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Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim

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1 The mechanisms involved in the mechano-inhibitory effects of acetylcholine (ACh) have been compared with those of sodium nitroprusside (SNP) and cromakalim on the rat isolated thoracic aorta.

2 Relaxations produced by ACh were endothelium-dependent, whereas those produced by SNP or cromakalim were endothelium-independent.

3 ACh, cromakalim and SNP relaxed established contractions produced by noradrenaline (NA) and KCl (20 mM) and these relaxations were well-maintained.

4 SNP was a relatively effective inhibitor of contractions produced by KCl (80 mM). ACh was relatively ineffective and cromakalim was without effect against such contractions.

5 Membrane potential and cyclic GMP concentrations were higher in tissues with an intact endothelium whereas rubbed tissues had a higher ^{86}Rb efflux rate coefficient.

6 ACh and cromakalim produced a transient and long-lasting hyperpolarization, respectively. These changes were accompanied by increases in the ^{86}Rb efflux rate coefficient with a time course comparable to that of the electrical changes.

7 Tissue cyclic GMP concentrations were significantly increased in the presence of ACh or SNP, whereas cromakalim had no effect.

8 Transmission electron microscopy showed the presence of endothelial cells on intact tissues. On rubbed preparations, such cells were absent and some damage to the underlying smooth muscle cells was detected.

9 It is concluded that at least two inhibitory substances are released from the endothelial cells by ACh. One of these increases tissue cyclic GMP concentrations and produces an electrically-silent relaxation. The other produces a transient hyperpolarization associated with the opening of ^{86}Rb-permeable K-channels. This event may serve to initiate relaxation processes and to close any open voltage-dependent Ca-channels.

Introduction

The work of Furchgott & Zawadzki (1980) revealed the importance of the vascular endothelium in mediating the inhibitory responses to acetylcholine (ACh) on rabbit aorta. Since these initial studies it has been shown that the inhibitory effects of mediators such as substance P, adenosine 5'-triphosphate (ATP) and histamine on a variety of blood vessels are also endothelium-dependent (Furchgott, 1984).

Although many studies have measured endothelium-dependent mechanical events, relatively little is known about the mechanism of action of endothelium-derived relaxing factor (EDRF). It is

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certainly clear that the endothelium-dependent inhibitory actions of acetylcholine at least are associated with an increase in tissue guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentrations (Furchgott, 1984; Ignarro & Kadowitz, 1985). However, the electrical changes associated with these events are not clear. Bolton *et al.* (1984) and Bolton & Clapp (1986) found that carbachol produced membrane hyperpolarization in guinea-pig mesenteric artery, whilst endothelium-dependent inhibition mediated by substance P was electrically silent. In contrast, Beny *et al.* (1986) were able to detect a substance P-induced hyperpolarization in pig coronary arteries and Mekata (1986) has shown that electrical stimulation of monkey coronary artery with an intact endothelium is associated with an increase in membrane potential.

The objective of the present study was to clarify the changes associated with the endothelium-dependent inhibitory action of acetylcholine in rat aorta. Measurements of membrane potential using microelectrodes were made together with changes in tissue cyclic GMP concentrations. In addition, K^+ flux was monitored using ^{86}Rb as a marker. For comparative purposes, parallel experiments were conducted using the nitrogen oxide-containing vasodilator, sodium nitroprusside, an agent known to increase cyclic GMP concentrations (Katsuki *et al.*, 1977; Ito *et al.*, 1978; Kukovetz *et al.*, 1979) and the K^+-channel opening drug cromakalim (BRL34915) (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). A preliminary account of some of the results has been presented to the Physiological Society (Southerton *et al.*, 1987).

Methods

All experiments were performed using male Wistar rats (300–500 g) supplied by the University of Manchester Animal Unit. The rats were killed by decapitation and the thoracic aorta was exposed, cleaned of surrounding tissue and removed. The aorta was cut into four segments which were cut along their longitudinal axis to form flat sheets each approximately 0.5 cm in length. In some experiments the endothelium on two of the sheets was removed by rubbing with a cotton bud to produce two matched pairs of rubbed (no endothelium) and intact (endothelium present) preparations.

Tissue bath studies

A small bent pin with thread attached was inserted through the preparation close to each longitudinally-cut edge of an aortic sheet preparation. This was then mounted isometrically under 1 g tension in a 20 ml tissue bath containing bicarbonate-buffered physiological salt solution (PSS). After 30 min, the load was readjusted to 1 g and after a further 1 h equilibration period, experiments were begun.

Inhibition of KCl-induced tension Intact preparations were exposed to KCl (20 or 80 mM). When the maximum mechanical effect had developed, the ability of acetylcholine (ACh), cromakalim or sodium nitroprusside (SNP) to relax the tissue was examined using a cumulative dose-response protocol. As soon as the maximum inhibitory effects of a given concentration of relaxant had been produced, tissues were exposed to further concentrations of relaxant.

Inhibition of tension induced by noradrenaline (NA) Matched pairs of rubbed/intact preparations were contracted with NA (1 μM). When contractions had fully developed, the inhibitory effects of ACh, cromakalim or SNP were examined using a cumulative protocol.

Microelectrode studies

An endothelium-intact segment of aorta was prepared as already described. It was placed, endothelial side uppermost in a recording bath and carefully pinned to the Sylgard base of the chamber. No attempt was made to record mechanical changes. After 1 h equilibration with physiological salt solution (PSS) at a flow rate of 2 ml min^{-1}, impalements were made using standard techniques through the internal elastic lamina with the following protocol. Initially, five impalements each for a period of 3 min were made. Deliberate pull-out of the electrode at the end of the time period yielded an oscilloscope deflection which was taken as the value of the resting membrane potential. The tissue was then re-impaled and exposed to either ACh, cromakalim or SNP, as appropriate. After approximately 10 min, the electrode was deliberately withdrawn to measure the extent of any membrane potential change. The endothelial layer was then removed with a cotton bud and after an interval of 1 h, a further five 3 min impalements were made to assess the resting membrane potential. Exposure to either ACh, cromakalim or SNP was then repeated as already described.

^{86}Rb efflux studies

In these experiments, ^{86}Rb was used as a marker for K^+ (Weir & Weston, 1986a). When rubbed aortic strips were used, they were prepared as already described. However, intact tissues consisted of unopened aortic rings since silver staining techniques showed that in such tissues the endothelial layer was

better maintained during these relatively long isotope experiments than if intact, opened preparations were employed. The anatomical position of each strip/unopened ring was noted (upper, upper middle, lower middle, lower) before it was assigned, using a balanced design, to the appropriate treatment group. Each tissue was impaled on a syringe needle attached to a perspex gassing manifold and then inserted into a test tube containing 5 ml PSS at 37°C bubbled with 95% O_2 plus 5% CO_2 via the needle. Tissues were then transferred to tubes containing 5 ml PSS + ^{86}Rb (5 μCi ml^{-1}) for a 90 min loading period. The ^{86}Rb was allowed to efflux from the tissues by transferring them to tubes containing 5 ml PSS alone for 7 successive 2 min periods. After this, the PSS into which the tissues were transferred contained either PSS alone (controls) or the appropriate concentrations of ACh, cromakalim or SNP, for a total of 8 min (four 2 min collection periods except in the case of ACh when 30 s and 1 min individual collection periods were employed). After this 8 min exposure to the modifying agent, tissues were transferred to tubes containing PSS alone for 3 further 2 min collection periods. The ^{86}Rb content of aliquots of PSS and segments of aorta was measured as described by Hamilton *et al.* (1986). The efflux data were expressed in terms of the rate co-efficient (fractional loss of ^{86}Rb from the tissue standardized for a 1 min period, expressed as a percentage).

Transmission electron microscopy

At the end of some experiments, some tissues were quickly removed from the bath and fixed for 3 h at room temperature in a 5% glutaraldehyde/8% formaldehyde (plus 0.2 mM $CaCl_2$ in 0.01 mM cacodylate buffer, pH 7.2) mixture. The preparations were rinsed and stored in 0.1 M cacodylate buffer overnight at 4°C before post-fixing for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). The tissues were then washed in 70% ethanol for 10 min and stored for up to two days at 4°C. The tissues were further dehydrated by placing in absolute ethanol at the temperature of melting ice for 15 min followed by a further 15 min in dried absolute ethanol at room temperature. To embed the tissues they were placed in propylene oxide for 15 min at room temperature followed by 4 h in a 50:50 mixture of resin (Epon/Araldite):propylene oxide. They were then transferred to pure resin for 24 h. Final embedding was in fresh resin with polymerization at 70°C for 3 days. The tissues were sectioned using an ultramicrotome (Reichert-Jung) with a diamond knife and stretched with xylene to give gold/silver ultrathin sections (i.e. approximately 75–100 nm thick). They were then mounted on gilded copper mesh grids (Veco, 200 HS) and stained with uranyl acetate for 15 min followed by lead citrate for 4 min. The sections were examined at 60 kV in a Philips transmission electron microscope (400T) and photographed as appropriate.

Determination of guanosine 3':5'-cyclic monophosphate levels

Rubbed aortic strips and intact aortic rings were used in these experiments and were prepared as described for the ^{86}Rb efflux studies. The anatomical position of each strip/ring was noted (as previously described) before it was assigned, using a balanced design, to its respective group. Each experimental group was equilibrated for 90 min in PSS at 37°C, bubbled with 95% O_2 plus 5% CO_2. During this time the PSS was changed once. The tissues were then exposed to either ACh, cromakalim or SNP for varying time periods before they were plunged into liquid nitrogen. Tissues were thawed in 1 ml 10% trichloroacetic acid and each homogenized separately in a glass/glass homogenizer. The homogenate was centrifuged at 3000 *g* for 15 min at 4°C and the supernatant extracted four times with three volumes of water-saturated ether, discarding the ether phases. Residual ether was removed with nitrogen gas. A portion of the extract was acetylated and assayed for cyclic GMP using a radio immunoassay kit (NEN). Precipitates were solubilized in 1 ml 1 M NaOH and a portion assigned for protein determination by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Drugs and solutions

The bicarbonate-buffered physiological salt solution (PSS) used had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose, 11. The following drugs and chemicals were used: acetylcholine chloride (Sigma); Araldite 502 (Emscope); bovine serum albumin (BDH), ascorbic acid (BDH), (±)-cromakalim (Beecham); sodium cacodylate (BDH); Epon 812 (Emscope); N,N-dimethylacetamide (Sigma); lead nitrate (BDH); (−)-noradrenaline bitartrate (BDH); potassium chloride (BDH); propylene oxide (BDH), $^{86}RbCl$ (Amersham); sodium nitrite (BDH); sodium nitroprusside (BDH); trichloroacetic acid (BDH); uranylacetate (BDH). Ascorbic acid, 100 μM was present in all experiments with noradrenaline.

Data presentation and analysis

Relaxations were calculated as a percentage of the induced tension which existed at the start of a relaxant dose-response experiment. Data are normally expressed as means ± s.e. mean. Tests of significance

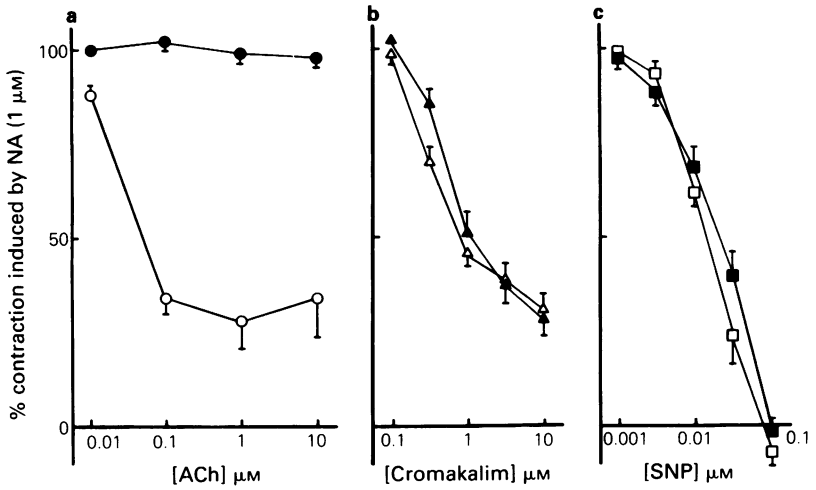

Figure 1 The inhibitory effects of (a) acetylcholine (ACh O, ●), (b) cromakalim (△, ▲) and (c) sodium nitroprusside (SNP, □, ■) on the contraction produced by noradrenaline (NA, 1 μM) in intact (open symbols) and rubbed (solid symbols) segments of rat aorta. Each point is the mean derived from 4 experiments; vertical lines show s.e.mean.

were made by use of Student's two-tailed unpaired *t* test. For the ^{86}Rb efflux experiments, a correction method to minimize the effects of tissue to tissue variation was used. For each tissue, the average efflux rate coefficient derived from the three collection periods immediately before drug addition was subtracted for each collection period during drug exposure, to yield the

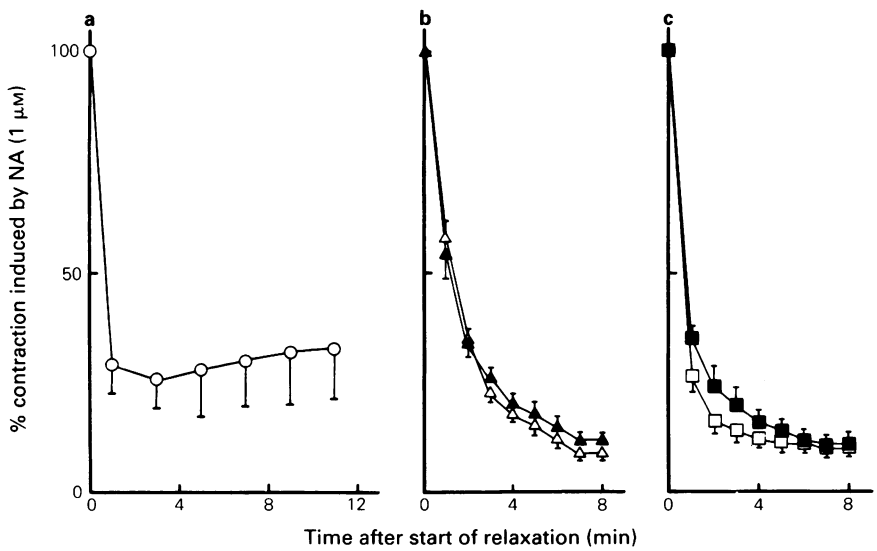

Figure 2 Time-course of the relaxation of noradrenaline (NA, 1 μM) contraction produced by (a) acetylcholine (ACh, 10 μM, O), (b) cromakalim (10 μM, △, ▲) and (c) sodium nitroprusside (SNP, 100 nM, □, ■) in intact (open symbols) and rubbed (solid symbols) segments of rat aorta. ACh produced no relaxations in rubbed segments. Each point is the mean derived from 4 experiments; vertical lines show s.e. mean.

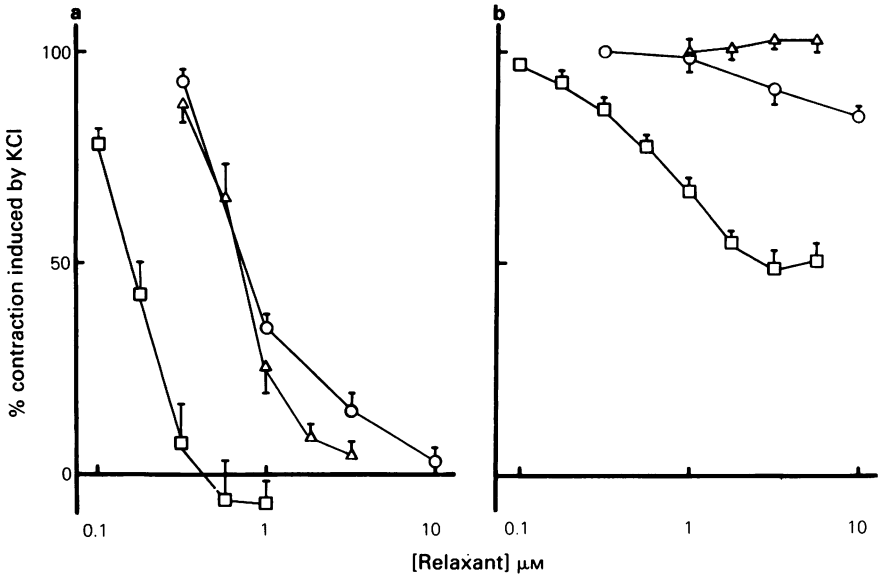

Figure 3 The inhibitory effects of acetylcholine (ACh, O), cromakalim (△) and sodium nitroprusside (SNP, □) on the contraction produced by (a) KCl, 20 mM and (b) KCl, 80 mM in intact segments of rat aorta. Ordinate scale: % of contraction produced by KCl, 20 mM or 80 mM as appropriate. Each point is the mean derived from 4 observations; vertical lines show s.e.mean.

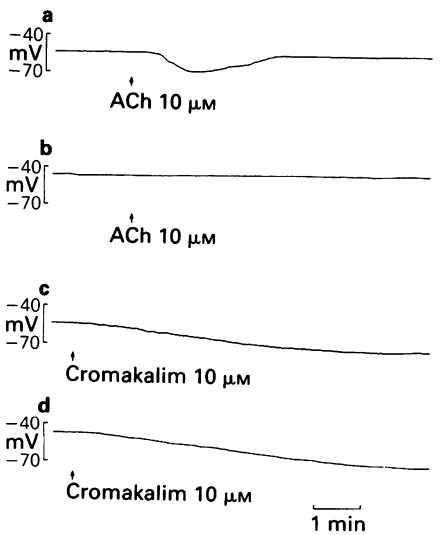

Figure 4 Effects of (a,b) acetylcholine (ACh) and (c,d) cromakalim on membrane potential in intact (a,c) and rubbed (b,d) segments of rat aorta. Responses to ACh and cromakalim were first obtained on the intact segment after which the endothelium was removed and exposure to ACh and cromakalim was repeated. All the records were from the same preparation, but not from the same cell.

change in efflux rate coefficient. Comparison of such changes was made by use of analysis of variance and differences between measurements were then assessed by the studentized range test.

Results

Tissue bath studies

Inhibition of responses to NA In intact preparations, ACh, cromakalim and SNP produced a concentration-dependent reduction of an established contraction to NA (1 μM). In matched, rubbed segments, however, ACh produced no inhibition of NA contractions while the relaxant effects of cromakalim and SNP were the same as those observed in intact tissues (Figure 1). The time-course of the inhibitory responses to the three agents was also compared and is shown in Figure 2. ACh, 10 μM, produced a rapid inhibition of responses to NA which was maximal at between 1 and 3 min. Thereafter, the inhibitory responses to ACh slowly faded and after 1 h were approximately 35–40% of the initial inhibitory response. This was unlikely to be due to ACh breakdown as similar fade occurred with carbachol (Taylor, unpublished observations). In contrast, the inhibitory effects of cromakalim and of SNP developed more slowly, but did not fade with time.

Table 1 Effects of acetylcholine (ACh), cromakalim and sodium nitroprusside (SNP) on membrane potential in intact and rubbed segments of rat aorta

	Membrane potential (mV)	
	Intact	*Rubbed*
Control	-57 ± 0.9 (40)	-48 ± 1.2 (40)
ACh 1 μM	-62 ± 1.7 (24)	-50 ± 2.1 (24)
ACh 10 μM	-76 ± 1.8 (24)	-51 ± 2.3 (24)
Cromakalim 10 μM	-82 ± 2.0 (24)	-78 ± 2.1 (24)
SNP 100 nM	-59 ± 1.4 (24)	-49 ± 1.4 (32)

For drug-treated aortae each value represents the membrane potential at its maximum change from the resting membrane potential and is the mean ± s.e.mean; numbers in parentheses show the number of determinations. The values are derived from 8 different tissues with at least three determinations on each tissue.

Inhibition of responses to KCl These experiments were performed using intact tissues. When the contractions induced by KCl (20 or 80 mM) had developed fully, the inhibitory effects of ACh, cromakalim and SNP were examined using a cumulative dose-response design. All three drugs were capable of totally relaxing a contraction induced by KCl 20 mM. However, when 80 mM KCl was used, ACh and SNP produced only approximately 14% and 53% inhibition, respectively. Cromakalim did not relax an 80 mM KCl-induced spasm (Figure 3).

Microelectrode experiments

In intact preparations, the resting membrane potential of rat aorta was -57 ± 0.9 mV ($n = 40$). The membrane potential was quite stable and only minor fluctuations were observed. On exposure to ACh (10 μM), a rapidly-developing hyperpolarization was detected. This reached a maximum in approximately 1 min and after 2–3 min, the membrane potential had returned to pre-exposure levels (Figure 4). The transient nature of the hyperpolarization was not restricted to high concentrations of ACh, but was also observed after exposure to ACh 1 μM, a concentration which produced significant mechanical relaxation in tissue bath experiments. These results are summarized in Table 1.

When intact tissues were exposed to cromakalim, 10 μM, a slowly-developing hyperpolarization was recorded and this reached a maximum in about 8 min. This was maintained in the continuing presence of cromakalim (Figure 4). After gentle rubbing to remove the endothelium, the resting membrane potential was -48 ± 1.2 mV ($n = 40$), and was significantly lower than when the endothelium was present ($P < 0.01$) (Table 1). Exposure to ACh failed to elicit any significant change in membrane potential (Figure 4) while the hyperpolarizing effects of cromakalim were unchanged (Figure 4, Table 1). Sodium nitroprusside had no effect on membrane potential in rubbed or intact tissues (Table 1).

^{86}Rb efflux experiments

In resting tissues, the average rate of ^{86}Rb exchange ($1.61 \pm 0.05\%\ min^{-1}$) in rubbed tissues was significantly greater than that ($1.05 \pm 0.04\%\ min^{-1}$) obtained for intact preparations ($P < 0.01$, $n = 8$, measured between the 14th and 22nd min of efflux). Because of this difference, subsequent data which show the effects of ACh, cromakalim and SNP are presented as the change in ^{86}Rb efflux (see Methods). These agents were used at concentrations which were associated with their maximum mechano-inhibitory effects.

In intact tissues ACh produced a marked transient increase in ^{86}Rb exchange (Figure 5). This was maximal between 30 s and 1 min and was essentially undetectable after 3 min. ACh had no effect in rubbed tissues. In contrast, cromakalim produced a long-lasting increase in ^{86}Rb efflux in both rubbed and intact preparations (Figure 5). The effects of SNP were examined only in rubbed tissues and no significant changes were detected (Figure 5).

Electron microscopy

The aortae from six different animals were used and many sections were cut and examined from each preparation. In intact tissues, smooth muscle cells lying between the elastic laminae could be seen, with the endothelial cells prominent above the internal elastic lamina (IEL). The internal structure of the endothelial cells was clearly visible (Figure 6). In rubbed tissues, the endothelial cells were absent and damage to the smooth muscle layer below the IEL was always apparent. Cell outlines were diffuse in this region and no clearly-defined contents could be seen. The smooth muscle cells below the damaged layer appeared normal (Figure 7).

Measurement of cyclic GMP concentrations

The resting basal concentration of cyclic GMP was lower in the absence than in the presence of the endothelium (0.076 ± 0.02 and $2.26 \pm 0.58\ pmol\ mg^{-1}$ protein, respectively). The

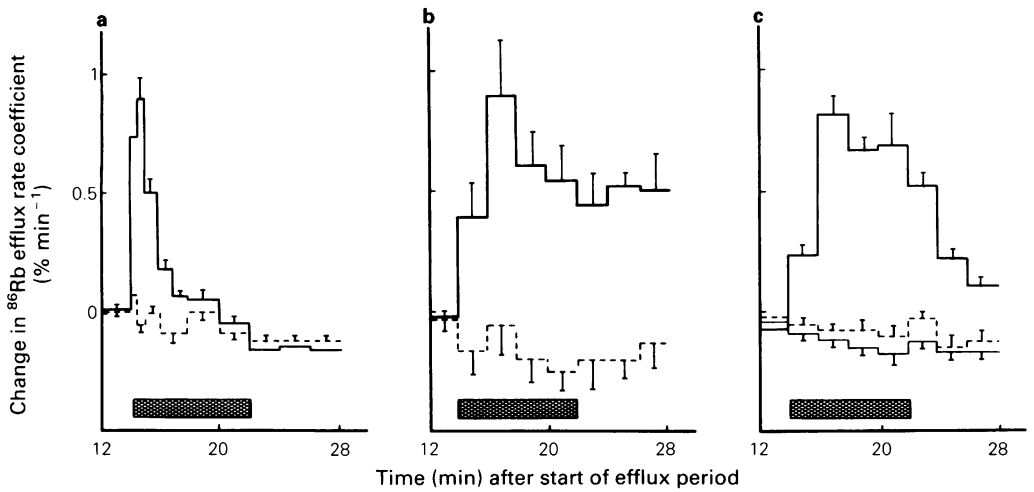

Figure 5 Effects of (a) acetylcholine (ACh, 10 μM), (b) cromakalim (10 μM) and (c) cromakalim (10 μM upper line) and sodium nitroprusside (SNP, 100 nM lower line) on the change in efflux of ^{86}Rb (continuous lines) from intact (a, b) and rubbed (c) segments of rat aorta compared with control, basal loss of ^{86}Rb (broken lines). Exposure to ACh, cromakalim or SNP is indicated by the hatched bar. Each point is the mean derived from 4 experiments; vertical lines show s.e.mean.

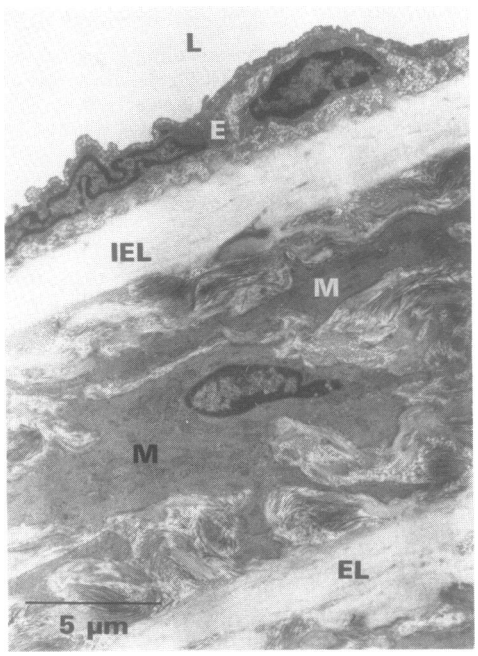

Figure 6 Transmission electron micrograph showing the normal appearance of a typical segment of untreated rat aorta in transverse section. The smooth muscle cells (M) lie between the elastic lamellae (EL). An endothelial cell (E) lying above the internal elastic lamina (IEL) lines the lumenal (L) aspect of the vessel wall.

time-course of changes in cyclic GMP was studied for both ACh and cromakalim in intact tissues. ACh produced a rapid increase in cyclic GMP concentrations which reached a maximum after about 1 min. The level of cyclic nucleotide then declined, but after 4 min, it was still some 400% higher than in controls. In contrast, cromakalim had no significant effect on cyclic GMP concentrations (Figure 8). After 2 min contact, SNP 100 nM increased cyclic GMP concentrations from 0.04 ± 0.0024 to 2.6 ± 0.3 pmol mg^{-1} protein in rubbed tissues ($P < 0.01$, $n = 4$) and from 0.55 ± 0.04 to 2.0 ± 0.2 pmol mg^{-1} protein in intact tissues ($P < 0.01$, $n = 4$).

Discussion

In the present study ACh produced a long-lasting inhibition of mechanical responses in rat aorta. This relaxant effect was associated with a transient hyperpolarization, a transient increase in ^{86}Rb exchange and a rise in tissue levels of cyclic GMP. These effects were all abolished by removal of the endothelium.

Mechanism of ACh-induced hyperpolarization

In a brief publication, Feletou & Vanhoutte (1986) concluded that endothelium-dependent hyperpolarization in rat aorta resulted from a stimulation of Na/K ATPase (see also Vanhoutte, 1987). The

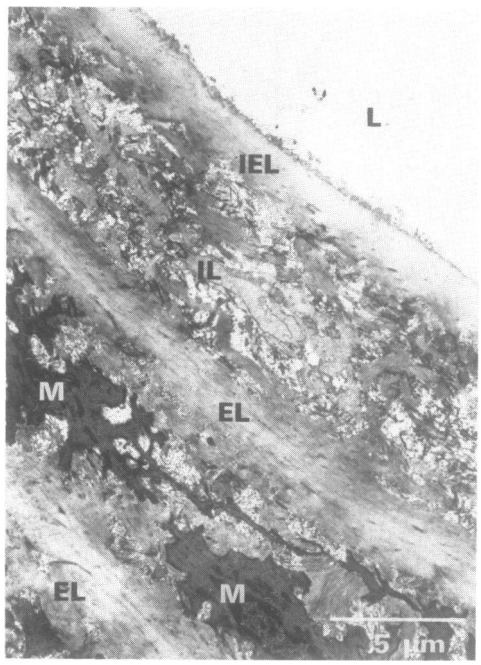

Figure 7 Transmission electron micrograph of a typical transverse section of a rubbed segment of rat aorta. Endothelial cells are absent on the lumenal (L) aspect of the vessel wall and the innermost layer of smooth muscle cells (IL) appears damaged with no clearly-defined cell membranes or contents. The next layer of muscle cells (M) shows the normal appearance of cells contracted with noradrenaline (1 μM). IEL = internal elastic lamina; EL = elastic lamellae.

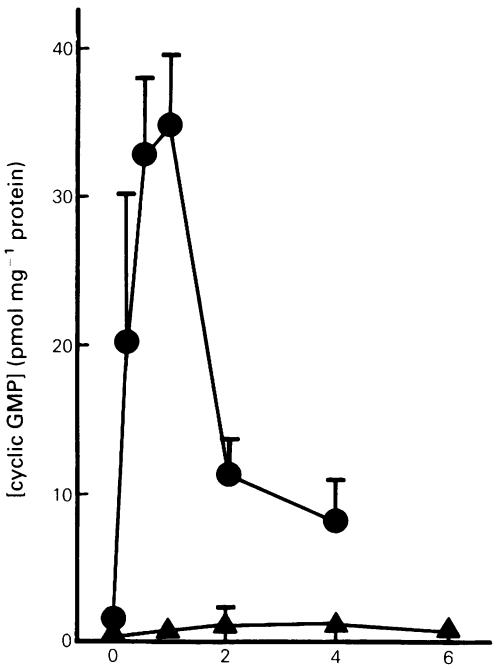

Figure 8 Time-course of changes in cyclic GMP concentrations produced by acetylcholine (10 μM, ●) and cromakalim (10 μM, ▲) in intact segments of rat aorta. Each point is the mean derived from 4 observations; vertical lines show s.e.mean.

results of the present study together with those of previous workers provide little or no support for this assertion. Using a combination of electrical measurements and the manipulation of ionic concentrations, Kuriyama & Suzuki (1978), Kitamura & Kuriyama (1979), Kajiwara (1982) and Bolton *et al.* (1984) provided evidence that cholinergic hyperpolarization was associated with the opening of membrane K^+-channels. The close temporal relationship between the stimulation of ^{86}Rb efflux and the hyperpolarization observed in the present study, strongly suggests that the cause of the ACh-induced hyperpolarization in rat aorta is the opening of membrane K^+-channels. Further evidence of the involvement of K^+-channels in endothelium-dependent relaxations was recently described by Gebremedhin *et al.* (1987).

Relationship between hyperpolarization and cyclic GMP changes

Time-course studies of the action of ACh showed that the maximum cyclic GMP changes coincided with the peak electrical hyperpolarization. However, several pieces of evidence suggest that there was no causal relationship between this electrical event and the changes in nucleotide levels. Firstly, cyclic GMP concentrations remained significantly higher than controls even after the membrane potential had returned to pre-ACh values. Furthermore, mechano-inhibitory concentrations of SNP raised cyclic GMP levels, yet SNP had no effect on membrane potential. Conversely, cromakalim produced a long-lasting hyperpolarization, but did not change tissue cyclic GMP concentrations. Several workers have studied the changes in membrane potential produced by SNP and glyceryltrinitrate (GTN) which are believed to act by stimulating guanylate cyclase. The observed electrical effects have been found to be species-specific, but in general only minor membrane changes accompany the actions of these substances in comparison with their powerful guanylate cyclase stimulating action (Ito *et al.*, 1978; 1983; Kukovetz *et al.*, 1979; Karashima, 1980; Ignarro & Kadowitz, 1985). These data therefore suggest that the observed ACh-induced increase in cyclic GMP concentrations

and the associated electrical hyperpolarization were not causally-related.

Role of hyperpolarization in ACn-induced relaxation

The relatively short-lived hyperpolarization produced by ACh contrasted with the accompanying long-lasting mechanical inhibition. This might suggest that these electrical changes were relatively unimportant in the observed relaxation. To investigate this, experiments were conducted in high K conditions to prevent any increase in membrane potential. Under these conditions, ACh produced a small relaxation, whereas SNP inhibited the K-induced contraction by approximately 50%. Cromakalim was without effect.

Furchgott (1983; 1984) and Murad (1986) have proposed that ACh-induced relaxation is independent of electrical changes and results from guanylate cyclase activation. In the present study, such relaxations should have been comparable with those produced by SNP, assuming that the release of factors from the endothelium is unaffected by depolarizing conditions. The results obtained with ACh, cromakalim and SNP collectively suggest that the full expression of the inhibitory actions of ACh requires both hyperpolarization and the activation of guanylate cyclase. The former may serve both to close any voltage-dependent Ca channels which may have been opened by an excitatory agonist (see Bolton & Large, 1986) and also to initiate relaxant processes distinct from those modulated by guanylate cyclase.

The explanation for the transient nature of the ACh-induced hyperpolarization in the present experiments is unclear. It is not, however, an inherent property of the cells of the aorta. Exposure to cromakalim produced a hyperpolarization of similar magnitude which was maintained, with no evidence of fade. In recent studies, Komori & Suzuki (1987a,b) showed that the hyperpolarization produced by ACh in rabbit saphenous artery was mediated by M_1-muscarinic receptors, whilst mechanical inhibition (and by inference guanylate cyclase activation) was associated with M_2-muscarinic receptors. Thus the transient increase in membrane potential changes observed in the present study might be associated with the development of selective tachyphylaxis of the response mediated by M_1-receptors. In a few experiments, a second exposure to ACh in the continuing presence of a concentration of ACh which had already produced a transient hyperpolarization generated only a small increase in membrane potential (Weston, unpublished). Further studies are required to clarify the possible role of tachyphylaxis in these electrical changes.

Membrane potential differences between rubbed and intact preparations

A striking feature of the present study was that the aortic cells from intact preparations had significantly higher membrane potentials than those from rubbed tissues. A similar finding has been obtained by Beny *et al.* (1986). It is therefore tempting to suggest that basal EDRF release (Rapoport & Murad, 1983; Bigaud *et al.*, 1984; Bullock *et al.*, 1986) holds the membrane in a partially hyperpolarized condition. If true, such an observation could contribute to vasospastic conditions associated with pathological changes to the internal lining of blood vessels.

However, the most likely explanation for these differences in membrane potential between rubbed and intact tissues can be attributed to damage following the rubbing procedure. Evidence for this was obtained from the electron micrographic examination of sections of rubbed tissues. Furthermore, the resting rate of ^{86}Rb exchange was greater in rubbed than in intact preparations, which is the opposite of what would be expected if the basal release of EDRF were holding the membrane in a hyperpolarized condition. These effects may be caused by leakage of intracellular contents from the damaged layer onto the intact muscle cells below.

Does ACh release more than one inhibitory substance?

In the present study, both ACh and SNP activated guanylate cyclase. In addition, ACh hyperpolarized the smooth muscle membrane by opening K-channels. It is possible that a single relaxing factor liberated by ACh could produce both enzyme activation and ion channel opening. An alternative explanation is that ACh liberates two different substances from the endothelium. One of these transiently hyperpolarizes the muscle cells by an action which results in the opening of membrane K^+-channels while the other factor produces an electrically-silent inhibitory response associated with the stimulation of soluble guanylate cyclase. Data consistent with the release by ACh of two inhibitory substances from the vascular endothelium of rabbit saphenous artery have recently been obtained by Komori & Suzuki (1987a, b).

The existence of two different endothelium-derived endogenous inhibitory substances could explain some anomalies in the results of workers in this field. In their study of endothelium-dependent inhibition, Bolton & Clapp (1986) demonstrated that the endothelium-dependent inhibitory action of carbachol in guinea-pig mesenteric artery was associated with a hyperpolarization, whilst the similar effects of substance P were electrically silent.

However, in intact porcine coronary artery, Beny *et al.* (1986) have shown that the inhibitory effects of substance P were associated with a hyperpolarization.

Following the evidence by Palmer *et al.* (1987) indicating that EDRF might be nitric oxide, Vanhoutte (1987) has speculated that the search for the identity of EDRF may be over. The results of the present study and those of other workers (Gebremedhin *et al.*, 1987; Komori & Suzuki, 1987a,b) suggest that whatever agent is responsible for the stimulation of guanylate cyclase, such an effect cannot fully account for the observed endothelium-dependent increase in membrane potential. Such changes are clearly a common feature of endothelium-dependent inhibitory responses in a variety of tissues and further studies to assess their importance are in progress.

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