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Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels

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1 The effects of haemoglobin and methylene blue on the acetylcholine (ACh)-induced electrical and mechanical responses of smooth muscle cells were investigated in rat aorta and rat main pulmonary artery.

2 When the endothelium was intact, ACh induced a transient hyperpolarization and sustained relaxation of tissues precontracted with noradrenaline. Both hyperpolarization and relaxation were absent in preparations without endothelium.

3 Haemoglobin and methylene blue inhibited the ACh-induced relaxation, but not the transient hyperpolarization.

4 In aorta with an intact endothelium, ACh produced an increase in both the rate of ^{86}Rb efflux and tissue cyclic GMP levels. The changes in ion flux were unaffected by either haemoglobin or methylene blue in concentrations which almost abolished the increase in cyclic GMP concentrations.

5 In arteries with an intact endothelium, indomethacin had no effect on the ACh-induced electrical and mechanical responses or on the increase in ^{86}Rb efflux and tissue cyclic GMP levels.

6 It is concluded that in the rat aorta and rat main pulmonary artery, ACh releases two different substances, an endothelium-derived relaxing factor (EDRF) and a hyperpolarizing factor (EDHF), from the endothelial cells. Neither substance appears to be derived from a pathway dependent on cyclo-oxygenase. EDHF seems to play a minor role in the relaxation of noradrenaline-induced contractions.

Introduction

Stimulation of muscarinic receptors by acetylcholine (ACh), in isolated arteries produces electrical membrane hyperpolarization and relaxation in tissues which have been pre-constricted by vasoactive agents (Bolton *et al.*, 1984; Komori & Suzuki, 1987a; Southerton *et al.*, 1987; Feletou & Vanhoutte, 1988; Taylor *et al.*, 1988). Such electrical and mechanical responses are observed only when the endothelial cells are intact, indicating that these events are generated by endothelium-derived substances, one of which has been named endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980; Furchgott, 1984).

In contrast to the electrical and mechanical effects of ACh, there is evidence that agents such as substance P (Bolton & Clapp, 1986) or oxotremorine (Komori & Suzuki, 1987a) are able to relax noradrenaline (NA)-induced contractions without the generation of membrane hyperpolarization. Such observations together with those of Taylor *et al.* (1988), could suggest that at least two different substances, a relaxing factor (EDRF) and a hyperpolarizing factor, are released by ACh from the vascular endothelial cells.

In the present study the effects of haemoglobin and methylene blue, agents which inhibit endothelium-dependent relaxations (Martin *et al.*, 1985), have been examined on segments of rat aorta and rat main pulmonary artery. Using a combination of electrical, mechanical and biochemical techniques, it was hoped to clarify the possible involvement of multiple factors in the relaxant effects of ACh in these blood vessels.

Methods

Albino rats of either sex, weighing 200–350 g, were killed by exsanguination from the carotid artery

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under ether anaesthesia. The main pulmonary artery and thoracic aorta were excised and immersed in Krebs solution at room temperature. After removal of connective tissue, the pulmonary artery and aorta were cut into rings each 1–1.5 mm and 5 mm, respectively. Transverse strips were then prepared by cutting along the longitudinal axis of each ring. During preparation, contact with the internal surface of the vessels was avoided as much as possible in order to preserve the endothelial cells. When necessary these were removed by rubbing with a cotton ball moistened with Krebs solution, as described by Furchgott & Zawadzki (1980). Light microscopic examination of cross sections of these vessels embedded in paraffin (Ibengwe & Suzuki, 1986), revealed that this method was sufficient to remove the endothelial cells.

Organ bath studies

Using silk threads tied at each end, the lower end of each strip was fixed at the bottom of the recording chamber while the upper end was connected to a force transducer for isometric tension recording. The recording chamber had a cylindrical shape (8 mm diameter, 15 mm high) with a volume of about 0.75 ml. Usually two strips (with and without endothelium) were suspended together in the same chamber, and superfused with Krebs solution at 35°C. A resting tension of 50–100 mg (pulmonary artery) or 1 g (aorta) was applied to the tissues which were incubated in this condition for about 1 h. Mechanical responses of the arterial strips were displayed on a pen recorder.

Microelectrode measurements

Electrical changes in the arterial smooth muscle cells were recorded using conventional microelectrode techniques; briefly, glass capillary microelectrodes filled with 3 M KCl (tip resistance, 30–60 MΩ) were inserted through the intimal layer of the vessel segment which was mounted in the recording chamber at 35°C with the endothelial layer uppermost. Tissue segments were superfused with Krebs solution at a flow rate of 2–3 ml min^{-1} and electrical responses were displayed on a pen recorder.

^{86}Rb efflux studies

The technique used was essentially that described by Taylor *et al.* (1988). Rings of rat aorta with an intact endothelium were prepared as previously described, but these were not opened along their longitudinal axis to minimize endothelial damage. The tissues were loaded with ^{86}Rb (5 μCi ml^{-1}) for 90 min after which the efflux of radioactivity was monitored using 30 s, 1 min or 2 min collection periods, as appropriate. Tissues were exposed to ACh for a total of 4 min in the presence or absence of indomethacin, haemoglobin or methylene blue, which were included in the Krebs solution throughout the efflux experiment. The ^{86}Rb content of efflux and tissue samples was determined by Cerenkov counting. The efflux data were expressed as the rate coefficient (fractional loss of ^{86}Rb from the tissue standardized for a 1 min period, expressed as a percentage).

Measurement of guanosine 3′: 5′-cyclic monophosphate levels

Changes in cyclic GMP levels were measured in intact aortic rings as described by Taylor *et al.* (1988). After exposure to ACh for various timed intervals, tissues were plunged into liquid nitrogen, thawed in 1 ml 10% trichloroacetic acid and homogenized separately in a glass/glass homogenizer. After centrifugation and ether extraction the cyclic GMP content was determined by radioimmunoassay (NEN). The method of Lowry *et al.* (1951) was used for tissue protein determination.

Drugs and solutions

The ionic composition of the Krebs solution was (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134, glucose 11.5. High potassium solution (K^+ = 118 mM) was prepared by replacing Na^+ with K^+. The solution was aerated with 97% O_2/3% CO_2 and the pH was kept at 7.3–7.4.

Drugs used were acetylcholine chloride, indomethacin, (−)-noradrenaline HCl, bovine haemoglobin and methylene blue (all from Sigma). Oxyhaemoglobin was prepared by the method of Martin *et al.* (1985) using sodium hydrosulphite as follows: to 10 ml commercial haemoglobin (1 mM) 10 ml of freshly prepared sodium hydrosulphite (10 mM) was added (the colour immediately changed from brown to red). The sodium hydrosulphite was then removed by dialysis (semipermeable tubing) against a sufficient volume of distilled water for 2 h at 4°C. The conversion of methaemoglobin to oxyhaemoglobin was checked spectrophotometrically using a spectrophotometer. If conversion had occurred satisfactorily, 1 ml aliquots were frozen at −20°C and stored for up to 2 weeks before use.

Results

Mechanical responses

Experiments were first carried out to demonstrate the involvement of endothelium-derived factors

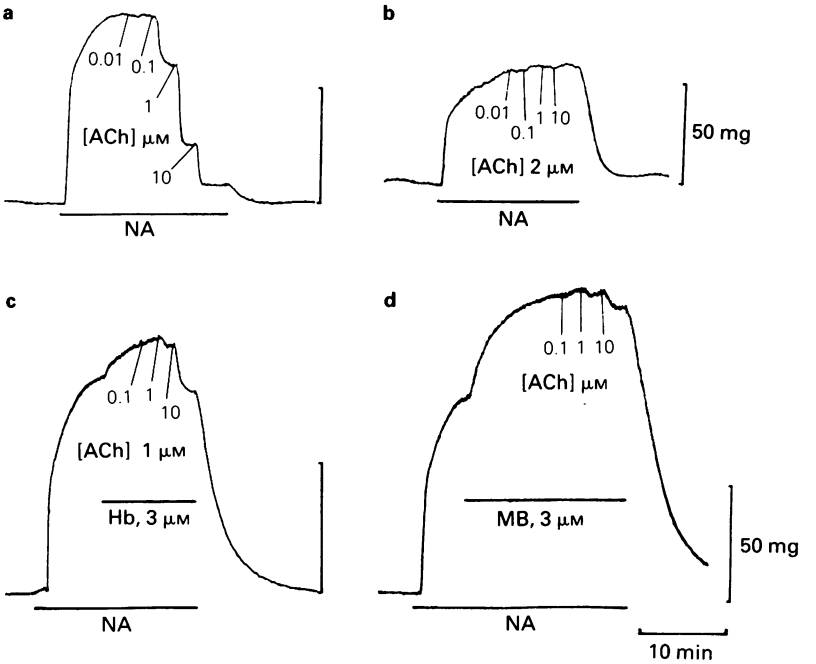

Figure 1 Acetylcholine (ACh)-induced inhibition of noradrenaline (NA)-induced contractions in rat main pulmonary artery. (a) Control with intact endothelium; (b) endothelium removed; (c) haemoglobin (Hb, 3 μM) with intact endothelium; (d) methylene blue (MB, 3 μM) with intact endothelium. ACh was added cumulatively during the NA (0.1 μM)-induced contraction. (a), (c) and (d), but not (b), show responses from the same tissue.

during ACh-induced inhibition of noradrenaline (NA)-induced contractions of the rat aorta and main pulmonary artery. Tissue segments were contracted by NA (0.1–0.3 μM) to 40–60% of the tension produced by 118 mM K^+ solution, and then increasing concentrations of ACh were added cumulatively in the continuing presence of NA. In pulmonary artery, ACh (0.01–10 μM) inhibited NA contractions in a concentration-dependent manner, and 10 μM ACh reduced the NA-induced contraction to about 10% of its peak amplitude (Figures 1a and 2). Similar results were obtained in rat aorta (Figure 2). In tissues with no endothelium, ACh (0.01–10 μM) did not modify the contraction induced by NA in rat aorta or in pulmonary artery occasionally enhanced the NA-contraction by 3–5% (Figure 1b).

Application of haemoglobin (Hb, 3 μM) contracted aortic and pulmonary artery segments to 6.1 $\pm$ 4.2% and 7.9 $\pm$ 3.3% ($n = 6$), respectively of the 118 mM K^+ contraction. When Hb was applied during NA-induced contractions, the muscle contracted further and tension was enhanced by 17.3 $\pm$ 5% ($n = 8$) and 27.6 $\pm$ 5.4% ($n = 17$) in aorta and pulmonary artery, respectively. Application of ACh in the presence of NA and Hb also relaxed the tissue, but to a much smaller extent than that seen in the absence of Hb (Figures 1c and 2). The inhibitory effect of Hb on ACh-induced relaxations was reversible and after about 1 h, recovery of the ACh relaxation was complete.

Methylene blue (3 μM) contracted the tissues to 8.1 $\pm$ 2.9% ($n = 6$) and 10.2 $\pm$ 4.4% ($n = 5$) of the 118 mM K^+ induced contraction in aorta and pulmonary artery, respectively. Application of methylene blue (3 μM) enhanced the NA-induced contraction by 34.3 $\pm$ 6.2% ($n = 6$) and 42.7 $\pm$ 7.7% ($n = 8$) in aorta and pulmonary artery, respectively, and in the presence of methylene blue, ACh-induced relaxations were greatly diminished (Figures 1d and 2). These inhibitory effects of methylene blue were essentially irreversible as they were still evident after prolonged washing.

In rat aorta, indomethacin (3 μM) had no effect on basal tension or on the ACh-induced inhibition of NA-induced contractions (Figure 2).

Electrical responses

The resting membrane potentials of the smooth muscle cells of rat aorta and rat main pulmonary artery were stable, as described previously (Suzuki & Twarog, 1982; Taylor *et al.*, 1988). The electrical

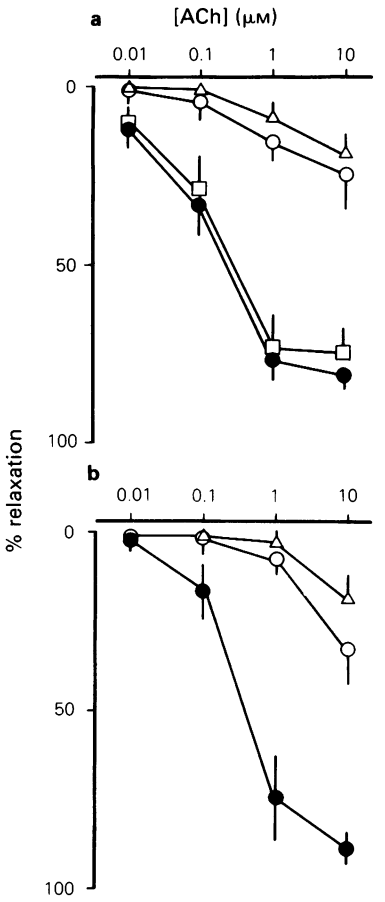

Figure 2 Effects of acetylcholine (ACh) on noradrenaline (NA)-induced contractions in intact preparations of (a) rat aorta and (b) rat main pulmonary artery. ACh was applied in the absence (control, ●) and after application of haemoglobin (3 μM, ○), methylene blue (3 μM, △) or indomethacin (3 μM, □). The amplitude of relaxation is expressed as % of the initial NA-induced contraction. Each point shows the mean. (n = 6–13) and vertical lines indicate s.d.

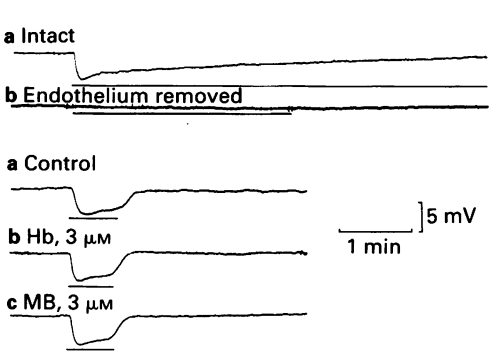

Figure 3 Effects of acetylcholine (ACh) on the membrane potential of smooth muscle cells in the rat main pulmonary artery. (A) ACh (10 μM) was applied to (a) intact and (b) endothelium-free tissues. (B) ACh (10 μM)-induced hyperpolarizations recorded in intact tissues before (a, control) and (b) during application of haemoglobin (Hb, 3 μM, for 10 min) or (c) methylene blue (MB, 3 μM, for 10 min). (Aa), (Ab) and (B) were recorded from different single cells. ACh was applied as indicated by the bar. The resting potentials were: (Aa) − 51 mV, (Ab) − 53 mV, (Ba) − 52 mV, (Bb) − 50 mV, (Bc) − 52 mV.

responses of aortic smooth muscle cells to ACh (>0.1 μM) have been described previously (Taylor *et al.*, 1988). Briefly, ACh produced a transient (2–3 min) hyperpolarization of the membrane. Occasionally, the hyperpolarization produced by relatively high concentrations of ACh (>10 μM) decayed slowly and required over 30 min for the membrane potential to revert to the resting level in the presence of ACh. After removal of the endothelial cells the resting membrane potential was depolarized by up to 9 mV and the ACh-induced hyperpolarization ceased.

In the main pulmonary artery, application of ACh (10 μM) produced hyperpolarization, the amplitude of which gradually diminished in the continuing presence of ACh. Within 5–7 min, the membrane potential had reverted to the previous resting level (Figure

Table 1 Membrane potential of smooth muscle cells of the rat main pulmonary artery in the presence and absence of the vascular endothelium

		Membrane potential (mV)
Endothelium intact	Control (resting membrane potential)	−52.0 ± 1.8 (125)
	ACh 10 μM (after 10 min)	−52.6 ± 2.1 (23)
	Haemoglobin 3 μM (after 3 min)	−50.4 ± 1.7* (12)
	Methylene blue 3 μM (after 5 min)	−51.5 ± 1.4 (9)
	Indomethacin 3 μM (after 5 min)	−51.6 ± 2.4 (12)
Endothelium removed	Control	−51.2 ± 1.6 (17)
	ACh 10 μM (after 5 min)	−50.8 ± 1.7 (16)

Each value is the mean ± s.d. with the number of observations in parentheses. *Statistically significantly different from the control (P < 0.05). ACh = acetylcholine.

3Aa). The membrane potential after application of ACh for more than 10 min, therefore, was not different from the resting potential (Table 1).

In pulmonary arteries devoid of endothelium, the resting membrane potential was the same as that in tissues with an intact endothelium (Table 1). Application of ACh (10 μM) did not change the membrane potential in the endothelium-free tissues (Figures 3Ab and 4) or on some occasions produced a small (1–3 mV) sustained depolarization. Application of haemoglobin (3 μM) depolarized the membrane by 1–3 mV (Table 1). The amplitude of the haemoglobin (Hb)-induced depolarization was enhanced by increasing the Hb concentration to 10 μM (control, -52.0 ± 1.5 mV, $n = 10$; Hb 10 μM, -48.7 ± 2.3 mV, $n = 7$, $P < 0.05$). In the presence of 3 μM Hb, ACh still produced hyperpolarization similar in amplitude to that seen in control conditions (Figures 3Ba,b and 4). Neither methylene blue (3 μM) nor indomethacin (3 μM) had any effect on the membrane potential or on the ACh-induced hyperpolarization (Figures 3Bc and 4, Table 1).

The concentration-response relationship of the ACh-induced hyperpolarization in smooth muscle cells of rat main pulmonary artery is shown in Figure 4, in which the peak amplitude of hyperpolarization is plotted. ACh (0.1–100 μM) produced a transient, concentration-dependent hyperpolarization only when the endothelial cells were intact, and this relationship was unaffected in the presence of Hb, methylene blue or indomethacin.

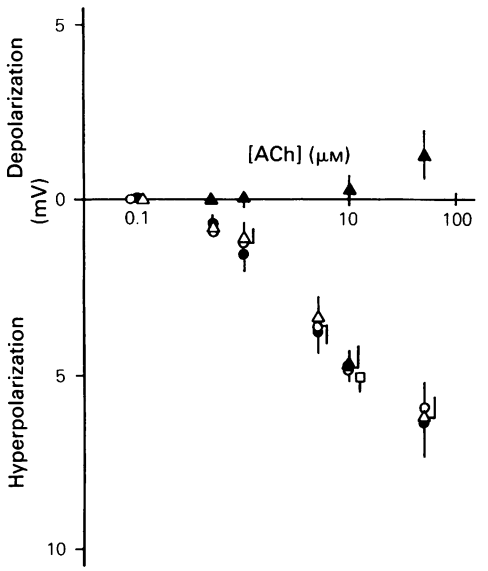

Figure 4 Acetylcholine (ACh)-induced hyperpolarizations in rat main pulmonary artery. (●) Control with intact endothelium, (▲) endothelium removed, (○) haemoglobin (3 μM) present, (△) methylene blue (3 μM) present, (□) indomethacin (3 μM) present; each with intact endothelium. Each point shows the magnitude of the change in membrane potential from the resting level as a mean. ($n = 6$–17); vertical lines indicate s.d. The resting membrane potentials in each situation are given in Table 1.

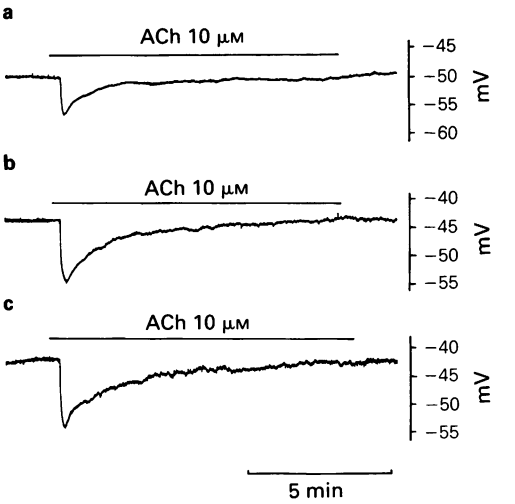

Figure 5 Effects of acetylcholine (ACh; 10 μM) on membrane potential of smooth muscle cells in the rat main pulmonary artery. (a) Control, (b) in the presence of 0.1 μM noradrenaline (NA), (c) in the presence of 0.1 μM NA plus 3 μM methylene blue (MB). Mean amplitude of the hyperpolarization at the peak was: (a) 5.0 ± 1.2 mV ($n = 10$), (b) 10.2 ± 1.2 mV ($n = 8$), (c) 10.5 ± 2.1 mV ($n = 5$). All the recordings were from the same tissue.

Experiments were further carried out to demonstrate that the ACh-induced hyperpolarization was a transient event even in conditions similar to those applied for mechanical experiments. Figure 5 shows that in the main pulmonary artery, ACh (10^{-5} M) hyperpolarized the membrane only transiently either in the absence or presence of NA or NA plus methylene blue, although the amplitude of the hyperpolarization was increased in the presence of NA plus methylene blue, possibly due to depolarization of the smooth muscle membrane.

Measurement of cyclic GMP levels

In untreated endothelium-intact segments of rat aorta, the basal cyclic GMP concentration was 0.145 ± 0.03 pmol mg^{-1} protein ($n = 6$). Exposure to ACh for 1 min produced a large increase in cyclic GMP levels to 31 ± 2.6 pmol mg^{-1} ($n = 6$). Pretreatment of tissues with either Hb or methylene blue

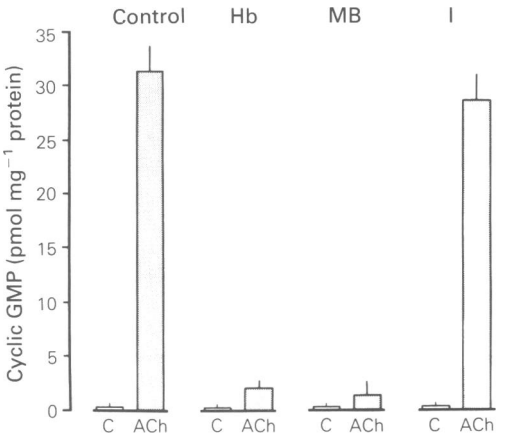

Figure 6 Effect of acetylcholine (ACh 10 μM, for 1 min, stippled columns) on tissue levels of cyclic GMP in intact segments of rat aorta in the absence and after 14 min exposure to either haemoglobin (Hb, 3 μM), methylene blue (MB, 3 μM) or indomethacin (I, 3 μM). C = appropriate control (open columns). Each column represents the mean of 6 determinations and bars show s.d.

(each 3 μM) for 14 min significantly reduced basal cyclic GMP concentrations to 0.062 ± 0.008 and 0.114 ± 0.01 pmol mg^{-1} protein ($P < 0.05$, $n = 6$). Subsequent exposure to ACh produced a significant increase in cyclic GMP levels (to 2.2 ± 0.09 and 1.8 ± 0.08 pmol mg^{-1} protein, respectively, $n = 6$), but these changes were much less than those seen in the absence of Hb or methylene blue. Exposure to indomethacin (3 μM) for 14 min had no significant effect on resting cyclic GMP levels or on the stimulant action of ACh. These results are summarized in Figure 6. ACh had no effect on cyclic GMP levels in endothelium-free segments of aorta (data not shown).

Measurement of ^{86}Rb efflux

In these experiments, ^{86}Rb was used as a marker for potassium. In untreated, endothelium-intact segments of rat aorta, the basal rate of ^{86}Rb exchange measured between the 14th and 18th min of the efflux period was $1.01 \pm 0.08\%$ min^{-1} ($n = 6$). Exposure to ACh produced a rapid transient increase in ^{86}Rb efflux (Figure 7a). Pretreatment with Hb or methylene blue (each 3 μM) from the start of the efflux period significantly reduced basal efflux to 0.85 ± 0.06 and $0.89 \pm 0.04\%$ min^{-1}, respectively ($P < 0.05$, $n = 6$). Under these conditions ACh was still able to produce its characteristic increase in ^{86}Rb exchange (Figure 7b and c). Exposure to indomethacin (3 μM) from the start of the efflux period had no effect on basal or ACh stimulated ^{86}Rb efflux (Figure 7d). ACh had no effect on ^{86}Rb efflux in endothelium-free segments of aorta (data not shown).

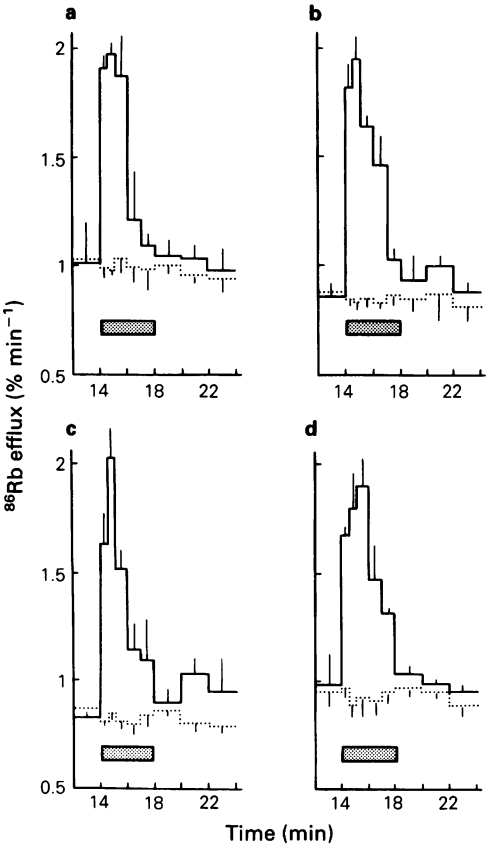

Figure 7 Effects of acetylcholine (ACh, 10 μM, stippled bar), (a) alone and in the presence of (b) haemoglobin (3 μM), (c) methylene blue (3 μM) and (d) indomethacin (3 μM), on the efflux of ^{86}Rb (continuous lines) from intact rings of rat aorta compared with control, basal loss of ^{86}Rb (broken lines). Each point is the mean derived from 6 experiments; vertical lines indicate s.d. Tissues were exposed to Hb, methylene blue or indomethacin from the start of the efflux period.

Discussion

The present experiments have demonstrated that in the rat aorta and main pulmonary artery, ACh produces two different responses in smooth muscle cells. These consisted of a transient electrical hyperpolarization and a sustained mechanical relaxation, observations which are similar to those previously

obtained in rabbit saphenous artery (Komori & Suzuki, 1987a), rat aorta (Southerton *et al.*, 1987; Taylor *et al.*, 1988; Weston *et al.*, 1988) and canine coronary artery (Feletou & Vanhoutte, 1988). Substance P also produces responses similar to those of ACh in the pig coronary artery (Beny *et al.*, 1986), but not in the guinea-pig mesenteric artery where this peptide inhibits NA-induced contractions in an endothelium-dependent manner but with no associated change in membrane potential (Bolton & Clapp, 1986).

Evidence that substance P (Bolton & Clapp, 1986) and oxotremorine (Komori & Suzuki, 1987a) produce endothelium-dependent, yet electrically silent relaxations, suggests that the transient hyperpolarization produced by ACh may be generated by substances other than the endothelium-derived relaxing factor (EDRF) described by Furchgott & Zawadzki (1980). In support of this, ACh produced an endothelium-dependent hyperpolarization which can be explained by the opening of membrane K^+ channels (Bolton *et al.*, 1984; Komori & Suzuki, 1987a; Taylor *et al.*, 1988). Such an effect was not observed following activation of guanylate cyclase which is thought to be involved in the mechanism by which the effects of EDRF are mediated (Furchgott, 1984). Furthermore, Komori & Suzuki (1987b) showed that the muscarinic receptors involved in the ACh-induced release of EDRF are of the M_2-subtype, whereas those involved in the ACh-induced hyperpolarization belong to the M_1-subtype. These data suggest that an endogenous substance which causes the opening of K^+-channels could be liberated by ACh from the vascular endothelium.

In the present experiments, Hb and methylene blue were found to reduce the ACh-induced endothelium-dependent relaxations without modifying the hyperpolarizing response. Hb inhibits endothelium-dependent relaxation by binding to EDRF (Martin *et al.*, 1985), while methylene blue indirectly inhibits the action of EDRF in vascular smooth muscle by inhibiting production of cyclic GMP through blockade of guanylate cyclase (Furchgott, 1984). This failure of Hb and methylene blue to block the ACh-induced hyperpolarization indicates that in addition to EDRF, a second endogenous factor is also released from the endothelium by ACh. The properties of this agent suggest that it should be designated endothelium-derived hyperpolarizing factor (EDHF). The inability of indomethacin to modify the hyperpolarization suggests that the cyclo-oxygenase pathway is not involved in the production of this factor.

Additional evidence in favour of the ability of ACh to release both EDHF and EDRF was obtained from the ion flux and cyclic GMP measurements in rat aorta. In this tissue the ACh-induced increase in ^{86}Rb efflux was unaffected by either Hb or MB. However, these agents almost completely abolished the ACh-induced increase in cyclic GMP concentrations, a well-known biochemical characteristic of the action of EDRF (Furchgott, 1984). Recently, it has been proposed that EDRF is nitric oxide (NO), since the actions of NO on the rabbit aorta are apparently identical to those of the EDRF released from porcine cultured aortic endothelial cells (Palmer *et al.*, 1987). In vascular smooth muscle, however, nitrocompounds produce relaxation with little or no change in membrane potential (Kuriyama *et al.*, 1982; Ousterhout & Sperelakis, 1987; Taylor *et al.*, 1988), providing further evidence that the observed ACh-induced hyperpolarization is produced by a factor distinct from EDRF.

Using procedures known to modify Na^+/K^+-ATPase, Rapoport *et al.* (1985) suggested that an increase in Na^+/K^+ pump activity (and hence changes in membrane potential) might directly or indirectly be involved in some endothelium-dependent inhibitory effects. Such a view has received apparent support from Feletou & Vanhoutte (1988). These workers showed that in dog coronary artery ACh produced a transient hyperpolarization which was inhibited by ouabain or by ionic conditions designed to inhibit Na^+/K^+-ATPase. Feletou & Vanhoutte (1988) thus concluded that the ACh-induced hyperpolarization resulted from stimulation of the Na^+/K^+ pump by endothelium-derived relaxing factor(s). However, the results of both the present study and those obtained earlier (Taylor *et al.*, 1988) provide no support for the involvement of Na^+/K^+-ATPase in the endothelium-dependent hyperpolarization produced by ACh. In these investigations, ACh generated a marked increase in ^{86}Rb efflux which was temporally associated with the membrane hyperpolarization. Such an increase in ^{86}Rb exchange is not consitent with stimulation of Na^+/K^+-ATPase and strongly suggests that EDHF produces hyperpolarization by opening ^{86}Rb-permeable K^+-channels. These data therefore confirm earlier suggestions of the involvement of K^+-channels rather than the Na^+/K^+-pump in cholinergic inhibitory responses (Kuriyama & Suzuki, 1978; Kitamura & Kuriyama, 1979; Kajiwara, 1982; Bolton *et al.*, 1984; Gebremedhin *et al.*, 1987). It is possible that the lack of ACh-induced hyperpolarization observed by Feletou & Vanhoutte (1988) in the presence of ouabain resulted from inhibition of EDHF release via an action of ouabain on the endothelial cells. Alternatively, inhibition of smooth muscle Na^+/K^+-ATPase should result in loss of intracellular K^+ and a shift of (E_K) (Casteels, 1970) to a less negative level. Thus any hyperpolarization produced by the K^+-channel opening action

of EDHF would be reduced and give rise to the conclusions of Feletou & Vanhoutte (1988) concerning the role of the Na^+/K^+-pump in the action of EDHF.

In both aorta and pulmonary artery the membrane potential reverted to the previous resting level during continued application of ACh. This suggests that either EDHF is released transiently or that the receptors for EDHF are desensitized rapidly. The diminishing amplitude of hyperpolarization observed during continued application of ACh is not due to a simultaneously developing depolarization of the smooth muscle membrane, as evidenced by the essential absence of any ACh-induced changes in membrane potential in endothelium-free preparations.

Removal of the endothelial cells produces a significant depolarization of the smooth muscle membrane in the pig coronary artery (Beny *et al.*, 1986) and in rat aorta (Taylor *et al.*, 1988). Beny *et al.* (1986) concluded that this depolarization resulted from the cessation of the spontaneous release of an endothelially-derived relaxant factor or from damage of the adjacent smooth muscle cells. However, in rabbit saphenous artery (Komori & Suzuki, 1987a), rabbit basilar artery (Nagao & Suzuki, 1987) and also in rat main pulmonary artery (present study), removal of the endothelium did not result in depolarization of the membrane. It remains to be determined whether these differences are due to tissue specificity or due to damage of the smooth muscle cells sustained during the rubbing procedure. The latter explanation was favoured by Taylor *et al.* (1988), based on electron microscope and ion flux measurements. However, in some endothelium-free preparations, NA (rabbit saphenous artery, Komori & Suzuki, 1987a) and 5-hydroxytryptamine (rabbit basilar artery, Nagao & Suzuki, 1987) depolarize the membrane to the same extent as they do in tissues which contain endothelial, suggesting that in these tissues the receptors for NA and 5-hydroxytryptamine are not damaged by the removal of endothelial cells.

Based on observations that drugs which inhibit the actions of EDRF enhance muscle tension and on measurements of cyclic GMP levels, a constant liberation of EDRF is believed to occur in many arteries (Vanhoutte *et al.*, 1986). In the rat aorta and main pulmonary artery, both Hb and methylene blue elevated resting tension, an effect which was even more marked during NA-induced contractions. This suggests that EDRF is liberated spontaneously at rest and that the release of EDRF is stimulated by NA (Cocks & Angus, 1983; Griffith *et al.*, 1984; Ibengwe & Suzuki, 1986). Hb generated a small but significant depolarization in pulmonary artery whereas methylene blue produced no change in membrane potential. It therefore seems likely that this action of Hb is not associated with the loss of a membrane effect of EDRF, but rather to an action of Hb itself on membrane potential. It is possible that this is related to contamination of the Hb with small amounts of sodium hydrosulphite used in the preparation of Hb (Weston, unpublished) and this is currently being investigated.

In the present study, Hb or methylene blue abolished the ACh-induced increase in cyclic GMP concentrations but had no effect on the associated hyperpolarization. Since Hb or methylene blue markedly inhibited the mechanical relaxation produced by ACh, it must be concluded that EDRF (possibly via guanylate cyclase) plays a more important role than EDHF (hyperpolarization via K^+-channel opening) in endothelium-dependent relaxations. Unless the hyperpolarization produced by EDHF triggers some long-lasting inhibitory process, the transient nature of the hyperpolarization further suggests that EDHF plays only a minor, perhaps facilitatory role, in mechanical inhibition. As suggested by Bolton & Large (1986), hyperpolarization may serve to close any voltage-dependent Ca^{2+} channels which may be open. In addition, hyperpolarization may influence the way in which intracellular Ca^{2+} stores are refilled (Bray *et al.*, 1988) or it may shift Na^+–Ca^{2+} exchange in the direction of Ca^{2+} extrusion from the cell (Kaczorowski *et al.*, 1988; Kaczorowski, personal communication).

It is concluded that in rat aorta and rat main pulmonary artery, ACh releases two different substances from the endothelium. One factor (EDRF) is largely responsible for muscle relaxation while the other (EDHF) hyperpolarizes the muscle membrane by opening ^{86}Rb-permeable K^+-channels. Only the latter response and a much-reduced relaxation is observed in the presence of Hb or methylene blue. These results also suggest that the actions of EDRF itself are not accompanied by electrical changes in the smooth muscle membrane of the tissues studied. The nature of EDHF is unknown, although the data obtained using indomethacin suggest that its production does not depend on cyclo-oxygenase activity. These results together with evidence for an endothelium-derived contractile factor, endothelin (Yanagisawa *et al.*, 1988) indicate the complex influence of the endothelium on vascular smooth muscle tone. The role of EDRF, EDHF and endothelin in normal and pathological conditions is a matter for conjecture and is currently under investigation.

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