Br. J. Pharmacol. (1992), **105**, 429–435

Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery

[1]Christopher J. Garland & [2]Grant A. McPherson

Baker Medical Research Institute, Commercial Rd, Prahran, 3181 Victoria, Australia

1 Acetylcholine caused a concentration-dependent smooth muscle hyperpolarization and relaxation in rat small mesenteric arteries (diameter at 100 mmHg 250–450 mm) stimulated with noradrenaline (3 μM).

2 Nitric oxide (NO), generated from either NO-gas or from acidified sodium nitrite, also induced smooth muscle hyperpolarization but only in the absence of active force. However, unlike the hyperpolarizations to acetylcholine, those to NO were abolished either by prior smooth muscle depolarization caused by noradrenaline, or by the K^+ channel blocker, glibenclamide (3 μM).

3 Hyperpolarization and relaxation to acetylcholine were unaffected by prior exposure of the mesenteric artery to either the cyclo-oxygenase inhibitor, indomethacin (10 μM), or the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NNA, 100 μM).

4 Haemoglobin (1.5 μM), which binds and inactivates NO, blocked the hyperpolarizing and vasorelaxant response to NO, but did not alter either response to acetylcholine.

5 These data show that, in the rat small mesenteric artery, membrane hyperpolarizations to NO and acetylcholine are mediated by different mechanisms, and that the hyperpolarization induced by NO is not involved in the responses to acetylcholine. In addition, they provide evidence that the acetylcholine responses in this artery, which are endothelium-dependent, are not mediated by the release of NO.

Keywords: Acetylcholine hyperpolarization; endothelium-derived hyperpolarizing factor (EDHF); rat small mesenteric artery; K^+ channels; nitric oxide

Introduction

Acetylcholine and related cholinomimetics cause both vasorelaxation and hyperpolarization in vascular smooth muscle cells by an endothelium-dependent mechanism (Bolton *et al.*, 1984; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; McPherson & Angus, 1991; Rand & Garland, 1991). Smooth muscle relaxation follows the stimulation of soluble guanylate cyclase by a diffusible factor which is released from endothelial cells (EDRF; see Angus & Cocks, 1989). EDRF is thought to be nitric oxide (NO) or a derivative, as the relaxation to acetylcholine is blocked in the presence of compounds such as haemoglobin, which 'capture' NO (Angus & Cocks, 1989). Hyperpolarization to acetylcholine also appears to be mediated, at least in part, by the release of a diffusible factor (Feletou & Vanhoutte, 1988). However, in contrast to the relaxation induced by acetylcholine, hyperpolarization is not blocked by either oxyhaemoglobin or methylene blue, which has led to the suggestion that a factor(s) distinct from NO is also released from the endothelium. This factor(s) has been termed endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.*, 1988; Huang *et al.*, 1988; Taylor & Weston, 1988).

The idea that acetylcholine-induced hyperpolarization reflects an endothelial cell action distinct from the release of NO, is supported by the failure to demonstrate hyperpolarization to NO in rabbit cerebral and canine coronary arteries (Brayden, 1990; Komori *et al.*, 1988). However, this is not a consistent observation, as Tare *et al.* (1990) have demonstrated a clear hyperpolarization and relaxation to NO in uterine arteries. These data suggest an important role for NO in both membrane and tension responses of the uterine artery to acetylcholine.

To date, the majority of studies indicate that an increase in membrane permeability to K^+ is responsible for the acetylcholine-induced hyperpolarization, although the type of K^+-channel involved is not clear (see Longmore & Weston, 1990). Standen *et al.* (1989) showed in the rabbit middle cerebral artery, that the hyperpolarizations to acetylcholine, and a number of other vasodilators, were sensitive to glibenclamide, a compound which they showed could inhibit ATP-sensitive K^+ channels opened by cromakalim in vascular smooth muscle cells. However, in isolated small mesenteric arteries from the rat we have recently shown (McPherson & Angus, 1991) that acetylcholine causes a marked glibenclamide-insensitive, endothelium-dependent hyperpolarization and relaxation, suggesting different mechanisms of action of acetylcholine in the two vascular segments.

The purpose of the present study was to investigate the possibility that the release of NO from endothelial cells was responsible for the smooth muscle hyperpolarization to acetylcholine in the rat small mesenteric artery. We showed that NO can produce hyperpolarization, but the mechanism underlying this hyperpolarization is different from that in response to acetylcholine. Further, the experiments indicate that NO does not contribute to either the acetylcholine-induced hyperpolarization or relaxation in this artery.

Methods

Isolation of resistance blood vessels

Wistar Kyoto (WKY) rat were killed by CO_2 asphyxia. The mesentery was rapidly removed and placed in ice cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) gassed with 5% CO_2 in O_2. A segment of rat small mesenteric artery, 2 mm in length, corresponding to a third to fourth order branch from the superior mesenteric artery, was mounted in a small vessel myograph as previously described (Angus *et al.*, 1988). Briefly, two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer, while the other was attached to an isometric force transducer. Data were recorded on a dual-channel flat bed recorder

[1] Permanent address, Department of Physiology and Pharmacology, University of Southampton, Southampton SO9 3TU, U.K.
[2] Author for correspondence.

(W&W Scientific Instruments, model 320) and on computer disk. Vessels were allowed to equilibrate under zero force for 30 min. Using the diameter of the vessel, calculated from the distance between the two mounting wires, a passive diameter-tension curve was constructed as previously described (Mulvany & Halpern, 1977). From this curve the effective transmural pressure was calculated. The vessel was set at a tension equivalent to that 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 by use of custom written programmes for the IBM PC (NORMALIZE, GA McPherson) which uses the Marquart-Levenberg modification of the Gauss-Newton technique (McPherson, 1985). Vessel diameters at an equivalent transmural pressure of 100 mmHg (D_{100}) are given in the text.

Electrophysiology

In experiments where the intracellular membrane potential was monitored, the vessel was mounted as described above. A conventional glass electrode (1 mm blanks, World Precision Instruments Inc., New Haven, U.S.A.) filled with 0.5 M KCl (tip resistance approximately 100 MΩ) was used to impale a single smooth muscle cell. The microelectrode was positioned by a Burleigh Inchworm motor driven by a 6000 series controller (Burleigh, U.S.A.). The microelectrode was advanced in 0.5 μm steps until a stable impalement was achieved.

The bath containing the vessel (7 ml volume) was part of a 25 ml recirculating system which contained a jacketed organ bath, where the Krebs solution was warmed and oxygenated. Drugs could also be added at this site. This design allowed cumulative steady-state concentration-effect curves to be constructed when assessing the electrophysiological effects of acetylcholine. In some experiments the vessel was activated with a sub-maximal concentration of noradrenaline (1–3 μM). In these experiments changes in membrane potential and active tension were recorded simultaneously. Concentration-relaxation curves to acetylcholine showed a time dependent decrease in sensitivity of about 3 fold between the first and second curves. Subsequent curves (up to four) could be superimposed on the second. The second concentration-response curve to acetylcholine was therefore used as the control response in all studies.

NO containing solutions were prepared from either NO gas or from acidified sodium nitrite. In the text these are referred to as NO_g and NO_a respectively (see later). In view of the unstable nature of NO, quantities of either were added to the inlet port of the tissue bath from a gas tight syringe. The amount added was expressed in terms of μmol injected in the port. In some experiments infusions (Terfusion STC-521 syringe pump) of NO_a were made into the inlet port of the bath to simulate a steady-state application of the compound.

Data collection and analysis

Force and membrane potential data were captured by use of a programme, DIGISCOPE (GA McPherson), custom written for the IBM PC. This programme uses a DASH16 A/D card (Metrabyte, U.S.A.) which collected and displayed data at 200 Hz. Data were saved on hard disk and reproduced on a Hewlett Packard 7470A plotter.

Concentration-effect curves were analysed by graphical procedures. Results in the text are the mean ± s.e.mean for the specified number of experiments.

Drugs

The following drugs were used: acetylcholine bromide, haemoglobin, (−)-noradrenaline (+)-bitartrate (arterenol), N^G-nitro-L-arginine (Sigma); glibenclamide (Hoechst); sodium nitrite (BDH, Analar grade). Nitric oxide and helium (research grade) were both purchased from CIG Australia.

Nitric oxide stock solutions were prepared by injecting NO gas into sealed vials containing cold distilled water which had been degassed (30 min) with helium (Palmer *et al.*, 1987). Subsequent dilutions were made in sealed vials with a gas-tight syringe. NO gas was injected into the stock solutions in quantities sufficient to produce a saturated solution based on the solubility constant for NO in water at 1 ATM and 0°C (7.4 ml/100 ml; Tracey *et al.*, 1990). NO solutions were also prepared from acidified sodium nitrite as previously described (Cocks & Angus, 1990). A 100 mM solution of sodium nitrite was prepared in a sealed container in ice cold acidified (pH less than 2 with HCl) distilled water. The resultant solution was allowed to stand for 10 min. Dilutions were made with the same acidified distilled water solution. In this case the concentration of NO was assumed to be equivalent to the final concentration of sodium nitrite in the solution.

Oxyhaemoglobin was prepared by reducing methaemoglobin in the presence of sodium dithionite, and then separating and collecting oxyhaemoglobin on a Sephadex column. Oxyhaemoglobin was then quantified spectrophotometrically, separated into aliquots and stored until required (for up to 14 days) at −20°C (Martin *et al.*, 1985).

Results

Electrophysiological and tension responses to acetylcholine and nitric oxide (NO)

Acetylcholine Smooth muscle cells in the rat small mesenteric artery (D_{100} = 337 ± 10 mm, n = 22) had a resting membrane potential of −57.0 ± 1.2 mV (n = 42 cells, 16 different vessels). In the absence of active tone, acetylcholine (0.01–10 μM) hyperpolarized these cells towards a membrane potential of −69 ± 1 mV; an effect that was not sensitive to the presence of glibenclamide (3 μM) (Figure 1). Noradrenaline (3 μM) caused membrane depolarization to −35 ± 2 mV (n = 12 cells) in association with an increase in active force. Figure 1 also shows simultaneous traces of changes in tension and membrane potential in response to acetylcholine, in a noradrenaline precontracted vessel (D_{100} = 336 μm). Acetylcholine caused a concentration-dependent hyperpolarization to −67 ± 3 mV in the presence of the highest concentration of acetylcholine used in this study (3 μM), representing hyperpolarization of approximately 30 mV. This hyperpolarization was associated with over 90% reversal of the contractile response. The absolute value for membrane potential attained in the presence of acetylcholine (3 μM) was similar whether

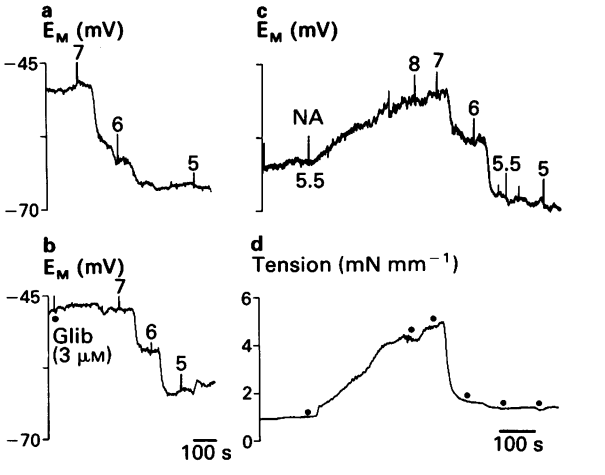

Figure 1 Effect of acetylcholine on rat small mesenteric artery in a non-activated (a,b) and activated vessel. (a,b) Concentration-dependent hyperpolarization to acetylcholine (−log M) in the absence (a) and in the presence (b) of glibenclamide (Glib, 3 μM). (c,d) The effect of acetylcholine on membrane potential (c) and tension development (d) in a vessel preactivated with noradrenaline (NA, 3 μM).

recorded in the absence or in the presence of depolarization induced by noradrenaline (i.e. −69 versus −67 mV respectively).

Nitric oxide In the absence of active tone, bolus applications of NO_g (0.1–1 μmol) caused a transient hyperpolarization of between 3 and 9 mV ($n = 16$) which was dependent on the dose injected into the bath (Figure 2a). A similar effect could be mimicked by NO_a generated from acidified sodium nitrite solution. The hyperpolarization observed with NO_a occurred over a similar concentration-range (0.05–5 μmol) and varied in amplitude to a maximum of 8.5 ± 0.7 mV ($n = 8$) (Figure 2b). The hyperpolarization to NO_g or NO_a was reversibly blocked by glibenclamide (3 μM, Figure 2). If NO_g or NO_a were applied against a background of noradrenaline-induced depolarization greater than approximately 20 mV, hyperpolarization to these compounds did not occur (Figure 3), although there was a pronounced smooth muscle relaxation. Concentration-relaxation curves constructed to either NO_g or NO_a (Figure 3), in the absence and presence of glibenclamide (3 μM), were not significantly different.

Because of the unstable nature of NO, the majority of the studies described involved applying bolus injections close to the vascular preparation. We wished to determine whether similar results where obtained under steady state conditions using a constant infusion of NO. Consequently, studies were repeated with a constant infusion of NO_a (2–16 μmol min^{-1}). Under these conditions NO_a caused a sustained hyperpolarization which, like the more transient responses to bolus injections of NO_a, could be blocked with glibenclamide (3 μM). (Figure 2c).

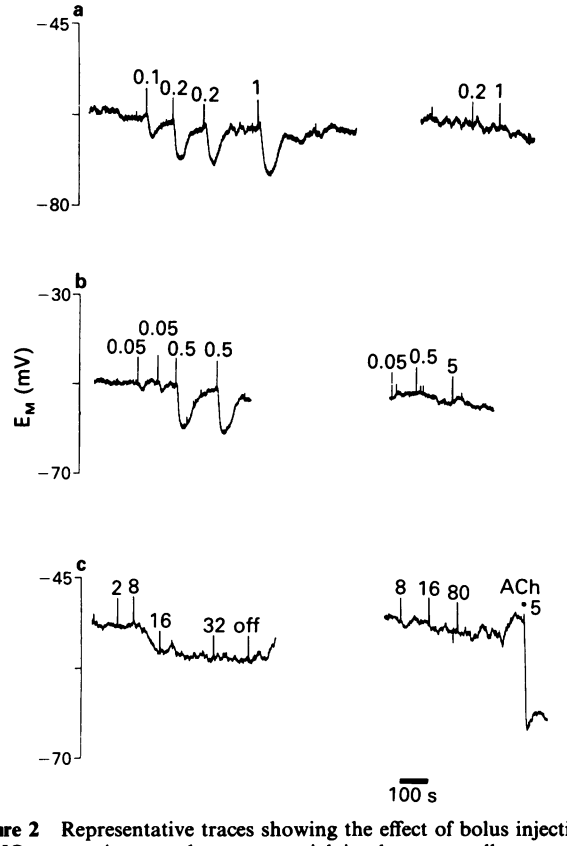

Figure 2 Representative traces showing the effect of bolus injections of NO on resting membrane potential in the rat small mesenteric artery. Responses were obtained in the absence (left side) and presence (right side) of glibenclamide (3 μM). (a) Dose-dependent hyperpolarizations to NO prepared from NO gas (NO_g; μmol). (b) Dose-dependent hyperpolarizations to NO generated from acidified $NaNO_2$ (NO_a; μmol). (c) Hyperpolarization produced by infusions of acidified $NaNO_2$. In this case quantities of NO_a are given as μmol min^{-1}. In this experiment the effect of acetylcholine (ACh 10 μM) was also assessed at the end of the experiment.

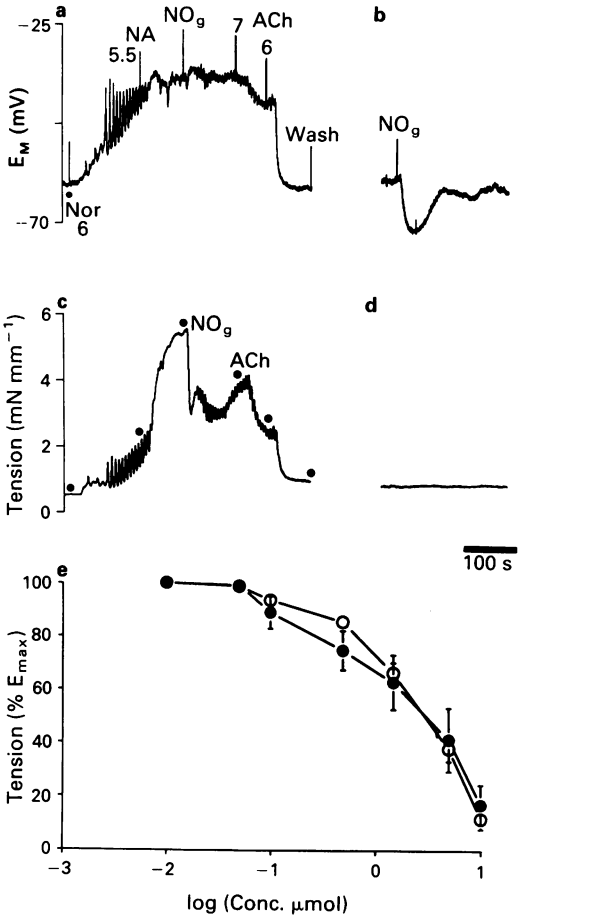

Figure 3 (a,b,c,d) Representative trace showing the responses to NO_g in the absence and presence (a,c) of noradrenaline (NA, 3 μM) to induce active force: (a,b) are the membrane potential and (c,d) tension. In the presence of active tone (a,c) NO_g caused a marked relaxation (c) in the absence of a change in membrane potential (a), while the application of acetylcholine (ACh) was followed by a marked hyperpolarization. Conversely, acetylcholine (−log M) caused a concentration-dependent hyperpolarization and relaxation. In the absence of active force (b,d) NO_g caused a marked hyperpolarizing response (b). (e) Mean dose-relaxation response curves constructed in rat small mesenteric arteries assessing the ability of nitric oxide (NO_a) to cause relaxation of tone induced by noradrenaline (3 μM). Results were obtained in the absence (○) or presence (●) of glibenclamide (3 μM). Results are the mean obtained from 6 separate experiments; s.e.mean shown by vertical bars.

Several control experiments were also performed to insure that the mechanical and electrophysiological effect observed with NO were not the result of the diluents, particularly in the case of NO_a which was made in acidified distilled water. Figure 4 shows the result of one such experiment examining the effect of NO_a on membrane potential. The injection of unacidified sodium nitrite or acidified distilled water were without effect, while bubbling of the acidified sodium nitrite solution (with 5% CO_2 in O_2 for 5 min at room temperature) markedly attenuated the hyperpolarizing response to NO_a (Figure 4).

Effect of N^G-nitro-L-arginine, indomethacin and oxyhaemoglobin on responses to acetylcholine

N^G-nitro-L-arginine (L-NNA) (100 μM) applied 20–60 min before noradrenaline and present during the subsequent application of acetylcholine, did not alter the depolarization to noradrenaline (membrane potential −39 ± 4 mV). Hyperpolarization and relaxation in response to acetylcholine was unaffected by the presence of L-NNA (Figure 5).

Indomethacin (10 μM), applied 10 min before noradrenaline and present throughout the subsequent application of acetyl-

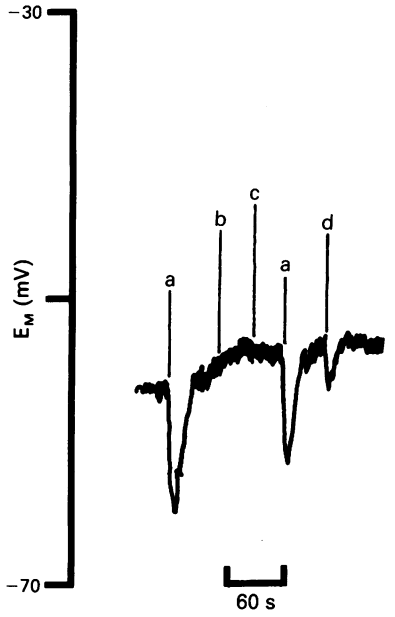

Figure 4 Representative trace showing the effect of diluents used to make the acidified nitrite-derived NO_a. Responses were obtained to acidified nitrite solution (50 μl of a 100 μM stock solution which is equivalent to a dose of 5 μmol) (a), non-acidified nitrite solution (50 μl of a 100 μM stock) (b) and acidified distilled water (50 μl) used to make up the acidified nitrite (c). In addition the response to acidified nitrite (5 μmol), which had been bubbled for 5 min at room temperature with 5% CO_2 in O_2, is also given (d).

choline, did not modify either the electrophysiological or the functional responses to noradrenaline or acetylcholine (Figure 5). This was the case if indomethacin was present alone or in the presence of L-NNA (100 μM). Noradrenaline depolarized (-34 ± 2 mV cf. -34 ± 5 mV) and contracted (2.3 ± 0.1 mN mm^{-1} cf. 2.3 ± 0.4 mN mm^{-1}) the rat small mesenteric artery in the absence and presence of indomethacin respectively, indicating that indomethacin did not alter the conditions from which the actions of acetylcholine were assessed.

Incubation of segments of mesenteric artery with oxyhaemoglobin (1.5 μM for 10 min) did not modify the vasorelaxant response ($n = 8$) to acetylcholine (Figure 6). Electrophysiological studies ($n = 3$) showed that the hyperpolarizing effects of acetylcholine were not sensitive to oxy-

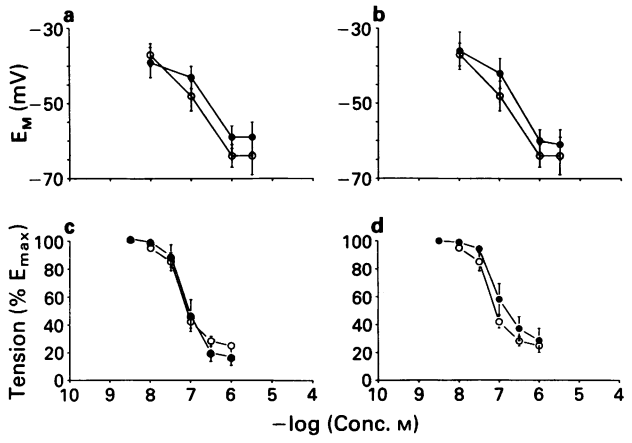

Figure 5 Mean concentration-effect curves constructed to acetylcholine in the rat small mesenteric artery in the absence (○) and presence (●) of (a,c) N^G-nitro-L-arginine (100 μM) or (b,d) indomethacin (10 μM). (a,b) Membrane potential changes induced by acetylcholine. Points are means of 8–14 observations in 6–8 experiments. (c,d). Relaxation of noradrenaline-induced contraction with acetylcholine in a separate series experiments. Results are mean from 6–8 separate experiments; s.e.mean shown by vertical bars.

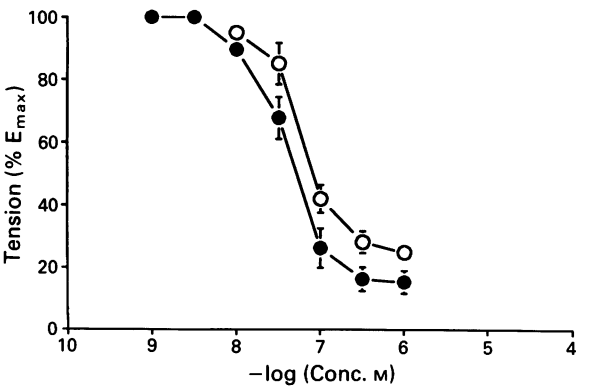

Figure 6 Mean concentration-response curve constructed to acetylcholine examining its ability to relax noradrenaline (3 μM) precontracted rat small mesenteric artery in the absence (○) and in the presence (●) of oxyhaemoglobin (1.5 μM). Results are the mean from 8 experiments; s.e.mean shown by vertical bars.

haemoglobin. Figure 7 shows an original trace where the effects of acetylcholine and NO_a on a noradrenaline (3 μM)-preactivated blood vessel were examined. While acetylcholine caused a marked relaxation and hyperpolarization, the pronounced relaxation to NO_a observed in absence of oxyhaemoglobin (Figure 3) was not observed in its presence (Figure 7b). In the absence of active tone, where NO_a caused a membrane hyperpolarizing effect, NO_a responses were also sensitive to the actions of oxyhaemoglobin (1.5 μM) (Figure 8).

Discussion

There are two main conclusions from this work. First, that in the rat small mesenteric artery, hyperpolarizations to acetylcholine and NO are caused by different mechanisms and second, that NO does not have an obligatory role in the endothelium-dependent responses to acetylcholine. The first conclusion is based on the ability to affect the hyperpolarization to either acetylcholine or NO differentially. The

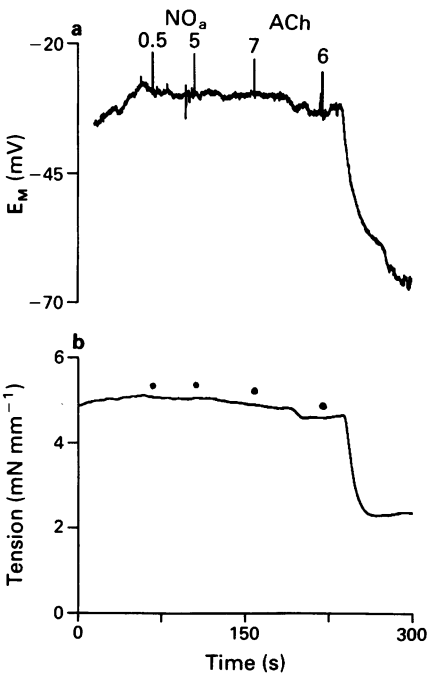

Figure 7 (a) Representative trace showing the effect of NO_a (0.5 and 5 μmol) and acetylcholine (ACh, 0.1 and 1 μM) on membrane (a) and tension (b) recorded simultaneously in a noradrenaline (3 μM)-precontracted rat small mesenteric artery pretreated with oxyhaemoglobin (1.5 μM).

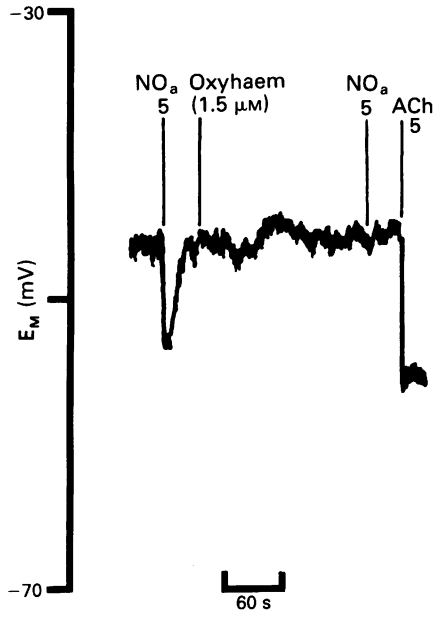

Figure 8 Representative trace showing the effect of NO_a on membrane potential in a rat small mesenteric artery. The experiment was performed in the absence of active tone. Responses to NO_a (5 μmol) were obtained in the absence and presence of oxyhaemoglobin (1.5 μM).

second is based on the persistence of both the hyperpolarization and relaxation to acetylcholine, after treatment of the mesenteric artery with compounds that affect NO synthesis or action (NO-synthase substrate inhibitor, oxyhaemoglobin or glibenclamide).

The hyperpolarization caused by NO, but not acetylcholine, could be blocked either by prior smooth muscle depolarization or in the presence of the potassium channel blocker glibenclamide. Why membrane depolarization should block the NO-induce hyperpolarization is not clear, but it is similar to the effect of depolarization on the responses to NO in the guinea-pig uterine artery (Tare *et al.*, 1990). In this artery, smooth muscle depolarization with phenylephrine, to around −30 mV, reduced the hyperpolarization to NO by over 70%. However, in contrast to the present study, relaxation to NO was also markedly reduced, as were both the hyperpolarization and relaxation to acetylcholine. Tare and coworkers (1990) suggested that their observations could be explained by an increase in membrane conductance, which is responsible for the depolarization, being sufficient to swamp the NO-induced potassium conductance. The similar depression they observed in both the acetylcholine-induced hyperpolarization and relaxation was one piece of evidence indicating that NO mediated the action of acetylcholine in the uterine artery. An alternative explanation is that prior membrane depolarization prevents the NO-activated K^+-channels from opening. In this situation, the channels would be similar to those comprising the potassium selective inward rectifier found in arteriolar smooth muscle (Edwards & Hirst, 1988). However, although hyperpolarization was blocked at membrane potentials which would prevent the opening of inwardly rectifying potassium channels, the channels are not identical to those in guinea-pig submucosal arterioles, as these latter channels were blocked in the presence of millimolar concentrations of barium (Edwards & Hirst, 1988) which failed to affect the acetylcholine-induced hyperpolarization in rat small mesenteric artery (data not shown). ATP-dependent K^+ channels in other cell types show inward rectification (Rorsman & Traube, 1990), which together with the glibenclamide-sensitivity of the hyperpolarizations to NO in this study, might indicate an involvement by this type of K^+ channel. Whatever the precise explanation for the reduction in hyperpolarization to NO, we did not observe a voltage-dependent block of either the relaxation to NO, or both the hyperpolarization and relaxation to acetylcholine. The magnitude of the hyperpolarizations to acetylcholine was in fact increased, as would be predicted by depolarization shifting the membrane potential away from the potassium equilibrium potential. These observations indicate that different mechanisms are responsible for the NO- and acetylcholine-induced hyperpolarizations in the mesenteric artery, and also that the hyperpolarization to NO is not an important mechanism in the relaxation response.

This idea is also supported by the different effect that glibenclamide displayed against the hyperpolarization to either NO or acetylcholine in the rat small mesenteric artery. In this and previous studies we have found that the hyperpolarization and relaxation to acetylcholine are not blocked by glibenclamide, although glibenclamide could block similar responses to cromakalim (McPherson & Angus, 1991). In addition, we now show that, in contrast to the hyperpolarization to acetylcholine, that to NO in this artery can be blocked with glibenclamide. Apart from indicating that acetylcholine and NO responses may be mediated by different membrane mechanisms, the result may also indicate that the NO-induced hyperpolarization occurs after activation of ATP-dependent K^+ channels. In cell types other than vascular smooth muscle, glibenclamide is known to block ATP-dependent K^+ channels (see Rorsman & Trube, 1990). ATP-dependent K^+-channels, which can be activated by cromakalim and blocked by glibenclamide, have been described in excised patches from rabbit and rat mesenteric artery. It has been suggested that these channels could provide an important mechanism for producing hyperpolarization and subsequent relaxation in vascular smooth muscle (Standen *et al.*, 1989). Support for this idea comes from experiments with the rabbit middle cerebral artery, where glibenclamide antagonizes both the hyperpolarization and relaxation to acetylcholine (Brayden, 1990). Some caution is needed however, before assuming that because hyperpolarization in vascular smooth muscle can be induced by cromakalim and blocked by glibenclamide, it is necessarily mediated by ATP-dependent K^+ channels. For example, glibenclamide is several orders of magnitude less potent (μM) at blocking hyperpolarization and relaxation in vascular smooth muscle than it is in stimulating the release of insulin from pancreatic β-cells, by blocking ATP-dependent K^+ channels (nM region, Sturgess *et al.*, 1988; Zunkler *et al.*, 1988). This suggests that in these two cell types, the channels are not identical. In addition in experiments using cells dispersed from the rabbit portal vein, cromakalim can activate a K^+ channel which is not ATP-dependent, and which closely resemble the K^+ channels that are responsible for delayed rectification (Beech & Bolton, 1989).

It would also appear that, in the rat mesenteric artery of the size we studied (approximately 350 μm), NO contributes very little in the responses to acetylcholine. Both L-NNA and oxyhaemoglobin had no significant effect on either the relaxation or the hyperpolarization to acetylcholine although the results from these studies should be viewed with some caution. While L-NNA has been shown to inhibit potently NO-synthase present in endothelial cells, both *in vitro* and *in vivo* (Rees *et al.*, 1990), one assumption that we have made is that L-NNA can enter the rat small mesenteric artery endothelial cell and that it is a substrate for the NO-synthase in this cell. From the present studies we cannot confirm this. However the results with oxyhaemoglobin also suggests that NO has a small role in the acetylcholine mediated relaxation and hyperpolarization in the rat small mesenteric artery. We found oxyhaemoglobin failed to effect the actions of acetylcholine at concentrations (1.5 μM) that abolished the effects of NO.

That acetylcholine-mediated hyperpolarization is oxyhaemoglobin-insensitive has been reported previously by Chen *et al.* (1988). The finding that the relaxation response shows a similar resistance is somewhat unusual and is suggestive of another factor (apart from NO) mediating either one or both of the effects of acetylcholine. In contrast to our findings,

previous work on rat mesenteric arterial preparations has provided some evidence that NO is at least partially involved in the relaxation response to acetylcholine. For example Furchgott and co-workers (Furchgott *et al.*, 1987), using a perfused rat mesenteric preparation, showed acetylcholine relaxation responses to be partially sensitive to haemoglobin (10 μM). In addition, other studies have shown acetylcholine responses in a blood-perfused mesenteric preparation, are sensitive to methylene blue (Randall & Hiley, 1988). The reasons for these differences are unclear at present. We have subsequently repeated some experiments with haemoglobin (4 μM, data not shown), the maximum concentration practically achievable in our system, and have found that vasorelaxation and hyperpolarizing responses to acetylcholine are resistant. The vessels characterized using the perfused mesenteric bed would be of a smaller diameter than those used by us. One possibility is that these smaller vessels use NO to a greater extent to cause the vasorelaxant action. We are currently testing this hypothesis. In any case, the finding that the concentration of haemoglobin used in the majority of our studies successfully abolished responses to NO suggests the concentration of haemoglobin was sufficient.

While it is likely that the acetylcholine-mediated hyperpolarization of the rat small mesenteric artery is not the result of NO liberation, it is currently unclear what factor or mechanism is responsible for the phenomenon. Previous studies have shown that the hyperpolarization and relaxation responses to acetylcholine can be blocked differentially (see introduction) by oxyhaemoglobin and methylene blue, leading to the suggestion that at least two factors are released from the endothelium on stimulation with acetylcholine (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Nishiye *et al.*, 1989; Taylor & Weston, 1990). One causes hyperpolarization (EDHF) and the other relaxation (EDRF). The results from this study suggest the possibility of a third factor/mechanism which does not rely on NO as the signal carrier. The fact that indomethacin did not reduce hyperpolarization to acetylcholine in this and other studies (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988), indicates that any unidentified hyperpolarizing factor is probably not a product of cyclo-oxygenase activity. An alternative or additional possibility, is that acetylcholine may directly hyperpolarize endothelial cells, and this hyperpolarization may spread electrotonically through the arterial media, closing voltage-operated calcium channels and inducing relaxation (Mekata, 1986). Such a process would, by definition, operate through voltage-dependent K^+ channels. Support for this idea comes from the finding that acetylcholine can directly hyperpolarize endothelial cells, and the knowledge that areas of close contact, which might support electrical transmission, are present between endothelial and vascular smooth muscle cells (Busse *et al.*, 1988; Spagnoli *et al.*, 1982; Taugner *et al.*, 1984). The only study to date which has directly investigated this possibility involved simultaneous intracellular recording from an endothelial cell and a neighbouring smooth muscle cell in pig coronary artery (Beny, 1990). Although bradykinin hyperpolarized both the endothelial and smooth muscle cell, this appeared to be, in both cells, a direct effect of bradykinin, as the injection of either current or lucifer yellow into endothelial cells, failed to provide any evidence for coupling between these cells. However, this does not mean that such coupling is not important in other vessels, particularly smaller resistance arteries. A variation in coupling between different arteries may go some way towards explaining the variation in both the size and duration of hyperpolarization caused by acetylcholine.

Lastly, some other technical considerations are worth considering. First we generated NO from two separate sources, NO gas and acidified nitrite, because we were concerned that the results obtained with NO generated from acidified nitrite, may be complicated by the presence of other nitro compounds. However, results obtained examining the actions of both NO preparations on the rat small mesenteric artery were similar, suggesting this was not the case. In addition we showed acidified distilled water and unacidified sodium nitrite, both major contaminants, were without effect on the rat small mesenteric artery. We were also concerned that NO liberated from the endothelium by acetylcholine has characteristics differing from NO injected near the vessel using a flow through perfusion system. In the first case, the smooth muscle cells underlying the endothelium would be exposed to a steady state level of NO released in close apposition to the muscle by acetylcholine. However, when injected into the bath the tissue would be exposed to a transient amount of NO. We feel this was not a complicating issue since, in experiments where NO was applied under steady state conditions via an infusion pump, the responses showed the same characteristics as when applied in a bolus fashion.

In summary, these data show that NO can hyperpolarize smooth muscle cells in the rat mesenteric artery, but by a different membrane mechanism to acetylcholine. Further, they indicate that any NO which is released by acetylcholine stimulating the endothelium has an insignificant role in the subsequent pronounced smooth muscle hyperpolarization and relaxation. The mechanisms underlying these responses are not clear and require further investigation.

This project was supported by an Institute Grant from the N.H. and M.R.C. of Australia and Glaxo Australia. C.J.G. was supported by a Wellcome-Ramaciotti Travel Fellowhip. We would like to thank Simon Keily for his skilled assistance during this study and Dr James Angus for support. We would also like to thank Pam Arnold and Dr Tom Cocks for preparing the oxyhaemoglobin.

References

ANGUS, J.A., BROUGHTON, A. & MULVANY, M.J. (1988). Role of a-adrenoceptors in constrictor responses of rat, guinea-pig and rabbit small arteries to neural activation. *J. Physiol.*, **403**, 495–510.

ANGUS, J.A. & COCKS, T.M. (1989). Endothelium derived relaxing factor. *Pharmacol. Ther.*, **41**, 303–351.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cell isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BENY, J.L. (1990). Endothelial and smooth muscle cells hyperpolarized by bradykinin are not dye coupled. *Am. J. Physiol.*, **258**, H836–H841.

BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J. Physiol.*, **351**, 549–572.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, **259**, H668–H673.

BUSSE, R., FICHTNER, H., LUCKHOFF, A. & KOHLHARDT, M. (1988). Hyperpolarization and increased free calcium in acetylcholine stimulated endothelial cells. *Am. J. Physiol.*, **255**, H965–H969.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.

COCKS, T.M. & ANGUS, J.A. (1990). Comparison of relaxation responses of vascular and non-vascular smooth muscle to endothelium derived relaxing factor (EDRF), acidified nitrite (NO) and sodium nitroprusside. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 364–372.

EDWARDS, F.R. & HIRST, G.D.S. (1988). Inward rectification in submucosal arterioles of guinea-pig ileum. *J. Physiol.*, **404**, 437–454.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary artery smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.

FURCHGOTT, R.F., CARVALHO, M.H., KHAN, M.T. & MATSUNAGA, K. (1987). Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. *Blood Vessels*, **24**, 145–149.

HUANG, A.H., BUSSE, R. & BASSENGE, E. (1988). Endothelium dependent hyperpolarization of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 438–442.

KOMORI, K., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

LONGMORE, J. & WESTON, A.H. (1990). The role of K^+ channels in the modulation of vascular smooth muscle tone. In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential.* ed. Cook, N.S. pp. 259–278. Chichester, West Sussex, U.K.: Halsted Press.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MEKATA, F. (1986). The role of hyperpolarization in the relaxation of the monkey coronary artery. *J. Physiol.*, **371**, 257–265.

McPHERSON, G.A. (1985). Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. *J. Pharmacol. Methods*, **14**, 213–228.

McPHERSON, G.A. & ANGUS, J. (1991). Evidence that acetylcholine mediated hyperpolarization of the rat small mesenteric artery does not involve the K^+ channel opened by cromakalim. *Br. J. Pharmacol.*, **103**, 1184–1190.

MULVANY, M.J. & HALPERN, W. (1977). Contractile responses of small resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

NISHIYE, E., NAKAO, K., ITOH, T. & KURIYAMA, H. (1989). Factors inducing endothelium-dependent relaxation in guinea-pig basilar artery as estimated from the actions of haemoglobin. *Br. J. Pharmacol.*, **96**, 645–655.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RAND, V.E. & GARLAND, C.J. (1991). Endothelium-dependent hyperpolarization and relaxation to acetylcholine in rabbit isolated cerebral arteries. *Br. J. Pharmacol.*, **102**, 128P.

RANDALL, M.D. & HILEY, C.R. (1988). Detergent and methylene blue affect endothelium-dependent vasorelaxation and pressure-flow relations in rat blood perfused mesenteric arterial bed. *Br. J. Pharmacol.*, **95**, 1081–1088.

REES, D.D., PALMER, R.M.J., SCHULTZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RORSMAN, P. & TRUBE, G. (1990). Biophysics and physiology of ATP-regulated K^+ channels (KATP). In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential.* ed. Cook, N.S. pp. 96–116. Chichester, West Sussex, U.K.: Halsted Press.

SPAGNOLI, L.G., VILLASCHI, S., NERI, L. & PALMIERI, G. (1982). Gap junctions in myoendothelial bridges of rabbit carotid arteries. *Experientia*, **38**, 124–125.

STANDEN, N.B., QUAYLE, J.M., DAVIS, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, T.M. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of sulfonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.*, **95**, 83–97.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **346**, 69–71.

TAUGNER, R., KIRCHHEIM, H. & FORSSMANN, W.G. (1984). Myoendothelial contacts in glomerular arterioles and in renal interglobular arteries of rat, mouse and *Tupaia belangeri*. *Cell Tiss. Res.*, **235**, 319–325.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium derived hyperpolarizing factor: an endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272–274.

TRACEY, W.R., LINDEN, J., PEACH, M.J. & JOHNS, R.A. (1990). Comparison of spectrophotometric and biological assays for nitric oxide and EDRF: Nonspecificity of the diazotization reaction for NO and failure to detect EDRF. *J. Pharmacol. Exp. Ther.*, **252**, 922–928.

ZUNKLER, B.K., LENZEN, S., MANNER, K., PANTEN, U. & TRUBE, G. (1988). Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic β-cells. *Naunyn Schmiedebergs Arch. Pharmacol.*, **337**, 225–230.

(Received July 12, 1991
Revised October 9, 1991
Accepted October 15, 1991)