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SOME ELECTRICAL PROPERTIES OF THE ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION RECORDED FROM RAT ARTERIAL SMOOTH MUSCLE CELLS

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SUMMARY

1. Electrical responses produced by acetylcholine (ACh) and histamine were recorded from smooth muscle cells of the intralobular small pulmonary artery (SPA), main pulmonary artery (MPA) and thoracic aorta of rats.

2. In MPA and SPA, ACh and histamine produced a transient hyperpolarization of the membrane, and the potential decayed exponentially with a time constant of 2–3 min. In aorta, ACh produced a sustained and histamine produced a transient hyperpolarization.

3. The ACh- and histamine-induced hyperpolarizations were blocked by atropine and mepyramine, respectively, or by removing the endothelial cells.

4. The amplitude of the hyperpolarization was increased in low $[K^+]_o$ solutions and decreased in high $[K^+]_o$ solutions. The ionic conductance of the membrane was increased during the hyperpolarization, suggesting an involvement of the increased potassium conductance.

5. A reproducible amplitude of hyperpolarization was generated when ACh or histamine was applied at intervals of over 10 or 30 min, respectively.

6. In aorta, after the transient hyperpolarization had ceased during continued application of histamine, ACh again produced a hyperpolarization, i.e. the transient nature of the hyperpolarization was not due to desensitization of the receptor upon which the hyperpolarizing substance acted, assuming histamine and ACh release the same hyperpolarizing substance.

7. ACh and histamine relaxed the tissues from SPA, MPA and aorta during the noradrenaline (NA)- or high $[K^+]_o$ solution-induced contraction, in a concentration-dependent manner, only when the endothelial cells were intact. Both ACh and histamine were potent relaxants in MPA and aorta, but showed weak relaxing actions in SPA.

8. In aorta, ACh and histamine produced a sustained relaxation for up to 10 min, and Methylene Blue diminished and altered it to a transient relaxation (for histamine) or an initial large, followed by a small sustained (for ACh), relaxation.

9. In the presence of NA and NA plus Methylene Blue, ACh and histamine also produced a hyperpolarization similar to that seen in the control

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10. It is concluded that in arteries of the rat, ACh and histamine release a hyperpolarizing substance from the endothelial cells. This substance may be different from the endothelium-derived relaxing factor (EDRF), and is released mainly transiently. The hyperpolarization is generated by an increase in potassium conductance of the membrane, and this has some contribution to the endothelium-dependent relaxation.

INTRODUCTION

The endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) is liberated from arterial tissues by many types of chemical and mechanical stimuli and modifies vascular smooth muscle tone, thus regulating cardiovascular haemodynamics (Furchgott, 1983, 1984; Vanhoutte, Rubanyi, Miller & Houston, 1986). Nitric oxide is a candidate for the EDRF (Palmer, Ferrige & Moncada, 1987), and similarities and dissimilarities between EDRF and nitric oxide are now being discussed (Ignarro, Byrns, Buga & Wood, 1987; Shikano, Ohlstein & Berkowitz 1987).

In arterial tissues, muscarinic agonists hyperpolarize the smooth muscle membrane (Kuriyama & Suzuki, 1978). This hyperpolarization is converted to depolarization after removal of the endothelial cells, thereby suggesting a release of hyperpolarizing substance from the endothelial cells (Bolton, Lang & Takewaki, 1984). In the rabbit saphenous artery, acetylcholine (ACh) and oxotremorine produce an endothelium-dependent relaxation through activation of muscarinic receptors, and the former, but not the latter, agonist effect is accompanied by a transient hyperpolarization of the membrane (Komori & Suzuki, 1987*a*). Furthermore in the rat main pulmonary artery, haemoglobin and Methylene Blue, which inhibit the actions of EDRF (Martin, Villani, Jothianandan & Furchgott, 1985), do not inhibit the ACh-induced hyperpolarization (Chen & Suzuki, 1988). These observations suggest that the substance involved in the endothelium-dependent hyperpolarization may be different from the EDRF.

We investigated the properties of the electrical responses (hyperpolarization) produced by ACh and histamine in smooth muscle cells of rat arteries. Both of these substances were found to release the hyperpolarizing substance from the endothelial cells and, although there were some regional differences in ability to release the hyperpolarizing substance, the properties of this substance were similar between arteries.

METHODS

Albino rats (Wister King) of either sex, weighing 200–300 g (8–16 weeks old) or 50–80 g (4–5 weeks old) were anaesthetized by diethylether and exsanguinated from the femoral artery. The main pulmonary artery and left lung were excised from the older rats and thoracic aorta from the younger rats. The isolated tissues were kept in Krebs solution at room temperature and the arteries cleaned by removing the surrounding tissues. Ring segments of the main pulmonary artery or aorta were made, and a transverse strip was prepared by cutting across the ring. The third branch of the intralobular pulmonary artery (external diameter, 100–300 μm; Suzuki & Twarog, 1982) was dissected by careful removal of the overlying lung parenchyma and bronchi. The vessels were mounted in an organ bath which was made from lucite plate with a capacity of about 2 ml. A silicon

rubber plate (Shin-etsu Kagaku, KE-66) was fixed at the bottom of the chamber, and the isolated tissues were mounted on the rubber plate by using tiny pins. The warmed Krebs solution (35 °C) was superfused over the tissue at a flow rate of about 3 ml min^{-1}. In the main pulmonary artery and aorta, the endothelial layer was mounted upwards while in the small pulmonary artery the vessel was mounted without cutting it open.

A glass capillary microelectrode filled with 3 M-KCl (the resistance being 40–80 MΩ) was inserted into smooth muscle cells, from the internal side in the aorta and main pulmonary artery and from the serosal side in the intralobular small pulmonary artery. The partition stimulating method (Abe & Tomita, 1968) was used to produce electrotonic potentials in smooth muscle cells. The electrical responses thus recorded were displayed on a cathode-ray oscilloscope (VC-9, Nihon Kohden) and also on a pen-writing recorder (Recticorder RJG-4024, Nihon-Kohden).

The endothelial cells of the main pulmonary artery and aorta were removed according to the methods of Furchgott & Zawadzki (1980), i.e. the internal surface of the arteries was rubbed gently with a moistened cotton ball. In the case of the small pulmonary artery, the inside of the vessel was rubbed with a fine steel needle (outer diameter, 0·2 mm) or internally perfused with distilled water for 10 min (Nagao & Suzuki, 1987). Light-microscopic examination of thin sections (20 μm thick) of these tissues (Ibengwe & Suzuki, 1987) indicated that either of the above-mentioned methods were able to remove the endothelial cells from the arteries, without any detectable change in the resting membrane potential and contractility of smooth muscle to noradrenaline and high $[K^+]_o$ solution (Table 1).

The ionic composition of the Krebs solution was as follows (mM): Na^+, 137·4; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·5; HCO_3^-. 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134; glucose, 11·5. Concentrations of K^+ were modified by replacing NaCl with KCl. The solutions were aerated with O_2 and 3% CO_2, and the pH of the solution was maintained at 7·2–7·4.

Drugs used were acetylcholine chloride, atropine sulphate, histamine hydrochloride, noradrenaline hydrochloride and mepyramine maleate from Sigma, St Louis, MO, USA.

The values recorded were expressed as mean ± S.D., and the statistical significances were determined using Student's *t* test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

RESULTS

Regional differences in the endothelium-dependent hyperpolarization

The effects of ACh and histamine on membrane potential of smooth muscle cells were investigated in isolated intralobular small pulmonary artery (SPA), the main pulmonary artery (MPA) and the thoracic aorta of the rat. Smooth muscle cells of these arteries were electrically quiescent, and their resting membrane potentials were much the same (Table 1). As shown in Fig. 1, ACh and histamine produced a transient hyperpolarization of the smooth muscle membrane in MPA and SPA. The peak amplitude of hyperpolarization and the time required to reach peak amplitude varied between arteries. The hyperpolarization ceased within 3–5 min in the presence of ACh or histamine. When the amplitude of the hyperpolarization produced by ACh (10^{-5} M) in MPA was plotted on a logarithmic scale against time, the decay of the hyperpolarization was exponential, with a time constant of 1·5–4 min (mean 2·3 ± 0·2 min, $n = 4$). In the aorta, however, ACh, but not histamine, produced a hyperpolarization which consisted of an initial large, and a following sustained amplitude, and when the muscles were stimulated with 10^{-5} M-ACh the amplitude of the hyperpolarization at 30 min was 2·8 ± 0·5 mV ($n = 5$), the value being about a quarter of the initial large hyperpolarization (Table 2). The histamine-induced hyperpolarization in the aortic smooth muscle was transient, as in the case of SPA.

The potency of ACh and histamine for generation the hyperpolarization varied

between arteries. When 10^{-5} M-ACh was applied, the amplitude of the hyperpolarization was larger in MPA and aorta (5–10 mV) than in SPA. Histamine (5×10^{-5} M) produced a large hyperpolarization in SPA and aorta and a negligibly small amplitude of hyperpolarization (up to 2 mV) in MPA (Fig. 1).

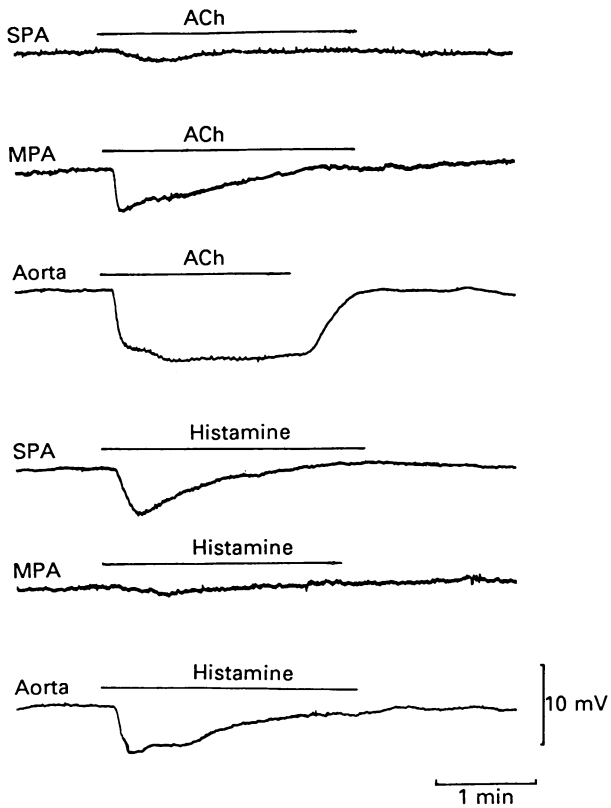

Fig. 1. Hyperpolarizations produced by ACh (10^{-5} M) and histamine (10^{-5} M) recorded from smooth muscle cells of SPA, MPA and aorta. ACh or histamine was applied at the bar above each recording. All the responses are from different tissues.

Figure 2 summarizes the concentration–response relationship of the ACh- or histamine-induced hyperpolarization in SPA, MPA and aorta. ACh was a potent hyperpolarizer in the aorta, and the action was about 100 times weaker in MPA. Smooth muscle cells of the SPA showed a negligibly small hyperpolarizing response to ACh. Histamine was equipotent in hyperpolarizing the membrane in SPA and aorta, and showed weak actions on MPA.

Experiments were carried out to observe the ACh- or histamine-induced hyperpolarization in solutions containing different $[K^+]_o$. In MPA, decreasing $[K^+]_o$ from 5·9 to 1·8 mM depolarized the membrane by about 2 mV (Table 1) and increased the amplitude of the ACh-induced hyperpolarization, while increasing $[K^+]_o$ to 10·7 mM depolarized the membrane by about 5 mV (Table 1) and decreased the amplitude of the ACh-induced hyperpolarization. Similar effects were observed in the

TABLE 1. Membrane potentials (mV) of smooth muscle cells recorded from the intralobular small pulmonary artery (SPA), the main pulmonary artery (MPA) and the thoracic aorta of rats, with intact or removed endothelial cells

	SPA	MPA	Aorta
		Endothelium intact	
Control (RMP)	$-52{\cdot}6 \pm 2{\cdot}7$ (49)	$-52{\cdot}0 \pm 1{\cdot}8$ (125)	$-53{\cdot}8 \pm 2{\cdot}3$ (35)
$[K^+]_o = 1{\cdot}8$ mM	$-50{\cdot}3 \pm 1{\cdot}0$ (10)*	$-49{\cdot}7 \pm 1{\cdot}2$ (8)*	—
$[K^+]_o = 3{\cdot}6$ mM	$-50{\cdot}8 \pm 1{\cdot}0$ (9)*	$-50{\cdot}3 \pm 1{\cdot}2$ (10)*	—
$[K^+]_o = 10{\cdot}7$ mM	$-50{\cdot}6 \pm 0{\cdot}9$ (10)*	$-46{\cdot}9 \pm 1{\cdot}6$ (9)*	—
NA (10^{-7} M)	$-45{\cdot}4 \pm 1{\cdot}3$ (6)*	$-42{\cdot}7 \pm 1{\cdot}3$ (11)*	$-47{\cdot}3 \pm 2{\cdot}0$ (10)*
MB (3×10^{-6} M)	$-51{\cdot}4 \pm 1{\cdot}6$ (6)	$-51{\cdot}6 \pm 1{\cdot}4$ (6)	$-46{\cdot}5 \pm 1{\cdot}2$ (10)*
NA (10^{-7} M) + MB (3×10^{-6} M)	$-44{\cdot}8 \pm 2{\cdot}1$ (5)*	$-40{\cdot}8 \pm 1{\cdot}0$ (5)*	$-40{\cdot}6 \pm 1{\cdot}8$ (6)*
Atropine (10^{-6} M)	—	$-51{\cdot}7 \pm 1{\cdot}7$ (8)	$-53{\cdot}2 \pm 1{\cdot}6$ (5)
Mepyramine (10^{-7} M)	$-52{\cdot}3 \pm 1.8$ (8)	—	$-53{\cdot}6 \pm 1{\cdot}8$ (6)
		Endothelium removed	
Control	$-51{\cdot}8 \pm 2{\cdot}2$ (46)	$-51{\cdot}2 \pm 1{\cdot}6$ (17)	$-50{\cdot}1 \pm 1{\cdot}4$ (9)*
NA (10^{-7} M)	$-44{\cdot}2 \pm 1{\cdot}5$ (14)*	$-41{\cdot}6 \pm 2{\cdot}2$ (10)*	$-41{\cdot}3 \pm 2{\cdot}1$ (5)*
MB (3×10^{-6} M)	$-50{\cdot}8 \pm 1{\cdot}8$ (5)	$-48{\cdot}7 \pm 1{\cdot}7$ (6)*	$-50{\cdot}4 \pm 1{\cdot}9$ (6)
NA (10^{-6} M) + MB (3×10^{-6} M)	$-42{\cdot}7 \pm 2{\cdot}0$ (3)*	$-39{\cdot}5 \pm 1{\cdot}9$ (5)*	$-42{\cdot}2 \pm 1{\cdot}7$ (4)*

NA = noradrenaline; MB = Methylene Blue; RMP = resting membrane potential. Results are given as mean ± S.D. with number of observations in parentheses. *, significantly different from the control ($P < 0{\cdot}05$).

TABLE 2. Effects of noradrenaline (NA) and Methylene Blue (MB) on the amplitude of hyperpolarization (mV) produced by ACh (10^{-5} M) and histamine (10^{-5} M) in rat arteries

	SPA	MPA	Aorta
		Control	
Histamine	3·7 ± 0·7 (15)	0·1 ± 0·2 (7)	4·5 ± 0·7 (15)
ACh	0·5 ± 0·6 (10)	4·8 ± 0·6 (17)	11·6 ± 0·8 (13)
		NA (10^{-7} M)	
Histamine	6·3 ± 1·1 (5)*	—	8·5 ± 1·2 (7)*
ACh	—	10·2 ± 2·4 (11)*	14·9 ± 2·3 (4)*
		NA (10^{-7} M) + MB (3×10^{-6} M)	
Histamine	6·7 ± 1·6 (4)*	—	8·1 ± 1·8 (3)*
ACh	—	11·5 ± 2·1 (5)*	12·7 ± 2·2 (3)

The peak amplitude was measured. SPA = small pulmonary artery; MPA = main pulmonary artery. Results are given as mean ± S.D. with number of observations in parentheses. A dash indicates that no experiment was done. *, significant difference from the control ($P < 0.05$).

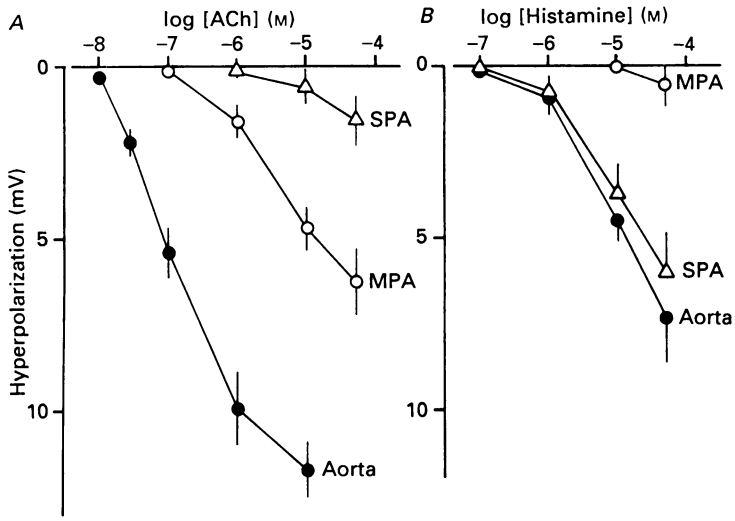

Fig. 2. Concentration–response relationship of the ACh- (*A*) and histamine-induced (*B*) hyperpolarization in SPA (△), MPA (○) and aorta (●). Mean ± S.D. ($n = 5$–10).

SPA (Fig. 3). Figure 4 shows that in SPA and MPA, the amplitude of the ACh- and histamine-induced hyperpolarizations had a linear relationship with the concentration of $[K^+]_o$ plotted on a logarithmic scale.

The amplitude of electrotonic potentials produced by the partition stimulating method (Abe & Tomita, 1968) decreased during the ACh- and histamine-induced hyperpolarizations (data not shown), suggesting that these hyperpolarizations were generated by an increase in potassium conductance of the membrane.

In SPA and MPA, but not in the aorta, from which the endothelial cells were removed, the membrane potential of smooth muscle cells was not significantly different from the endothelium-intact tissues (Table 1). In the absence of the endothelial cells, ACh and histamine did not produce the hyperpolarization.

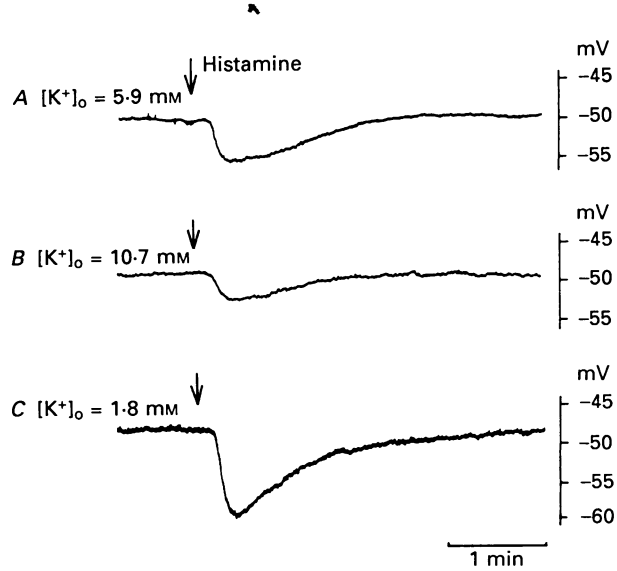

Fig. 3. The histamine-induced hyperpolarizations recorded from SPA in different $[K^+]_o$ solutions (*A*, 5·9 mM; *B*, 10·7 mM; *C*, 1·8 mM). Histamine (5×10^{-5} M) was added into the superfusate at the arrow. All responses from the same tissue.

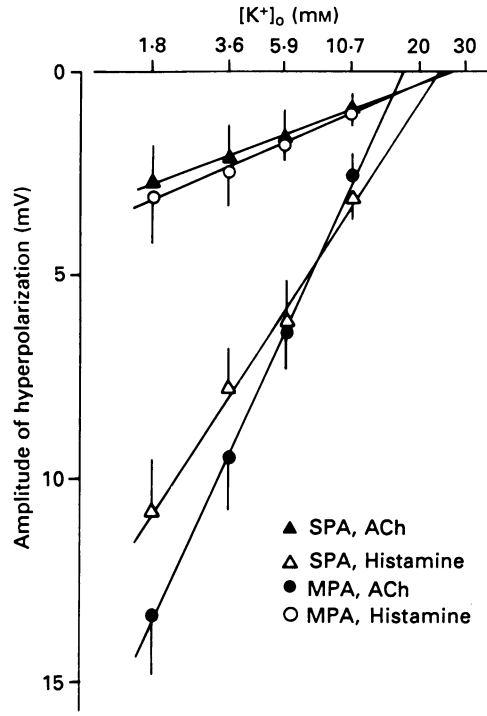

Fig. 4. Relationship between $[K^+]_o$ concentration and amplitude of hyperpolarization produced by ACh (5×10^{-5} M) and histamine (5×10^{-5} M) in pulmonary arteries of the rat. ACh (▲) and histamine (△) in SPA; ACh (●) and histamine (○) in MPA. Mean ± S.D. (n = 5–8). The lines in the figure were drawn by eye.

The hyperpolarizing responses elicited by ACh and histamine were also blocked by pre-treatment with atropine (10^{-6} M) and mepyramine (10^{-7} M), respectively, which did not alter the membrane potential (Table 1).

Desensitization experiments

Attempts were made to determine the time required to generate a reproducible amplitude of hyperpolarization by application of ACh and histamine in smooth

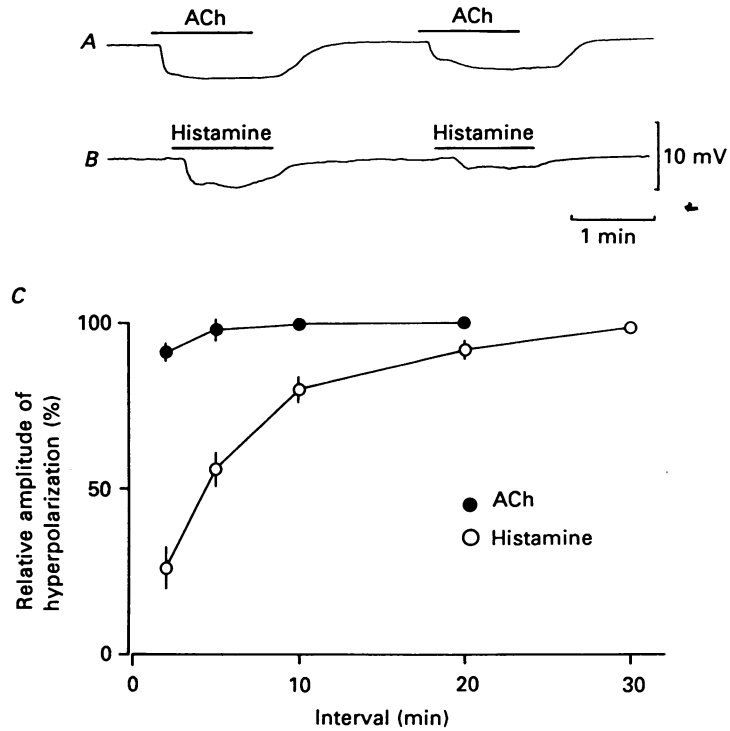

Fig. 5. Decrease in ACh- and histamine-induced hyperpolarization recorded from aortic smooth muscle cells. ACh, 5×10^{-6} M (*A*), or histamine, 5×10^{-5} M (*B*), was applied twice at varying intervals (1–30 min), and the amplitude of hyperpolarization produced by the second application was expressed as percentage of the first (*C*). Mean ± S.D. (n = 5–10).

muscle cells of the aorta. A fixed concentration of ACh (5×10^{-6} M) or histamine (5×10^{-5} M) was applied twice at varying intervals, and the amplitude of the hyperpolarization (measured at the peak) produced by the second stimulus relative to the first was plotted as a function of time between the two stimuli (Fig. 5). A reproducible amplitude of hyperpolarization was generated when the interval between ACh applications was over 10 min, while it required more than a 30 min interval for histamine to generate a similar amplitude of hyperpolarization.

It was interesting to know why the ACh- and histamine-induced hyperpolarizations were generated only transiently. Figure 6 shows membrane hyperpolarizations produced by histamine and ACh in smooth muscle cells of the aorta. After the histamine-induced hyperpolarization ceased, application of ACh again

hyperpolarized the membrane. The ACh-induced hyperpolarization was still generated in the presence of histamine but the membrane potential had reverted to the resting level (Fig. 6*B*). Thus, the decline in amplitude of the hyperpolarization during continued application of histamine was not due to desensitization of receptors upon which the hyperpolarizing substance acts or alternatively ACh and histamine release different hyperpolarizing substances.

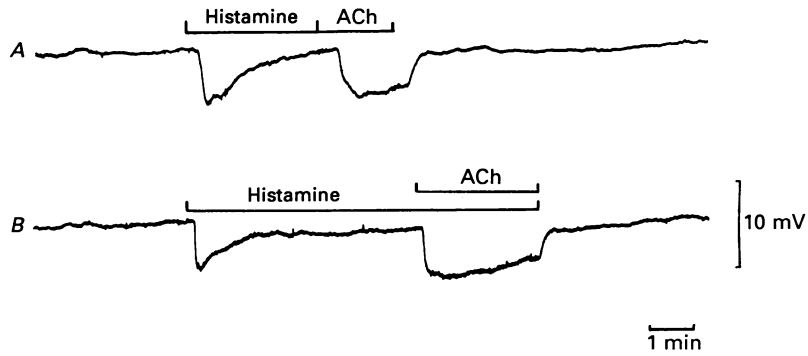

Fig. 6. Hyperpolarization by ACh of the aortic smooth muscle cells generated after the histamine-induced transient hyperpolarization had ceased. ACh (10^{-6} M) was applied just after histamine (5×10^{-5} M) (*A*) or together with histamine (*B*).

Quantitative experiments were designed to determine whether or not ACh and histamine release the same hyperpolarizing substance from the endothelial cells. ACh and histamine, at concentrations which were equipotent in producing a given amplitude of hyperpolarization (5–7 mV), were applied successively at different intervals, in order to observe the effects of conditioning hyperpolarization on the ACh- or histamine-induced hyperpolarization. The stimulant was applied for 1 min, which was long enough to obtain the maximum amplitude of hyperpolarization. The recovery from hyperpolarization after washing out the stimulant required 10–20 s. The peak amplitude of the hyperpolarization produced by the test stimulus was expressed as a percentage of the control response which was produced over 10 min (for ACh) or 30 min (for histamine) before application of the test stimulus.

As shown in Fig. 7*A* and *B*, the hyperpolarization generated by histamine or ACh 1 min before a second application of ACh or histamine reduced the responses generated by the test stimulus to about 70% of the control. The amplitude of the hyperpolarization produced by the test stimulus was identical to the control when the interval between the conditioning and the test stimuli was over 5 min. The recovery of the response was similar for ACh and histamine and independent of which agent was chosen as the conditioning or test stimulus (Fig. 7*C*).

Relationship between hyperpolarization and relaxation

In isolated segments of SPA, MPA and aorta, endothelium-dependent relaxations of smooth muscle tissue by ACh and histamine were demonstrated during noradrenaline (NA)- or high $[K^+]_o$ solution-induced contractions. The tissues from MPA and aorta were contracted by NA (10^{-7} M) and those from SPA were contracted

by 24·6 mM $[K^+]_o$ solution, because NA produced sustained contracture in MPA and aorta but not in SPA. Both NA and 24·6 mM $[K^+]_o$ solution elevated the tension to 40–60% of the maximum contraction produced by 118 mM $[K^+]_o$ solution. After the tension reached a stable level, increasing concentrations of ACh or histamine were applied cumulatively, and the amplitude of the relaxation was expressed as a percentage of the contraction generated before application of stimulant.

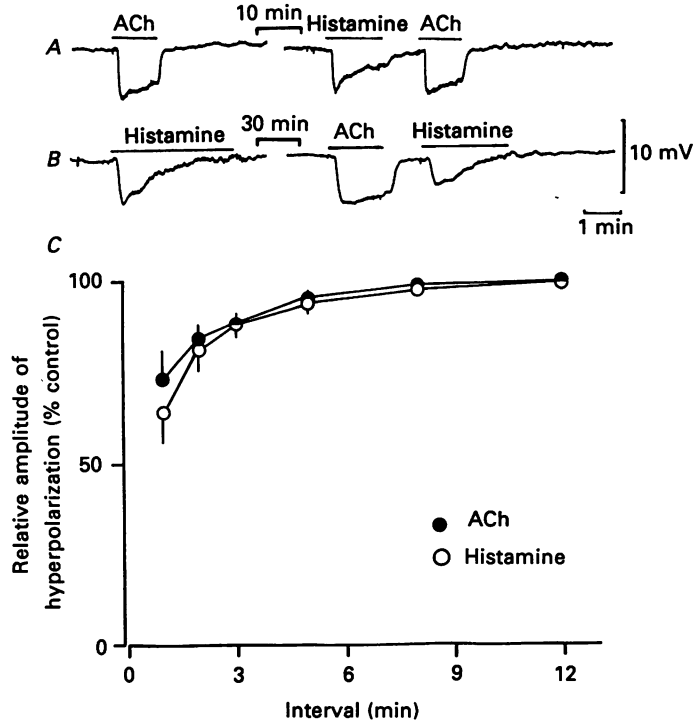

Fig. 7. Effects of conditioning hyperpolarization by histamine (2×10^{-5} M) and ACh (10^{-6} M) on the ACh- (*A*) and histamine-induced (*B*) hyperpolarization of aortic smooth muscle cells. *C*, effects of previously generated hyperpolarization on subsequently generated ACh- (●) or histamine-induced (○) hyperpolarization in the rat aorta. Relative value is plotted with mean ± S.D. (n = 5–8), as a function of time between the conditioning and test stimulations.

Figure 8 shows the concentration–relaxation relationship of SPA, MPA and aorta to ACh and histamine. ACh was a potent relaxant in MPA and aorta, and the latter was about 10 times more sensitive than the former. A similar relationship was also found in the case of histamine. In SPA, ACh and histamine were weak relaxants, and high concentrations of histamine (above 10^{-5} M), but not ACh, generated a biphasic response, i.e. an initial transient relaxation followed by a sustained contraction, the amplitude being 10–30% of the NA-induced contraction (Fig. 8*B* shows only the peak amplitude of the transient relaxation by not the contraction).

The ACh- and histamine-induced relaxations were blocked by atropine (10^{-6} M) and mepyramine (10^{-7} M), respectively. Removal of the endothelial cells also caused

disappearance of the ACh- or histamine-induced relaxation, and produced a contraction at higher concentrations (above $1–5 \times 10^{-5}$ M). The concentrations of SPA produced by high concentrations of histamine were further enhanced by 20–40%, after removal of the endothelial cells.

We reported (Chen & Suzuki, 1988) that Methylene Blue inhibits the endothelium-dependent relaxation, with no alteration of the endothelium-dependent hyperpolarization generated by ACh. In the aorta, application of ACh or histamine

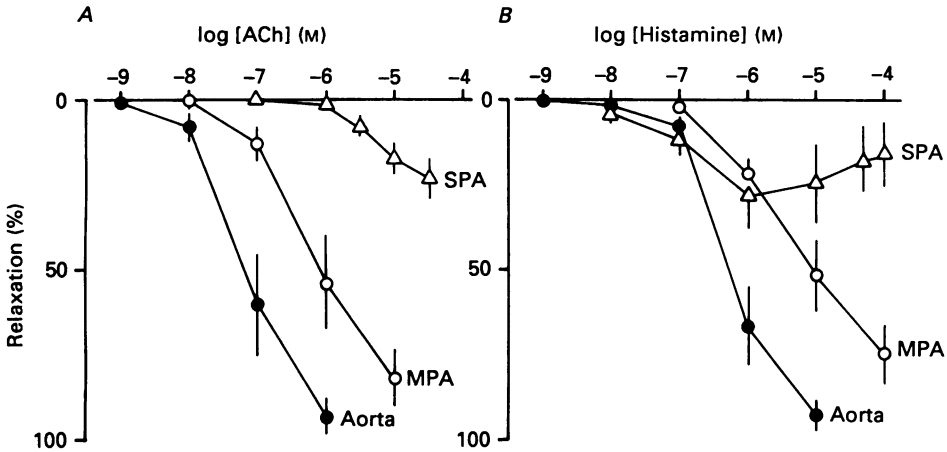

Fig. 8. Concentration–response relationship of the ACh- (*A*) and histamine-induced (*B*) relaxation of tissues which were contracted by 10^{-7} M-noradrenaline in MPA and aorta or by 24·6 mM $[K^+]_o$ solution in SPA. After the tension had reached a stable level, increasing concentrations of ACh (10^{-9}–3×10^{-5} M) or histamine (10^{-9}–10^{-4} M) were added cumulatively, and the maximum amplitude of relaxation measured from the tension before application of these stimulants was expressed as a percentage of the tension. Mean ± S.D. (n = 6–10).

produced a sustained relaxation of tissues which were contracted by NA. Application of Methylene Blue (3×10^{-6} M) enhanced the NA-induced contraction by 30–60%, and the ACh- or histamine-induced relaxation was greatly reduced. In the absence of Methylene Blue, both ACh and histamine produced a large sustained relaxation (the amplitude being nearly 100% of the NA-induced contraction) for up to 10 min. In the presence of Methylene Blue, the histamine-induced relaxation was transient, with the peak amplitude about a quarter of the control, and ceased within 10 min. The time required to reach the maximum relaxation was 64 ± 16 s (n = 7). The ACh-induced response in the presence of Methylene Blue consisted of an initial large transient relaxation followed by a sustained component of relaxation. The maximum amplitude was about 40% of the control. The time required to reach the maximum relaxation was 113 ± 10 s (n = 4).

Electrical responses of smooth muscle cells of the aorta to histamine and ACh were recorded in conditions similar to those used for recording the mechanical responses, i.e. histamine or ACh was applied in the presence of NA and NA plus Methylene Blue. The transient hyperpolarization was also generated by ACh and histamine in these conditions (Fig. 9). NA depolarized the membrane by 6–10 mV (Table 1) and

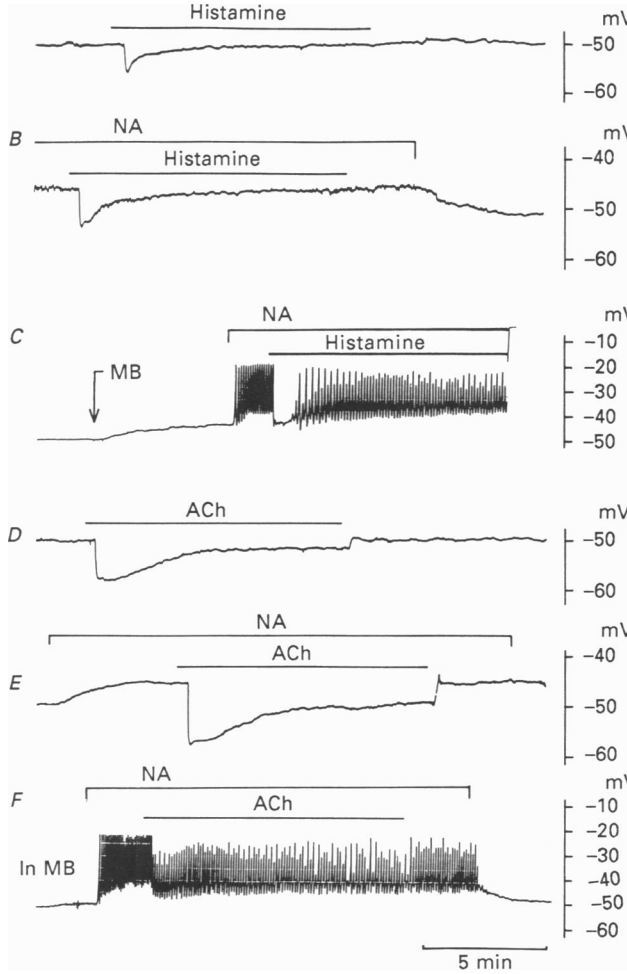

Fig. 9. Electrical responses recorded from aortic smooth muscle cells during application of histamine (10^{-5} M, *A–C*) and ACh (10^{-6} M; *D–F*) before (*A* and *D*) and after application of noradrenaline (NA, 10^{-7} M; *B* and *E*) and noradrenaline plus Methylene Blue (MB, 3×10^{-6} M; *C* and *F*). *A–C* and *D–F* are recordings from different tissues.

increased the amplitude of the peak of the transient hyperpolarization (Table 2). Application of Methylene Blue depolarized the membrane of aortic smooth muscle cells by about 8 mV, but did not alter the membrane potential in MPA and SPA. Application of NA together with Methylene Blue further depolarized the membrane in the aorta and MPA but not in SPA. Application of NA to the aorta, either in the presence or absence of Methylene Blue, often generated spike potentials with the amplitude being 20–30 mV. Application of ACh or histamine in the presence of NA and Methylene Blue also produced a transient hyperpolarization and often blocked the generation of spikes transiently (1–3 min).

After removal of the endothelial cells, the amplitude of the NA-induced depolarization was increased in aorta but not in MPA and SPA. Methylene Blue

(3×10^{-6} M) depolarized the membrane in MPA but not in SPA and aorta (Table 1). In tissues with no intact endothelial cells, ACh and histamine did not produce hyperpolarization. In SPA, but not in MPA and aorta, histamine (above 10^{-5} M) depolarized the smooth muscle membrane by 3–5 mV after removal of endothelial cells.

DISCUSSION

The present experiments confirmed that in arteries, the hyperpolarization produced by muscarinic agonists is mainly due to substances released from the endothelium (Bolton *et al.* 1984). The endothelium-dependent hyperpolarization of arterial smooth muscle cells is also produced by substance P (Beny, Brunet & Huggel, 1986), but it remains unclear whether the hyperpolarization is generated by EDRF or by other unknown substances. Komori & Suzuki (1987*a*) showed that in the rabbit saphenous artery ACh relaxes the tissue which is contracted by NA, with a transient hyperpolarization, while oxotremorine relaxes with no alteration of the membrane potential, i.e. the endothelium-dependent relaxation by muscarinic agonists is independent of the endothelium-dependent hyperpolarization. The muscarinic receptors involved in the release of EDRF also differ from those of the hyperpolarizing substance, and stimulation of M_1- and M_2-subtypes of the receptor releases the hyperpolarizing and relaxing substances, respectively (Komori & Suzuki, 1987*b*). Furthermore in the rat main pulmonary artery, the ACh-induced transient hyperpolarization which is produced endothelium-dependently, is insensitive to Methylene Blue and haemoglobin (Chen & Suzuki, 1988), but both of these agents are inhibitors of the actions of EDRF (Martin *et al.* 1985). All these observations suggest that the endothelium-dependent hyperpolarization is generated by a substance which is different from the EDRF.

In the rat pulmonary artery, there was a regional difference in potency of ACh and histamine to release hyperpolarizing substance and EDRF, and the relaxing actions of these agonists were not always related to the hyperpolarizing actions. In particular, histamine released the hyperpolarizing substance in the aorta and SPA and to a lesser extent in MPA, while ACh released this substance mainly in the aorta and MPA but to a lesser extent in SPA. Such regional differences in releasing the hyperpolarizing substance would explain the different actions of substance P in guinea-pig mesenteric artery (Bolton & Clapp, 1986) and pig coronary artery (Beny *et al.* 1986).

The agonist-induced release of the hyperpolarizing substance seems to be transient, although rather sustained release of this substance could be elicited by ACh in the rat aorta. The transient nature of the hyperpolarizing response may not be due to desensitization of the receptors for hyperpolarizing substance, because application of ACh after the cessation of the histamine-induced hyperpolarization again hyperpolarized the membrane. The desensitization of ACh or histamine receptors at the endothelial cell membrane is also unlikely, since stimulation of these receptors could produce sustained relaxation.

The recovery from the desensitization of receptors was rapid for ACh, in comparison with histamine which required up to 30 min to recover from desensitization. Thus, the nature of the receptors at the endothelial cell membrane

(or possibly the biochemical processes between the receptor activation and the exocytosis of the hyperpolarizing substance in the cell) differs between ACh and histamine. However, the experiments in which ACh and histamine were applied alternatively suggest that the hyperpolarizing substance released by ACh may be identical to that released by histamine, because the recovery process of the receptors for the hyperpolarizing substance for the desensitization was not different, irrespective of whether ACh or histamine was used as conditioning or test stimulant.

In many arteries, constant liberation of EDRF from the endothelial cells has been suggested from the evidence that drugs which inhibit the actions of EDRF enhance muscle tension (Furchgott, 1984; Vanhoutte *et al.* 1986). A contribution by the hyperpolarizing substance to maintenance of the resting membrane potential of arterial smooth muscle cells is also suggested for some arteries (Beny *et al.* 1986; Southerton, Taylor & Weston, 1987), although this is not the case in other arteries (Nagao & Suzuki, 1987; Komori & Suzuki, 1987*a*). The present experiments revealed that in rat pulmonary arteries, the removal of the endothelium does not cause depolarization of arterial smooth muscle cells. i.e. it is unlikely that the hyperpolarizing substance is liberated constantly from the endothelial cells to maintain the membrane potential at more negative level. Electron-microscopic examination demonstrates that mechanical removal of the endothelial cells often damages the innermost layer of smooth muscle cells, and such tissues show a higher resting rate of ^{86}Rb exchange than the intact tissues (Taylor, Southerton, Weston & Baker, 1988). Thus, it is likely that depolarization of smooth muscle membrane after removal of the endothelial cells may be mainly due to damage of the adjacent smooth muscle cells.

In vascular smooth muscle cells, hyperpolarization of the membrane is sometimes related to relaxation; for example, β-adrenoceptor-mediated hyperpolarization in the rabbit facial vein (Prehn & Bevan, 1983) or cat cerebral arteries (Harder, Abel & Hermsmeyer 1981), muscarinic receptor-mediated hyperpolarization of the guinea-pig lingual artery (Brayden & Large, 1986) and cromakalim (a potassium channel opener)-induced hyperpolarization in the rat portal vein (Weir & Weston, 1986) are related to the relaxation of the tissue. However, vasodilatation induced by compounds which contain a nitro group is mediated by an increase in cyclic GMP production in the cell (Ignarro & Kadowitz, 1985) and is not accompanied by membrane hyperpolarization (Kuriyama, Ito, Suzuki, Kitamura & Itoh, 1982). A possible candidate for EDRF is nitric oxide (Palmer *et al.* 1987), and if this is the case, such a chemical substance would relax the muscle with no alteration of the membrane potential. This would also support the concept that the hyperpolarizing substance differs from EDRF.

When we consider the contribution of membrane hyperpolarization to endothelium-dependent relaxation, the effects of Methylene Blue may be informative. The ACh- or histamine-induced relaxation of muscles which were contracted by NA, were sustained until these agonists were removed, and inhibition by Methylene Blue of the actions of EDRF (Martin *et al.* 1985) resulted in a transient relaxation (histamine) or an initial large, followed by small sustained (ACh), relaxation; the time course of the relaxation being similar to that of the membrane potential. Thus, the endothelium-dependent relaxations induced by ACh or histamine consist of two

components, the EDRF-induced and the hyperpolarization-induced relaxations, and the former is the major contributor for relaxation while the latter contributes only transiently and does not exceed 40% of the endothelium-dependent relaxation.

In summary, the differences and similarities between EDRF and the hyperpolarizing substance are that (1) both substances are released from the endothelial cells during stimulation of ACh or histamine receptors, (2) the hyperpolarizing substance is released mainly transiently, in contrast with the sustained release of EDRF during stimulation with ACh or histamine, (3) EDRF relaxes the tissue with no alteration of the membrane potential, while the hyperpolarizing substance relaxes the tissue with alteration of the membrane potential, (4) the release of EDRF is not necessarily related to that of the hyperpolarizing substance, (5) EDRF is liberated continuously in the absence of stimuli while the hyperpolarizing substance is released only upon stimulation by agonists, and (6) Methylene Blue blocks the actions of EDRF but not those of the hyperpolarizing substance. Thus, the hyperpolarizing substance is indeed different from EDRF, and we tentatively call this substance the endothelium-derived hyperpolarizing factor (EDHF).

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