Role of membrane potential in endothelium-dependent relaxation of guinea-pig coronary arterial smooth muscle

Helena C. Parkington, M. A. Tonta, H. A. Coleman and M. Tare

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

1. Membrane potential and tension were measured simultaneously in ring segments of main coronary artery of guinea-pigs. The synthetic thromboxane A_2 analogue U46619 depolarized the tissues from -58 ± 2 to -40 ± 1 mV and increased tension by 12 ± 1 mN mm^{-1}. Nitric oxide (NO) and Iloprost, the stable analogue of prostacyclin, evoked hyperpolarization and relaxation.

2. The concentration of NO required to evoke half-maximal hyperpolarization (EC_{50} of 2×10^{-5} M) was 40-fold higher than that which was required to induce relaxation (EC_{50} of 5×10^{-7} M). The EC_{50} for Iloprost-induced hyperpolarization (3×10^{-8} M) was similar to that for relaxation (4×10^{-8} M).

3. Glibenclamide (10^{-6} M) abolished the hyperpolarization in response to both NO and Iloprost but was without effect on the amplitudes of the relaxations over the complete concentration–response curves.

4. Acetylcholine evoked concentration-dependent hyperpolarization and relaxation in the presence of N^{ω}-nitro-L-arginine methyl ester (NAME; 10^{-5} M) and indomethacin (10^{-6} M), and these responses were attributed to endothelium-derived hyperpolarizing factor (EDHF). The hyperpolarization produced by EDHF always preceded relaxation, and relaxation never occurred at concentrations of acetylcholine that were insufficient to evoke hyperpolarization.

5. The concentration–hyperpolarization and concentration–relaxation curves in response to acetylcholine were not affected by glibenclamide or barium (1–3 mM) but were shifted to the right 4- and 5-fold, respectively, by 1 mM tetraethylammonium. The hyperpolarization and relaxation evoked by acetylcholine were also reduced in a parallel manner when the potassium concentration in the superfusate was increased.

6. Hyperpolarizing current steps, applied to spiral strips of coronary artery denuded of endothelium and depolarized and constricted with U46619, caused relaxation. The relationship between hyperpolarization and relaxation evoked electrotonically was similar to that which was due to EDHF in intact tissues stimulated with acetylcholine.

7. It is concluded that the ability of NO or Iloprost to relax guinea-pig coronary artery does not depend upon hyperpolarization of the smooth muscle. In contrast, hyperpolarization is likely to play a major, if not the only, role in the relaxation in response to EDHF in this tissue.

Muscarinic agonists evoke hyperpolarization of the smooth muscle in intact segments of arteries (Kuriyama & Suzuki, 1978; Bolton, Lang & Takewaki, 1984; Komori & Suzuki, 1987) via endothelium-derived hyperpolarizing factor (EDHF) (Feletou & Vanhoutte, 1988; Chen, Suzuki & Weston, 1988; Taylor & Weston, 1988), endothelium-derived relaxing factor (EDRF) (Tare, Parkington, Coleman, Neild & Dusting, 1990*a*,*b*; Garland & McPherson, 1992; Parkington, Tare, Tonta & Coleman, 1993) and prostacyclin (Siegel, Stock, Schnalke & Litza, 1987; Parkington *et al.* 1993). The calcium that enters vascular smooth muscle through voltage-dependent calcium channels is involved in raised vascular tone (Mulvany, Nilsson & Flatman, 1982; Nelson, Standen, Brayden & Worley, 1988; Edwards & Weston, 1990; Nelson, Patlak, Worley & Standen, 1990*b*) and drugs that block these channels are highly effective in controlling hypertension (Janis, Silver & Triggle, 1987). Hyperpolarization of the membrane decreases the likelihood of these calcium channels being open and hence decreases calcium entry (Nelson *et al.* 1988, 1990*b*). Hyperpolarization has been invoked to explain the

relaxation induced by some hormones (Standen, Quayle, Davies, Brayden, Huang & Nelson, 1989; Nelson, Huang, Brayden, Hescheler & Standen, 1990*a*; Brayden, 1991) and drugs (Nakao, Okabe, Kitamura, Kuriyama & Weston, 1988; Taylor, Southerton, Weston & Baker, 1988; Leblanc, Wilde, Keef & Hume, 1989; Videbaek, Aalkjaer, Hughes & Mulvany, 1990) that cause hyperpolarization of vascular smooth muscle. However, the hyperpolarization induced by cromakalim and pinacidil, which open ATP-sensitive potassium channels, is abolished in the presence of solutions containing high concentrations of potassium, yet these drugs continue to cause relaxation of the contraction in response to high external potassium (Nakao *et al.* 1988; Erne & Hermsmeyer, 1991). This suggests that a component of the relaxation in response to these drugs is other than via hyperpolarization alone. The proportion of the endothelium-dependent relaxation that has been attributed to EDHF has varied widely between different vessels and amongst various groups. The techniques that have been used to study EDHF include: no measurement of membrane potential (Adeagbo & Triggle, 1993); measurement of membrane potential in the absence of, and relaxation in the presence of, spasmogen (Feletou & Vanhoutte, 1988; Chen & Suzuki, 1989); and simultaneous recording of membrane potential and tension (Garland & McPherson, 1992; Rand & Garland, 1992; Parkington *et al.* 1993). We set out to determine the role of hyperpolarization in the relaxation evoked by NO, prostacyclin and EDHF in the smooth muscle of guinea-pig coronary artery that was depolarized and constricted with the thromboxane A_2 analogue, U46619. Membrane potential and tension were measured simultaneously in order to facilitate a better understanding of the relationship between these two parameters. A brief description of some of these results has been presented (Parkington, Tonta, Tare & Coleman, 1992).

METHODS

Guinea-pigs of both sexes, but mostly males, were killed by decapitation and the heart removed. The left descending main coronary artery, from the coronary sinus to its entry into the muscle of the heart, was removed. Ring segments (1–2 mm in length) were prepared as described previously (Parkington *et al.* 1993). Briefly, each segment was mounted on a Mulvany–Halpern-style myograph for simultaneous recording of membrane potential and tension development in the smooth muscle. 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 and 5% CO_2 at 3 ml min^{-1} and maintained at 35 °C. The tissues were stretched until the tension was equivalent to a transmural pressure of 60 mmHg. In some experiments hyperpolarizing and depolarizing current steps were applied to spiral strips of main coronary artery. One end of each strip was introduced into a small suction electrode and secured, endothelium uppermost, to the silicone base of a recording chamber with 25 μm pins. The other end of the strip was attached to a force transducer (AE 801; SensoNor, Horten, Norway) that was mounted on a micrometer screw allowing stretch to be applied to the strips. The strips were superfused with physiological solution at 35 °C. The endothelium was removed as required with a small roughened wire. The membrane potential of the smooth muscle cells was recorded using conventional glass microelectrodes, filled with 1 M KCl, and having resistances of around 100 MΩ.

When the concentration of potassium in the superfusate was increased, sodium was omitted on a molar basis. N^{ω}-nitro-L-arginine methyl ester (NAME), indomethacin, tetraethylammonium (TEA) (Sigma Chemicals, USA), and the synthetic thromboxane A_2 analogue U46619 (Cayman Chemical Co., Ann Arbor, MI, USA) were added to the superfusate as required. Glibenclamide (a kind gift from Hoechst, Melbourne, Australia) was dissolved in dimethyl sulphoxide and a 1 : 10000 dilution made into the physiological solution of the superfusate. Stock solutions of nitric oxide (NO) were made by bubbling vials of 0·9% sterile NaCl with argon for approximately 1 h to displace oxygen. The appropriate volume of NO gas (Matheson Gas Products Inc., TX, USA) was then injected from a gas-tight syringe into the vial through the injection septum. Due to its lability, NO was applied to the tissues for 10 s by direct injection into the superfusion line, with a 1 s delay between the point of injection and the tissue. The concentration of NO reached a steady state within 3–4 s of injection (see Parkington *et al.* 1993). In order to facilitate comparison, acetylcholine (Sigma) and Iloprost, a stable analogue of prostacyclin (a generous gift from Schering, Berlin, Germany), were also applied for 10 s in most experiments. In some experiments acetylcholine was added to the superfusate for 1 min (see Results).

The mean ± S.E.M., based on the number of animals (n) studied, is quoted. Student's t test was used to test for significance between groups. A significance level of $P < 0·05$ was used throughout.

RESULTS

Hyperpolarization and relaxation evoked by NO and Iloprost

Ring segments of coronary artery were denuded of endothelium to prevent the release of endogenous vasodilators. The resting membrane potential in these preparations was -58 ± 2 mV ($n = 48$). U46619 (10^{-8}–10^{-7} M) depolarized the smooth muscle to -40 ± 1 mV ($n = 48$) and increased tension by 12 ± 1 mN mm^{-1}. The membrane potential was not stable in the presence of U46619 and small (less than 2 mV) fluctuations occurred continuously. In about 30% of tissues, spike action potentials occurred on the depolarization evoked by U46619. In these cases, oscillations in membrane potential were more pronounced and the action potentials usually occurred in bursts of one to five, with troughs of repolarization between each burst (see Fig. 4 for example).

In denuded and depolarized tissues, exogenous NO evoked concentration-dependent hyperpolarization and relaxation (Fig. 1*A*). It is clear from Fig. 1*A* that the

lowest concentrations of NO caused relaxation in the absence of hyperpolarization. As the concentration of NO was increased, hyperpolarization emerged. Relaxation preceded hyperpolarization in many tissues. Upon cessation of the hyperpolarization, the return of the membrane potential to the depolarized level was often sufficiently brisk to initiate action potentials (Fig. 1*A*, last panel). The EC_{50} for NO-induced relaxation was 5×10^{-7} M (the pD_2 (−log EC_{50}) was $6{\cdot}32 \pm 0{\cdot}01$, $n = 5$) and was 40 times higher for the hyperpolarization (2×10^{-5} M; pD_2, $4{\cdot}70 \pm 0{\cdot}04$, $n = 5$) measured simultaneously (Fig. 1*B*).

We have previously confirmed that the ability of the stable analogue of prostacyclin, Iloprost, to hyperpolarize guinea-pig coronary artery at rest is indistinguishable from that of pure prostacyclin (Parkington *et al.* 1993). In the present study, Iloprost evoked concentration-dependent hyperpolarization and relaxation in denuded and depolarized tissues (Fig. 2*A*). In contrast to NO, the concentrations of Iloprost at which the onset of hyperpolarization and relaxation occurred were not easily separated. Furthermore, the EC_{50} values for the hyperpolarization (3×10^{-8} M; pD_2, $7{\cdot}53 \pm 0{\cdot}08$; $n = 7$) and relaxation (4×10^{-8} M; pD_2, $7{\cdot}41 \pm 0{\cdot}03$) in response to Iloprost were not different (Fig. 2*B*). However, as with NO, the onset of the relaxation often preceded that of the hyperpolarization.

The amplitudes of the largest hyperpolarizations evoked by NO ($10{\cdot}1 \pm 1{\cdot}0$ mV; $n = 5$) and Iloprost ($12{\cdot}9 \pm 1{\cdot}6$ mV; $n = 7$) were not significantly different. However, the durations of the largest responses to Iloprost exceeded those evoked by NO (Figs 1*A* and 2*A*).

Effects of glibenclamide on the responses to NO and Iloprost

The hyperpolarization induced by some vasodilators is blocked by the oral hypoglycaemic agent glibenclamide (Beech & Bolton, 1989; Standen *et al.* 1989; Nelson *et al.* 1990*a*; Brayden, 1991). In fourteen coronary arteries that had been denuded of endothelium and depolarized and constricted with U46619, glibenclamide (10^{-6} M) abolished the hyperpolarization evoked by NO (10^{-5} M) and Iloprost (10^{-6} M) with no effect on the amplitudes of the relaxations, although it can be appreciated that the durations of the relaxations were reduced in the absence of hyperpolarization (Fig. 3*A*). The effect of glibenclamide on the amplitude of the relaxation was tested over the full concentration–relaxation curves for NO ($n = 4$) and Iloprost ($n = 6$). Glibenclamide did not alter the EC_{50} values (Fig. 3*B*).

Hyperpolarization and relaxation evoked by acetylcholine

In the presence of U46619, stimulation of the endothelium of intact preparations with acetylcholine evoked hyper-

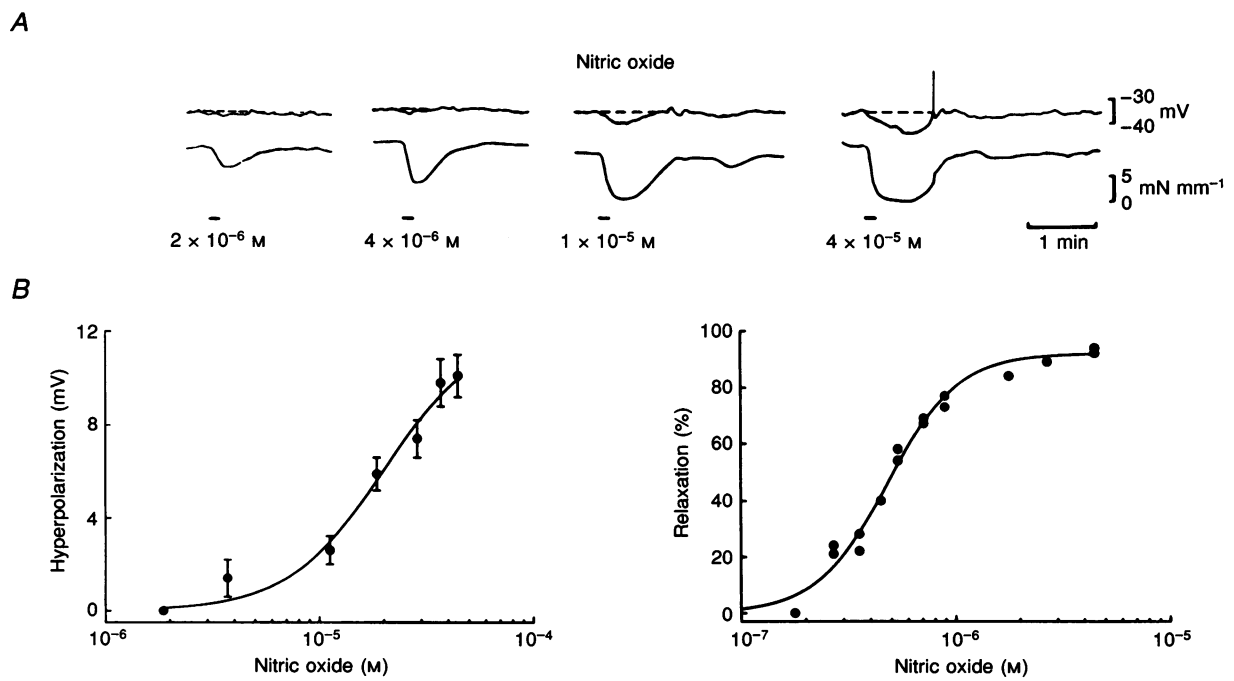

Figure 1. Hyperpolarization and relaxation evoked by NO

A, NO evoked concentration-dependent hyperpolarization and relaxation in segments of guinea-pig coronary artery denuded of endothelium and depolarized and constricted with U46619 (10^{-8} M). Relaxation appeared at lower concentrations of NO, and hyperpolarization emerged as the concentration of NO was increased. The dashed line indicates the mean value of the membrane potential in U46619. A continuous impalement. *B*, the pD_2 for hyperpolarization (left) was $4{\cdot}70 \pm 0{\cdot}04$ and for relaxation (right) it was $6{\cdot}32 \pm 0{\cdot}01$ ($n = 5$).

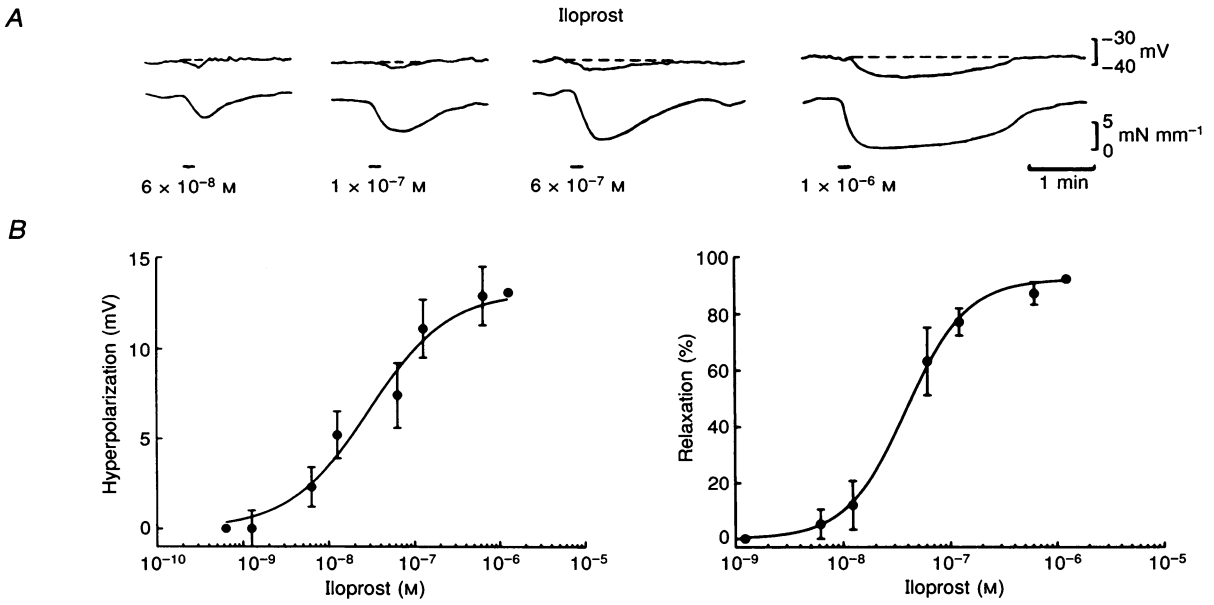

Figure 2. Hyperpolarization and relaxation evoked by Iloprost

A, Iloprost evoked concentration-dependent hyperpolarization and relaxation in segments of guinea-pig coronary artery denuded of endothelium and depolarized and constricted with U46619 (10^{-8} M). The dashed line indicates the mean value of the membrane potential in U46619. A continuous impalement. *B*, the pD_2 for hyperpolarization (left) was 7·53 ± 0·08 and for relaxation (right) it was 7·41 ± 0·03 ($n = 7$).

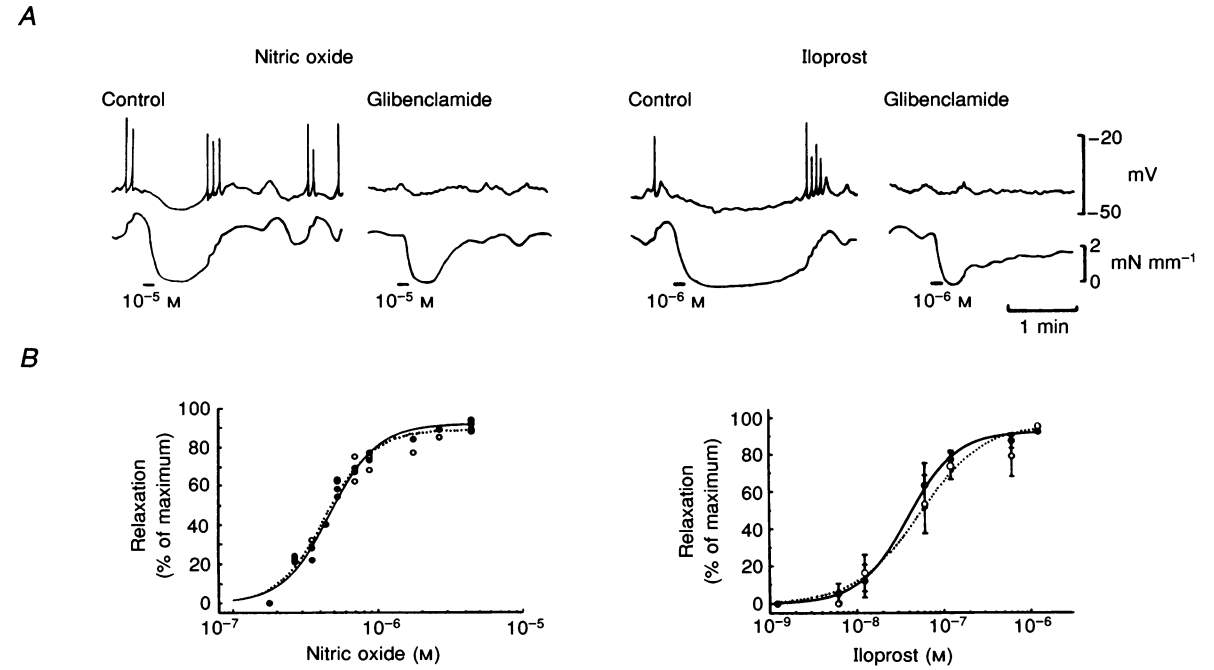

Figure 3. The influence of glibenclamide on the responses to NO and Iloprost

A, the hyperpolarizations evoked by both NO and Iloprost were abolished by glibenclamide (10^{-6} M). Glibenclamide had no effect on the amplitudes of the relaxations evoked by NO or Iloprost, although their durations were reduced. A continuous impalement in segments denuded of endothelium and in the presence of U46619 (2×10^{-8} M). *B*, the EC_{50} values for the relaxations evoked by NO (left; $n = 4$) or by Iloprost (right; $n = 6$) were not affected by glibenclamide. ●, control; ○, glibenclamide.

polarization that consisted of a transient component, followed by a more prolonged component. This membrane potential response was accompanied by a prolonged relaxation (Fig. 4*A*). In a previous study we demonstrated in coronary arteries at rest, that is, in the absence of constrictor, that the prolonged component of the hyperpolarization in response to acetylcholine resulted from the combined release of NO and a prostaglandin, most probably prostacyclin, from the endothelium (Parkington *et al.* 1993). In the present study the prolonged components of both the hyperpolarization and relaxation of twelve arteries depolarized and constricted with U46619 were abolished in the presence of NAME (2×10^{-5} M), which suppresses the production of NO, and indomethacin (5×10^{-7} or 10^{-6} M), which blocks cyclo-oxygenase and hence suppresses the production of prostaglandins (Fig. 4*B*). The hyperpolarization evoked by acetylcholine in the presence of NAME and indomethacin was brief, as was the corresponding relaxation.

In the presence of U46619, NAME and indomethacin, glibenclamide had no effect on either the hyperpolarization or relaxation evoked by acetylcholine (Fig. 4*C*) in four of these tissues. Barium (1–3 mM), which is also thought to block ATP-sensitive potassium channels (Standen *et al.* 1989), was without effect on the hyperpolarization or relaxation elicited by acetylcholine in five tissues (data not shown).

The onset of the hyperpolarization induced by acetylcholine always occurred before the relaxation and, as can be appreciated from the first panel of Fig. 5*A*, low concentrations of acetylcholine that did not evoke hyperpolarization also failed to elicit relaxation. The EC_{50} for the relaxation evoked by acetylcholine was 7×10^{-8} M (pD_2, 7·15 ± 0·07; $n = 7$), and this was significantly lower than the EC_{50} for the hyperpolarization, $1{\cdot}7 \times 10^{-7}$ M (pD_2, 6·77 ± 0·06; $n = 7$), recorded simultaneously (Fig. 5*B*).

Since the hyperpolarization was likely to reduce the probability of opening of voltage-dependent calcium channels it was of interest to determine the voltage dependence of the relaxation. In tissues from eleven animals, the tension at the peak of the relaxation was plotted against the membrane potential at the peak of the hyperpolarization for various concentrations of acetylcholine (Fig. 5*C*). The exponential of best fit indicates that tension increased e-fold per 11·8 mV depolarization, between −70 and −40 mV.

Effects of tetraethylammonium

Intact tissues from eight animals were continuously superfused with solution containing U46619, NAME and indomethacin. The EC_{50} for the hyperpolarization was shifted 4-fold to the right, and the EC_{50} for the relaxation, measured simultaneously, was shifted 5-fold to the right

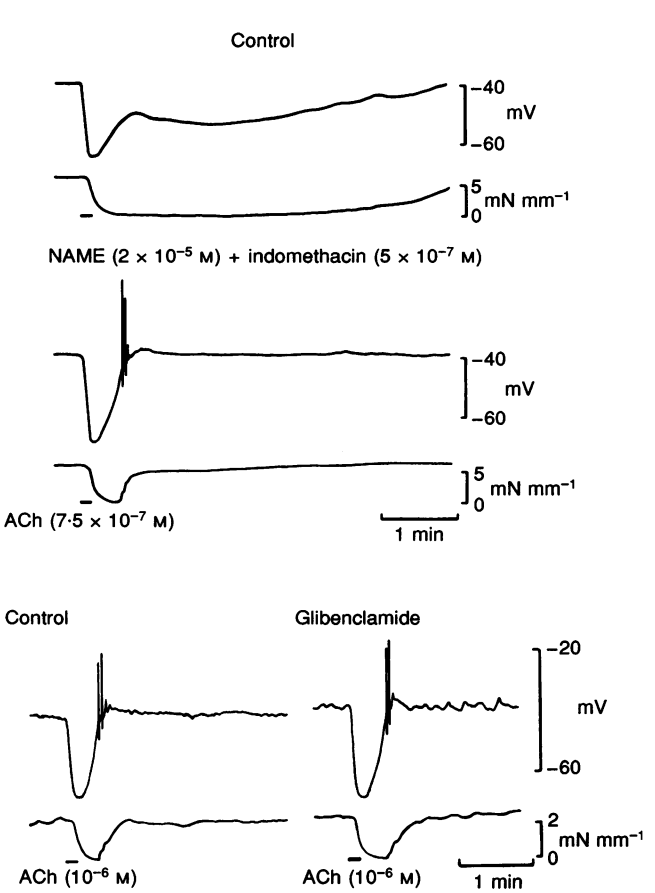

Figure 4. Hyperpolarization and relaxation resistant to NAME, indomethacin and glibenclamide

A, in segments of coronary artery depolarized and constricted with U46619 (2×10^{-8} M), acetylcholine (ACh, $7{\cdot}5 \times 10^{-7}$ M) evoked hyperpolarization and relaxation, both of which were prolonged. *B*, a combination of NAME (2×10^{-5} M) and indomethacin (5×10^{-7} M) abolished the slow component of the hyperpolarization and markedly reduced the duration of the relaxation. *C*, the brief responses that persisted in the presence of NAME and indomethacin were not blocked by 10^{-6} M glibenclamide.

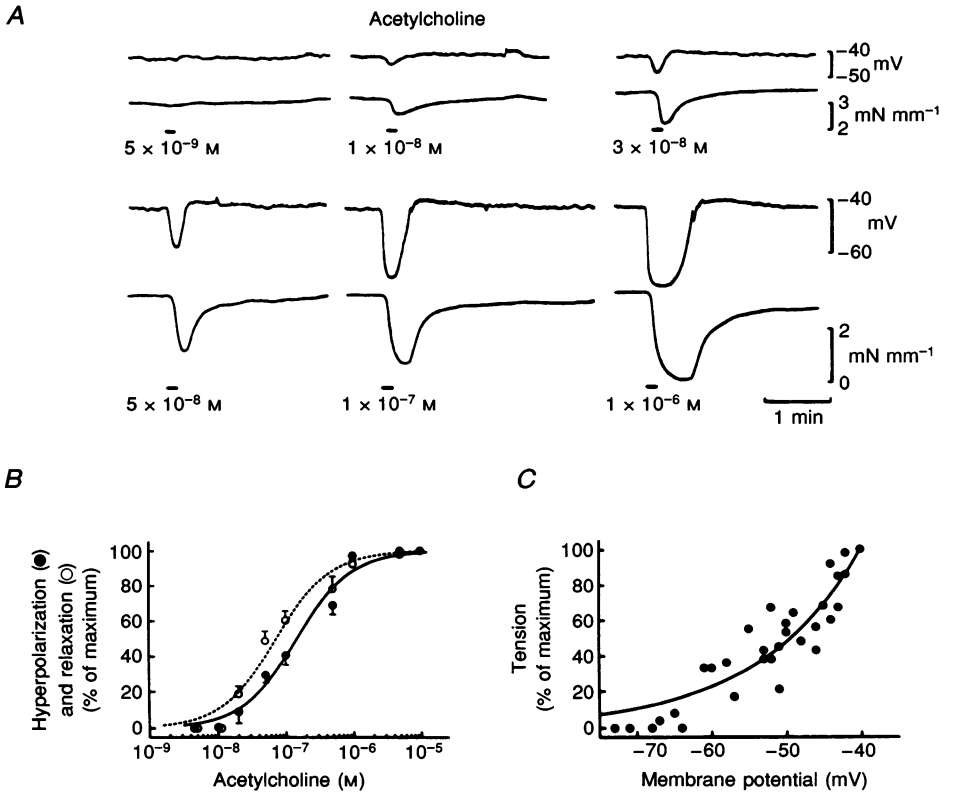

Figure 5. Membrane potential dependence of EDHF-induced relaxation

A, the brief hyperpolarization and relaxation evoked by acetylcholine in intact segments, in the presence of U46619 (2×10^{-8} M), NAME and indomethacin, increased in amplitude as the concentration of acetylcholine was increased. *B*, the pD_2 for hyperpolarization (●) was 6·77 ± 0·06 and for relaxation (○) it was 7·15 ± 0·07 ($n = 7$). Although these values were significantly different, relaxation never occurred in the absence of hyperpolarization. Furthermore, hyperpolarization always preceded relaxation. *C*, in tissues from 11 animals, the level of tension at the peak of the relaxation was expressed as a percentage of the maximal contraction evoked by U46619 and was plotted against the value of the membrane potential at the peak of the repolarization.

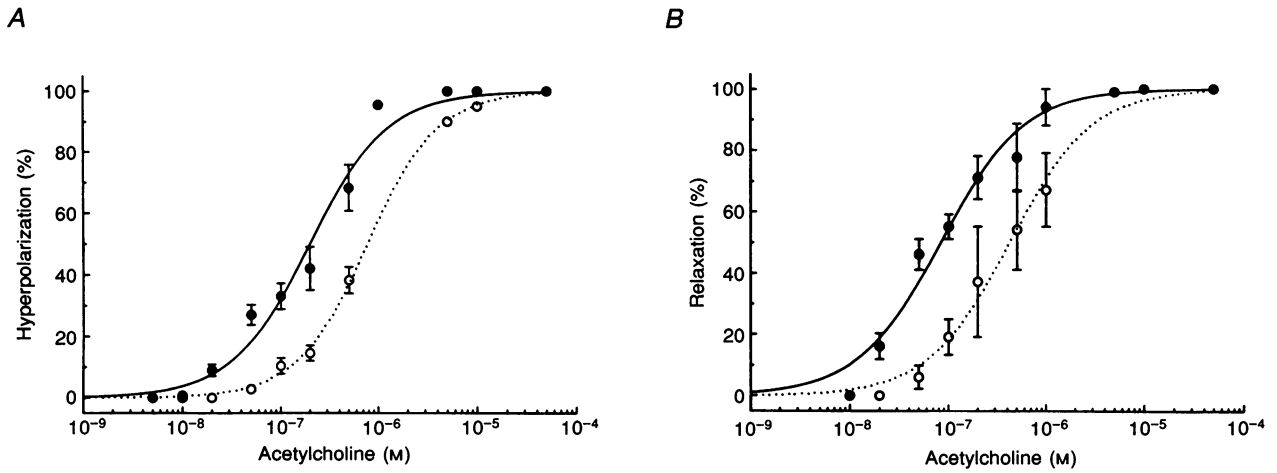

Figure 6. Effects of TEA on the hyperpolarization and relaxation induced by the release of EDHF

In tissues from 8 animals, the pD_2 for hyperpolarization (*A*) was reduced from 6·70 ± 0·05 in the control to 6·11 ± 0·02 by 1 mM TEA. The pD_2 for relaxation (*B*) was reduced from 7·08 ± 0·05 in the control to 6·37 ± 0·04 in 1 mM TEA. U46619 (2×10^{-8} M), NAME (2×10^{-5} M) and indomethacin (10^{-6} M) were present throughout. ●, control; ○, 1 mM TEA.

by 1 mM TEA (Fig. 6). The effects of TEA on the EC_{50} values for hyperpolarization (difference between pD_2 values, 0·59 ± 0·05) and relaxation (difference between pD_2 values, 0·71 ± 0·06) were not significantly different. Again, relaxation was not observed in response to concentrations of acetylcholine in which hyperpolarization was blocked by TEA. Acetylcholine (3×10^{-8} to 3×10^{-7} M) was applied to tissues from four animals for 1 min. TEA (1 mM) shifted the curves for hyperpolarization and relaxation to the right (Fig. 7).

At concentrations of 1 mM, TEA did not affect the hyperpolarizations or relaxations evoked by exogenous NO or Iloprost ($n = 6$). However, the hyperpolarizations in response to these agents were reduced and finally abolished when the concentration of TEA was increased to 5 and 10 mM (data not shown).

Increasing external potassium

Since the hyperpolarization evoked by acetylcholine is likely to result from an increase in potassium conductance, the effects of reducing the potassium gradient on the responses to acetylcholine were investigated. Increasing external potassium from the control concentration of 6 mM to 15, 25, 50 and 100 mM resulted in depolarizations of 7·7 ± 1·3, 17·0 ± 1·0, 30·5 ± 0·6 and 40·0 ± 1·2 mV ($n = 4$), respectively. In the presence of NAME and indomethacin, and in these concentrations of external potassium, the amplitudes of the hyperpolarizations in response to acetylcholine decreased progressively (Fig. 8). As the amplitude of the hyperpolarization decreased so did the amplitude of the relaxation, and relaxation did not occur in the absence of hyperpolarization (Fig. 8). U46619 was also included in the superfusate as external potassium was increased in two additional preparations and the observations were similar to those in which potassium was raised in the absence of U46619.

Ability of hyperpolarization alone to cause relaxation

The ability of hyperpolarization alone to directly relax vascular smooth muscle depolarized and constricted with agonist has not been demonstrated previously. Spiral strips of main coronary artery from seven guinea-pigs were denuded of endothelium, to prevent the release of endogenous vasorelaxants. One end of each was introduced into a suction electrode (see Methods) with approximately 4 mm of tissue protruding into the recording chamber, and cells were impaled in the centre of the strip. The strip was then depolarized and constricted with U46619. Hyperpolarizing current steps, applied via a suction electrode placed at one end of the strips, relaxed the smooth muscle (Fig. 9*A*). In three additional spiral preparations, the endothelium was retained intact and NAME and indomethacin were included in the superfusate. Stimulation of the endothelium with

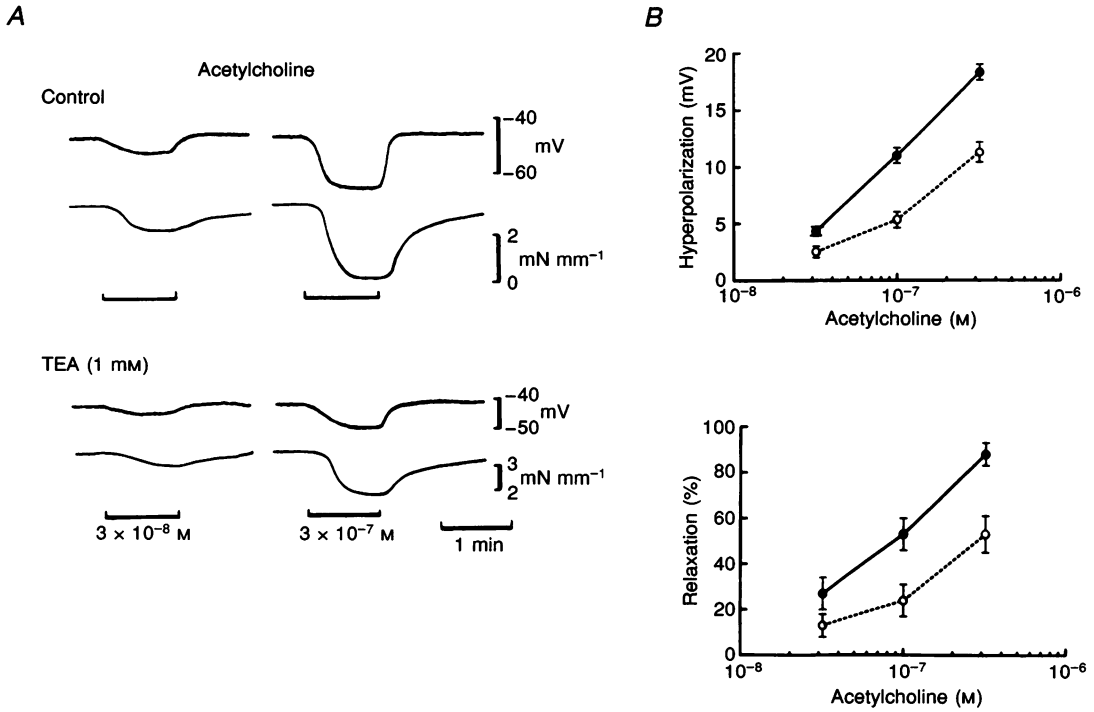

Figure 7. Effects of TEA on the responses evoked by 1 min application of ACh

A, TEA (1 mM) reduced the amplitude of the hyperpolarization and relaxation evoked by 1 min application of acetylcholine. *B*, in tissues from 4 animals, the hyperpolarization (top) and relaxation (bottom) evoked by acetylcholine were shifted to the right by 1 mM TEA . U46619 (4×10^{-8} M), NAME (2×10^{-5} M) and indomethacin (10^{-6} M) were present throughout. ●, control; ○, 1 mM TEA.

acetylcholine evoked concentration-dependent hyperpolarization and relaxation. The relationship between hyperpolarization and relaxation evoked by acetylcholine was remarkably similar to the relationship between these parameters in response to electrotonic hyperpolarization in the same tissues (Fig. 9*B*).

DISCUSSION

The results presented here demonstrate that the hyperpolarizations evoked by NO or the prostacyclin analogue, Iloprost, in guinea-pig coronary artery depolarized and constricted with U46619, are not essential for relaxation in this artery. Essentially: (1) relaxation occurred at concentrations of NO that were too low to evoke hyperpolarization; (2) relaxation often preceded hyperpolarization; (3) the relaxation was often more prolonged than the hyperpolarization, especially in response to NO; and (4) when hyperpolarization was abolished by glibenclamide, relaxation persisted, although its duration was reduced. The preparations used to study the effects of exogenous NO and Iloprost were free of endothelium and this circumvents the possibility of indirect actions via the release of vasoactive agents from the endothelium. The tissues were capable of relaxing in response to concentrations of NO that were too low to elicit a detectable change in membrane potential. A similar effect has been reported for other arteries (Tare *et al.* 1990*b*; Rand & Garland, 1992). The cyclic nucleotides that are induced in smooth muscle by NO and Iloprost are capable of increasing calcium uptake into the endoplasmic reticulum (Twort & van Breemen, 1988), decreasing the sensitivity of the contractile apparatus to calcium (Rapoport, Draznin & Murad, 1983; Nishimura & van Breemen, 1989) and stimulating calcium extrusion (Suematsu, Hirata & Kuriyama, 1984; Vrolix, Raeymaekers, Wuytack, Hofmann & Casteels, 1988). All of these mechanisms could contribute to relaxation that was not dependent upon hyperpolarization of the membrane. The hyperpolarizations evoked by exogenous NO and Iloprost were blocked by the sulphonylurea, glibenclamide, thus providing the definitive test of the role of membrane potential in relaxation evoked by these two agents. Glibenclamide had no effect on the

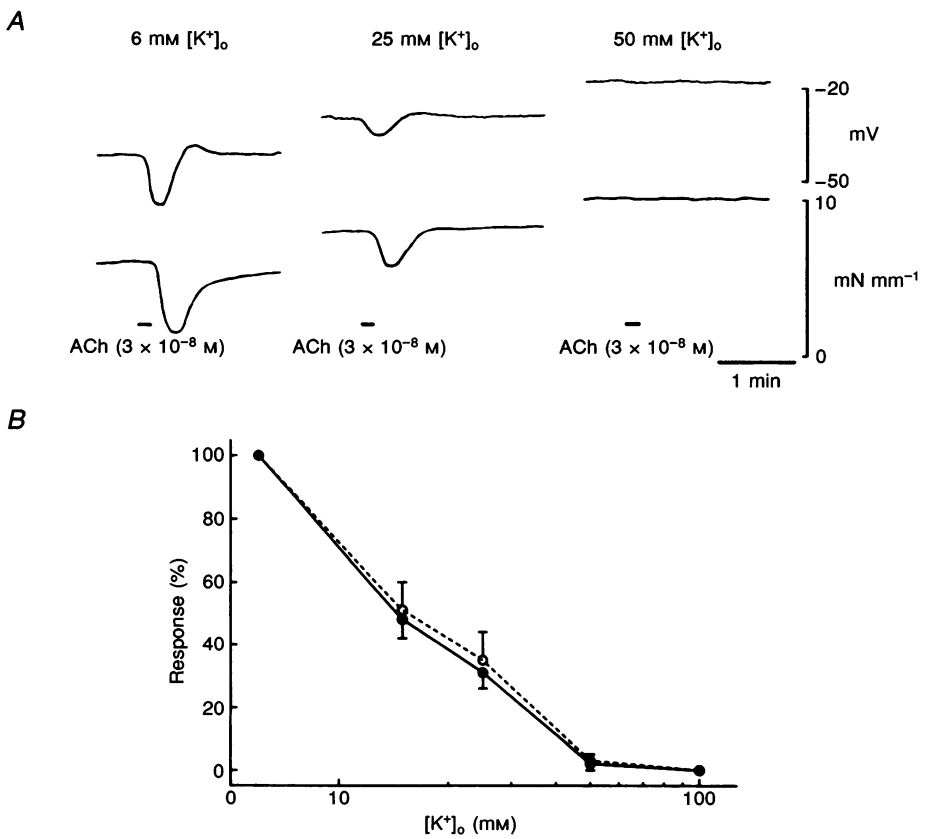

Figure 8. Effects of increasing concentrations of extracellular potassium

A, the responses to acetylcholine were reduced when the concentration of potassium in the bathing solution was increased from 6 mM (control) to 25 and 50 mM. *B*, the hyperpolarization and relaxation obtained in 15, 25, 50 and 100 mM potassium in 4 tissues were expressed as a percentage of the response in 6 mM potassium and were plotted against the external potassium concentration. ●, hyperpolarization; ○, relaxation.

amplitudes of the relaxations. Nevertheless, in abolishing the hyperpolarization, glibenclamide markedly reduced the duration of the relaxation, especially in response to Iloprost. In rabbit cerebral arteries, glibenclamide blocked the hyperpolarizations and also reduced the amplitudes of the relaxations evoked by both vasoactive intestinal polypeptide, which stimulates adenylate cyclase, and acetylcholine (Standen *et al.* 1989). The hyperpolarizations evoked by ADP in mesenteric arteries and in the resistance arteries supplying skeletal muscle of rabbits were also abolished by glibenclamide and the relaxations reduced (Brayden, 1991). In rabbit mesenteric artery glibenclamide caused complete abolition of the hyperpolarization in response to calcitonin gene-related peptide, while reducing the relaxation by about 50% (Nelson *et al.* 1990*a*). This indicates that, in a variety of arteries, the hyperpolarization that is sensitive to glibenclamide contributes to either the amplitude and/or the duration of the relaxation.

The characteristics of the component of hyperpolarization and relaxation evoked by acetylcholine that was resistant to NAME and indomethacin in the coronary artery contrast with the responses evoked by exogenous NO and Iloprost. The response to acetylcholine was sensitive to the concentration of extracellular potassium and was completely resistant to glibenclamide or barium; this is consistent with the observations of others which led them to conclude that these responses were likely to be due to EDHF (Chen *et al.* 1988; Feletou & Vanhoutte, 1988; Taylor & Weston, 1988). The hyperpolarization evoked by acetylcholine in guinea-pig coronary artery was resistant to a variety of other potassium channel blockers, namely apamin, quinine, 4-aminopyridine and caesium (authors' unpublished observations). The temporal and quantitative relationships between the hyperpolarization and relaxation might prompt the interpretation that the former caused the latter: decreasing the amplitude of the hyperpolarization, by TEA or high extracellular potassium, was associated with a concomitant decrease in the ability of acetylcholine to relax the tissue. The hyperpolarization induced by potassium channel openers such as cromakalim and pinacidil is abolished in high potassium solution (>40 mM), as expected. However, these drugs continue to relax vascular smooth muscle contracted by high potassium (Nakao *et al.* 1988; Erne & Hermsmeyer, 1991) indicating that a component of the relaxation in response to these drugs is other than via hyperpolarization alone. In contrast, the hyperpolarization evoked in guinea-pig coronary artery by EDHF was

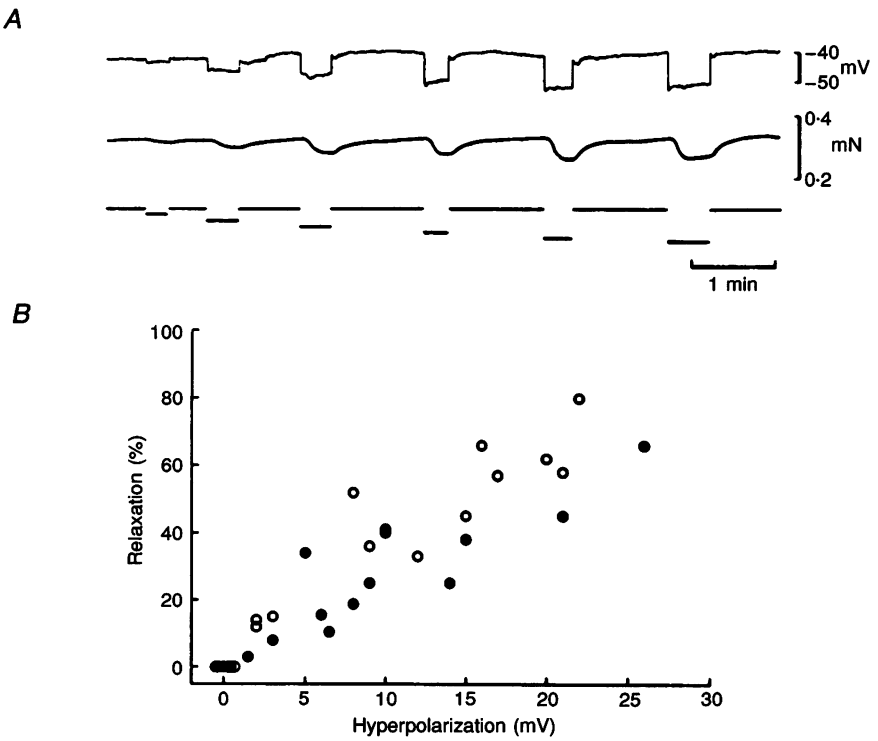

Figure 9. Effects of hyperpolarizing electrotonic potentials on tension

A, in spiral strips of coronary artery denuded of endothelium, hyperpolarizing current steps evoked relaxations that increased in amplitude as the amplitude of the hyperpolarization increased. *B*, in three tissues with intact endothelia, and in the presence of NAME and indomethacin, the relationship between the hyperpolarization and relaxation evoked electrotonically (●) was remarkably similar to their relationship when evoked by acetylcholine (○). U46619 (2×10^{-8} M) was present throughout.

abolished by high potassium and relaxation no longer occurred. Furthermore, relaxation by EDHF correlated well with both the amplitude and duration of the hyperpolarization. In caution, an effect of manoeuvres such as increasing extracellular potassium, and drugs that block potassium channels, on the production of EDHF cannot be ruled out with certainty and provides an alternative interpretation of our observations. The available evidence suggests that EDHF production requires an increase in cytoplasmic calcium which results both from release of internal stores and influx (Chen & Suzuki, 1990). The involvement of voltage-operated calcium channels in calcium influx into endothelial cells is unresolved and sustained calcium influx may be predominantly via poorly selective cation channels (Lückhoff & Busse, 1990; Nilius, Schwartz, Oike & Droogmans, 1993). The actions of TEA on the endothelial cells (Chen & Cheung, 1992; Marchenko & Sage, 1993) and increasing extracellular potassium are likely to result in a decrease in the sustained phase of the production of EDHF. However, the brief stimulation of the endothelium with our 10 s application of acetylcholine might be expected to depend predominantly on calcium release rather than influx and hence be resistant to inhibition of EDHF production by TEA. Thus, the effectiveness of TEA in decreasing the hyperpolarization evoked by acetylcholine may reflect an action of TEA on potassium channels in the smooth muscle. Previous observations have suggested that endothelium-dependent hyperpolarization evoked by acetylcholine is likely to result from an increase in potassium conductance in vascular smooth muscle cells (Kuriyama & Suzuki, 1978; Bolton *et al.* 1984; Taylor *et al.* 1988; Chen & Suzuki, 1989; Kauser, Stekiel, Rubanyi & Harder, 1989) and a sensitivity of the response to TEA has been reported (Chen, Yamamoto, Miwa & Suzuki, 1991). Nonetheless, a final resolution of events awaits chemical identification of EDHF and study of the responses evoked by its exogenous application.

In view of the close correlation between EDHF-induced hyperpolarization and relaxation, the voltage dependence of the tension was estimated in the present study from the responses evoked by acetylcholine in the presence of NAME and indomethacin. Tension increased e-fold per 11·8 mV depolarization between −70 and −40 mV. This rate of change is somewhat less than the e-fold increase in tension per 7–9 mV obtained for the voltage dependence of calcium channels in isolated vascular smooth muscle cells (Nelson *et al.* 1990*b*). This difference may well reflect the dependence of the contractile apparatus on cytoplasmic calcium, as well as the myriad, and poorly understood, processes involved in the regulation of cytoplasmic calcium, including pumps and exchange mechanisms. The possibility that the relaxation produced by membrane hyperpolarization may be a consequence of inhibition of the production of second messengers or inhibition of receptor activation of ion channels cannot be ruled out (Itoh, Seki, Suzuki, Ito, Kajikuri & Kuriyama, 1992). However, the voltage dependence of receptor-activated signal transduction is likely to be weaker than that of voltage-dependent calcium channels (see Nilius *et al.* 1993).

Suppression of spontaneous tension development, that is, in the absence of agonist, by hyperpolarization has been described for monkey coronary artery (Mekata, 1986). However, the ability of hyperpolarization to relax vascular smooth muscle depolarized and constricted with agonist could not be assumed since calcium availability other than by entry through voltage-operated calcium channels is likely to be involved in the contraction. The relaxations obtained in response to electrotonic stimulation were consistently smaller than those obtained in response to acetylcholine. This is most likely to be explained by the fact that, while EDHF hyperpolarized all of the smooth muscle cells within the preparation to a similar extent, the amplitude of the electrotonic hyperpolarization was not so homogeneously distributed, being maximal in the vicinity of the stimulating electrode and declining exponentially with distance along the preparation. However, our results demonstrate that hyperpolarization can cause significant relaxation of guinea-pig coronary artery in the presence of U46619.

In conclusion, it is clear that coincident hyperpolarization and relaxation in vascular smooth muscle cannot automatically be interpreted in terms of cause and effect. This is particularly true in the case of EDRF/NO and prostacyclin in the guinea-pig coronary artery, both of which can cause significant relaxation after the hyperpolarization they normally evoke has been blocked. There was a close correlation between EDHF-induced hyperpolarization and relaxation but causality, while attractive, should be viewed with caution at present. However, this study demonstrates directly that hyperpolarization *per se* is capable of causing relaxation of coronary artery that is depolarized and contracted by U46619.

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