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CALCIUM DEPENDENCY OF THE ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION IN SMOOTH MUSCLE CELLS OF THE RABBIT CAROTID ARTERY

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SUMMARY

1. In smooth muscle cells of the rabbit carotid artery, ACh ($> 10^{-8}$ M) generated a hyperpolarization with two components (transient followed by sustained), only in the tissues with an intact endothelium. There were no detectable changes in the membrane potential, as elicited by ACh (up to 10^{-5} M) in tissues with no endothelium or in the presence of atropine (10^{-6} M).

2. Reduction of $[Ca^{2+}]_o$ inhibited the sustained component which was not apparent in $[Ca^{2+}]_o$ below 0·16 mM. In Ca^{2+}-free (EGTA-containing) solution, the generation of the transient component of the hyperpolarization remained sustained but with a substantially reduced amplitude.

3. Procaine ($> 10^{-6}$ M) inhibited the ACh-induced hyperpolarization in a concentration-dependent manner, and at a concentration of procaine (10^{-3} M) which caused substantial depolarization of the membrane, no detectable change was elicited by ACh.

4. Caffeine (10^{-6}–10^{-3} M) produced a transient hyperpolarization, independent of the presence or absence of the endothelium, and inhibited the sustained component of the ACh-induced hyperpolarization more so than the initial component.

5. A23187 ($> 10^{-8}$ M) hyperpolarized the smooth muscle membrane in a concentration-dependent manner, and this hyperpolarization was not generated in Ca^{2+}-free solution or in the absence of endothelial cells.

6. In intact tissues, pre-treatment with A23187 resulted in a reduction of the subsequently generated ACh-induced hyperpolarization, in an irreversible manner.

7. It would thus appear that in the rabbit carotid artery, the endothelium-dependent hyperpolarization induced by ACh has Ca^{2+}-dependent and Ca^{2+}-independent components, and each may be related to the increase in endothelial $[Ca^{2+}]_i$ by release from the intracellular store and by influx from the extracellular medium, respectively. The increased $[Ca^{2+}]_i$ would trigger a release of an endothelium-derived hyperpolarizing factor (EDHF) from the endothelial cells.

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INTRODUCTION

Acetylcholine (ACh) releases an endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) and hyperpolarizing factor (EDHF; Chen, Suzuki & Weston, 1988) from vascular endothelial cells. The electrophysiological and pharmacological properties of EDRF differ from EDHF in that (1) actions of EDRF estimated from mechanical responses are sustained whereas those of EDHF are mostly transient (Komori & Suzuki, 1987*a*; Chen *et al.* 1988; Feletou & Vanhoutte, 1988; Chen & Suzuki, 1989); (2) EDRF relaxes smooth muscle, with an associated increase in cyclic GMP production (Ignarro & Kadowitz, 1985), and methylene blue or haemoglobin, an inhibitor of the action of EDRF (Martin, Villani, Jothianandan & Furchgott, 1985), cannot modify the ACh-induced hyperpolarization (Chen *et al.* 1988; Huang, Busse & Bassenge, 1988); (3) nitric oxide, a candidate for EDRF (Palmer, Ferrige & Moncada, 1987), relaxes arterial smooth muscles with no alteration of the membrane potential (Komori, Lorenz & Vanhoutte, 1988); (4) in the rabbit saphenous artery, EDRF and EDHF are released by ACh through activation of M_2 and M_1 muscarinic receptors, respectively (Komori & Suzuki, 1987*b*); and (5) the tachyphylaxis occurring in the ACh-induced relaxation is less in comparison with the ACh-induced hyperpolarization in the dog coronary artery (Chen, Hashitani & Suzuki, 1989).

The release of EDRF requires an increase in $[Ca^{2+}]_i$ in the endothelial cells (Long & Stone, 1985; Griffith, Edwards, Newby, Lewis & Henderson, 1986; Peach, Singer, Izzo & Loeb, 1987; Lückhoff, Pohl, Mülsch & Busse, 1988), and the main sources of Ca^{2+} are both the intracellular store (probably in the endoplasmic reticulum, ER) and the extracellular medium (Danthuluri, Cybulsky & Brock, 1988). Stimulation of muscarinic receptors on the endothelial cells by ACh elevates phosphatidylinositol metabolism by activating phospholipase C (Hong & Deykin, 1982; Derian & Moskowitz, 1986; Lambert, Kent & Whorton, 1986; Martin & Whysolmerski, 1987). The metabolite of phosphatidyl inositol activates phospholipase A_2 which facilitates production of prostacyclin (PGI_2) by metabolizing arachidonic acid. However, in cultured endothelial cells, the production by ACh of PGI_2 is independent of $[Ca^{2+}]_i$, thereby indicating that the releasing mechanism differs between PGI_2 and EDRF (Lückhoff *et al.* 1988).

We investigated the role of Ca^{2+} in relation to generation of the endothelium-dependent hyperpolarization by ACh in smooth muscle cells of the rabbit carotid artery. The electrophysiological properties of the ACh-induced hyperpolarization of this artery were similar to those observed in other arteries, and the hyperpolarization was modified by $[Ca^{2+}]_o$ and by drugs acting on the intracellular Ca^{2+} stores. The release of EDHF seems to require an increase in $[Ca^{2+}]_i$ in the endothelial cells. ACh increases $[Ca^{2+}]_i$ probably by releasing Ca^{2+} from ER and by accelerating influx from the extracellular medium.

METHODS

Male albino rabbits, weighing 1·9–2·1 kg, were anaesthetized by injection of sodium pentobarbitone (40 mg kg^{-1}, I.V.), and then were exsanguinated from the femoral artery. The common

carotid artery was excised and cleaned by removing the surrounding connective tissues in Krebs solution at room temperature. The artery (1·0–1·5 cm long) was opened by cutting vertically and mounted in an organ bath made of lucite plate (capacity, about 2 ml). A silicon rubber plate (KE-66, Shin-Etsu Kagaku, Tokyo) was fixed at the bottom of the organ bath, and the tissue was immobilized on the plate, using tiny pins and with the endothelial layer facing up. The tissue was superfused with warmed (35 °C) Krebs solution.

The endothelial cells were removed by gentle rubbing of the internal surface of the vessel with a moistened cotton ball (see Furchgott & Zawadzki, 1980).

A glass capillary microelectrode filled with 3 M-KCl was made from a borosilicate glass tube (o.d., 1·2 mm with a core inside, Hilgenberg, FRG). The resistance of the electrode ranged between 40 and 80 MΩ. The electrode was impaled into the smooth muscle cells through the endothelial cell layer and presumably through the elastic lamina located between the medial and intimal layers of the artery (Rhodin, 1980). Electrical responses thus recorded were displayed on a cathode-ray oscilloscope (VC-9A, Nihon–Kohden, Tokyo) and a pen-writing recorder (Recticorder RJG-4024, Nihon–Kohden, Tokyo).

The ionic composition of Krebs solution was as follows (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. Low $[Ca^{2+}]_o$ solutions were prepared by replacing $CaCl_2$ with $MgCl_2$. In Ca^{2+}-free solution, $MgCl_2$ was added at the level of 3 times the normal Krebs solution.

The drugs used were acetylcholine chloride, caffeine, procaine hydrochloride, atropine sulphate (all from Sigma Chemical Co., St Louis, MO, USA). A23187 (free acid, Calbiochemicals, San Diego, CA, USA) and ethyleneglycol-bis-(aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA, Dozin Lab., Kumamoto, Japan).

Experimental values were expressed as mean ± standard deviation (S.D.), and statistical significance was tested using Student's t test. Probabilities of less than 5% ($P < 0·05$) were considered to have a statistical significance.

RESULTS

Endothelium-dependent hyperpolarization by ACh

In smooth muscle cells of the rabbit carotid artery, the resting membrane potential ranged between −55 and −60 mV (mean value, $-57·2 \pm 2·6$ mV, $n = 37$), and this potential remained stable in the absence of stimulation. Figure 1 shows the membrane responses elicited by ACh in the absence or presence of endothelial cells. In tissues with intact endothelial cells, the application of 10^{-7} M-ACh produced a transient hyperpolarization which ceased within 5–7 min (Fig. 1*A*). Increasing the concentration of ACh to 10^{-5} M produced a hyperpolarization with two components, i.e. an initial transient and following sustained hyperpolarization (Fig. 1*B*). The time required to reach the peak amplitude of the initial transient hyperpolarization was 20–30 s, whereas the sustained hyperpolarization was reached within 3–5 min and remained unchanged for up to 30 min.

Application of 10^{-5} M-ACh for 1 min produced a hyperpolarization which was restored to the resting level within 1 min after the removal of ACh. When the recovery of the ACh-induced hyperpolarization was measured with a 1 min application of ACh twice at various intervals, a 5 min period between the two applications was required to obtain a reproducible amplitude of hyperpolarization.

Mechanical removal of the endothelial cells produced no detectable change in the resting membrane potential of the smooth muscle cells (mean value, $-56·8 \pm 3·5$ mV, $n = 25$, $P > 0·2$). In de-endothelialized tissues, the application of ACh (up to 10^{-5} M) produced no (Fig. 1*C*) or only a substantial depolarization of the membrane

(Fig. 1*D*). In five of eleven tissues used, depolarizations by 1–3 mV were observed in response to 10^{-5} M-ACh.

Atropine (10^{-6} M) had no detectable effect on the resting membrane potential, and the absence or presence of the endothelium made no difference in the response to this alkaloid. However, atropine did block the ACh-induced change in the membrane potential, both in the presence or absence of endothelial cells.

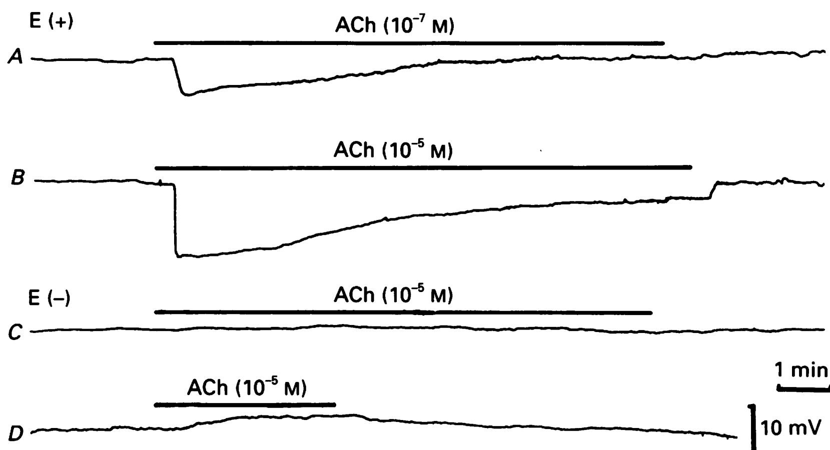

Fig. 1. Membrane potential changes produced by ACh in smooth muscle cells of the rabbit carotid artery. Intact endothelium (E(+), *A* and *B*), no endothelium (E(–), *C* and *D*). Membrane potential: *A* and *B*, –58 mV; *C*, –56 mV; *D*, –58 mV.

Figure 2 summarizes the effects of ACh on the membrane potential of smooth muscle cells in the rabbit carotid artery. In arteries with an intact endothelium, ACh produced a transient hyperpolarization in concentrations above 10^{-8} M, and 10^{-5} M was required to reach the maximum amplitude. The sustained hyperpolarization measured at 7–10 min in ACh was detected in concentrations above 10^{-6} M.

$[Ca^{2+}]_o$ *and ACh-induced hyperpolarization*

Removal of all $CaCl_2$ from Krebs solution (Ca^{2+}-free solution), but not decreasing $[Ca^{2+}]_o$ to 0·16 mM, caused a significant depolarization of the membrane. Figure 3 shows the ACh-induced hyperpolarization recorded in the case of three different $[Ca^{2+}]_o$ solutions (2·5, 0 and 0 mM containing 0·2 mM-EGTA). In Ca^{2+}-free solution, ACh produced a transient hyperpolarization which was shorter in duration than that in the control, and the sustained component of the hyperpolarization was not generated (Fig. 3*A* and *B*). The time required for the hyperpolarization to decay to half the peak amplitude (half-time, $t_{1/2}$) was 3–4 min in the control condition with $[Ca^{2+}]_o = 2{\cdot}5$ mM (Fig. 3*A*), and was reduced to 20–30 s in Ca^{2+}-free solution (Fig. 3*B*). The duration of the ACh-induced hyperpolarization was further shortened in Ca^{2+}-free, EGTA-containing solution, the half-time being about 15 s (Fig. 3*C*).

Figure 4 summarizes the effects of $[Ca^{2+}]_o$ on the peak amplitude and the half-time of the ACh-induced hyperpolarization, and the membrane potential of smooth muscle cells. The membrane was depolarized significantly in Ca^{2+}-free (without EGTA) and

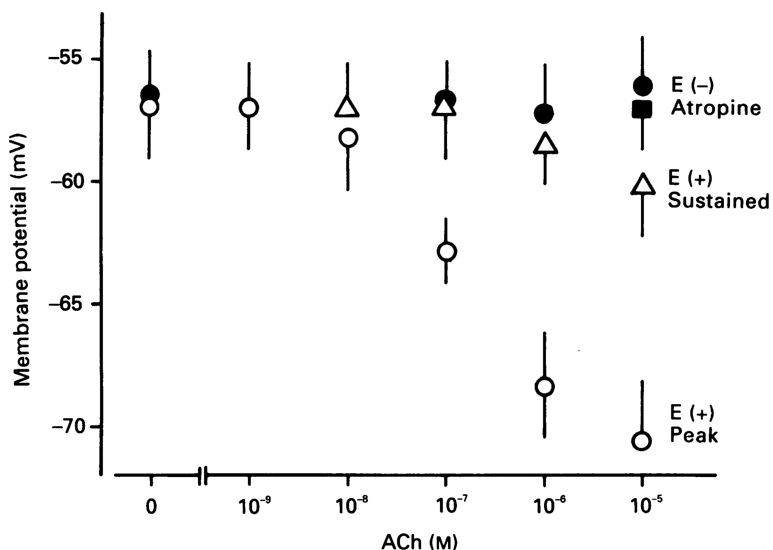

Fig. 2. Effects of ACh on membrane potential of smooth muscle cells in the rabbit carotid artery. ○, endothelium-intact tissue; peak = the initial transient hyperpolarization; △, the sustained hyperpolarization; ●, endothelium-removed tissue; ■, in the presence of atropine (10^{-6} M). Mean ± S.D. (n = 12–36).

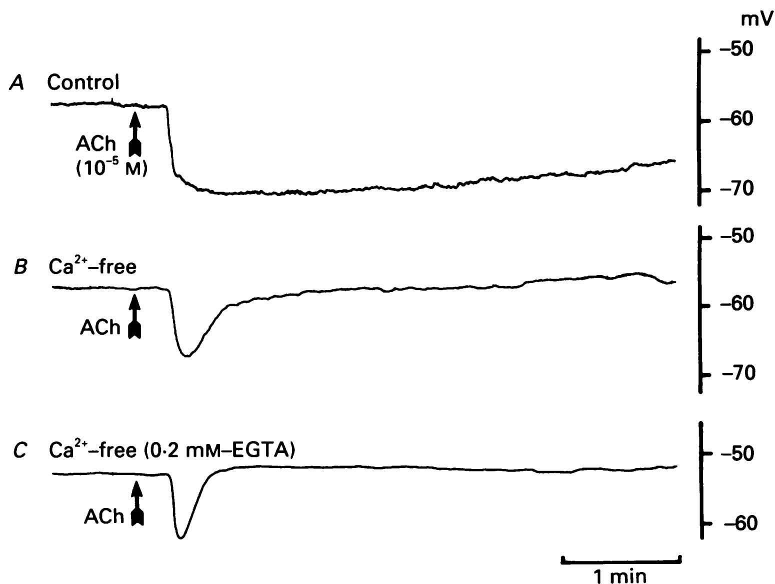

Fig. 3. ACh-induced hyperpolarization generated in different $[Ca^{2+}]_o$ solutions. $[Ca^{2+}]_o$ = 2·5 mM (A), 0 mM (B) and 0 mM with 0·2 mM-EGTA (C). ACh (10^{-5} M) was applied at the arrow in each trace.

Ca^{2+}-free (EGTA-containing) solutions. The amplitude and duration of the ACh-induced hyperpolarization were not changed in high $[Ca^{2+}]_o$ solution (7·5 mM) but were decreased significantly in low $[Ca^{2+}]_o$ solutions (below 0·16 mM). The half-time of the hyperpolarization was shortened in proportion to the decrease in $[Ca^{2+}]_o$.

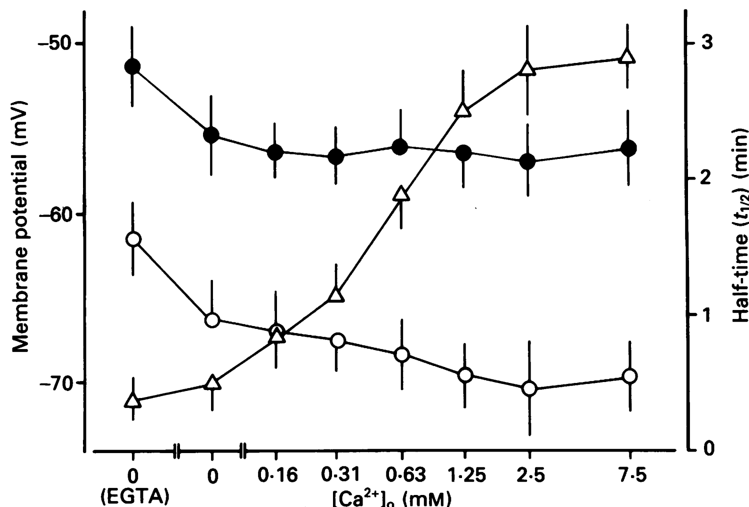

Fig. 4. Effects of $[Ca^{2+}]_o$ on the membrane potential in the absence (●) and presence (○) of 10^{-5} M-ACh and half-time ($t_{1/2}$, △) of the ACh-induced hyperpolarization in smooth muscle cells of the rabbit carotid artery. Mean ± S.D. (n = 12–25 for the membrane potential and 4–12 for the hyperpolarization).

Intracellular Ca^{2+} store and endothelium-dependent hyperpolarization

Experiments were carried out to observe the effects of chemical agents which modify intracellularly stored Ca^{2+} in vascular smooth muscle cells on the ACh-induced hyperpolarization in the rabbit carotid artery. In arterial smooth muscles, caffeine facilitates and procaine inhibits the release of Ca^{2+} from intracellular store sites (Itoh, Kuriyama & Suzuki, 1981), while the calcium ionophore, A23187, accelerates leakage of Ca^{2+} from the intracellular store sites, in concentrations below those which permeate Ca^{2+} channels at the plasma membrane (below 10^{-7} M; Itoh, Kanmura & Kuriyama, 1985). Therefore, it was of interest to test actions of these agents on the ACh-induced hyperpolarization.

Figure 5 shows the effects of procaine or caffeine on the ACh-induced hyperpolarization, and Fig. 6 summarizes the membrane potential and amplitude of the hyperpolarization in the presence of procaine or caffeine. Procaine in concentrations above 3×10^{-4} M significantly depolarized the membrane. The ACh-induced hyperpolarization was inhibited by procaine ($> 10^{-6}$ M) in concentrations below those depolarizing the smooth muscle membrane; the sustained component of the hyperpolarization was first blocked, whereas the initial transient component was evident in the case of exposure to procaine up to 10^{-4} M (Figs 5*A* and 6).

Caffeine (10^{-5}–10^{-3} M) transiently hyperpolarized the membrane (2–3 min), as in the case of other arteries (mesenteric artery; Fujii, Miyahara & Suzuki, 1985; aorta; Kajiwara, 1982), and this action of caffeine was observed irrespective of the absence

or presence of the endothelium. The amplitude of hyperpolarization (measured at the peak) produced by 1 mM-caffeine was $3{\cdot}1 \pm 0{\cdot}6$ mV ($n = 8$) and $3{\cdot}3 \pm 0{\cdot}4$ mV ($n = 8$) in tissues with and without endothelial cells, respectively.

Application of ACh in the presence of 1 mM-caffeine (after the membrane potential had reverted to the resting level, usually 5–7 min), produced a hyperpolarization with two components but with reduced amplitude (Fig. 5*B*).

These effects of procaine and caffeine on the ACh-induced hyperpolarization were reversible, and 7–10 min was required for the recovery.

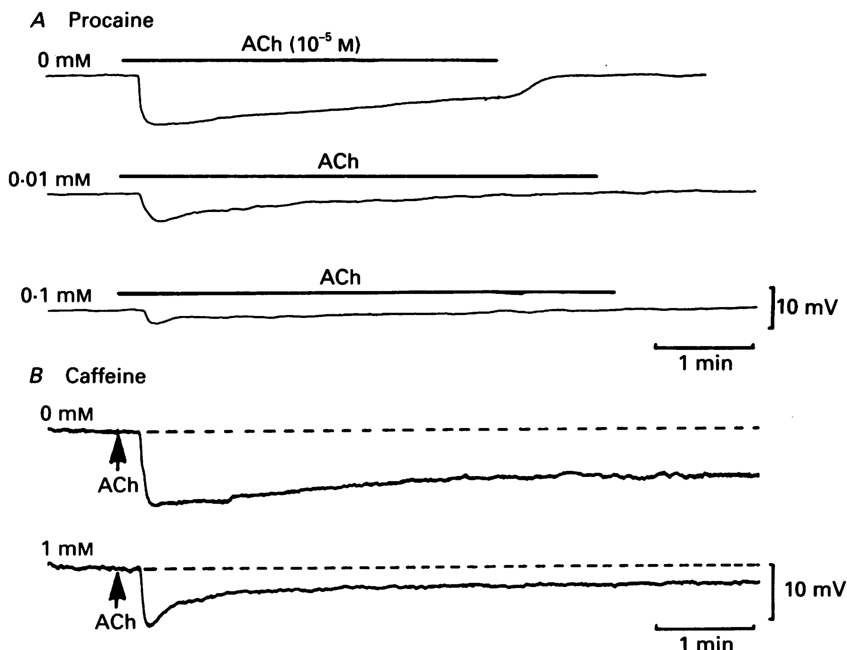

Fig. 5. Modulation by procaine (*A*) and caffeine (*B*) of the ACh (10^{-5} M)-induced hyperpolarization in smooth muscle cells of the rabbit carotid artery. Procaine (0, 0·01 or 0·1 mM) or caffeine (0 or 1 mM) was applied 5–7 min before application of ACh. The resting membrane potential: *A*, -60 mV; *B*, -57 mV.

A23187 ($> 10^{-8}$ M) hyperpolarized the smooth muscle membrane, in a concentration-dependent manner, and this hyperpolarization was not generated in tissues from which the endothelium had been mechanically removed. The membrane potential of smooth muscle cells from the de-endothelialized tissue was not changed by A23187 in concentrations up to 3×10^{-7} M (Fig. 7). The generation of the endothelium-dependent hyperpolarization by A23187 required $[Ca^{2+}]_o$, and in Ca^{2+}-free solutions either with or without EGTA, A23187 (up to 10^{-6} M) produced no detectable change in the membrane potential.

Figure 8 demonstrates the endothelium-dependent hyperpolarizations produced by ACh or A23187 in the rabbit carotid artery. The onset and initial phase of the hyperpolarization were slower in the case of A23187 (10^{-7} M) than in the case of ACh (10^{-5} M) (Fig. 8*A* and *B*). The hyperpolarization produced by A23187 reached the maximum amplitude at 1·5–2·5 min, and decayed slowly with a half-time of over

10 min. Removal of ACh from the superfusate resulted in a restoration of the membrane potential to the resting level within 1–3 min, with an associated small transient depolarization (Fig. 8*A*). Recovery of the membrane potential to the resting level after removal of A23187 from the superfusate required a very slow time course

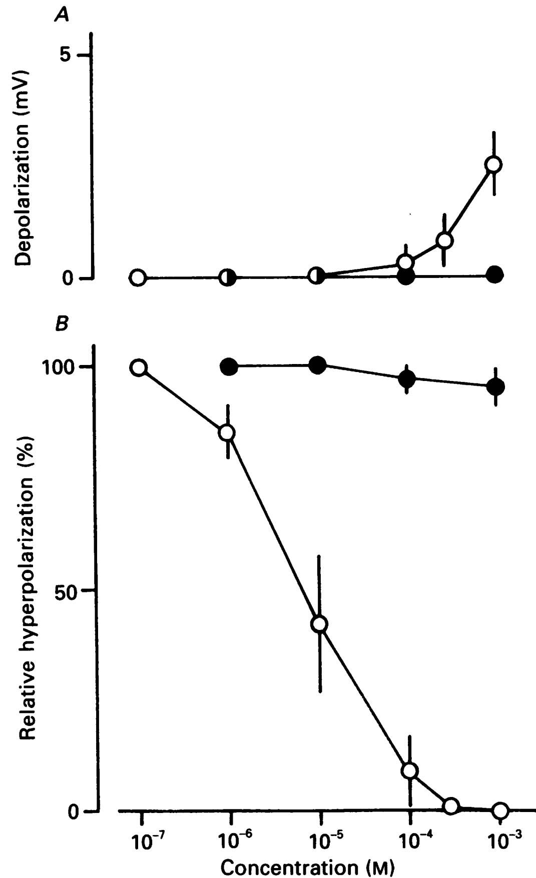

Fig. 6. Effects of caffeine (●) and procaine (○) on the membrane depolarization from the resting level (*A*) and amplitude of the ACh (10^{-5} M)-induced hyperpolarization (*B*) in smooth muscle of the rabbit carotid artery. In *B*, amplitude of the initial transient component of the hyperpolarization is expressed as a percentage of the control (= 100%). Mean ± S.D. (n = 7–15 for *A*, 5–8 for *B*).

(5–8 min, Fig. 8*B*). After recovery of the membrane potential, application of ACh produced a hyperpolarization with two components, but with a drastically reduced amplitude (Fig. 8*C*). As a reproducible amplitude of hyperpolarization was generated when ACh was applied twice with intervals over 5 min in the control condition, the reduced amplitude of the ACh-induced hyperpolarization after A23187 was probably not due to the desensitization of receptors for ACh. The inhibitory effects of the pre-treatment of A23187 on the ACh-induced hyperpolarization remained irreversible for up to 2 h, and also depended on the concentration of A23187. Pre-treatment with 3×10^{-8} M and 10^{-7} M-A23187 for 5 min resulted in a reduction in the amplitude of

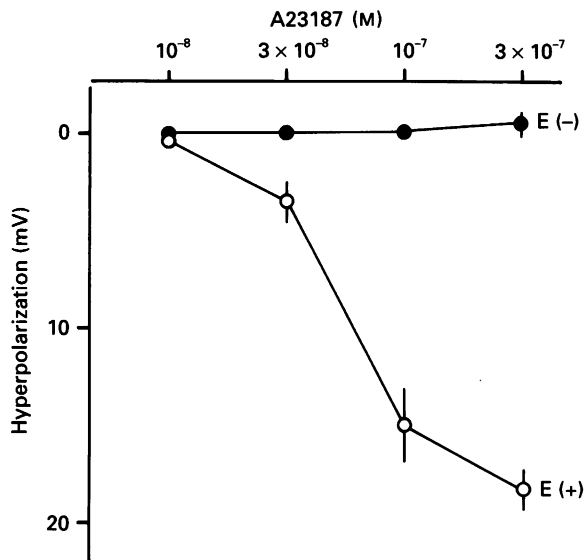

Fig. 7. Hyperpolarization of smooth muscle membrane elicited by A23187 in the rabbit carotid artery. ○, endothelium-intact tissue, (E(+)). ●, endothelium-removed tissue (E(−)). Amplitude of hyperpolarization at the peak was measured. Mean ± S.D. (n = 5–7).

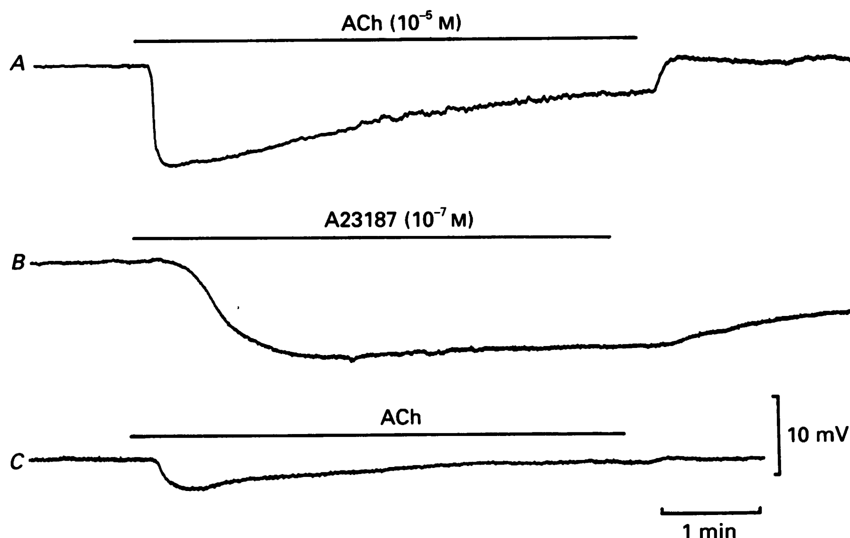

Fig. 8. Endothelium-dependent hyperpolarizations produced by ACh (10^{-5} M, *A*) and A23187 (10^{-7} M, *B*) in the rabbit carotid artery smooth muscles. *C*, ACh (10^{-5} M)-induced hyperpolarization elicited after A23187 had been removed for 20 min.

hyperpolarization produced by subsequently applied ACh to 42·6 ± 5·6 % ($n = 8$) and 23·5 ± 5·0 % ($n = 6$) of the control, respectively.

DISCUSSION

The present experiments demonstrated that ACh produced an endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery, as seen in other arteries from the rabbit (Bolton, Lang & Takewaki, 1984; Komori & Suzuki, 1987*a*; Bény & Brunet, 1988; Suzuki, 1988), rat (Chen *et al.* 1988; Chen & Suzuki, 1989) or dog (Feletou & Vanhoutte, 1988; Komori *et al.* 1988). The hyperpolarizations generated in many of these arteries are transient in response to continued application of ACh, and 3–7 min are required to complete the hyperpolarization (Komori & Suzuki, 1987*a*; Chen *et al.* 1988; Feletou & Vanhoutte, 1988; Suzuki, 1988; Taylor, Southerton, Weston & Baker, 1988). The transient nature of the endothelium-dependent hyperpolarization is also apparent in the rat aorta and intrapulmonary artery in response to histamine and this is in good contrast with the sustained relaxation by EDRF of smooth muscles pre-contracted with noradrenaline (Chen & Suzuki, 1989). Experiments done to determine the recovery of receptors from desensitization revealed that the transient nature of the hyperpolarization by ACh or histamine may be mainly due to the slow recovery of the receptors on the endothelial cell membrane from desensitization and not to depletion of the hyperpolarizing factor (EDHF, Chen *et al.* 1988) in the endothelial cells or to desensitization of receptors on the muscle membrane to EDHF (Chen & Suzuki, 1989).

In the rabbit carotid artery, we found that the endothelium-dependent hyperpolarization induced by ACh consisted of two components. The transient and sustained components were less sensitive and sensitive to $[Ca^{2+}]_o$, respectively. In tissues with no endothelial cells, ACh produced no detectable change in the membrane potential, and therefore both components of the ACh-induced hyperpolarization may be mainly related to the production or liberation of EDHF from the endothelial cells. Estimation of $[Ca^{2+}]_i$ in cultured endothelial cells using various Ca^{2+}-sensitive dyes (Quin-2: Lückhoff & Busse, 1986; Indo-1: Busse, Fichtner, Lückhoff & Kohlhardt 1988, Lückhoff *et al.* 1988; Fura-2: Hallam & Pearson, 1986; Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Danthuluri *et al.* 1988) demonstrates that agents which enhance the liberation of EDRF elevate $[Ca^{2+}]_i$ with two components (transient largely followed by sustained maintenance at an elevated level), and only the initial transient component is generated in Ca^{2+}-free solution (Hallam & Pearson, 1986; Lückhoff & Busse, 1986; Colden-Stanfield *et al.* 1987; Danthuluri *et al.* 1988; Lückhoff *et al.* 1988). Therefore, the calcium dependencies in the endothelium-dependent hyperpolarization and endothelial $[Ca^{2+}]_i$ strongly suggest that the release of EDHF requires an increase in $[Ca^{2+}]_i$ in the endothelial cells, as in the case of EDRF (Long & Stone, 1985; Griffith *et al.* 1986; Peach *et al.* 1987); the increase in $[Ca^{2+}]_i$ for the release of the latter factor is mediated by the release from the intracellular stores and by influx from the extracellular medium. The endothelial cell membrane possesses Ca^{2+} channels

sensitive to ACh or ATP (i.e. the receptor-operated Ca^{2+} channel) but not the voltage-dependent Ca^{2+} channel (Colden-Stanfield *et al.* 1987; Johns, Lategan, Lodge, Ryan, van Breemen & Adams, 1987), while the release of intracellularly stored Ca^{2+} by ACh or bradykinin is accompanied by the degradation of membrane phosphatidylinositol (Hong & Deykin, 1982; Derian & Moskowitz, 1986; Lambert *et al.* 1986; Martin & Wysolmerski, 1987). These two processes may be also involved in the elevation of $[Ca^{2+}]_i$ in endothelial cells during the release of EDHF by ACh.

In smooth muscles, caffeine and procaine have opposite actions on intracellularly stored Ca^{2+}; the former facilitates and the latter inhibits the release of Ca^{2+} from the store sites (Itoh *et al.* 1981). Actions of caffeine are mainly due to acceleration of the Ca^{2+}-induced Ca^{2+} release mechanism (Endo, 1977), and therefore in the presence of caffeine, the amount of Ca^{2+} in the intracellular store site is decreased (Itoh *et al.* 1981). These actions of caffeine and procaine also seem to be applicable to endothelial cells, i.e. modulation by these agents of the ACh-induced endothelium-dependent hyperpolarization is closely related to the function of Ca^{2+} stores in the endothelial cell. The observation that the sustained component of the hyperpolarization is more sensitive to caffeine and procaine than is the transient component, suggests that the intracellular store site of Ca^{2+} is an important pathway for the influx of Ca^{2+} from extracellular medium. This concept could be supported if the action of A23187 on the endothelial Ca^{2+} store sites is similar to actions on the SR in vascular smooth muscle, i.e. A23187 at low concentrations selectively damages SR membrane and causes disfunction of the Ca^{2+} storage systems in the cell (Itoh *et al.* 1985). A23187 itself accelerates the influx of Ca^{2+} from extracellular medium and increases $[Ca^{2+}]_i$ in the endothelial cells. It also damages intracellular Ca^{2+} store sites, thereby inhibiting the ACh-induced hyperpolarization irreversibly. It should be mentioned here that A23187 irreversibly inhibits ACh-induced relaxation in the rabbit aorta (Furchgott, 1983).

Direct actions of caffeine and procaine on the smooth muscle membrane also occur in some species of arteries, and these two agents inhibit K^+ permeability and depolarize the membrane (Itoh *et al.* 1981; Kajiwara, 1982; Fujii *et al.* 1985). The transient hyperpolarization elicited by caffeine was also a direct action on smooth muscles, with no relation to the endothelial cells. This caffeine-induced hyperpolarization may be due to opening of the K^+ channels activated by the caffeine-induced increase in $[Ca^{2+}]_i$ in smooth muscle cells (Schwarz & Passow, 1983).

The endothelium-dependent hyperpolarization by ACh is mainly due to an increase in K^+ permeability of smooth muscle membrane, as deduced from evidence that (1) the amplitude of the hyperpolarization is increased in low $[K^+]_o$ solution and decreased in high $[K^+]_o$ solution (Chen *et al.* 1988; Chen & Suzuki, 1989); (2) efflux of incorporated ^{86}Rb is increased by ACh in an endothelium-dependent manner (Chen *et al.* 1988; Taylor *et al.* 1988); and (3) ionic conductance of the membrane estimated from the amplitude of the electrotonic potential is increased during the ACh-induced hyperpolarization (Komori & Suzuki, 1987*a*; Chen & Suzuki, 1989). ACh also hyperpolarizes the membrane of the cultured endothelial cells by an increase in K^+ conductance (Busse *et al.* 1988), probably by activating Ca^{2+}-dependent K^+ channels (Colden-Stanfield *et al.* 1987; Johns *et al.* 1987; Takeda, Schini & Stoeckel, 1987; Olesen, Davies & Clapham, 1988). As there are gap junctions

between endothelial and adjacent smooth muscle cells (Rhodin, 1967; Taugner, Kirchheim & Forssmann, 1984), electrical coupling between these two types of cells may occur (Busse *et al.* 1988). In fact, the non-neuronal depolarizing responses elicited by electrical field stimulation in smooth muscle cells of the rabbit basilar artery are not generated after removal of the endothelium (Nagao & Suzuki, 1987). However, evidence that the hyperpolarization of smooth muscle cells in tissues with no endothelium can be produced indirectly (i.e. in cascade experiments, Feletou & Vanhoutte, 1988) also indicates that the EDHF is indeed released together with EDRF, in response to ACh.

It is concluded that the endothelium-dependent hyperpolarization by ACh consists of Ca^{2+}-dependent and Ca^{2+}-independent components. These electrical responses can be explained if the release of EDHF by ACh requires an increase in $[Ca^{2+}]_i$ in the endothelial cell, probably by release from intracellular store sites and influx from extracellular medium, and if the available Ca^{2+} produces the initial transient and following sustained components of the ACh-induced hyperpolarization respectively. Although the action of EDHF on electrical and biochemical reactions of vascular smooth muscle differs from that of EDRF, the release of both factors is likely to be triggered by an increase in $[Ca^{2+}]_i$, in contrast with the $[Ca^{2+}]_i$-independent release of PGI_2 from endothelial cells (Lückhoff *et al.* 1988). The physiological roles of these factors, however, seem to be different in that EDRF is liberated continuously under physiological conditions (Furchgott, 1983), whereas EDHF is released upon stimulation, since removal of endothelial cells does not depolarize the smooth muscle membrane (Komori & Suzuki, 1987*a*; Nagao & Suzuki, 1987; Chen *et al.* 1988).

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