Journal of Physiology (1993), **465**, *pp.* 459–476
With 9 figures
Printed in Great Britain

STRETCH REVEALED THREE COMPONENTS IN THE HYPERPOLARIZATION OF GUINEA-PIG CORONARY ARTERY IN RESPONSE TO ACETYLCHOLINE

BY HELENA C. PARKINGTON, M. TARE, M. A. TONTA AND H. A. COLEMAN

From the Department of Physiology, Monash University, Clayton, Victoria 3168, Australia

(*Received 24 February 1992*)

SUMMARY

1. Membrane potential was recorded with intracellular microelectrodes from the smooth muscle of coronary arteries of guinea-pigs, and the responses to endothelium-derived relaxants were studied under a variety of conditions.

2. Stimulation of the endothelium with brief applications of acetylcholine or substance P evoked concentration-dependent hyperpolarizations that were complex in nature. A transient component, which is likely to result from endothelium-derived hyperpolarizing factor (EDHF), was followed by a slow component that resulted from the production of nitric oxide (NO) and a prostaglandin.

3. The ability of exogenous and endogenous NO and prostacyclin to hyperpolarize the membrane depended upon the smooth muscle being under stretch. Unstretched preparations responded to acetylcholine with only the transient component of hyperpolarization; NO and prostacyclin were without effect.

4. In stretched preparations exogenous NO and prostacyclin, and its synthetic analogue methyl prostacylin (Iloprost), evoked hyperpolarization, and the slow component of the response induced by acetylcholine appeared. The amplitudes of these responses reached maximum when the tissues were stretched to the equivalent of approximately 50 mmHg.

5. From a resting membrane potential of $-61 \pm 0{\cdot}6$ mV, exogenous NO and Iloprost hyperpolarized the smooth muscle to around -80 mV. The EC_{50} values for NO- and Iloprost-induced hyperpolarization were $2{\cdot}6 \times 10^{-6}$ and $1{\cdot}3 \times 10^{-8}$ M, respectively.

6. Coronary arterial smooth muscles from rats, rabbits and sheep also hyperpolarized in response to exogenous NO, although their sensitivities were less than those of preparations obtained from guinea-pigs. Iloprost hyperpolarized tissues from rabbits and sheep but not those obtained from rats.

7. It is concluded that the endothelial lining of coronary arteries can release three factors, EDHF, NO and prostacyclin, all of which can hyperpolarize the membrane of the smooth muscle. The relative proportions and significance of each factor depends on the amount of stretch, on the artery and on the species of animal.

MS 1148

INTRODUCTION

The level of tone in vascular smooth muscle is determined by the concentration of free calcium in the cytoplasm. Influx of calcium from the external environment is essential for maintained tone and may also contribute to more transient constriction, particularly in resistance vessels (see Hirst & Edwards, 1989; Mulvany & Aalkjaer, 1990; Nelson, Patlak, Worley & Standen, 1990). Dihydropyridines, which block calcium influx through potential-dependent calcium channels, are highly effective in lowering vascular tone and reducing blood pressure. Thus, influx through potential-sensitive calcium channels is likely to be an important route of calcium entry in resistance arteries. Low concentrations of agonist evoke depolarization that is accompanied by contraction in small arteries (Bolton, Lang & Takewaki, 1984). Small depolarizations, 5–10 mV in amplitude and taking the membrane potential to −55 to −45 mV, have been shown to be sufficient to activate a sustained inward current in arterioles which is accompanied by constriction and which is blocked by dihydropyridines (Hirst, Silverberg & Van Helden, 1986). It appears that the most polarized level of membrane potential attained in arterial smooth muscle *in vivo* may be in the range −55 to −40 mV (Speden, 1964; Steedman, 1966; Keef & Neild, 1985), and this is within the range of potential-dependent calcium entry. Thus, it was not surprising that local application of tetrodotoxin to block activity of nerves resulted in hyperpolarization that was accompanied by dilatation (Keef & Neild, 1985). From a consideration of the voltage dependence of potential-sensitive calcium channels from a variety of arteries, Nelson *et al.* (1990) have concluded that even modest changes in membrane potential within the physiological range of values can have profound repercussions in relation to calcium influx.

In view of the importance of membrane potential in determining calcium influx and tone in vascular smooth muscle it is timely to reconsider, in detail, the well-established ability of acetylcholine to hyperpolarize arterial smooth muscle as a result of stimulating the endothelium (Kuriyama & Suzuki, 1978; Kitamura & Kuriyama, 1979; Bolton *et al.* 1984; Mekata, 1986; Komori & Suzuki, 1987; Chen, Suzuki & Weston, 1988; Feletou & Vanhoutte, 1988). The endothelium can produce nitric oxide (NO) and prostacyclin, two potent vasodilators. Although NO- and prostacyclin-induced vasodilatation may involve a reduction in cytoplasmic calcium (Popescu, Panoiu, Hinescu & Nutu, 1985; Kobayashi, Kanaide & Nakamura, 1985) and/or a reduction in the sensitivity of the contractile apparatus (Adelstein, Conti, Hathaway & Klee, 1978; Nishimura & Van Breemen, 1989), their ability to hyperpolarize as a possible means of reducing calcium entry, and hence assist in relaxation, must be explored. The role of these agents in endothelium-dependent hyperpolarization is so controversial (Chen *et al.* 1988; Feletou & Vanhoutte, 1988; Huang, Büsse & Bassenge, 1988; Komori, Lorenz & Vanhoutte, 1988) that the existence of another factor, called endothelium-derived hyperpolarizing factor (EDHF), has been proposed (Bolton *et al.* 1984; Komori & Suzuki, 1987; Taylor & Weston, 1988). The present study was undertaken in an attempt to elucidate further the effect of acetylcholine on the membrane potential of vascular smooth muscle, and the role of NO, prostacyclin and EDHF in the response. Acetylcholine-induced relaxation of rat aorta appears to be influenced by the degree of stretch on the

preparation (Dainty, McGrath, Spedding & Templeton, 1990). We examined the possible influence of stretch on the membrane potential response to acetylcholine, NO and prostacyclin using coronary arteries of several species.

Some aspects of this work have been presented previously (Parkington, Tare & Coleman, 1990).

METHODS

Guinea-pigs of both sexes, but mostly males, and male rats were killed by decapitation and the heart removed. The left descending main coronary artery, from the coronary sinus to its entry into the cardiac muscle underneath the pulmonary artery, was used. In the case of sheep, hearts were obtained at the abattoir within minutes of death and were transported to the laboratory in ice-cold physiological solution. Rabbits were killed by a blow to the head. Small branches of the main coronary artery from sheep and rabbits were used.

For most experiments, ring segments (1–2 mm in length) were threaded with a pair of wires (40 μm in diameter) and secured to two supports (Mulvany & Halpern, 1977; Tare, Parkington, Coleman, Neild & Dusting, 1990*a*). One support was attached to a micrometer screw and the other to a force transducer. The segments were continuously superfused with physiological solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 2·5; KH_2PO_4, 1; $MgSO_4$, 1·2; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2–5% CO_2 and maintained at 35 °C. The segments were stretched in increments until their tension was equivalent to a transmural pressure that approximated the mean blood pressure. The endothelium which lined the segment was removed when required by rubbing the lumen with a small roughened wire. Preparations were rested for at least 1 h following removal of the endothelium (Kotecha & Neild, 1988). Removal of the endothelium was deemed to be successful if the transient component of hyperpolarization did not occur following application of a high concentration of acetylcholine. In some experiments, segments 5 mm in length were pinned without tension to the silicone rubber base of a recording chamber. In other experiments, one end of a spiral strip was pinned, endothelium uppermost, to the base of the bath, while the other end was attached to a force transducer (AE801, SensoNor, Horten, Norway). The membrane potentials of the smooth muscle cells in both ring segments and strips were recorded using conventional glass intracellular microelectrodes filled with 1 M KCl and having resistances of around 100 MΩ (Tare *et al.* 1990*a*).

Drugs were applied using two methods: for prolonged exposure, a drug was added to the superfusate; for short exposures, drugs were applied for 10 s periods by direct injection into the superfusion line, with a 1 s delay between the point of injection and the preparation. The time course of the changes in potential in response to increasing chloride in the bathing solution, recorded with a silver–silver chloride electrode placed at the position of the preparation, confirmed that the concentration of drug reached a steady state within 3–4 s of injection and thus the 10 s injection period was sufficient. Solutions to be injected at close range to the preparations were dissolved in 0·9% saline. Solutions of NO were prepared by dissolving the appropriate volume of NO gas in a sealed vial of saline that had been deoxygenated by bubbling with argon for 1 h.

The mean ± standard error of the mean of responses are quoted throughout and a significance level of 0·05 was used in testing. The n values quoted represent the number of preparations studied and not the number of cells impaled.

The drugs used were NO (Matheson Gas Products Inc., TX, USA); prostacyclin, N^{ω}-nitro-L-arginine methyl ester (NAME), N^{G}-monomethyl-L-arginine (NMMA), bradykinin, indomethacin and substance P (Sigma Chemicals); acetylcholine (Merck, UK); Iloprost (methyl prostacyclin) was a kind gift from Schering (Germany).

RESULTS

Stimulation of the endothelium with acetylcholine

Stimulation of the endothelium of unstretched spiral strips with a 10 s application of acetylcholine resulted in a transient hyperpolarization in three preparations of guinea-pig coronary artery (Fig. 1*A*). Exogenous NO was essentially without effect

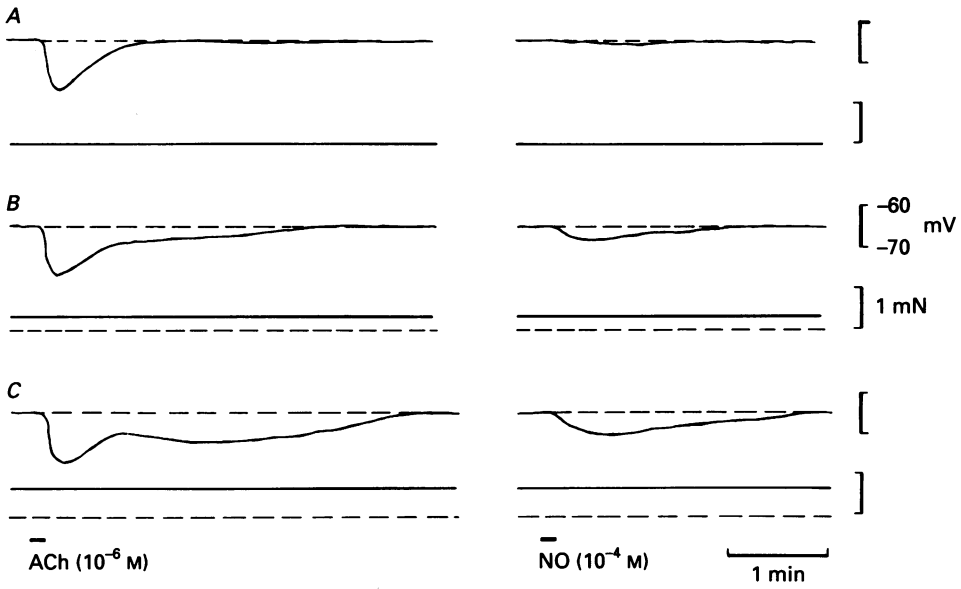

Fig. 1. *A*, only one component of hyperpolarization was recorded in an unstretched spiral strip of coronary artery in response to a 10 s exposure to acetylcholine (10^{-6} M), and exogenous NO (10^{-4} M) was essentially without effect on membrane potential. *B*, as the preparation was stretched progressively a slow component of hyperpolarization was evoked by acetylcholine, and a hyperpolarization in response to NO emerged. *C*, the slow component in response to acetylcholine and NO-induced hyperpolarization increased in amplitude as stretch was increased. In each panel the top trace depicts membrane potential and the bottom trace tension. The dashed lines represent the level of membrane potential and tension in the unstretched state.

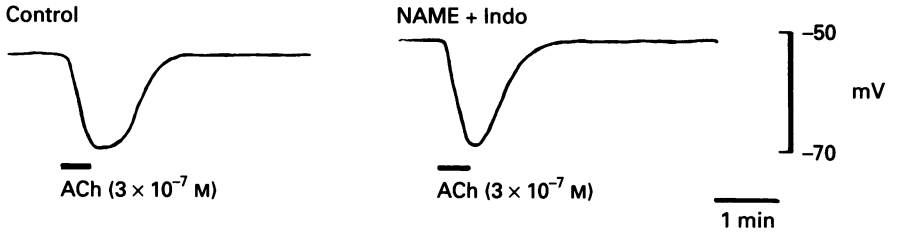

Fig. 2. Only one component of hyperpolarization was observed when unstretched preparations of guinea-pig coronary artery were perfused with acetylcholine (3×10^{-7} M) for half a minute. The duration of the response was only slightly reduced in the presence of a combination of NAME (2×10^{-5} M) and indomethacin (5×10^{-7} M) (Indo). The preparation consisted of a ring (~ 5 mm long) which was pinned at each end to the base of an organ bath.

on membrane potential (Fig. 1*A*). As the tissues were stretched, a second, slow component emerged in the hyperpolarization induced by acetylcholine, and exogenous NO caused hyperpolarization. These responses increased in amplitude as the amount of stretch was increased (Fig. 1*B* and *C*).

When five unstretched ring segments of coronary artery were exposed for 0·5–2 min to acetylcholine (5×10^{-8} to 10^{-6} M) only one component of hyper-

polarization was observed. The duration of this response was only slightly reduced by the presence of N^{ω}-nitro-L-arginine methyl ester (NAME), to block synthesis of NO, and/or indomethacin, to block production of prostaglandins (Fig. 2). At high concentrations of acetylcholine (above 10^{-6} M) a small second phase of hyperpolarization emerged and this was blocked by NAME and indomethacin.

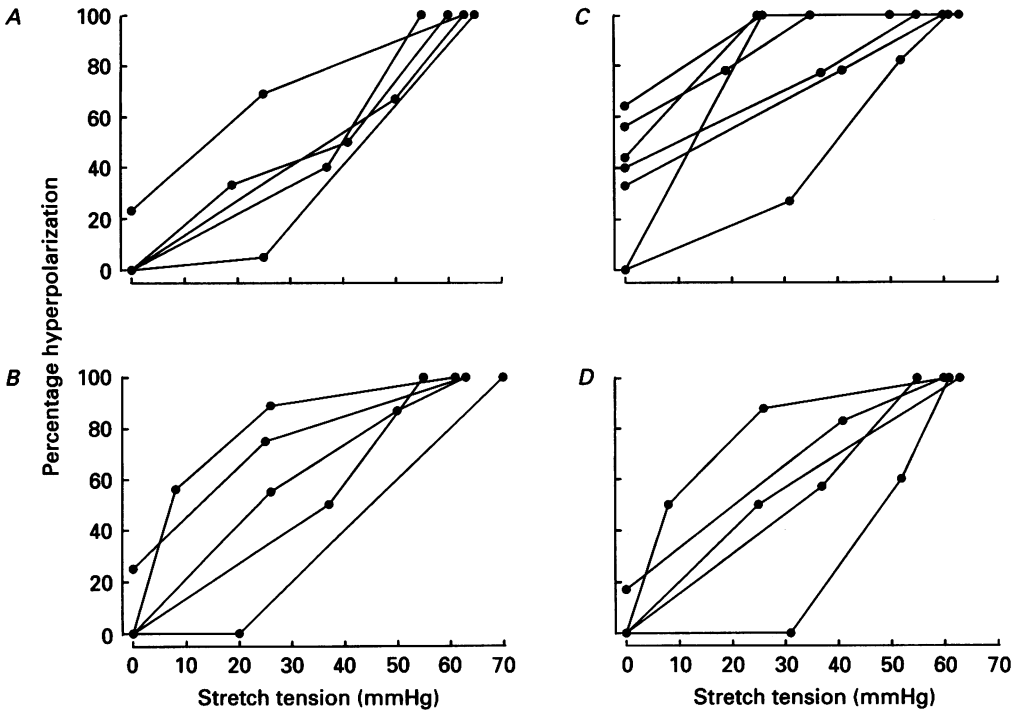

Fig. 3. *A*, NO (10^{-5} M) or *B*, Iloprost (10^{-8} M) did not evoke hyperpolarization in 4 of 5 unstretched preparations of coronary artery mounted on a myograph, and (*D*) the slow component of the hyperpolarization in response to acetylcholine (10^{-7} M) was not observed. Maximal hyperpolarization was not recorded until the preparations had been stretched to the equivalent of 50–60 mmHg. *C*, the transient component of the hyperpolarization induced by acetylcholine (10^{-7} M) was observed in 5 of 7 unstretched preparations. A maximal response occurred when the preparations were stretched to 20–50 mmHg.

Exogenous NO (10^{-5} M) did not evoke hyperpolarization in four of five unstretched ring segments that were threaded with wires and mounted in a myograph (Fig. 3*A*). Hyperpolarization appeared following stretch of the preparations, and increased in amplitude as the amount of stretch was increased, with a maximum hyperpolarization recorded when the tension in the preparations was equivalent to approximately 50 mmHg. The hyperpolarization evoked by Iloprost (10^{-8} M) (a stable analogue of prostacyclin) (Fig. 3*B*) and the slow component of the response to acetylcholine (10^{-7} M; Fig. 3*D*) followed a similar pattern. The initial transient component of the hyperpolarization in response to acetylcholine (10^{-7} M) was present in five of seven completely unstretched tissues, and this component of the response reached its maximum amplitude at lower levels of stretch (20–50 mmHg; Fig. 3*C*).

Endothelium-derived NO

It is clear that a complex hyperpolarization was evoked by acetylcholine in preparations of coronary artery that were stretched to the equivalent of around 60 mmHg and hence the identity of the agent(s) that might be responsible for the different components of the response was studied in preparations thus stretched.

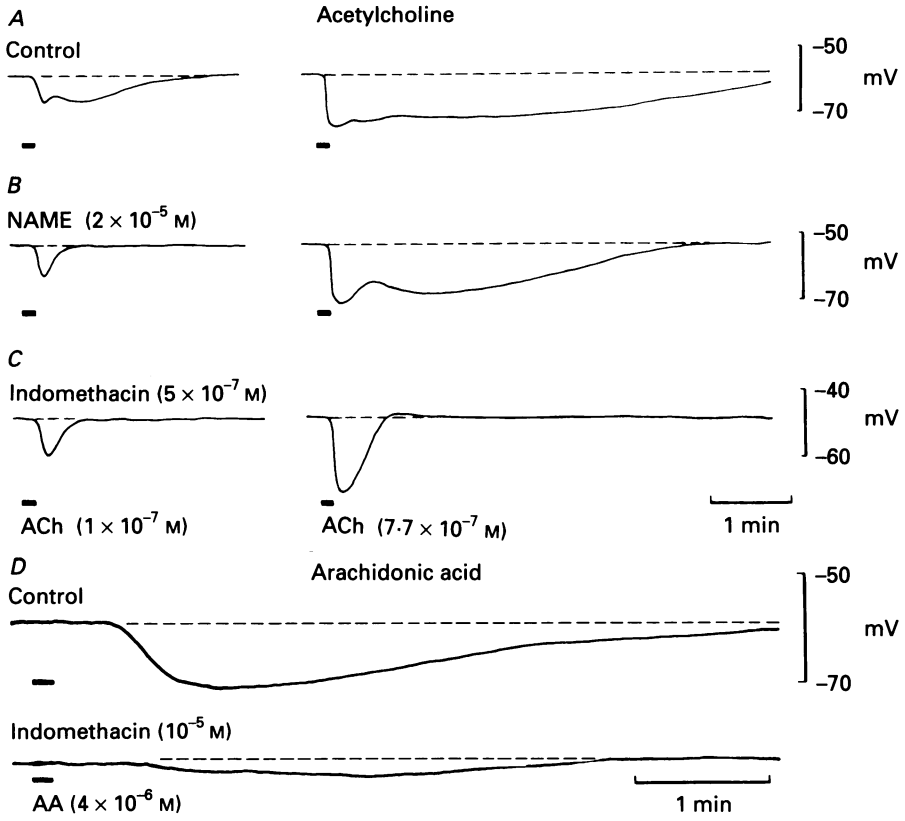

Fig. 4. The slow component of the hyperpolarization in response to a low (1×10^{-7} M) concentration of acetylcholine (*A*) was abolished by NAME (2×10^{-5} M; *B*). The duration of this component of the response to a higher concentration of acetylcholine ($7{\cdot}7 \times 10^{-7}$ M) was reduced (*B*). *C*, in the continued presence of NAME, indomethacin (5×10^{-7} M) abolished the remainder of the slow component, leaving the transient component intact. *D*, arachidonic acid (4×10^{-6} M; AA) induced hyperpolarization after a delay. This response was all but abolished by indomethacin.

To determine which component of the hyperpolarization evoked by acetylcholine might be due to the release of NO from the endothelium, preparations were superfused with 2 or 5×10^{-5} M NAME, or N^G-monomethyl-L-arginine (NMMA) to block biosynthesis of NO (Palmer, Rees, Ashton & Moncada, 1988). Exposure to these blockers for 10 min depolarized the membrane by some 5 ± 1 mV in ten of fifteen preparations. The depolarization was associated with an increase in tension of $0{\cdot}6 \pm 0{\cdot}3$ mN/mm length of the segment (approximately 6% of maximal tension).

The slow component of hyperpolarization evoked by low concentrations (10^{-7} M or

less) of acetylcholine was inhibited by NAME and NMMA (Fig. 4*B*). Since NMMA and NAME caused depolarization, the amplitudes of the transient and slower components were corrected for the change in driving force based on the Goldman–Hodgkin–Katz current equation for potassium ions. NMMA and NAME reduced the second component of the hyperpolarization evoked by low concentrations of acetylcholine (10^{-7} M or less) to $11 \pm 3\%$, while leaving the response to exogenous NO unchanged ($107 \pm 5\%$) (15 preparations). The effect of NAME and NMMA was reversed in all of six preparations when the tissues were perfused with 10^{-4} M L-arginine for 10 min before and during the application of acetylcholine.

Endothelium-derived prostaglandins

Only the duration of the slow component of the hyperpolarization evoked by higher concentrations of acetylcholine was reduced by blockers of NO synthesis (Fig. 4*B*). The amplitude of the response remained intact. Indomethacin was used to test the possibility that a prostaglandin contributed to the slow component of the hyperpolarization evoked by high concentrations of acetylcholine. Indomethacin (5×10^{-7} to 10^{-6} M) depolarized twelve preparations by 6 ± 1 mV and increased tension by $0{\cdot}7 \pm 0{\cdot}3$ mN/mm (approximately 7% of maximal tension). It was without effect on membrane potential or tension in four preparations.

In the continued presence of NAME, addition of indomethacin (5×10^{-7} M) reduced the slow component of the hyperpolarization evoked by high concentrations of acetylcholine (5×10^{-7} to 2×10^{-6} M) to $10 \pm 3\%$ (Fig. 4*C*). The transient component remained intact in the presence of a combination of these blockers ($103 \pm 6\%$, after correction; Fig. 4*C*). The effects of aspirin (10^{-3} M), another blocker of cyclo-oxygenase, were similar to those of indomethacin.

Infusion of arachidonic acid (10^{-7} to 10^{-5} M), a precursor in prostaglandin synthesis, for 10 s, caused a dose-dependent hyperpolarization that was markedly reduced by indomethacin (5×10^{-7} to 10^{-5} M; Fig. 4*D*). There was a delay between the time of application of arachidonic acid and the appearance of the hyperpolarization.

Removal of the endothelium

The response of the smooth muscle alone to acetylcholine was examined in twenty-two preparations in which the endothelium had been removed by rubbing with a roughened wire. This procedure resulted in significant depolarization of the smooth muscle cells to -57 ± 1 mV. The transient component of hyperpolarization was never observed following removal of the endothelium.

In six of the twenty-two preparations studied acetylcholine, up to 10^{-4} M, had no detectable effect on membrane potential, although a small contraction was evoked. A slow component of hyperpolarization was recorded in eight tissues (Fig. 5*B*). This occurred after a delay, was around 5 mV in amplitude and was blocked by indomethacin, suggesting that it resulted from the production of prostaglandins by the smooth muscle. Its amplitude decreased following repeated application of high concentrations of acetylcholine. In the remaining eight preparations 2–10 mV depolarization preceded a 2–5 mV hyperpolarization (Fig. 5*C*), which was significantly smaller than the slow component of hyperpolarization that was recorded

from preparations in which the endothelium was intact (see Fig. 5*A*). Again, the hyperpolarization was abolished by indomethacin leaving only depolarization (Fig. 5*D*). In no instance was the hyperpolarization affected by NAME. High concentrations (10^{-5} M) of arachidonic acid induced a small (around 5 mV)

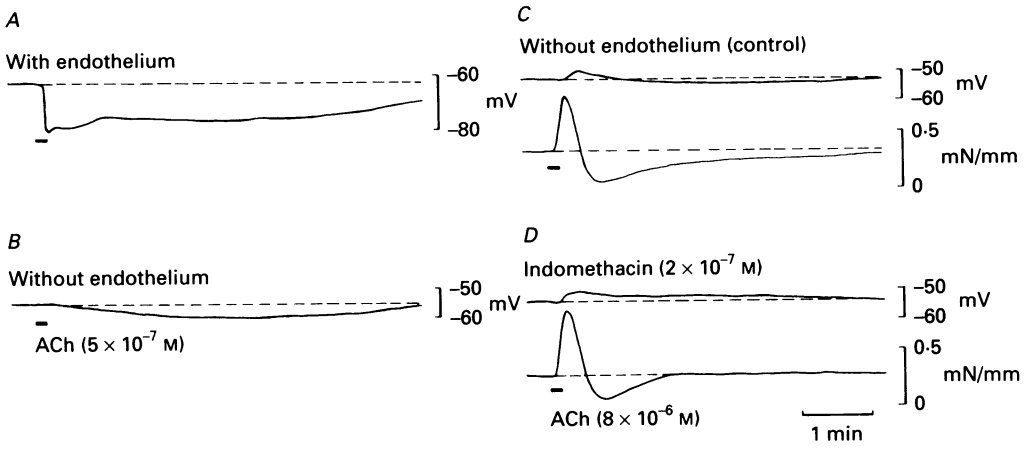

Fig. 5. The typical complex hyperpolarization in response to acetylcholine (ACh) recorded from an intact segment of guinea-pig coronary artery (*A*) was replaced by a small hyperpolarization that was slow in onset in the same preparation following removal of the endothelium (*B*). The transient component of hyperpolarization was never observed following removal of the endothelium. *C*, an example of acetylcholine-induced depolarization and contraction followed by a small hyperpolarization and relaxation in a denuded preparation. *D*, in the same cell, indomethacin (2×10^{-7} M) abolished the hyperpolarization and shortened the relaxation induced by acetylcholine.

hyperpolarization in the absence of endothelium, demonstrating that the smooth muscle cells were capable of producing prostaglandin.

Exogenous NO and prostacyclin

Exogenous NO, applied for 10 s, evoked concentration-dependent hyperpolarization of the smooth muscle in segments of coronary artery that had been denuded of endothelium (Fig. 6*A*). The maximum level of hyperpolarization recorded in response to NO was to −79 mV. Sodium nitroprusside, which is thought to release NO within smooth muscle cells, also induced concentration-dependent hyperpolarization in these preparations (Fig. 6*B*). The delay between the application of the drug and the onset of hyperpolarization was greater for nitroprusside than it was for NO. The hyperpolarization in response to exogenous NO and nitroprusside was unaffected by NAME or NMMA. The effective concentration of NO required to achieve half-maximal hyperpolarization (EC_{50}) was $2{\cdot}6 \times 10^{-6}$ M (Fig. 6*F*).

Application of the vasodilator prostaglandins, prostacyclin (Fig. 6*C*) or its stable analogue Iloprost (Fig. 6*D*), or prostaglandin E_2 (Fig. 6*E*) for 10 s, also caused concentration-dependent hyperpolarization in segments denuded of endothelium. The maximum level of hyperpolarization recorded in response to Iloprost was to −82 mV and was not significantly different from that in response to NO. The hyperpolarization induced by exogenously applied prostacyclin, Iloprost or prostaglandin E_2 was resistant to indomethacin and aspirin (97 ± 4 % for Iloprost in the

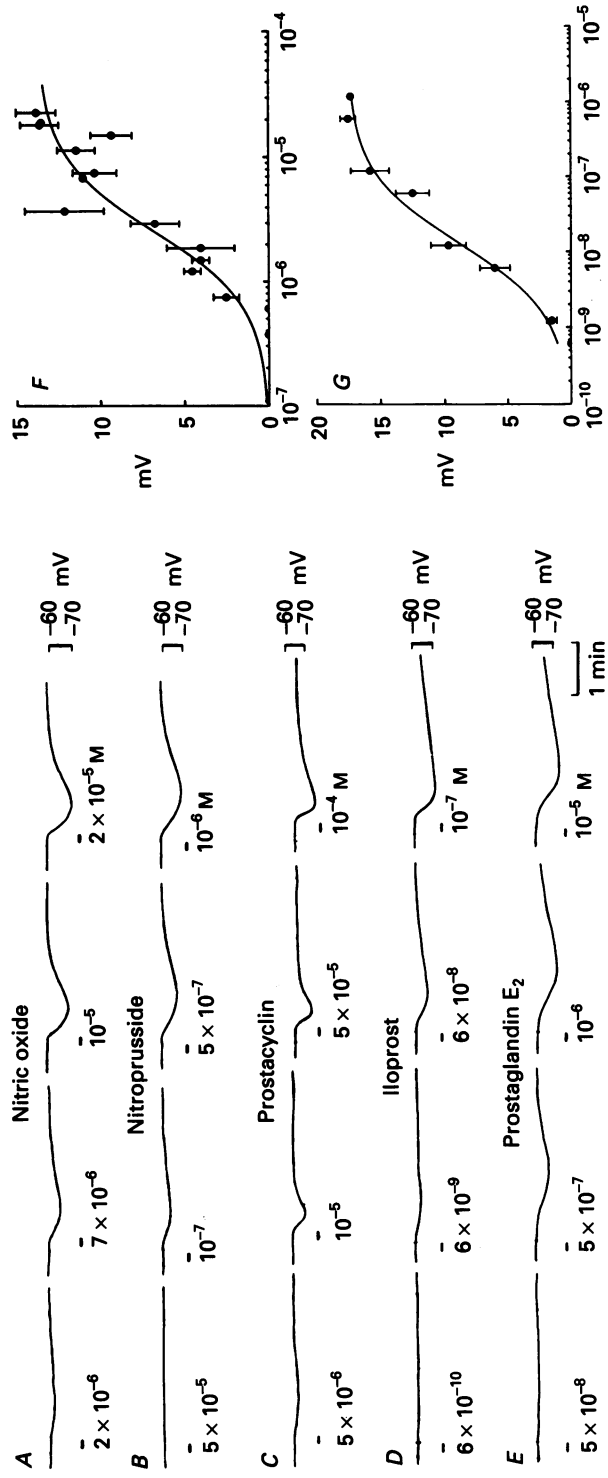

Fig. 6. In segments of coronary artery denuded of endothelium, application of NO (*A*), nitroprusside (*B*), prostacyclin (*C*), Iloprost (*D*) and prostaglandin E_2 (*E*) for 10 s induced concentration-dependent hyperpolarization. Concentration–response curves were constructed for NO (*F*) and Iloprost (*G*). The EC_{50} for NO-induced hyperpolarization was $2{\cdot}6 \times 10^{-6}$ M ($n = 15$) and for Iloprost-induced hyperpolarization was $1{\cdot}3 \times 10^{-8}$ M ($n = 16$).

presence of indomethacin). The EC_{50} for Iloprost-induced hyperpolarization was $1{\cdot}3 \times 10^{-8}$ M (Fig. 6*G*).

The preparations of coronary artery used in this study possessed sympathetic nerves that contained noradrenaline, and the β-adrenoceptor agonists noradrenaline and isoprenaline have been shown to hyperpolarize coronary arteries of dogs (Itoh, Kitamura & Kuriyama, 1980). We found that isoprenaline also induced concentration-dependent hyperpolarization of guinea-pig coronary arterial smooth muscle. Thus, it was considered that the hyperpolarization evoked by NO or Iloprost might represent release of noradrenaline from the nerves. Hyperpolarizations evoked by 10^{-7} to 10^{-5} M isoprenaline were abolished by pretreatment with 10^{-7} M propranolol in five preparations. In contrast, hyperpolarizations that were equivalent in amplitude but induced by exogenous NO or Iloprost in the same tissues were not influenced by this concentration of propranolol.

Cyclic nucleotides

NO increases intracellular levels of cGMP and prostacyclin increases cAMP in vascular smooth muscle. The possibility was considered that these cyclic nucleotides might mediate the effects of NO and prostacyclin on membrane potential in coronary arteries. In eight of eleven preparations, application of the membrane-permeable analogue 8-bromo-cGMP (10^{-4} M) for 6–13 min caused hyperpolarization of $5{\cdot}5 \pm 0{\cdot}9$ mV. It was without effect on three preparations. Application of 8-bromo-cAMP (10^{-4} M for 6–12 min) hyperpolarized each of three preparations by $5{\cdot}3 \pm 2{\cdot}0$ mV. Thus, it is possible that cyclic nucleotides may mediate the hyperpolarization in response to NO and prostacyclin.

Stimulation of the endothelium with substance P

Many substances, other than acetylcholine, stimulate vascular endothelium to release relaxants. Application of substance P to coronary arteries from eleven guinea-pigs resulted in complex hyperpolarization (Fig. 7*A*) which resembled that in response to acetylcholine. As with acetylcholine, the hyperpolarization evoked by substance P consisted of a transient component, followed by a slow component. NAME reduced the amplitude and duration of the slow component evoked by low concentrations of substance P (Fig. 7*B*). A combination of NAME and indomethacin completely abolished this component (Fig. 7*C*). Substance P did not evoke a detectable change in membrane potential in preparations that had been denuded of endothelium.

Responses of coronary arteries in other species

The responses to stimulation of the endothelium and to exogenous NO and Iloprost, were studied in the coronary arteries of other species. The preparations were stretched to the appropriate mean blood pressure. The resting membrane potential of the smooth muscle cells in preparations of coronary arteries from rats was $-45 \pm 0{\cdot}5$ mV ($n = 5$), $-56 \pm 0{\cdot}1$ mV ($n = 3$) in rabbits, and $-57 \pm 0{\cdot}9$ mV ($n = 5$) in sheep. These values were significantly more depolarized than the $-61 \pm 0{\cdot}6$ mV ($n = 48$) recorded in preparations from guinea-pigs.

Acetylcholine elicited the transient component of hyperpolarization in prepar-

ations from rats and rabbits, although this component was small in tissues from rabbits (Fig. 8). The slow component was much reduced in duration in preparations from rats and this may reflect the inability of the smooth muscle to respond to prostacyclin in this species. Despite the fact that acetylcholine evoked only

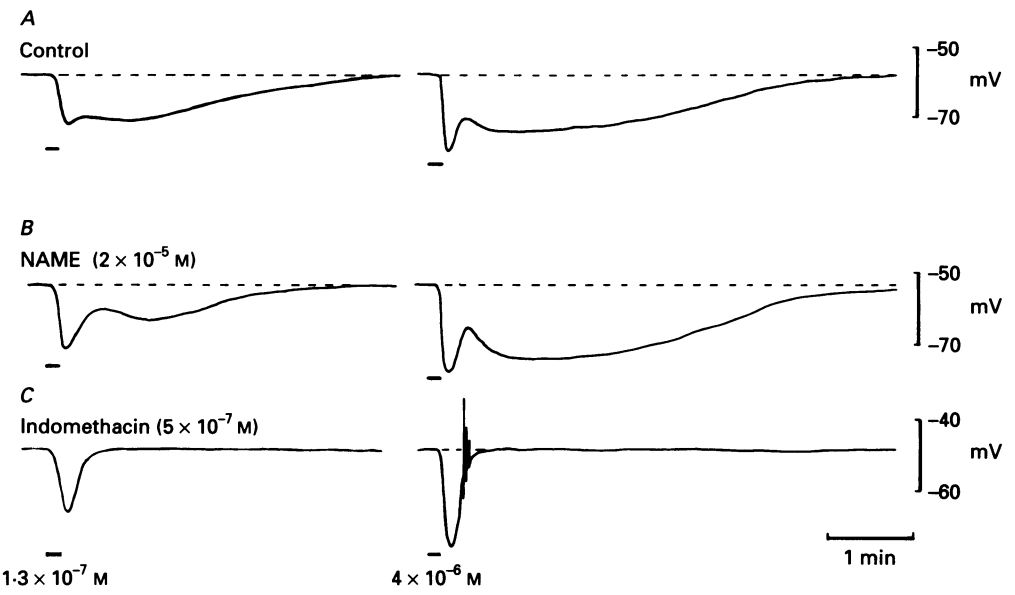

Fig. 7. *A*, the hyperpolarization induced by substance P ($1{\cdot}3 \times 10^{-7}$ and 4×10^{-6} M) consisted of a transient and a slow component . *B*, the amplitude and duration of the slow component evoked by the low concentration was reduced by NAME (2×10^{-5} M). *C*, this component was abolished in the presence of a combination of NAME and indomethacin (5×10^{-7} M).

depolarization and contraction in all five preparations from sheep, bradykinin, which also stimulates the endothelium, caused hyperpolarization (Fig. 8).

Coronary arterial smooth muscle of rats and sheep hyperpolarized in response to exogenously applied NO. However, these responses were small compared with observations in tissues from guinea-pigs. In rabbits, NO-induced hyperpolarization was preceded by a transient depolarization (Fig. 8) which was associated with a brief contraction (data not shown).

Iloprost elicited hyperpolarization in coronary arterial smooth muscle of rabbits and sheep which was similar to that which occurred in guinea-pig tissues. However, the smooth muscle of rat coronary did not respond to Iloprost (Fig. 8) or to pure prostacyclin (data not shown).

Prolonged stimulation of the endothelium

In many previous studies the effects of prolonged exposure to acetylcholine on membrane potential have been examined (Komori & Suzuki, 1987; Komori *et al.* 1988; Chen *et al.* 1988; Feletou & Vanhoutte, 1988; Nishiye, Nakao, Itoh & Kuriyama, 1989; Chen & Suzuki, 1989). Continuous superfusion of guinea-pig

coronary arteries with acetylcholine for 10 min resulted in hyperpolarization that declined only slowly during the period of exposure, and upon removal of acetylcholine there was transient depolarization followed by a second phase of hyperpolarization which declined gradually with time (Fig. 9*A*). The responses following 2 min

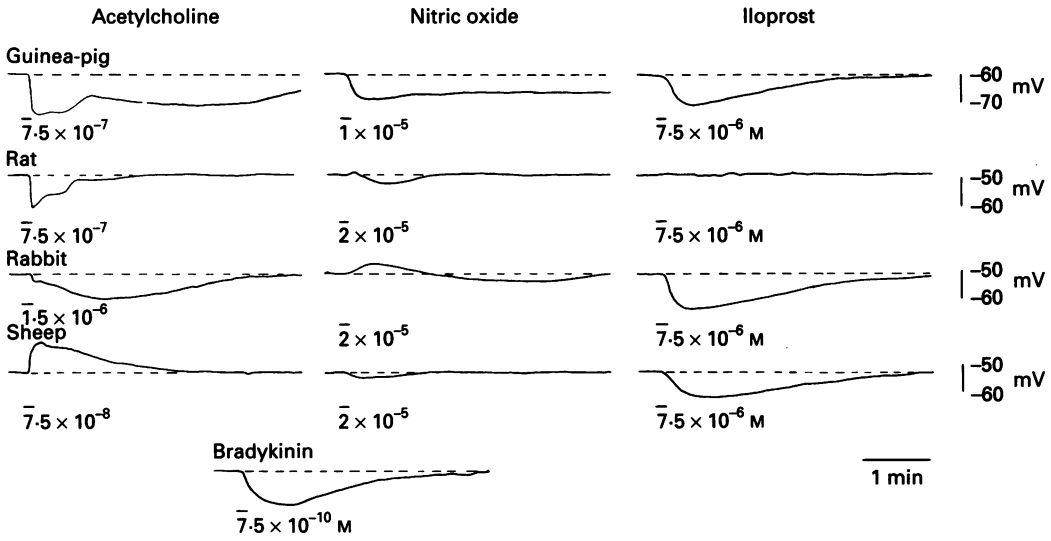

Fig. 8. Comparisons were made between the responses of coronary arteries of guinea-pigs, rats, rabbits and sheep to 10 s applications of acetylcholine, NO and Iloprost. Tissues from sheep depolarized in response to acetylcholine but bradykinin elicited endothelium-dependent hyperpolarization in these preparations. In tissues from rabbits, an initial depolarization was followed by hyperpolarization in response to NO. Preparations from rats did not respond to Iloprost and the response to acetylcholine was brief.

exposure to a range of concentrations of acetylcholine were studied in more detail. Hyperpolarization was sustained during the 2 min of exposure to acetylcholine in control solution (Fig. 9*B*). Upon removal of acetylcholine the membrane repolarized briefly, after which the second phase of hyperpolarization was observed. The amplitude and duration of the second phase of hyperpolarization were reduced by NMMA (3×10^{-5} M; Fig. 9*B*). When NMMA and indomethacin (5×10^{-7} M) were both included in the superfusing solution the initial phase of the hyperpolarization, occurring during the period of exposure to the lower concentrations of acetylcholine (up to 3×10^{-7} M), became more transient and the second phase of hyperpolarization was abolished (Fig. 9*B*).

The possibility was considered that the early phase of repolarization, that which occurred immediately upon removal of acetylcholine, might represent the direct excitatory action of acetylcholine on the smooth muscle (see Fig. 5*C* and *D*). If this were the case then the transient repolarization should not occur if substance P were used since the latter stimulates the endothelium but does not appear to have a direct action on the smooth muscle of guinea-pig coronary artery. Exposure of segments to a range of concentrations of substance P evoked hyperpolarization that consisted of a transient component followed by a more sustained component. No early phase of

repolarizations was observed. Only the initial transient hyperpolarization evoked by substance P persisted in the presence of a combination of NAME and indomethacin (Fig. 9*C*).

DISCUSSION

The results presented in this study demonstrate that the degree of tension on the smooth muscle influences the membrane potential response to acetylcholine, NO and

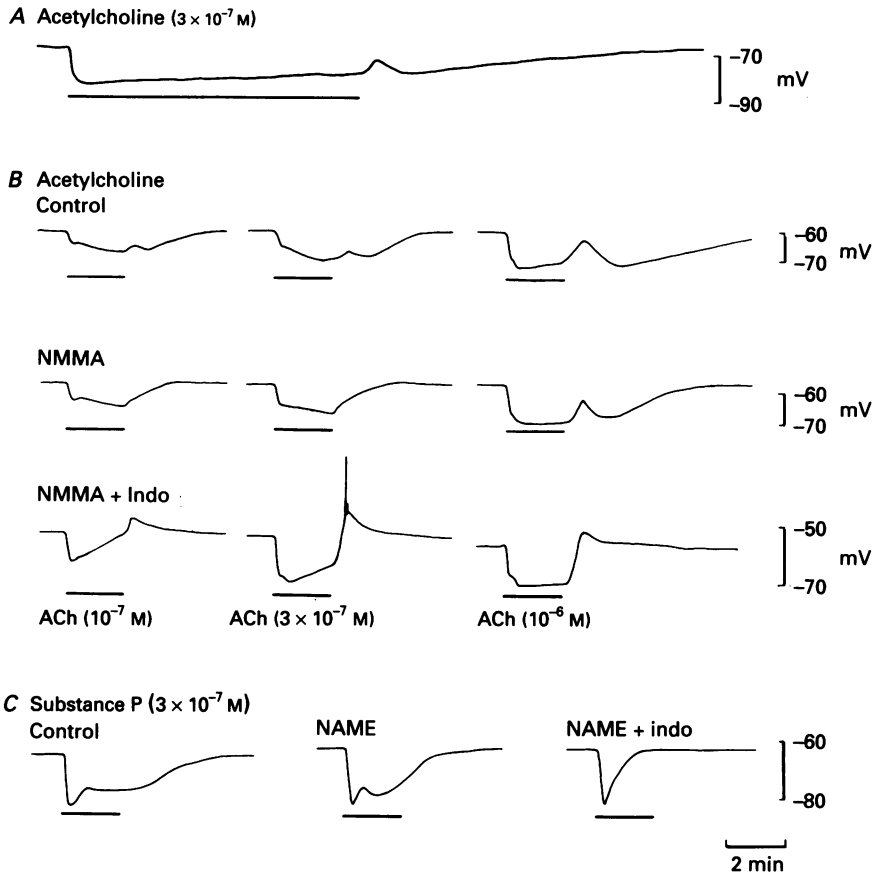

Fig. 9. *A*, exposure to acetylcholine for 10 min resulted in hyperpolarization that declined only slowly. *B*, application of acetylcholine for 2 min induced sustained hyperpolarization. Upon removal of acetylcholine the membrane repolarized briefly and this was followed by a second phase of hyperpolarization. The second phase was abolished by a combination of NMMA (3×10^{-5} M) and indomethacin (5×10^{-7} M). These blockers reduced the duration of the hyperpolarization which occurred during exposure to low concentrations of acetylcholine (10^{-7} and 3×10^{-7} M). *C*, the hyperpolarization in response to superfusion of substance P (3×10^{-7} M) for 2 min consisted of a transient component followed by a slow component. The slow component was blocked by NAME (2×10^{-5} M) and indomethacin (5×10^{-7} M), leaving the transient component.

Iloprost. In unstretched tubes the response to acetylcholine resulted in transient hyperpolarization that was only slightly affected by blockers of NO and prostacyclin synthesis. Similarly, exogenous NO and Iloprost were without effect on membrane potential in unstretched preparations when applied at concentrations that caused maximal hyperpolarization in stretched preparations. As these tissues were progressively stretched the component of the acetylcholine-induced hyperpolarization attributable to NO and prostacyclin emerged and exogenous NO and Iloprost elicited hyperpolarizations of increasing amplitude. It has recently been demonstrated that the relaxation of phenylephrine-induced contraction by acetylcholine increases as rat aorta is stretched to 'optimal length' (Dainty *et al.* 1990). The involvement of stretch in the hyperpolarization evoked by NO and prostacyclin may result from an increase in tension on membrane channels by the cytoskeleton and/or the extracellular connective tissue. This may facilitate the gating of channels by an agonist. Perhaps an analogous situation is that which has been reported for chick skeletal muscle wherein a class of channel was sensitive not only to stretch but also to membrane potential, in an additive way (Guharay & Sachs, 1985).

It is clear from this study that the sensitivity of the smooth muscle of the coronary arteries of different species to NO, prostacyclin and EDHF can vary. In previous studies in which tension was measured, Christie & Lewis (1988) demonstrated that the sensitivity of the smooth muscle of pig coronary artery to endothelium-derived NO exceeded that of rabbit aortic smooth muscle. In the present study Iloprost did not hyperpolarize the coronary artery of rats. This may be due to a lack of receptors for prostacyclin. It is interesting that isoprenaline, which also stimulates the production of cAMP, was without effect on membrane potential in coronary arteries from this species (authors' unpublished observations). The membrane potential of coronary arteries from guinea-pigs was most sensitive to hyperpolarization by NO. In preparations from rabbits, depolarization preceded hyperpolarization when exogenous NO was applied. We have previously reported differences in sensitivity to exogenous NO in its ability to hyperpolarize a variety of arteries (Tare *et al.* 1990*b*). While the transient component of hyperpolarization induced by acetylcholine, most likely due to EDHF, was prominent in tissues from rats and guinea-pigs, it was small or not detectable in tissues from rabbits and sheep.

A contribution of NO to the hyperpolarization induced by acetylcholine has previously been dismissed and the observations giving rise to this conclusion can be summarized: (1) some investigators have failed to observe hyperpolarization in response to exogenous application of NO (Komori *et al.* 1988) or nitroprusside (Huang *et al.* 1988; Taylor, Southerton, Weston & Baker, 1988); (2) the hyperpolarization induced by acetylcholine was found to be transient while relaxation was prolonged (Chen *et al.* 1988; Feletou & Vanhoutte, 1988); (3) acetylcholine evoked an increase in rubidium efflux while nitroprusside did not (Chen *et al.* 1988; Taylor *et al.* 1988); (4) the relaxation, but not the hyperpolarization, evoked by acetylcholine was blocked by Methylene Blue and oxyhaemoglobin (Chen *et al.* 1988). (Methylene Blue reduces NO and is also thought to inactivate guanylate cyclase (Gruetter, Kadowitz & Ignarro, 1981), while oxyhaemoglobin complexes with and inactivates NO (Martin, Villani, Jothianandan & Furchgott, 1985).) It may be possible to explain some of these apparent anomalies in terms of either the degree

of stretch on the preparation, and/or species differences. In many earlier reports unstretched preparations were studied (Komori *et al.* 1988; Huang *et al.* 1988; Chen *et al.* 1988; Nishiye *et al.* 1989; Chen & Suzuki, 1989). The results reported in the present study support our earlier findings of endothelium-dependent hyperpolarization of uterine and other arteries that can be attributed to NO (Tare *et al.* 1990*a*, *b*) and that of Garland & McPherson (1992) in rat mesenteric artery. The tissues in both of these studies were stretched to the mean blood pressure of the animal from which they were obtained.

The method of stimulating the endothelium may also contribute to the ease with which the various components of the hyperpolarization in response to acetylcholine can be detected. The various components merged during prolonged exposure (1 min or more). Notwithstanding, several components in the responses to 1–2 min application of acetylcholine in preparations of guinea-pig coronary artery can be distinguished in records reported by Keef & Bowen (1989; their Fig. 8) and by Nishiye, Chen, Hirose & Kuriyama (1990; their Fig. 10). These records are remarkably similar to those in response to prolonged exposure to acetylcholine and illustrated here in Fig. 9.

It has previously been reported that, while the hyperpolarization and relaxation evoked by acetylcholine are both blocked by the general muscarinic antagonist atropine with equal potency, the hyperpolarization is more sensitive than the relaxation to the M_1 antagonist pirenzepine (Komori & Suzuki, 1987). This observation, together with the transient nature of acetylcholine-induced hyperpolarization, the failure of nitroprusside to stimulate rubidium efflux and the lack of effect of Methylene Blue on this hyperpolarization (all discussed above) led to the proposal of the existence of another factor, called endothelium-derived hyperpolarizing factor (EDHF) (Komori & Suzuki, 1987; Taylor & Weston, 1988). The present demonstration that NO and prostacyclin of endothelial origin can hyperpolarize vascular smooth muscle does not preclude the existence of EDHF. On the contrary, the transient component of acetylcholine-induced hyperpolarization, which is resistant to blockers of NO and prostacyclin synthesis, can most likely be ascribed to EDHF, whose chemical identity remains to be clarified.

Acetylcholine-induced hyperpolarization is likely to result from an increase in potassium conductance (Kuriyama & Suzuki, 1978; Bolton *et al.* 1984). Kitamura & Kuriyama (1979) concluded that the reversal potential for acetylcholine-induced hyperpolarization in guinea-pig coronary artery was around -75 mV. In preparations of rat tail artery in which the resting membrane potential was close to -70 mV, no hyperpolarization occurred in response to nitroprusside, while in tissues that were less polarized hyperpolarization was recorded (Cheung & MacKay, 1985). In the latter study nitroprusside never hyperpolarized the membrane beyond -75 mV. The resting membrane potential of uterine arterial smooth muscle (guinea-pig) is also around -70 mV and neither acetylcholine nor NO elicit hyperpolarization when these preparations are at rest, although hyperpolarization does occur in the presence of agonist-induced depolarization (Tare *et al.* 1990*a*). Thus, the ability of acetylcholine or NO to hyperpolarize vascular smooth muscle also appears to be dependent upon the level of the membrane potential.

In conclusion, endothelium-derived NO, prostacyclin and EDHF are all capable of

contributing to the hyperpolarization that results from stimulating the endothelium of the coronary artery in a variety of species. The relative importance of each of these factors in the response of the membrane potential to stimulating the endothelium varies between arteries and between species. Their ability to hyperpolarize also depends upon the concentration applied, the level of membrane potential and the state of stretch of the tissue. Our results reconcile some of the apparent discrepancies within the literature. The observations provide an insight into a possible mechanism whereby vascular tone may be regulated. The next step towards a greater understanding of the modulation of tone in vascular smooth muscle lies in an elucidation of the role of these various components of hyperpolarization in the control of tension.

The authors thank Professor M. E. Holman, Dr T. O. Neild and Dr R. J. Lang for helpful discussion of the manuscript and Ms D. Clare for preparing the illustrations. The work was supported by the National Heart Foundation of Australia.

REFERENCES

ADELSTEIN, R. S., CONTI, M. A., HATHAWAY, D. R. & KLEE, C. B. (1978). Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3′:5′-monophosphate-dependent protein kinase. *Journal of Biological Chemistry* **253**, 8347–8350.

BOLTON, T. B., LANG, R. J. & TAKEWAKI, T. (1984). Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea pig anterior mesenteric artery. *Journal of Physiology* **351**, 549–572.

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

CHEUNG, D. W. & MACKAY, M. J. (1985). The effects of sodium nitroprusside and 8-bromo-cyclic GMP on electrical and mechanism activities of the rat tail artery. *British Journal of Pharmacology* **86**, 117–124.

CHRISTIE, M. I. & LEWIS, M. J. (1988). Vascular smooth muscle sensitivity to endothelium-derived relaxing factors is different in different arteries. *British Journal of Pharmacology* **95**, 630–636.

DAINTY, I. A., MCGRATH, J. C., SPEDDING, M. & TEMPLETON, A. G. B. (1990). The influence of the initial stretch and the agonist-induced tone on the effect of basal and stimulated release of EDRF. *British Journal of Pharmacology* **100**, 767–773.

FELETOU, M. & VANHOUTTE, P. M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *British Journal of Pharmacology* **93**, 515–524.

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.

GRUETTER, C. A., KADOWITZ, P. J. & IGNARRO, L. J. (1981). Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerine, sodium nitrate, and amyl nitrate. *Canadian Journal of Physiology and Pharmacology* **59**, 150–156.

GUHARAY, F. & SACHS, F. (1985). Mechanotransducer ion channels in chick skeletal muscle: the effects of extracellular pH. *Journal of Physiology* **363**, 119–134.

HIRST, G. D. S. & EDWARDS, F. R. (1989). Sympathetic neuroeffector transmission in arteries and arterioles. *Physiological Reviews* **69**, 546–604.

HIRST, G. D. S., SILVERBERG, G. D. & VAN HELDEN, D. F. (1986). The action potential and underlying ionic currents in proximal rat middle cerebral arterioles. *Journal of Physiology* **371**, 389–404.

HUANG, A. H., BÜSSE, 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.

ITOH, T., KITAMURA, K. & KURIYAMA, H. (1980). Nitroglycerine and catecholamine actions in smooth muscle cells of the canine coronary artery. *Journal of Physiology* **309**, 171–183.

KEEF, K. D. & BOWEN, S. M. (1989). Effect of acetylcholine on electrical and mechanical activity in guinea-pig coronary arteries. *American Journal of Physiology* **257**, H1096–1103.

KEEF, K. D. & NEILD, T. O. (1985). Measurements of the membrane potential of arterial smooth muscle in anaesthetized animals and its relationship to changes in artery diameter. *Microvascular Research* **30**, 19–28.

KITAMURA, K. & KURIYAMA, H. (1979). Effects of acetylcholine on the smooth muscle cell of isolated main coronary artery of the guinea-pig. *Journal of Physiology* **293**, 119–133.

KOBAYASHI, S., KANAIDE, H. & NAKAMURA, M. (1985). Cytosolic-free calcium transients in cultured vascular smooth muscle cells: Microfluorometric measurements. *Science* **229**, 553–556.

KOMORI, K., LORENZ, R. R. & VANHOUTTE, P. M. (1988). NO, ACh, 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.

KOTECHA, N. & NEILD, T. O. (1988). Relaxation and hyperpolarization of the smooth muscle of the rat tail artery following electrical stimulation. *Journal of Physiology* **397**, 489–501.

KURIYAMA, H. & SUZUKI, H. (1978). The effects of acetylcholine on the membrane and contractile properties of smooth muscle cells of the rabbit superior mesenteric artery. *British Journal of Pharmacology* **64**, 493–501.

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

MEKATA, F. (1986). The role of hyperpolarization in relaxation of smooth muscle of monkey coronary arteries. *Journal of Physiology* **371**, 257–265.

MULVANY, M. J. & AALKJAER, C. (1990). Structure and function of small arteries. *Physiological Reviews* **70**, 921–961.

MULVANY, M. J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circulation Research* **41**, 19–26.

NELSON, M. T., PATLAK, J. B., WORLEY, J. F. & STANDEN, N. B. (1990). Calcium channels, potassium channels and voltage dependence of arterial smooth muscle tone. *American Journal of Physiology* **259**, C3–18.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochemical and Biophysical Research Communications* **163**, 929–935.

NISHIYE, E., CHEN, G., HIROSE, T. & KURIYAMA, H. (1990). Mechanical and electrical properties of smooth muscle cells and their regulations by the endothelium-derived factors in the guinea-pig coronary artery. *Japanese Journal of Pharmacology* **54**, 391–405.

NISHIYE, E., NAKAO, K., ITOH, T. & KURIYAMA, H. (1989). Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. *British Journal of Pharmacology* **96**, 645–655.

PALMER, R. M. J., REES, D. D., ASHTON, D. S. & MONCADA, S. (1988). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochemical and Biophysical Communications* **153**, 1251–1256.

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. *Proceedings of the Australian Physiological and Pharmacological Society* **21**, 122P.

POPESCU, L. M., PANOIU, C., HINESCU, M. & NUTU, O. (1985). The mechanism of cGMP-induced relaxation in vascular smooth muscle. *European Journal of Pharmacology* **107**, 393–394.

SPEDEN, R. N. (1964). Electrical activity of single smooth muscle cells of the mesenteric artery produced by splanchnic nerve stimulation in the guinea-pig. *Nature* **202**, 193–194.

STEEDMAN, W. M. (1966). Microelectrode studies on mammalian vascular smooth muscle cells. *Journal of Physiology* **186**, 382–400.

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

TARE, M., PARKINGTON, H. C., COLEMAN, H. A., NEILD, T. O. & DUSTING, G. J. (1990*b*). Actions of endothelium-derived nitric oxide include hyperpolarization of vascular smooth muscle. In *Nitric Oxide from L-Arginine: A Bioregulatory System*, ed. MONCADA, S. & HIGGS, E. A., pp. 73–80. Elsevier, Amsterdam.

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.

TAYLOR, S. G. & WESTON, A. H. (1988). Endothelium-derived hyperpolarising factor: a new endogenous inhibitor from the vascular endothelium. *Trends in Pharmacological Sciences* **9**, 272–274.