscle relaxation follows membrane hyperpolarization, either induced by current injection (Makata, 1986) or by potassium channel openers (Bray *et al.*, 1987; Nakao *et al.*, 1988; McPherson & Angus, 1990), hyperpolarization to acetylcholine will also provide an important drive to relaxation. This concept is supported by the finding that smooth muscle hyperpolarization to acetylcholine, which reflects an increase in membrane potassium conductance, can be abolished in the presence of raised extracellular potassium (Chen & Suzuki, 1989). Raised extracellular potassium, in addition to abolishing hyperpolarization, also blocked relaxation by 30–50% (Taylor & Weston, 1988; Nagao & Vanhoutte, 1991). The fact that both the smooth muscle hyperpolarization and the relaxation were suppressed, is a further indication that both events are causally linked. Extrapolating from this evidence to the basilar artery, hyperpolarization would most probably cause relaxation were it not for the fact that it is not induced, except with high concentrations of acetylcholine, concentrations which stimulate maximal relaxation. At this stage, relaxation driven by a parallel process, presumably the activation of guanylyl cyclase, will mask any influence exerted by hyperpolarization.

The majority of the evidence available from other arteries, suggests that the hyperpolarization observed in response to cholinomimetics is not stimulated by nitric oxide. This assertion is based on a number of observations, including the fact that endothelium-dependent smooth muscle relaxation can be blocked independently of hyperpolarization, with either methylene blue (blocks guanylyl cylase which is activated by nitric oxide) or oxyhaemoglobin (binds and inactivates nitric oxide). Also, Chen *et al.* (1991) demonstrated that the relaxation, but not the hyperpolarization to acetylcholine in the guinea-pig coronary artery, could be significantly reduced by the nitric oxide synthase inhibitor, nitroarginine; while a number of studies, including the present investigation, have failed to show any significant hyperpolarization in response to exogenous nitric oxide concentrations which stimulate maximal smooth muscle relaxation (Komori *et al.*, 1988; Huang *et al.*, 1988; Brayden, 1990). Although these observations indicated that nitric oxide had very little effect on smooth muscle membrane potential, recent work has clearly demonstrated hyperpolarization to exogenous nitric oxide in vessels from a number of different species (Tare *et al.*, 1990a, b; Garland & McPherson, 1991; 1992). This raises the possibility that, at least in some vessels, nitric oxide may contribute to the acetylcholine-induced hyperpolarization, and hence to smooth muscle relaxation. In the guinea-pig uterine artery, this does seem to be the case, as both the hyperpolarization and relaxation in response to acetylcholine were reduced with the nitric oxide synthase inhibitor, L-NMMA (Tare *et al.*, 1990a).

However, the ability of nitric oxide to induce smooth muscle hyperpolarization, and thereby contribute to relaxation, is not a general property of the endothelium-dependent responses of arteries to acetylcholine. A variation between arteries was demonstrated by observations on the rat mesenteric artery, where the hyperpolarizations to either acetylcholine or nitric oxide were shown to have very different characteristics. Principally, the nitric oxide-induced hyperpolarization could be blocked, either by prior membrane depolarization or with glibenclamide, whereas depolarization increased the amplitude of the hyperpolarization to acetylcholine, and hyperpolarization was not modified in the presence of glibenclamide (McPherson & Angus, 1991;

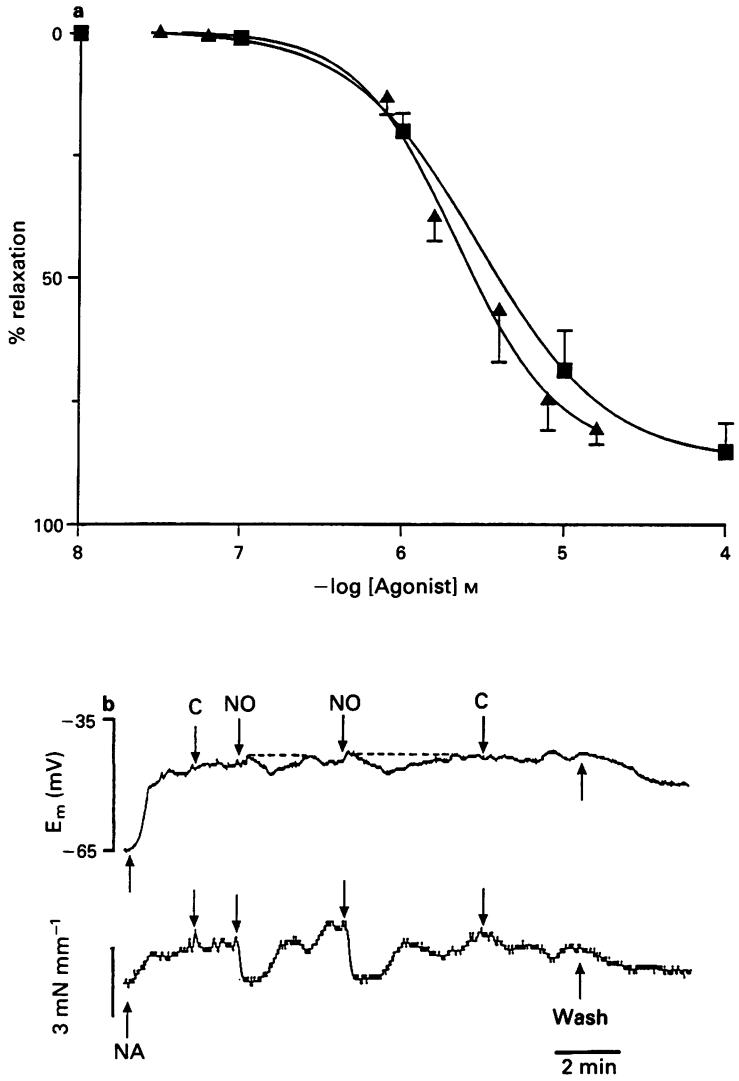

Figure 4 (a) Mean concentration-relaxation curve for the action of sodium nitroprusside (■, $n = 6$) and nitric oxide gas (▲, $n = 8$) against contraction to noradrenaline in the rabbit basilar artery. Points show means with s.e. mean indicated by vertical bars. (b) Representative trace showing a simultaneous record of membrane potential change and relaxation to nitric oxide gas, injected in PSS, in a basilar artery stimulated with noradrenaline (NA, 100 μM). Injection of PSS alone (200 μl, C) did not modify the membrane potential or the tension, while PSS-containing nitric oxide (200 μl of a 15 μM NO solution) reversed contraction and induced a small, reproducible hyperpolarization.

Garland & McPherson, 1992).

It may be the case that high concentrations of nitric oxide can induce hyperpolarization in most blood vessels, as suggested by Tare *et al.* (1990a), but that a separate endothelium-derived hyperpolarizing factor also contributes to a variable extent in different blood vessels. A general failure to demonstrate hyperpolarization to exogenous nitric oxide, may then reflect the concentration applied and/or the method of application. The concentration of exogenous nitric oxide which is available to stimulate smooth muscle cells will be continually decreasing as nitric oxide is oxidised. So while the available concentration of nitric oxide is still sufficient to stimulate marked relaxation, it may have fallen below the threshold for activating membrane hyperpolarization.

In the present study, the highest concentration of nitric oxide gas tested was equivalent to adding around 0.1 μmol to the tissue chamber. This amount was only sufficient to induce a small hyperpolarization of about 2 mV and is close to the threshold for hyperpolarization (3 mV increase in potential) to nitric oxide in the rat mesenteric artery, in which tissue 1 μmol was required to increase the membrane potential by nearly 10 mV (Garland & McPherson, 1992). If nitric oxide and EDHF do make a variable contribution to the hyperpolarization in different arteries, this may then partly explain the somewhat variable hyperpolarizations to acetylcholine which have been recorded in arteries. For example, in the rabbit ear and femoral artery (Suzuki, 1988; Huang *et al.*, 1988), the rat pulmonary (Chen *et al.*, 1988) and the canine mesenteric arteries (Komori *et al.*, 1988) acetylcholine induces only a small, transient hyperpolarization, whereas in the rat mesenteric artery (McPherson & Angus, 1991), feline middle cerebral artery (Brayden & Wellman, 1989), canine coronary artery (Chen *et al.*, 1989) and the guinea-pig basilar artery (Nishiye *et al.*, 1989) large hyperpolarizations of as much as 25 mV have been reported.

If this hypothesis is correct, the rabbit basilar artery may represent an artery which releases only nitric oxide in response to acetylcholine. Exposure to a high concentration of acetylcholine will release enough nitric oxide to stimulate marked smooth muscle relaxation and hyperpolarization, with both responses susceptible to the blocking action of nitric oxide synthase inhibitors. Acetylcholine-induced relaxation and hyperpolarization were blocked or modified by the nitric oxide synthase inhibitors in this tissue, although the action of L-NMMA was somewhat variable, when compared with the blocking action of L-NAME. The reason for the

relatively variable effect of L-NMMA is not clear, but this may be related to the ability of nitric oxide synthase to utilize L-NMMA, but not nitro-arginine, as a substrate (Hecker *et al.*, 1990). If the release of nitric oxide from endothelial cells in the basilar artery is slightly reduced on a second exposure to acetylcholine, the nitric oxide concentration may be sufficient to stimulate maximal smooth muscle relaxation, but the concentration fall below the threshold for hyperpolarization. Feedback control of the release of nitric oxide has been shown in other studies. Nitric oxide can increase the concentration of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in endothelial cells, and elevated levels of cyclic GMP may be responsible for the reduced release of EDRF from endothelial cells in culture, when they are stimualted with atrial natriuretic peptide (Busse *et al.*, 1988; Boulanger *et al.*, 1990).

In contrast to the rabbit basilar artery, vessels such as the rat small mesenteric artery, may represent arteries which release only, or predominantly EDHF. In the mesenteric artery, acetylcholine stimulates marked and reproducible endothelium-dependent hyperpolarization, which is mediated by membrane mechanisms different from those responsible for the hyperpolarization induced with exogenous nitric oxide. In such a tissue, hyperpolarization to acetylcholine is closely linked to, and probably largely responsible for the smooth muscle relaxation. Furthermore, both the hyperpolarization and the relaxation to acetylcholine are resistant to the blocking action of L-nitroarginine, oxyhaemoglobin and glibenclamide (Garland & McPherson, 1992).

In summary, muscarinic stimulation of the rabbit basilar artery is followed by smooth muscle hyperpolarization and relaxation. Both events appear to be mediated by the endothelium-dependent release of nitric oxide, or a related substance. In contrast to other blood vessels, the smooth muscle hyperpolarization appears to make a negligible contribution to relaxation. In the context of studies on other vessels, this raises the intriguing possibility that nitric oxide and a separate EDHF may vary in their contribution to the endothelium-dependent relaxation which occurs in different parts of the circulation.

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