Contribution of nitric oxide to the endothelium-dependent hyperpolarization in rat aorta

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1. The effect of endogenous and exogenous nitric oxide on the membrane potential (E_m) of smooth muscle cells of the thoracic aorta of rats was investigated.

2. In tissues with intact endothelium, application of ACh or carbachol generated a change of the membrane potential consisting of an initial hyperpolarization by 10–12 mV, followed by a partial recovery toward a level which was at 10 min still 6–8 mV more negative than in control conditions.

3. Application of N^G-nitro-L-arginine methylester (L-NAME), an inhibitor of endogenous NO production, had no significant effect on the resting membrane potential. The initial peak endothelium-dependent hyperpolarization elicited by ACh or carbachol was not significantly diminished. However, the recovery was more accentuated. Similarly, N^G-monomethyl-L-arginine (L-NMMA) significantly diminished the second component of the endothelium-dependent hyperpolarization without affecting the magnitude of the first transient peak E_m change.

4. Nitroglycerin produced a small sustained hyperpolarization of 1–2 mV, and the NO donor SIN-1, the active metabolite of molsidomine, similarly increased E_m by about 1 mV. Infusion of high doses of acidified $NaNO_2$ solution caused a hyperpolarization smaller than that evoked by ACh or carbachol.

5. 8-Bromo-cyclic GMP caused little change of membrane potential. In the presence of 8-Br-cGMP, ACh evoked a membrane electrical response similar to that observed in the absence of the nucleotide.

6. It is concluded that, in the rat aorta, the initial peak endothelium-dependent hyperpolarization observed under the influence of ACh or carbachol is not directly related to the synthesis of NO. Endothelium-derived NO does, however, contribute for a small part to the second, more sustained component of the ACh- or carbachol-induced hyperpolarization.

The endothelium of blood vessels has been shown to influence the tone of the underlying smooth muscle layer. This is due in a large part to the action of a diffusable substance, the endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), which is released in larger amounts in response to endothelium-dependent vasodilators such as acetylcholine. The endothelial relaxing factor has been identified as nitric oxide or at least a very similar NO-containing molecule (Ignarro, Buga, Wood, Byrns & Chaudhuri, 1987; Palmer, Ferrige & Moncada, 1987). Endothelium-derived NO (EDNO) is synthesized from L-arginine by a Ca^{2+}-dependent NO synthase, while the biosynthesis can be inhibited by synthetic analogues of L-arginine, such as nitro-L-arginine, its methylester (L-NAME) or monomethyl-L-arginine (L-NMMA) (Rees, Palmer, Schulz, Hodson & Moncada, 1990). Application of these analogues strongly depresses the endothelium-dependent relaxation. Under the influence of EDRF, which activates guanylate cyclase, cyclic GMP levels in muscle increase (Rapoport & Murad, 1983; Ignarro & Kadowitz, 1985).

Several endothelium-dependent vasodilators have also been shown to cause a hyperpolarization of the membrane of the vascular smooth muscle cells (Komori & Suzuki, 1987; Taylor, Southerton, Weston & Baker, 1988; Suzuki & Chen, 1990). The nature of the factor inducing hyperpolarization is still unclear. While some studies suggest that the membrane electrical change is due to NO derived from the endothelium (Tare, Parkington, Coleman, Neild & Dusting, 1990), the existence of a humoral factor different from NO, the endothelium-dependent hyperpolarizing factor (EDHF), was postulated (Chen, Suzuki & Weston, 1988; Komori, Lorenz & Vanhoutte, 1988; Chen & Suzuki, 1989).

In the present experiments on the rat aorta, the influence of two inhibitors of endogenous NO production, L-NAME and L-NMMA, on the resting membrane potential and on the course of the acetylcholine-induced

endothelium-dependent hyperpolarization was analysed, and the effect of NO and of the exogenous NO donors nitroglycerin and SIN-1, the active metabolite of molsidomine, on the resting E_m was investigated.

METHODS

The thoracic aorta was isolated from Wistar rats anaesthetized with an intraperitoneal injection of a lethal dose of sodium pentobarbitone. The preparation was dissected free of connective tissue. Great care was taken not to injure the endothelium. Aortic ring segments (5–7 mm long) were slit along the longitudinal axis. The strips were pinned down to the bottom of an experimental chamber, where the preparation was continuously superfused with oxygenated Krebs–Ringer solution containing (mM): NaCl, 135; KCl, 5; $NaHCO_3$, 20; $CaCl_2$, 2·5; $MgSO_4.7H_2O$, 1·3; KH_2PO_4, 1·2; EDTA, 0·026; and glucose, 10. The temperature was maintained at 35 °C unless otherwise explicitly mentioned.

The transmembrane potential was measured by penetrating the muscle strip with conventional microelectrodes, filled with 3 M KCl. The microelectrodes were pulled with a vertical pipette puller (model 750, David Kopf Instruments, Tujunga, CA, USA) from filamented borosilicate glass tubing (1 mm o.d., Hilgenberg Gmbh, Malsfeld, Germany). The electrical resistance of the microelectrodes, measured in normal Krebs–Ringer solution, ranged from 25 to 60 MΩ. The electrical signal was followed on an oscilloscope, traced with a pen recorder, and in some experiments also digitized (DAP 800/2, Microstar Laboratories Inc., Bellevue, WA, USA).

Primary criteria for successful cellular impalement were an extremely sharp voltage deflection on entering the cell, and a rapid return to the baseline value on exit. Values of the membrane potential were taken as the difference of the stabilized potential and the zero potential on withdrawal or dislodgement of the microelectrode from the cell.

Drugs

Acetylcholine chloride (ACh), carbamylcholine chloride (carbachol), L-arginine and N^G-nitro-L-arginine methylester hydrochloride (L-NAME) were obtained from Sigma Chemical Co., St Louis, MO, USA. N^G-monomethyl-L-arginine acetate (L-NMMA) was purchased from Calbiochem Co., La Jolla, CA, USA. Nitroglycerin was obtained from Merck, Darmstadt, Germany. These substances were added from the appropriate stock solutions a few minutes before use. The concentrations are expressed as final molar concentrations in the fluid superfusing the preparation. In some experiments, NO-containing solution was applied to the muscle strip by continuous infusion of acidified sodium nitrite ($NaNO_2$, pH 2·0) directly into the inlet port of the experimental chamber.

Statistics

Results are expressed as means ± S.E.M. When the effect of L-NAME or L-NMMA on the hyperpolarization was tested, values for the peak and the later (5 and 10 min) change of the membrane potential both in control conditions and after drug application were taken from experiments in which the microelectrode was kept in the same cell throughout the whole experimental protocol. Statistical significance was evaluated using Student's t test for paired observations. Values of $P < 0·05$ were considered to indicate a significant difference between means; n indicates the number of aorta preparations, each obtained from a different rat.

RESULTS

Effect of acetylcholine and carbachol

During superfusion with the control Krebs–Ringer solution, a stable mean membrane potential (E_m) of $-51·9 \pm 0·6$ mV was measured in the aortic smooth muscle ($n = 22$). Addition of either acetylcholine (10^{-5} M) or carbachol (10^{-5} M) produced a rapidly developing increase of the E_m by $12·7 \pm 0·6$ ($n = 16$) or $10·8 \pm 1·6$ mV ($n = 7$) respectively. This peak hyperpolarization was followed by a partial recovery of E_m toward a potential value which remained more negative than in the absence of the vasodilator. After wash-out of acetylcholine or carbachol, E_m completely recovered. Typical examples of this well-known electrical response to acetylcholine (Chen & Suzuki, 1989) can be found in the experimental traces of Figs 3–7.

During repeated 10 min exposures to acetylcholine or carbachol, we usually noted a small decrease of the amplitude of the induced peak hyperpolarization. To quantify this 'run-down', the results of all experiments in which, during the same cellular impalement, more than one application of the vasodilator was performed in control conditions were plotted against time. The data, shown in Fig. 1, indicate that a second hyperpolarization evoked within 2 h after the first application of acetylcholine or carbachol is usually decreased by about 20–25 % of the original magnitude. We noted in preliminary experiments that the 'run-down' was somewhat more expressed at 37 than at 35 °C. We therefore performed most experiments at the slightly lower temperature.

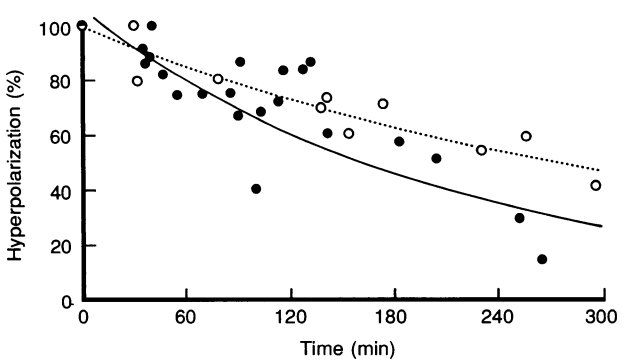

Figure 1. Decrease with time of the hyperpolarization produced by carbachol (10^{-5} M) or acetylcholine (10^{-5} M) in control conditions

Experiments were performed at 37 (●) or 35 °C (○). Time axis indicates the duration of cellular impalement, with the time of the first peak hyperpolarization taken as 0 min. All values were normalized to the amplitude of the first hyperpolarization obtained in that particular cell. Curves are exponential fits to the data points, with time constants of −0·0048 (37 °C) and −0·0026 (35 °C).

Table 1. Effect of L-NAME (10^{-5} M) and L-NMMA (10^{-4} M) on the resting E_m (ΔE_m) and the endothelium-dependent hyperpolarization (EDH) elicited by acetylcholine (ACh, 10^{-5} M) or carbachol (Carb, 10^{-5} M) as measured at the peak and after 5 and 10 min in the presence of the vasodilator

	ΔE_m (mV)	EDH peak (mV)	EDH at 5 min (mV)	EDH at 10 min (mV)
ACh control	—	$-12{\cdot}2 \pm 1{\cdot}0$	$-10{\cdot}3 \pm 1{\cdot}0$	$-8{\cdot}1 \pm 0{\cdot}7$
L-NAME	$+0{\cdot}4 \pm 0{\cdot}4$ (4)	$-10{\cdot}2 \pm 1{\cdot}2$ (6)	$-6{\cdot}5 \pm 0{\cdot}4$* (5)	$-3{\cdot}8 \pm 0{\cdot}4$** (5)
Carb control	—	$-10{\cdot}8 \pm 1{\cdot}6$	$-9{\cdot}9 \pm 2{\cdot}0$	$-5{\cdot}9 \pm 1{\cdot}3$
L-NAME	$+0{\cdot}4 \pm 0{\cdot}4$ (6)	$-8{\cdot}3 \pm 1{\cdot}5$ (7)	$-4{\cdot}7 \pm 2{\cdot}0$* (5)	$-2{\cdot}5 \pm 1{\cdot}6$ (5)
ACh control	—	$-12{\cdot}4 \pm 1{\cdot}1$	$-10{\cdot}2 \pm 1{\cdot}8$	$-8{\cdot}4 \pm 1{\cdot}4$
L-NMMA	$+1{\cdot}2 \pm 0{\cdot}4$* (5)	$-11{\cdot}2 \pm 1{\cdot}0$ (5)	$-7{\cdot}2 \pm 1{\cdot}8$* (4)	$-4{\cdot}4 \pm 1{\cdot}0$** (4)

Values are means ± S.E.M. The number of different preparations is given in parentheses. * and **, mean significantly different from the value in the absence of the inhibitor at $P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively.

Influence of inhibition of endogenous NO synthesis

The membrane electrical response to either acetylcholine or carbachol was compared before and after inhibition of NO biosynthesis, effected by superfusion of the preparations for at least 10 min with L-NAME- or L-NMMA-containing fluid. On average, the second exposure to the vasodilator was made $56{\cdot}0 \pm 7{\cdot}4$ min (acetylcholine experiments) and $43{\cdot}7 \pm 4{\cdot}8$ min (carbachol experiments) after eliciting the first response. The mean response (± S.E.M.) to 10^{-5} M acetylcholine in control conditions and after inhibition of NO synthesis with L-NAME obtained from five experiments is indicated in Table 1 and presented in Fig. 2. Typical records of such experiments are shown in Fig. 3.

The addition of L-NAME (10^{-5} M) had no measurable effect on the resting membrane potential. In the presence of L-NAME, the peak hyperpolarization induced by acetylcholine was not significantly different from that observed in the absence of the inhibitor (Fig. 2*B*). A consistent observation in these experiments, however, was that, after inhibition of NO synthesis, the recovery from the peak hyperpolarization was faster and a less hyperpolarized potential in the continuous presence of acetylcholine was reached, the difference in mean E_m reaching significant levels between 5 and 10 min (Fig. 2*B* and Table 1). This effect was not seen during repeated acetylcholine applications in the absence of L-NAME, as can be verified from the curves of Fig. 2*A* depicting the mean first and the mean second response (evoked at

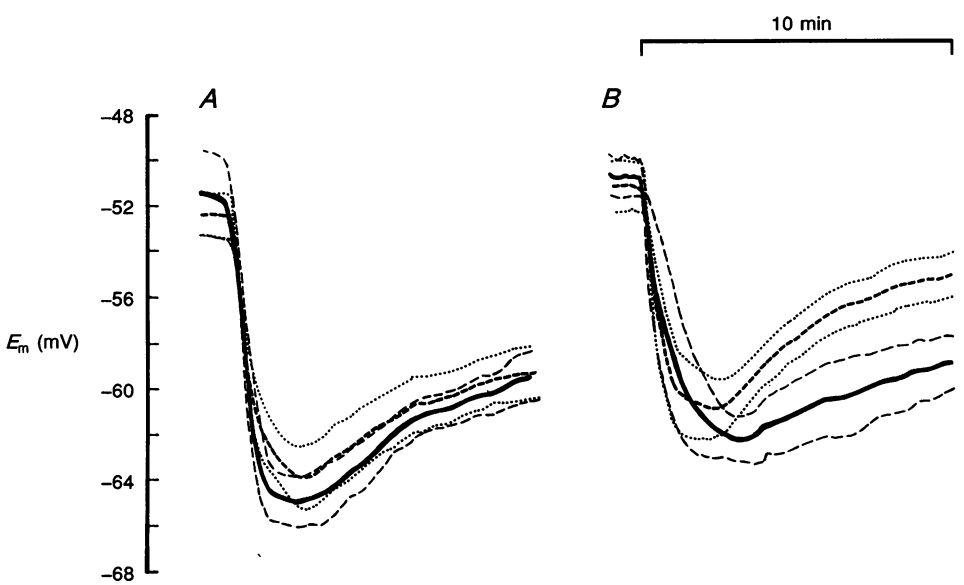

Figure 2. Average E_m responses to acetylcholine
The mean ± S.E.M. is given from 5 experiments, in which ACh was applied twice to the same muscle strip. *A*, first (continuous line; S.E.M. shown as long dashed lines) and second (short dashed lines; S.E.M. shown as dotted lines) control responses. *B*, after the control response (continuous line; S.E.M. shown as long dashed lines), L-NAME (10^{-5} M) was applied for at least 10 min, and a second ACh application in the presence of L-NAME (short dashed lines; S.E.M. shown as dotted lines) caused a hyperpolarization which declined significantly faster than in control conditions.

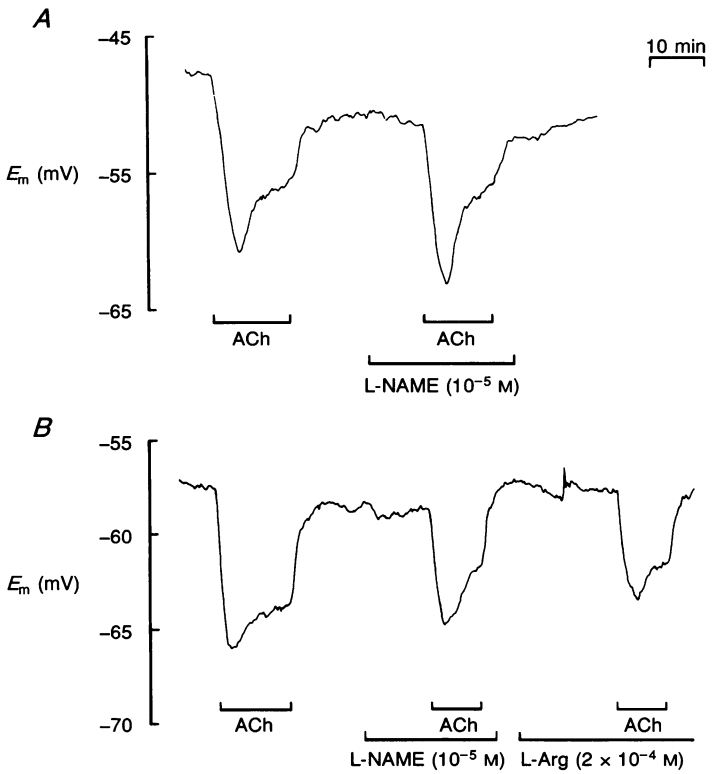

Figure 3. Traces from two experiments in which the influence of acetylcholine (10^{-5} M) on the membrane potential (E_m) was investigated in the absence and presence of L-NAME (10^{-5} M)
In *B*, L-arginine (L-Arg, 2×10^{-4} M) was added after wash-out of the drug.

40–60 min) to acetylcholine from five aorta preparations in control conditions.

In some experiments, a small decrease of the amplitude of peak hyperpolarization in the presence of L-NAME was noted (cf. Fig. 3). In these preparations, washing out of the drug and superfusion with a high dose (2×10^{-4} M) of L-arginine, the substrate for NO biosynthesis, could not restore the peak hyperpolarization to the magnitude of the first response (Fig. 3*B*). This suggests that the decline of the peak response in the presence of L-NAME was due to the normal 'run-down', which was also observed during prolonged superfusion with the control fluid.

In a series of experiments ($n = 5$) in which the endothelium-dependent hyperpolarization was elicited by 10^{-5} M carbachol, a synthetic stable analogue of acetylcholine, a similar tendency for the membrane potential in the prolonged presence of the vasodilator to become smaller after L-NAME application than in the control conditions was observed. The effect was significant when measured 5 min after addition of carbachol (Table 1).

In a further series of experiments, L-NMMA, another inhibitor of NO synthase, had a similar influence on the endothelium-dependent hyperpolarization (Table 1). The E_m change was significantly smaller at 5 and 10 min in the presence of L-NMMA. Figure 4 shows a representative trace from such an experiment.

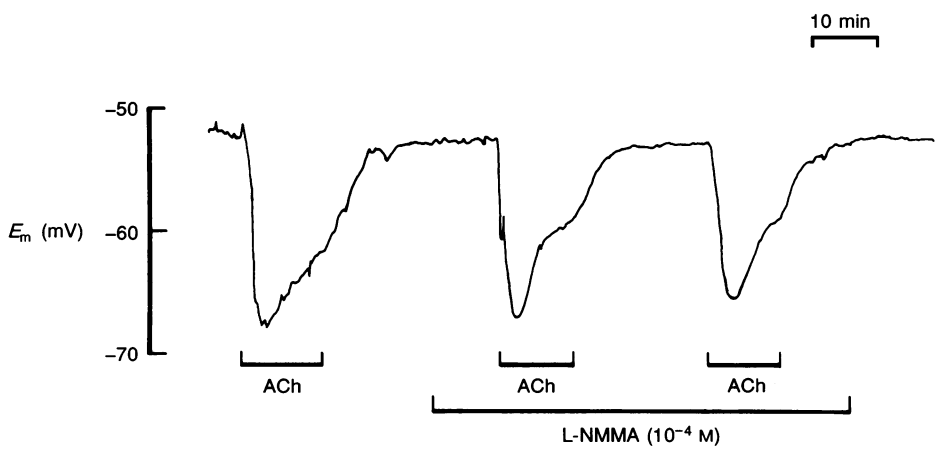

Figure 4. Recordings from an experiment showing the influence of L-NMMA (10^{-4} M) on the acetylcholine-induced endothelium-dependent hyperpolarization
Note the more extensive recovery of E_m during ACh exposure in the presence of L-NMMA.

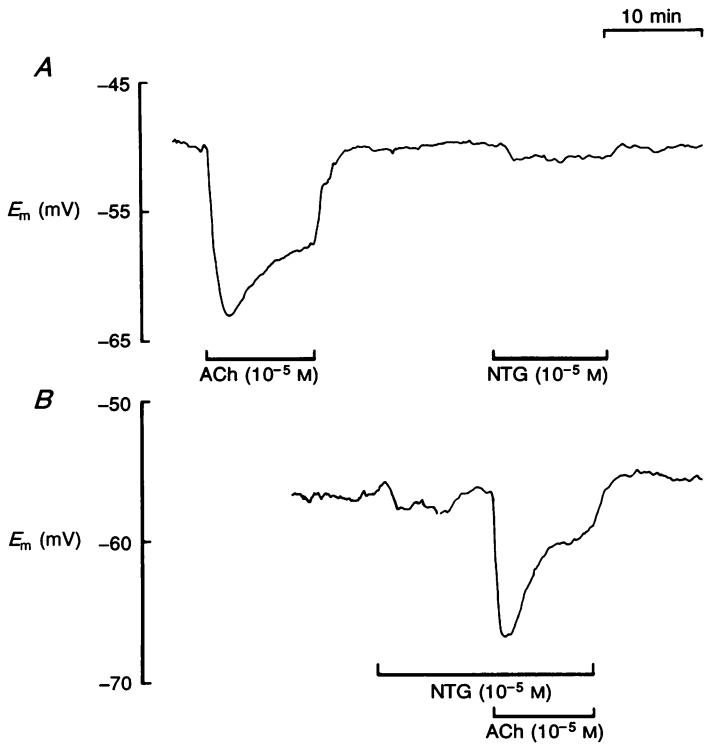

Figure 5. Influence of acetylcholine (ACh, 10^{-5} M) and of nitroglycerin (NTG, 10^{-5} M) on the resting membrane potential (E_m) of a rat aortic smooth muscle cell
In *B*, ACh was applied in the continuous presence of NTG. *A* and *B* are from different rats.

Since the L-NAME and L-NMMA experiments suggested that endogenously formed NO influences to some extent the later part of the endothelium-dependent hyperpolarization to acetylcholine and carbachol, the effect of exogenous NO on the resting E_m of the aortic smooth muscle cell was investigated.

Effect of NO donors

Superfusion with nitroglycerin (NTG)-containing fluid at 10^{-5} M caused a small sustained hyperpolarization, but no transient peak hyperpolarization was present (Fig. 5*A*). In five muscle strips, E_m increased by $1{\cdot}9 \pm 0{\cdot}2$ mV.

In the presence of NTG, the addition of acetylcholine still elicited a hyperpolarization (Fig. 5*B*). Superfusion with the NO donor SIN-1 had a comparable effect on the resting membrane potential (Fig. 6). In three experiments, application of 3×10^{-6} M SIN-1 hyperpolarized E_m by $1{\cdot}3 \pm 0{\cdot}2$ mV.

Infusion of acidified $NaNO_2$ solution in the superfusate upstream of the muscle strip, producing a HNO_2 concentration in the tissue bath of up to $3{\cdot}3 \times 10^{-3}$ M, caused a slowly developing small hyperpolarization, the latter concentration changing E_m by 4 mV (Fig. 7).

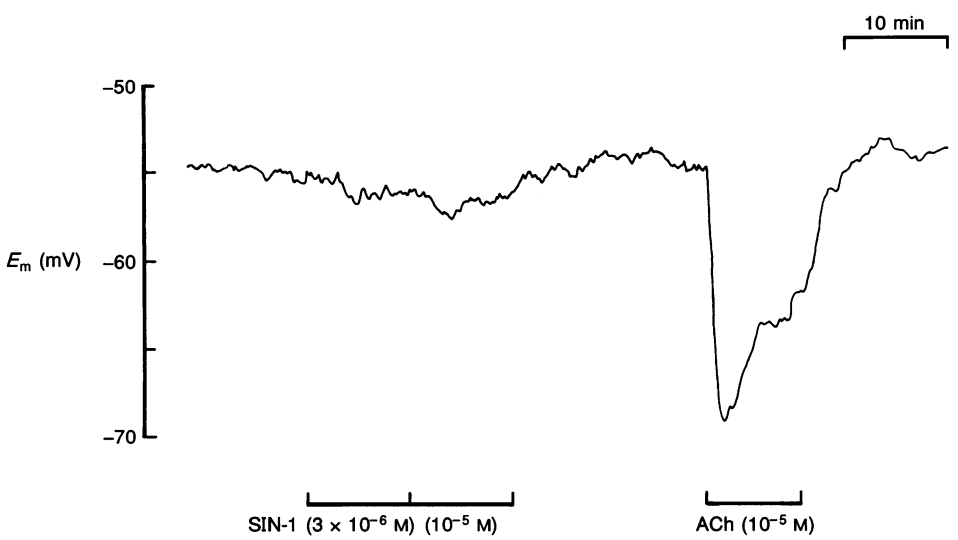

Figure 6.
Influence of SIN-1 (3×10^{-6} M, 10^{-5} M) and acetylcholine (10^{-5} M) on the membrane potential of the rat aortic smooth muscle cell.

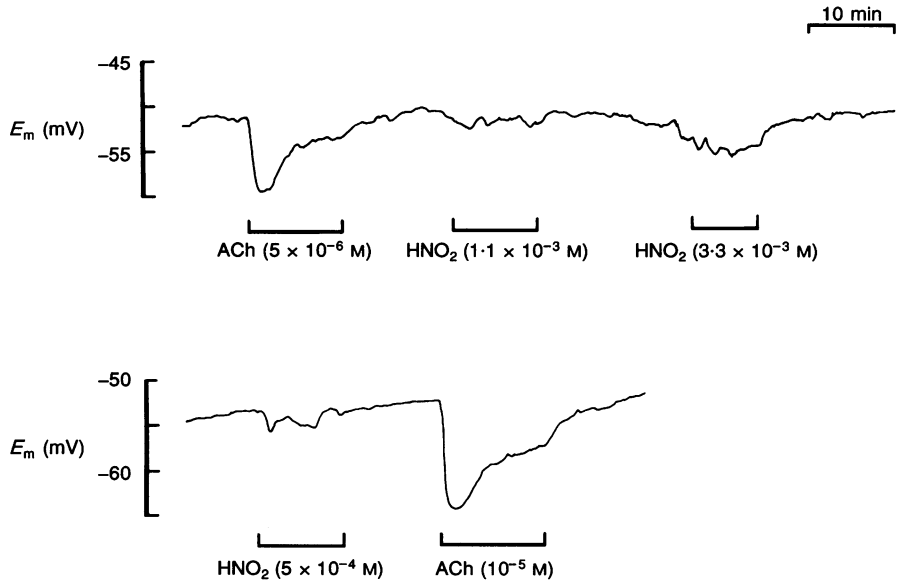

Figure 7.
Traces from two experiments showing the influence of direct infusion of acidified $NaNO_2$ solution in the experimental chamber upstream of the superfused muscle strip, in comparison with the effect of acetylcholine.

Influence of 8-Br-cGMP

Application of 8-Br-cGMP, at 10^{-4} M, caused a barely detectable change of the membrane potential. The addition of acetylcholine in the continuous presence of the nucleotide elicited a transient peak hyperpolarization similar to that observed in control conditions (Fig. 8).

DISCUSSION

The *in vitro* application of acetylcholine to isolated rat aorta preparations initially produces a fast peak hyperpolarization of the smooth muscle cells followed by a return to a value more negative than in control conditions (Chen & Suzuki, 1989). In the absence of the endothelium, this hyperpolarization does not occur. In the present experiments, acetylcholine (10^{-5} M) or carbachol (10^{-5} M) elicited a fast hyperpolarization of, on average, 10–12 mV. It was followed by a progressive increase of the membrane potential to a level which was still 6–8 mV below the control value after 10 min.

In recent years, it has been demonstrated that the hyperpolarization of the smooth muscle layer is due to an endothelial humoral factor which does not appear to be endothelium-derived nitric oxide (EDNO). The presence of haemoglobin or of Methylene Blue, both of which prevent the effect of NO on guanylate cyclase in muscle (Gruetter, Gruetter, Lyon, Kadowitz & Ignarro, 1981; Martin, Williams, Jothianandan & Furchgott, 1985), did not inhibit the hyperpolarization elicited by acetylcholine in the rat

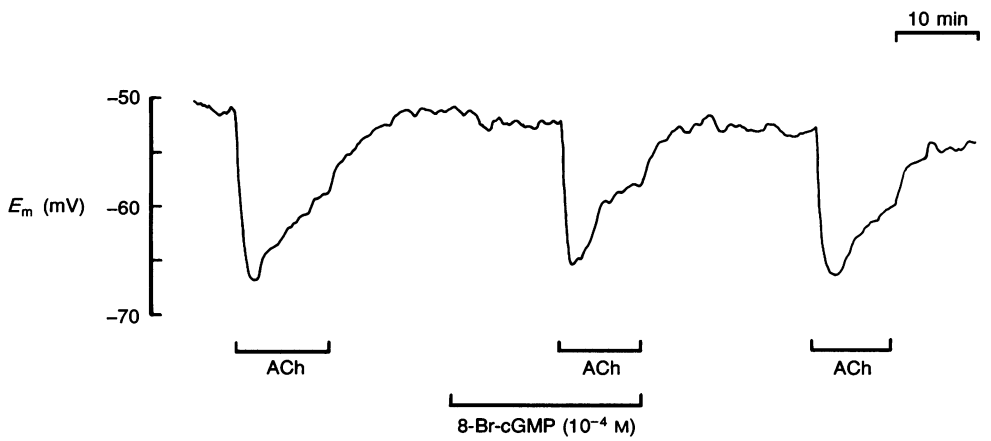

Figure 8.
Recording of the membrane potential during application of acetylcholine (10^{-5} M) before, in the presence of, and after 8-Br-cGMP (10^{-4} M).

aorta and pulmonary artery (Chen *et al.* 1988), the rabbit femoral artery (Huang, Busse & Bassenge, 1988) or the dog mesenteric artery (Komori *et al.* 1988).

More recent reports also showed that inhibition of NO synthesis using nitro-L-arginine had no effect, either on the resting membrane potential or on the endothelium-dependent hyperpolarization in the coronary artery (Chen, Yamamoto, Miwa & Suzuki, 1991; Nagao & Vanhoutte, 1992), mesenteric artery (Fujii *et al.* 1992) and in the rat femoral vein (Nagao & Vanhoutte, 1991). In the present experiments on the rat aorta, NO synthase inhibition with L-NAME or L-NMMA indeed did not influence the early peak hyperpolarization elicited by acetylcholine or carbachol (Table 1). This indicates that most of the endothelium-dependent hyperpolarization in response to ACh in this preparation is not directly related to the endothelial synthesis of nitric oxide. Support for such a view is found in the observation that the presence of nitroglycerin (10^{-5} M) or 8-Br-cGMP (10^{-4} M) did not modify the E_m transient in response to acetylcholine (Figs 5*B* and 8).

The two inhibitors of endogenous NO production, however, appeared to influence the later phase of the endothelium-dependent hyperpolarization. The time course of the E_m recovery after the peak hyperpolarization was accelerated after treating the preparations with L-NAME, resulting in the development of a slight difference of E_m from that observed in control conditions, reaching significant levels between 5 and 10 min (Fig. 2). The difference in membrane potential measured after 10 min was on average 3–4 mV (Table 1). This effect was not seen when a first and a second response of the muscle strips to acetylcholine were compared in the absence of L-NAME (Fig. 2). Similar findings were obtained with L-NMMA. These observations suggest that endogenously formed NO, stimulated by acetylcholine application, influences the hyperpolarization induced by this vasodilator, the effect becoming more apparent during the second phase of the membrane electrical response, but as a whole being rather limited. Such an interpretation finds support from the rather small effects seen with nitroglycerin, SIN-1, or HNO_2 on the steady-state membrane potential (Figs 5, 6 and 7).

An acetylcholine-induced endothelium-dependent hyperpolarization consisting of several components has been suggested in the guinea-pig coronary artery, the second component being caused by NO (Parkington, Tare & Coleman, 1990). Moreover, in the rabbit carotid artery, it was shown that the normal response to acetylcholine, which in this preparation consists equally of a rapid large and a smaller sustained hyperpolarization, was differentially affected by removal of external calcium or inhibition of calcium release from internal stores (Chen & Suzuki, 1990). In low extracellular [Ca^{2+}] solutions, the initial transient component was only slightly decreased in amplitude, whereas the latter component was not generated. Since the release of EDRF is also dependent upon the calcium concentration (Griffith, Edwards, Newby, Lewis & Henderson, 1986), and in the present study we found that inhibition of NO synthesis has an effect on the hyperpolarization similar to that described for reduction of extracellular calcium, we suggest that the second component of the hyperpolarization elicited by acetylcholine is related to the more sustained release of endothelium-derived nitric oxide.

A sustained and small hyperpolarizing effect upon stimulation of EDNO release by ACh could also imply that in the resting muscle strip a continuous, albeit very limited, hyperpolarizing influence could arise from the basal release of this factor from the endothelial cells. A number of observations corroborates this view, at least in the rat aorta. In this preparation, removal of the endothelium produces a slight depolarization (Taylor *et al.* 1988; Chen & Suzuki, 1989; Van de Voorde, Vanheel & Leusen, 1992); in other vessels the influence of de-endothelialization on the resting E_m was less pronounced or absent (Komori & Suzuki, 1987; Chen & Suzuki, 1989). In addition, the application of Methylene Blue has been reported to depolarize rat aortic strips by ~8 mV, while this substance did not affect the E_m of the pulmonary artery and of the de-endothelialized aorta (Chen & Suzuki, 1989). Acetylcholine caused a biphasic (peak and sustained) hyperpolarization in the rat aorta, but only a transient change of E_m in the pulmonary artery (Chen & Suzuki, 1989). In line with these results, it was recently shown that nitro-L-arginine decreased the resting E_m of the rat aortic strip by 4–10 mV, and that addition of SIN-1 or 8-Br-cGMP could restore the membrane potential to its original value (Krippeit-Drews, Morel & Godfraind, 1991). In our experiments, L-NAME produced no significant depolarization while L-NMMA slightly decreased E_m (Table 1).

Experiments on different types of blood vessels indicate that the influence of NO on muscle membrane potential may vary. In the guinea-pig uterine artery, exogenously applied nitric oxide caused a hyperpolarization of about 6 mV in noradrenaline-precontracted muscle strips, and the hyperpolarization elicited by ACh was reduced in the presence of L-NMMA (Tare *et al.* 1990). In rat small mesenteric artery, NO has also been shown to increase the membrane potential by 3–9 mV; unlike the hyperpolarization induced by acetylcholine, the NO-induced E_m change could be blocked by inhibition of the ATP-regulated K^+ channels with glibenclamide (Garland & McPherson, 1992). Recently, high concentrations of exogenous NO were found to cause a hyperpolarization of about 2 mV in the rabbit basilar artery, while L-NMMA and L-NAME blocked or modified the hyperpolarization evoked by acetylcholine (Rand & Garland, 1992). The latter authors suggested that nitric oxide and EDHF make a variable contribution to the hyperpolarization in different vessels.

Our results show that, in the rat aorta, EDHF is the main factor producing the E_m change. In our experiments the contribution of NO to the acetylcholine-induced hyperpolarization was dissected out as part of the more sustained component of the electrical response to this vasodilator. The variable duration of the hyperpolarization to acetylcholine observed in different arteries, as well as the variable time course of E_m change produced in the same artery by different endothelium-dependent vasodilating agents, might therefore be another expression of the variable contribution of nitric oxide to the membrane electrical response.

REFERENCES

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *Journal of Physiology* **410**, 91–106.

CHEN, G. & SUZUKI, H. (1990). Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. *Journal of Physiology* **421**, 521–534.

CHEN, G., SUZUKI, H. & WESTON, A. H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *British Journal of Pharmacology* **95**, 1165–1174.

CHEN, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1991). Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *American Journal of Physiology* **260**, H1888–1892.

FUJII, K., TOMINAGA, M., OHMORI, S., KOBAYASHI, K., KOGA, T., TAKATA, Y. & FUJISHIMA, M. (1992). Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. *Circulation Research* **70**, 660–669.

FURCHGOTT, R. F. & ZAWADZKI, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373–376.

GARLAND, C. J. & MCPHERSON, G. A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *British Journal of Pharmacology* **105**, 429–435.

GRIFFITH, T. M., EDWARDS, D. H., NEWBY, A. C., LEWIS, M. J. & HENDERSON, A. H. (1986). Production of endothelium derived relaxant factor is dependent on oxidative phosphorylation and extracellular calcium. *Cardiovascular Research* **20**, 7–12.

GRUETTER, C. A., GRUETTER, D. Y., LYON, J. E., KADOWITZ, P. J. & IGNARRO, L. J. (1981). Relationship between cyclic guanosine 3′,5′-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. *Journal of Pharmacology and Experimental Therapeutics* **219**, 181–186.

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

IGNARRO, L. J., BUGA, G. M., WOOD, K. S., BYRNS, R. E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the USA* **84**, 9265–9269.

IGNARRO, L. J. & KADOWITZ, P. J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annual Review of Pharmacology and Toxicology* **25**, 171–191.

KOMORI, K., LORENZ, R. R. & VANHOUTTE, P. M. (1988). Nitric oxide, acetylcholine, and electrical and mechanical properties of canine arterial smooth muscle. *American Journal of Physiology* **255**, H207–212.

KOMORI, K. & SUZUKI, H. (1987). Electrical responses of smooth muscle cells during cholinergic vasodilation in the rabbit saphenous artery. *Circulation Research* **61**, 586–593.

KRIPPEIT-DREWS, P., MOREL, N. & GODFRAIND, T. (1991). Effect of the endothelium on the membrane potential of smooth muscles of rat aorta. *Pflügers Archiv* **419**, R128.

MARTIN, W., WILLIAMS, G. M., JOTHIANANDAN, D. & FURCHGOTT, R. F. (1985). Selective blockade of endothelium-dependent and glyceryl-trinitrate-induced relaxation by hemoglobin and methylene blue in the rabbit aorta. *Journal of Pharmacology and Experimental Therapeutics* **232**, 708–716.

NAGAO, T. & VANHOUTTE, P. M. (1991). Hyperpolarization contributes to endothelium-dependent relaxations to acetylcholine in femoral veins of rats. *American Journal of Physiology* **261**, H1034–1037.

NAGAO, T. & VANHOUTTE, P. M. (1992). Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *Journal of Physiology* **445**, 355–367.

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

PARKINGTON, H. C., TARE, M. & COLEMAN, H. A. (1990). More than one agent is involved in the hyperpolarization induced by acetylcholine in coronary arteries of guinea-pigs. *Blood Vessels* **27**, 52.

RAND, V. E. & GARLAND, C. J. (1992). Endothelium-dependent relaxation to acetylcholine in the rabbit basilar artery: importance of membrane hyperpolarization. *British Journal of Pharmacology* **106**, 143–150.

RAPOPORT, R. M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. *Circulation Research* **52**, 352–357.

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

SUZUKI, H. & CHEN, G. (1990). Endothelium-derived hyperpolarizing factor (EDHF): an endogenous potassium-channel activator. *News in Physiological Sciences* **5**, 212–215.

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

TAYLOR, S. G., SOUTHERTON, J. S., WESTON, A. H. & BAKER, J. R. J. (1988). Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. *British Journal of Pharmacology* **94**, 853–863.

VAN DE VOORDE, J., VANHEEL, B. & LEUSEN, I. (1992). Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circulation Research* **70**, 1–8.

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