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ELECTRICAL PROPERTIES OF RESTING AND ACETYLCHOLINE-STIMULATED ENDOTHELIUM IN INTACT RAT AORTA

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SUMMARY

1. The passive electrical properties and the effects of acetylcholine on the membrane potential of the endothelium of intact rat aorta were investigated using the whole cell mode of the patch clamp technique.

2. Unstimulated endothelium had a membrane potential of -58 ± 8 mV (S.E.M., $n = 193$; range -47 to -76 mV). The input resistance was 43 ± 13 MΩ (S.E.M., $n = 8$; range 26–64 MΩ). KCl and $BaCl_2$, but not tetraethylammonium (2 mM), 4-aminopyridine (5 mM) or 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; 100 μM) depolarized the endothelium.

3. Acetylcholine (0·2–4 μM) evoked in most preparations a biphasic response with a transient hyperpolarization to a value close to the K^+ reversal potential, followed by depolarization beyond the resting potential. In 46% of recordings, the depolarization was followed by oscillations in membrane potential. The duration of the hyperpolarization and magnitude of the depolarization was similar in all recordings from a given aorta, but varied greatly between different preparations.

4. Hyperpolarization of the endothelium below the K^+ reversal potential reversed the direction of the first phase of the acetylcholine-evoked response, which was unaffected by tetraethylammonium, 4-aminopyridine, or DIDS.

5. The removal of extracellular Ca^{2+} evoked a depolarization of the endothelium from -61 ± 3 to -34 ± 3 mV (S.E.M., $n = 9$) over 2–15 min. Restoration of external Ca^{2+} evoked a transient hyperpolarization.

6. ACh applied in nominally Ca^{2+}-free medium shortly after Ca^{2+} removal evoked only a transient hyperpolarization. After the establishment of a stable membrane potential in Ca^{2+}-free medium, acetylcholine was without effect.

7. $NiCl_2$ (2 mM) evoked a small depolarization of the endothelium (6 ± 2 mV; S.E.M., $n = 7$). The subsequent removal of Ni^{2+} evoked a transient hyperpolarization.

8. In the presence of Ni^{2+}, acetylcholine evoked a short-lived hyperpolarization. Both the application of Ni^{2+} and the removal of extracellular Ca^{2+} immediately blocked oscillations in membrane potential evoked by acetylcholine.

9. The blockers of voltage-operated Ca^{2+} channels, nifedipine (1–10 μM) and verapamil (20 μM) were without effect on the biphasic acetylcholine-evoked responses.

10. In preparations in which acetylcholine evoked large (20–45 mV) oscillations in

membrane potential, depolarization of the endothelium alone, by current injection or application of KCl, did not evoke oscillations.

11. The activator of protein kinase C, phorbol 12, 13-dibutyrate (200 nM) depolarized and greatly increased the input resistance of the endothelium, presumably due to an effect on gap junctions. The blockers of protein kinase C, staurosporine (200 nM) and H-7 (200 nM), were without effect on acetylcholine-evoked responses.

12. These results suggest that acetylcholine evokes complex changes in endothelial membrane potential following changes in cytosolic calcium concentration. The initial agonist-evoked hyperpolarization appeared dependent on the release of Ca^{2+} from intracellular stores, whilst the prolonged phase of the hyperpolarization, depolarization and oscillations in membrane potential are dependent upon the entry of Ca^{2+} through Ni^{2+}-sensitive channels. Protein kinase C does not appear to be involved in any phase of the acetylcholine-evoked response.

INTRODUCTION

Endothelium is composed of a monolayer of thin, flat cells which line the internal surfaces of the blood vessels and heart. Under normal conditions, endothelial cells are the only cell type which contact the blood. Following the demonstration that the acetylcholine-evoked relaxation of blood vessels is dependent on the presence of endothelium (Furchgott & Zawadzki, 1980), much work has indicated the important role played by endothelium in the control of vascular vessel tone and in the regulation of the activity of platelets and possibly other blood cells. Many of the functions of endothelium are mediated by the release of physiologically active substances such as prostacyclin, endothelium-derived relaxing factor (EDRF) and endothelin (for reviews, see Ånggård, Botting & Vane, 1990; Moncada, Palmer & Higgs, 1990).

Stimulation with muscarinic agonists evokes the release of EDRF from endothelial cells (Furchgott & Zawadzki, 1980). Muscarinic agonists have also been shown to evoke increases in cytosolic calcium concentration ($[Ca^{2+}]_i$) in freshly isolated endothelial cells (Busse, Fichtner, Lückhoff & Kohlhardt, 1988; Lückhoff & Busse, 1990). Recent investigations have shown that freshly isolated bovine aortic endothelial cells express M1, M2 and M3 muscarinic receptor subtypes (Tracey & Peach, 1992). The signal transduction pathways activated by muscarinic agonists in endothelium have not been elucidated, but in many cells types, stimulation of M1 and M3 receptors leads to the activation of phospholipase C and the production of inositol 1,4,5-trisphosphate ($InsP_3$; Hulme, Birdsall & Buckley, 1990). $InsP_3$ evokes the release of Ca^{2+} from intracellular stores. In many cell types, the depletion of the intracellular stores in turn activates the entry of Ca^{2+} from the extracellular space (Putney, 1990). Store-regulated Ca^{2+} entry has been demonstrated in endothelial cells (Hallam, Jacob & Merritt, 1989; Jacob, 1990). The elevation of $[Ca^{2+}]_i$ is a major feature of endothelial signal transduction, stimulating the release of prostacyclin and EDRF (see Jacob, Sage & Rink, 1990).

It has been shown that membrane potential affects $[Ca^{2+}]_i$ (Cannell & Sage, 1989) and EDRF release (Lückhoff & Busse, 1990) in endothelial cells, indicating a possible role for membrane potential in the function of endothelium. To date, the

electrophysiology of endothelium has been investigated mainly in cells dissociated from vessels and grown in culture (Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Bregestovski, Bakhramov, Danilov, Moldobaeva & Takeda, 1988; Daut, Merke, Nees & Newman, 1988; Olesen, Davies & Clapham, 1988; Sauve, Parent, Simoneau & Roy, 1988; Cannell & Sage, 1989; Mehrke & Daut, 1990; Merke, Pohl & Daut, 1991). However, changes which occur in cultured cells make interpretation of some results difficult and in any case, the situation is rather unphysiological since the endothelial cells have no contacts with other cells of the vessel wall. Data concerning the electrophysiological effects of endothelium-dependent vasodilators are contradictory, which may at least in part reflect different culture procedures and conditions. A particular difficulty is that acetylcholine (ACh) receptors are rapidly lost by endothelial cells in culture (Tracey & Peach, 1992) and they consequently do not respond to muscarinic agonists with either a rise in $[Ca^{2+}]_i$ or EDRF release (Peach, Singer & Loeb, 1985; Loeb, Johns, Milner & Peach, 1987).

In an attempt to overcome these difficulties, we have employed the whole cell mode of the patch clamp technique to study the electrophysiological properties of resting and acetylcholine-stimulated endothelium of intact rat aorta. A preliminary report of some of this work has been published (Marchenko & Sage, 1992).

METHODS

Rats aged 1–2 months were anaesthetized with diethyl ether and killed by cervical dislocation. The thoracic aorta was dissected out and placed in modified Krebs solution aerated with 95% O_2 and 5% CO_2. The modified Krebs solution contained (mM): 118·3 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·2 $MgSO_4$, 1 $CaCl_2$, 11·1 glucose, 0·02 phenol red, pH 7·4 at 20 °C. The aorta was dissected free of adventitia and cut into rings of 3–4 mm. Aortic rings were stored in gassed modified Krebs solution with 50 μg/ml gentamycin for up to 3 days. Before an experiment, a ring was cut open and held with nylon mesh, lumen face up, in a chamber of volume 150–200 μl. The chamber was perfused with modified Krebs solution at a rate of 1–2 ml/min.

Membrane potential was measured using the whole cell configuration of the patch clamp technique, with an EPC-7 patch clamp amplifier (List, Darmstadt, Germany) in current clamp mode. Electrical contact with the cytosol was established using the polyene antibiotic, amphotericin B (Rae, Cooper, Gates & Watsky, 1991), which was included in the pipette filling solution at a concentration of 100 μg/ml. Pipettes were filled with a solution of composition (mM): 140 KCl, 10 Hepes, pH adjusted to 7·3 at room temperature with NaOH. Amphotericin B was added from stock in dimethyl sulphoxide just before pipette filling.

The pipette was lowered towards the luminal surface of the vessel using a Narishige micromanipulator (Narashige, Minamikarasuyama, Setagaya-Ku, Tokyo, Japan) and viewed with a stereo microscope. The pipette was then further lowered to touch the surface of the vessel, which was detected by a sharp rise in pipette resistance. Gigohm contact was established by suction. In most experiments, electrical contact with the cytosol was established within 1–2 min of seal formation and was stable after 5–10 min. Stable recordings could be made for up to 2 h. Pipettes had resistances of less than 5 MΩ.

The input resistance of the endothelium was measured by hyperpolarization of the cells by 2–5 mV by injecting a current of 10–100 pA through the recording pipette. Just after this, the recording pipette was removed from the endothelium and briefly lifted out of the medium to destroy the outer part of the resulting ball of membrane which formed on the pipette tip. The pipette resistance was then measured and this value used to correct the input resistance of the endothelium determined in that recording. Experiments were conducted at room temperature (17–20 °C).

ADP, ATP, amiloride, 4-aminopyridine (4-AP), bradykinin, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), histamine, 1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine (H-7), nifedipine, platelet activating factor (PAF), staurosporine, TEA and verapamil were obtained from Sigma (Poole, Dorset). EGTA was obtained from Fluka (Basle, Switzerland).

RESULTS

Electrical properties of unstimulated endothelium

In vessels with intact endothelium, contact of the patch pipette with the surface of the vessel led to a sharp rise in resistance and, after light suction, the establishment of a gigohm seal and subsequently the recording of a potential below −40 mV. Further lowering of the pipette led to a sharp rise in potential reflecting damage to the surface cell. If the endothelium was removed from the vessel by gently rubbing with filter paper prior to the experiment, approaching the vessel with the pipette resulted in a gradual increase in resistance with no sudden jumps in potential and no successful gigohm seals were formed. Thus it could be established when a preparation had an intact, undamaged endothelium and when a seal with the endothelium had been formed.

After formation of low-resistance electrical contact by amphotericin B, the endothelium showed a membrane potential of -58 ± 8 mV (S.E.M., $n = 193$; range −47 to −76 mV). There were differences in membrane potential both between preparations and between different samples from the same preparation. Fig. 1*A* shows a histogram of membrane potentials from unstimulated endothelium, which indicates a single mode distribution. The input resistance of the endothelium was 43 ± 13 MΩ (S.E.M., $n = 8$; range 26–64 MΩ). In two out of twenty-seven aortic preparations, oscillations in membrane potential were observed in unstimulated endothelium (see Fig. 2*I*). This behaviour was characteristic for all recordings from these preparations.

Partial substitution of NaCl by KCl in the extracellular medium depolarized the endothelium. Over the range of $[K^+]$ from 14–80 mM, the membrane potential was less negative than the K^+ equilibrium potential (Fig. 1*B*). $BaCl_2$ (5 mM), which blocks many types of potassium channel (Rudy, 1988), depolarized the endothelium. Tetraethylammonium (2 mM, $n = 7$), 4-AP (5 mM, $n = 3$), amiloride (100 μM, $n = 2$) and DIDS (100 μM, $n = 2$) were without effect on membrane potential.

Effects of agonists on endothelial membrane potential

Application of ACh (0·2–4 μM) evoked an initial hyperpolarization in all preparations (Fig. 2). Subsequent stages of the ACh-evoked response varied in different preparations. However, within a particular aortic preparation, all recordings showed a very similar pattern of response to ACh, with very little change over the 3 days the preparation was used.

In one preparation only, ACh evoked a sustained hyperpolarization, with only a very slow recovery (Fig. 2*A*). In three preparations, the ACh-evoked hyperpolarization was followed by a gradual return to the resting potential (Fig. 2*B*). In the majority of preparations, however, the initial ACh-evoked hyperpolarization was followed by depolarization to above the resting potential, which in some cells reached 0 mV (Fig. 2*C* and *D*). In twelve out of twenty-seven aortic preparations, the depolarization phase of the ACh-evoked response was followed by oscillations in membrane potential with varying amplitudes and time courses (Fig. 2*E–H*). In some preparations, the amplitude of the oscillations was as large as 30–45 mV. In preparations which showed oscillations in membrane potential before stimulation,

ACh greatly increased the amplitude and somewhat reduced the frequency of oscillation (Fig. 2*I*).

ACh-evoked changes in membrane potential were accompanied by changes in the input resistance of the endothelium, which usually increased under depolarization

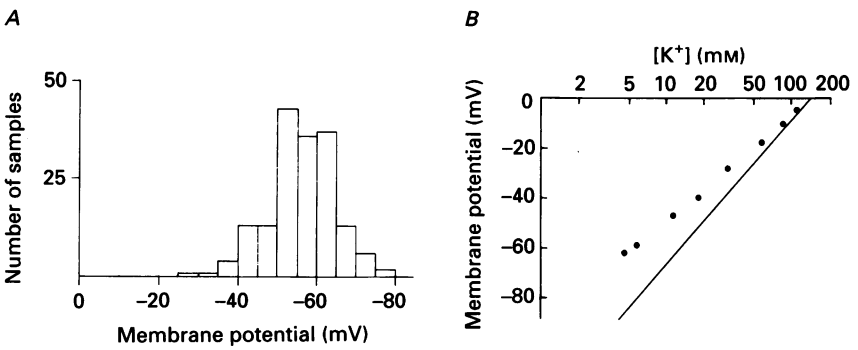

Fig. 1. Membrane potential of endothelium in intact rat aorta. *A*, histogram showing the distribution of membrane potentials in unstimulated endothelium. *B*, dependence of endothelial membrane potential on extracellular K^+ concentration. The K^+ concentration was changed by partial substitution of NaCl by KCl in modified Krebs solution. The K^+ equilibrium potential is shown by the straight line. Values for one preparation, typical of five experiments.

and decreased under hyperpolarization in the range 10–30%, but in some experiments no reliable changes in input resistance were detected.

The ACh-evoked response was reduced in magnitude with repetitive application of the agonist. Fig. 3 shows that the initial response of endothelium after the application of 2 μM ACh is larger than that evoked by a second application after a 10 min wash period.

Several other agonists were also tested. ATP (20–100 μM), ADP (100 μM), histamine (100 μM), PAF (10 nM) and bradykinin (1 μM) all evoked responses similar to those evoked by ACh (not shown).

Analysis of the ACh-evoked membrane potential response

Effects of hyperpolarization and channel blockers

Hyperpolarization of the cell by current injection reversed the hyperpolarization phase of the ACh-evoked response to a depolarization (Fig. 4). In preparations which showed oscillatory responses to ACh, depolarization of the endothelium by current injection through the recording pipette did not itself evoke oscillations in membrane potential (not shown).

The blockers of voltage-operated Ca^{2+} channels, nifedipine (1–10 μM; $n = 5$) and verapamil (20 μM, $n = 2$) did not affect ACh-evoked responses (not shown). Similarly, the K^+ channel blockers TEA (2–5 mM, $n = 7$) and 4-AP (5 mM, $n = 2$), the Cl^- channel blocker DIDS (100 μM, $n = 2$) and amiloride (1 mM, $n = 2$), which blocks a number of cation channels, were all without effect on the ACh response (not shown).

Role of Ca^{2+} entry in the ACh-evoked response

The application of medium containing 5 mM EGTA and no added Ca^{2+} evoked a gradual depolarization of the endothelium from -61 ± 3 mV to -34 ± 3 mV (S.E.M., $n = 9$) over a period of 2–15 min (Fig. 5*A*). Return to a Ca^{2+}-containing medium

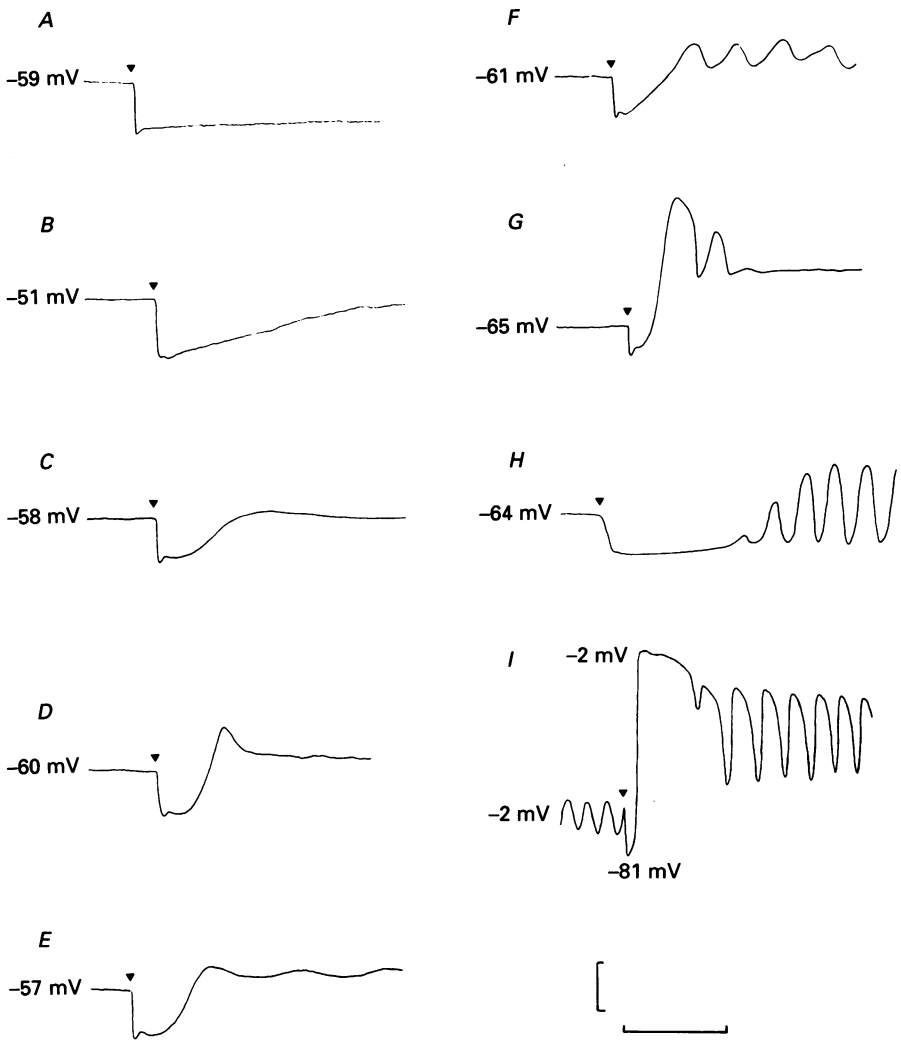

Fig. 2. Variation in responses evoked by acetylcholine in endothelium of intact rat aorta. Preparations were perfused with Krebs solution containing 2 μM ACh from the time indicated by the arrowheads. The value of the membrane potential before ACh addition is shown to the left of each record. In *I*, the endothelium showed oscillations in membrane potential at rest. Scale bars indicate, for traces *A–H*, 20 mV (vertical) and 10 min (horizontal). For *I*, the bars indicate 18 mV and 15·8 min.

evoked a transient hyperpolarization followed by restoration of the initial resting potential (Fig. 5*A*). The application of ACh in Ca^{2+}-free medium shortly after the removal of external Ca^{2+}, evoked a short-lived hyperpolarization with no plateau.

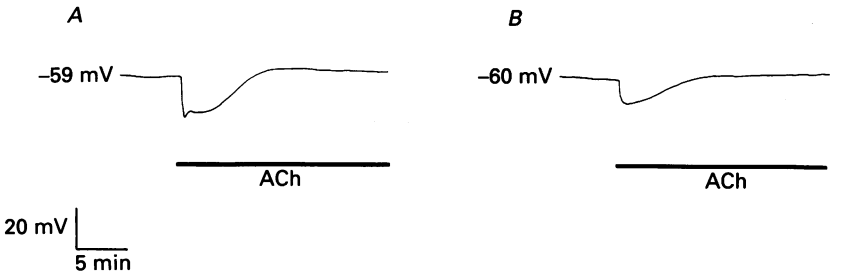

Fig. 3. Desensitization of the acetylcholine-evoked response. *A* shows the first stimulation with 2 μM ACh and *B* a second application to the same preparation after a 10 min wash period. The durations of ACh application are indicated by the horizontal bars.

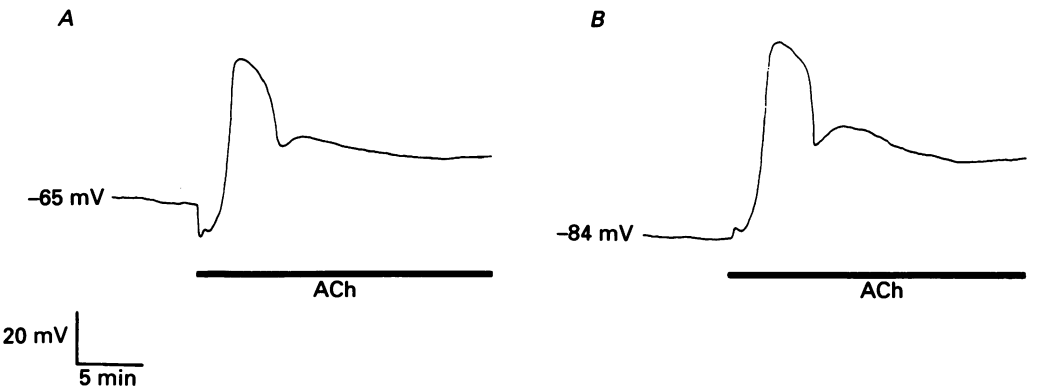

Fig. 4. Voltage-dependence of the acetylcholine-evoked response. *A* shows a control response evoked by 2 μM ACh. *B* shows the response of the same preparation after hyperpolarization by current injection through the recording pipette. In the hyperpolarized endothelium, the initial response evoked by ACh was depolarization rather than hyperpolarization. In both cases, ACh shifts the potential towards the K^+ equilibrium potential, which was $-79{\cdot}8$ mV in this case. Initial membrane potentials are indicated on the left of the traces; that in *B* has been corrected for the value of the potential drop on the pipette. The records shown are representative of three experiments.

There was no depolarization beyond the resting potential, nor any oscillation in membrane potential (cf. Fig. 5*C* and *D*, and Fig. 5*E* and *F*). Application of ACh in Ca^{2+}-free medium after a new stable potential had been established was without effect (Fig. 5*B*).

The application of $NiCl_2$ (2 mM), which blocks agonist-evoked Ca^{2+} entry in many cell types, depolarized the endothelium by 6 ± 2 mV (S.E.M., $n = 7$) (Fig. 6*A*). The subsequent removal of Ni^{2+} evoked a transient hyperpolarization after which the initial resting potential was re-established (Fig. 6*A*). Application of ACh in the presence of Ni^{2+} evoked only a short-lived hyperpolarization (cf. Fig. 6*B* and *C*). The removal of Ni^{2+} in the continued presence of ACh led to similar changes in membrane potential to those evoked by ACh in control medium (Fig. 5*D*).

In preparations in which ACh evoked an oscillatory response, the application of $NiCl_2$ (Fig. 7*A*) or removal of external Ca^{2+} (change of medium to one containing 2–5 mM EGTA and no added Ca^{2+}; Fig. 7*B*) immediately and reversibly blocked the oscillations in membrane potential.

Effects of modulators of protein kinase C

The application of the protein kinase C activator, phorbol 12, 13-dibutyrate (200 nM) resulted in a large increase in the input resistance of the endothelium (Fig. 8*A*), which was accompanied by depolarization.

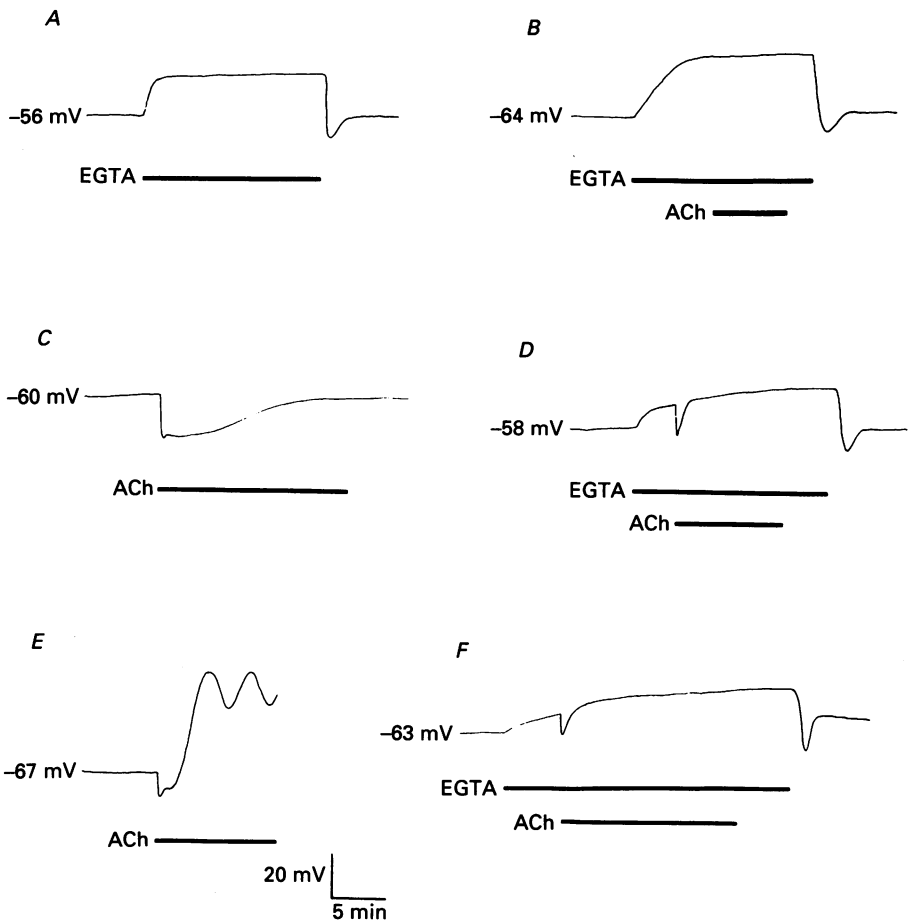

Fig. 5. Effects of calcium-free solutions on endothelial membrane potential and response to acetylcholine. *A*, depolarization of endothelium in Ca^{2+}-free solution. *B*, ACh (2 μM) failed to evoke any response after a stable potential was established in Ca^{2+}-free medium. *C–F*, responses of two preparations to 2 μM ACh in control medium (*C* and *E*) and in Ca^{2+}-free medium (*D* and *F*), before the potential had stabilized (cf. *B*). In both cases in Ca^{2+}-free medium, ACh evoked a short-lived hyperpolarization, regardless of the pattern of the control response of the preparation. Initial membrane potentials indicated on the left of each trace. Duration of perfusion with Ca^{2+}-free medium (containing 2–5 mM EGTA) and ACh (2 μM) indicated by the horizontal bars.

The ACh-evoked response was not affected by pretreatment with either of the protein kinase C blockers, staurosporine (200 nM; Fig. 8*B*) or H-7 (200 nM; not shown) compared with controls in the same preparation.

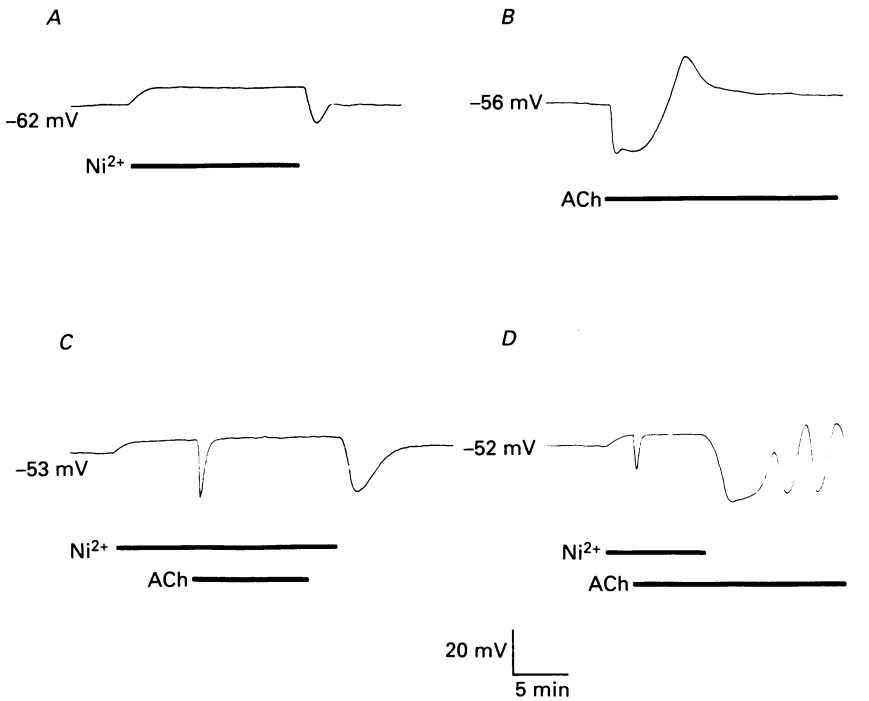

Fig. 6. Effects of nickel on endothelial membrane potential and response to acetylcholine. *A*, depolarization of endothelium evoked by application of 2 mM $NiCl_2$. Removal of Ni^{2+} evoked a transient hyperpolarization. Control response (*B*) and that in the presence of 2 mM Ni^{2+} (*C*) of the same preparation. Ni^{2+} blocked all phases of response except the initial hyperpolarization. *D*, effect of removal of Ni^{2+} in continued presence of ACh (2 μM). ACh evoked only a short-lived hyperpolarization in the presence of Ni^{2+}. Removal of Ni^{2+} resulted in a more typical ACh response.

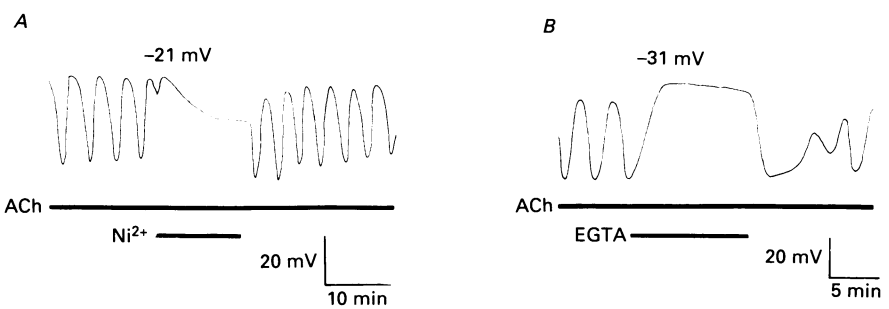

Fig. 7. Dependence of ACh-evoked oscillations in membrane potential on external calcium. *A*, inhibition of ACh-evoked oscillations by application of 2 mM $NiCl_2$ (same preparation as Fig. 2*I*). Representative of three experiments. *B*, inhibition of ACh-evoked oscillations in Ca^{2+}-free solution (2 mM EGTA) (same preparation as Fig. 6*D*). Application of ACh, $NiCl_2$ or Ca^{2+}-free medium (EGTA) shown by horizontal bars. Representative of two experiments. Minimum potential reached indicated above traces.

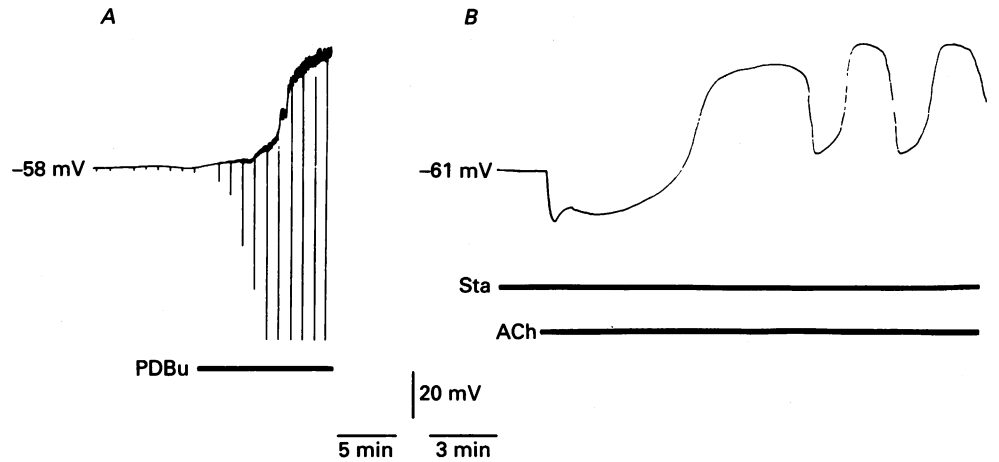

Fig. 8. Effects on endothelial membrane potential and response to acetylcholine of protein kinase C modulators. *A*, application of the protein kinase C activator, phorbol 12,13-dibutyrate (PDBu, 200 nM). Depolarization and a rise in input resistance resulted. Hyperpolarization pulses of 50 pA were injected into the endothelium once per minute. Representative of two experiments. *B*, response evoked by 2 μM ACh after application of the protein kinase C inhibitor, staurosporine (Sta, 200 nM). Staurosporine was applied 10 min before ACh. Staurosporine was itself without effect on membrane potential and did not affect any phase of the ACh-evoked response (compared with controls in the same preparation). Representative of four experiments.

DISCUSSION

Properties of unstimulated endothelium in intact rat aorta

The endothelium of intact rat aorta had a relatively large mean resting potential of −58 mV. Reported membrane potentials for cultured endothelial cells show wide variations with no obvious correlation with source or culture conditions (Colden-Stanfield *et al.* 1987; Fichtner, Frobe, Busse & Kohlhardt, 1987; Bregestovski *et al.* 1988; Daut *et al.* 1988; Olesen *et al.* 1988; Bossu, Feltz, Rodeau & Tanzi, 1989; Mehrke & Daut, 1990; Merke *et al.* 1991). Recent microelectrode measurements of membrane potential in endothelium of intact guinea-pig coronary artery gave a mean value of −47·7 mV (Chen & Cheung, 1992). This lower value than that obtained in rat aorta may reflect the different vessel and species, or the result of damage incurred in the complex preparation of the guinea-pig tissue (see below). Further work on intact vessels will be necessary to establish whether there are characteristic differences in resting potential in endothelia of different vessels and whether these correlate with those measured in cells from the same source in culture.

The input resistance of the endothelium in intact vessels was low and indicates that the cells were electrically coupled. This is supported by morphological studies which show that endothelial cells are connected by gap junctions. Electrical coupling has been demonstrated in cultured endothelial monolayers (e.g. Bregestovski *et al.* 1988). The presence of electrical coupling in the endothelium of intact rat aorta is

supported by the large rise in input resistance when the vessel was treated with phorbol ester, a potent inhibitor of gap junctions (Yotti, Chang & Trosko, 1979). The input resistance in phorbol ester-treated endothelium reached values similar to those reported by Bregestovski *et al.* (1988) for human umbilical vein endothelial cells in non-confluent monolayers. The existence of electrical coupling between cells indicates that the endothelium is a functional syncytium in which electrical and possibly chemical signals may pass from cell to cell.

The resting potential of the endothelium in intact vessels was found to vary between recordings from a given preparation. The reason for these differences is not clear. The most simple explanation is that differing values of membrane potential within a preparation result from electrical leakage through damaged parts of the endothelium. Morphological studies have indicated widespread damage to more than 40% of the endothelium in excised vessels (e.g. Lewis, Loomis & Segal, 1991), presumably resulting from contraction and shortening of the vessel after isolation. Our observations do not support such massive damage in our aortic preparations. Sealing of pipettes onto the luminal surface of the vessels indicated a layer of cells with large membrane potentials in almost 100% of attempts. Staining of vessels with Trypan Blue revealed damaged cells only near the edges, not the central parts of the luminal surface of the preparations (not shown). Determinations of membrane potential showed no clear correlation with the distance of the sampled cell from the edge of the preparation. Thus, although we cannot exclude damage from the formation of gradients in membrane potential in endothelium, it is likely that such gradients are an intrinsic property. Differences in potential may reflect differences in the properties of the plasma membranes of cells or groups of cells in the same clone in the endothelium. Local stimulation might increase these intercellular differences in potential.

The physiological significance of possible differences in potential within the endothelium is unclear. Intercellular currents could involve nearby unstimulated areas of endothelium in physiological responses both by changing the membrane potential and thereby affecting $[Ca^{2+}]_i$ (Cannell & Sage, 1989), and by transporting charged second messengers (Ca^{2+}, cyclic nucleotides, InsP_3) between cells.

Effects of calcium removal

The removal of external Ca^{2+} resulted in depolarization of the endothelium. The underlying mechanism of this response is uncertain, but it may reflect a fall in $[Ca^{2+}]_i$ and consequent reduction in a Ca^{2+}-activated K^+ conductance. Application of Ni^{2+} evoked a similar, though smaller depolarization. This might reflect a relatively high rate of Ca^{2+} leakage across the endothelial plasma membrane in the intact aorta, with a consequent fall in $[Ca^{2+}]_i$ if this is blocked by Ni^{2+}.

If Ca^{2+} was added back to the medium after a period of absence, or if Ni^{2+} was removed, a transient hyperpolarization of the endothelium occurred, followed by a return to the original resting potential. This hyperpolarization most likely reflects a transient rise in $[Ca^{2+}]_i$ and consequent activation of Ca^{2+}-activated K^+ channels. A transient rise in $[Ca^{2+}]_i$ on Ca^{2+} replacement has been demonstrated in cultured bovine aortic endothelial cells (Gillespie, Greenwell & Johnson, 1991; Gillespie, Johnson, Nicholls, Lynch & Greenwell, 1992). This rise in Ca^{2+} may, at least in part,

reflect Ca^{2+} entry activated by depletion of the intracellular Ca^{2+} stores (Putney, 1990; Hallam *et al.* 1989). The rapid failure of the ACh-evoked response after removal of external Ca^{2+} in the present experiments suggests that the agonist-releasable intracellular Ca^{2+} store is rapidly depleted. In the cultured bovine cells, the rise in $[Ca^{2+}]_i$ which occurred on Ca^{2+} replacement was reduced in Na^+-free medium, suggesting that reversed Na^+–Ca^{2+} exchange may account for some of the Ca^{2+} entry (Gillespie *et al.* 1992). Na^+ may accumulate in the cells during the exposure to low-Ca^{2+}medium. A sizeable Ca^{2+} entry following the elevation of cytosolic Na^+ has been demonstrated in cultured bovine pulmonary artery endothelium in low Na^+ medium (Sage, van Breemen & Cannell, 1991).

Agonist-evoked responses

Endothelium-dependent vasodilators such as ATP, bradykinin, histamine and thrombin are widely reported to evoke a biphasic rise in $[Ca^{2+}]_i$ in a variety of cultured endothelial monolayers. The initial peak in $[Ca^{2+}]_i$ is largely independent of extracellular Ca^{2+}, being accounted for by release from intracellular stores. The subsequent plateau in $[Ca^{2+}]_i$ is dependent on Ca^{2+} entry (for review, see Jacob *et al.* 1990). Oscillations in $[Ca^{2+}]_i$ in cultured monolayers have also been reported (Sage, Adams & van Breemen, 1989; Neylon & Irvine, 1990). In contrast, there is no general agreement concerning the electrical responses of endothelium to endothelium-dependent vasodilators. Agonists have been reported to evoke depolarization and inward currents (Johns, Lategan, Lodge, Ryan, van Breemen & Adams, 1987; Bregestovski *et al.* 1988), hyperpolarization and the activation of K^+ channels (Colden-Stanfield *et al.* 1987; Busse *et al.* 1988; Olensen *et al.* 1988; Sauve *et al.* 1988; Merke *et al.* 1991; Chen & Cheung, 1992), biphasic responses (Merke & Daut, 1990) or late developing inward currents lagging well behind the rise in $[Ca^{2+}]_i$ (Cannell & Sage, 1989). These differences are difficult to explain alongside the general agreement over the pattern of action of the same agonists on $[Ca^{2+}]_i$, but differences in source of endothelium and culture conditions are probably involved.

Acetylcholine-evoked hyperpolarization

In all tested preparations of endothelium in intact rat aorta, we observed an initial hyperpolarization evoked by ACh. In the majority of preparations, the hyperpolarization was followed by depolarization to above the initial resting potential and in some cases reached 0 mV. In many preparations the depolarization was followed by pronounced oscillations in membrane potential. The ACh-evoked hyperpolarization reversed near the K^+ reversal potential and was apparently evoked by activation of Ca^{2+}-dependent K^+ channels as suggested by others (Colden-Stanfield *et al.* 1987; Fichtner *et al.* 1987). The ACh-evoked hyperpolarization was not blocked by TEA, but this compound has been reported to fail to block Ca^{2+}-activated K^+ channels in several cell types (Latorre, Oberhauser, Labarca & Alvarez, 1989).

Although ACh is an important physiological agonist, the response of endothelium to ACh has not been widely studied because cultured cells rapidly cease to synthesize messenger RNA for muscarinic receptors (Tracey & Peach, 1992). Therefore, few

investigations have been devoted to the electrophysiology of the ACh-evoked response. In patch clamp experiments on freshly isolated rabbit aortic endothelial cells, ACh was found to evoke hyperpolarizations which lasted for less than 1 min (Busse *et al.* 1988). The hyperpolarization was attributed to an increased K^+ conductance, but never reached the K^+ reversal potential. A rise in $[Ca^{2+}]_i$ evoked by ACh was demonstrated in suspensions of fura-2-loaded cells from the same preparations (Busse *et al.* 1988). The rapid time course and relatively small amplitude of the ACh-evoked hyperpolarizations in these experiments were apparently due to the buffering of $[Ca^{2+}]_i$ by EGTA, which was included in the pipette solution.

In microelectrode recordings from the endothelium of intact guinea-pig coronary artery, ACh was reported to evoke a large maintained hyperpolarization which only slowly decreased with time (Chen & Cheung, 1992). These authors observed neither depolarization nor oscillations evoked by ACh. The ACh-evoked hyperpolarization was shown to be Ca^{2+} dependent. Charybdotoxin, relatively low concentrations of TEA and, surprisingly, 4-AP, all depolarized the endothelium and blocked the ACh-evoked hyperpolarization in the guinea-pig coronary artery. The differences between these results and those presented here may be the result of anatomical differences and/or the different species of origin of the vessels used. Further work comparing the responses of endothelium in different intact vessels is needed to reveal whether or not there is a genuine difference in response.

In one report of the responses of cultured bovine aortic endothelial cells to ACh, the results differed significantly from those reported in the endothelium of intact vessels here and by Chen & Cheung (1992). In the cultured cells, ACh was reported to activate a K^+ current which showed strong inward rectification and was independent of extracellular Ca^{2+}. In suspensions of these cells loaded with fura-2, ACh was reported not to increase $[Ca^{2+}]_i$ (Olesen *et al.* 1988). It has been shown that cultured endothelial cells cease to produce messenger RNA for M1 and M3 muscarinic receptor subtypes, but continue to produce that for the M2 subtype (Tracey & Peach, 1992). It may be that the response observed in the cultured bovine cells is mediated by M2 receptors, whilst this response is masked in the endothelium of intact vessels by responses mediated by the other muscarinic receptor subtypes.

Acetylcholine-evoked depolarization

The events which underlie the depolarization phase of the ACh-evoked response are uncertain. Merke & Daut (1990), who have reported a biphasic response evoked by bradykinin in cultured guinea-pig coronary artery endothelium, have suggested that the depolarization phase may result from K^+ channel closure following the activation of protein kinase C. However, in the endothelium of intact rat aorta, we found the protein kinase inhibitors staurosporine and H-7 did not block the depolarization phase of the ACh-evoked membrane potential responses. Our results thus do not implicate a role for protein kinase C in the ACh-evoked response.

When ACh was applied in Ca^{2+}-free medium or in the presence of Ni^{2+}, it evoked only a short-lived hyperpolarization, which was not followed by depolarization beyond the resting potential. However, the subsequent addition of extracellular Ca^{2+} or removal of Ni^{2+} resulted in typical ACh responses, including a depolarization

phase. These results indicate that the ACh-evoked depolarization, as well as the sustained plateau in the hyperpolarization phase, require the entry of extracellular Ca^{2+}. How Ca^{2+} entry results in depolarization is not certain and is discussed below.

Acetylcholine-evoked oscillations in membrane potential

A major difference between our results and those reported by others who have studied responses evoked by ACh (Busse *et al.* 1988; Olesen *et al.* 1988; Chen & Cheung, 1992) and other agonists (Colden-Stanfield *et al.* 1987; Johns *et al.* 1987; Bregestovski *et al.* 1988; Sauve *et al.* 1988; Cannell & Sage, 1989; Merke & Daut, 1990; Merke *et al.* 1991) is that in our experiments, ACh evoked large oscillations in membrane potential in a significant proportion of preparations. Merke & Daut (1990) did report bradykinin-evoked oscillations in cultured guinea-pig coronary artery endothelium, but these were small, with an amplitude of only a few millivolts. Small oscillations in membrane potential have also been reported in response to bradykinin in cultured bovine atrial valve endothelial cells, but only under unphysiological conditions when K^+ was removed from the external medium (Laskey, Adams, Cannell & van Breemen, 1992). It is not clear whether these oscillations occur under physiological conditions and in any case, their small amplitude renders their functional significance questionable.

In contrast, the large oscillations in membrane potential observed in the endothelium of the intact rat aorta should greatly affect the transmembrane driving force for Ca^{2+} and so $[Ca^{2+}]_i$ (Cannell & Sage, 1989). This appears difficult to reconcile with the report that depolarization of single human umbilical vein endothelial cells with K^+ did not inhibit histamine-evoked oscillations in $[Ca^{2+}]_i$ and only slightly reduced the peak values of the spikes (Jacob, Merritt, Hallam & Rink, 1988). It may be that these cells did not exhibit oscillations in membrane potential. Furthermore, these cells may, as reported in many cultured preparations, have had relatively small membrane potentials (see above). This would be expected to greatly reduce agonist-evoked Ca^{2+} entry (Cannell & Sage, 1989; Lückhoff & Busse, 1990).

The oscillations in endothelial membrane potential in intact rat aorta were absolutely dependent on Ca^{2+} entry. ACh only evoked oscillations in the presence of external Ca^{2+} and they were blocked immediately when external Ca^{2+} was removed or when the Ca^{2+} entry blocker, Ni^{2+}, was added. Oscillations in $[Ca^{2+}]_i$ in cultured bovine pulmonary artery endothelial monolayers have also been shown to depend on extracellular Ca^{2+} (Sage *et al.* 1989). So, in the present experiments, the entry of external Ca^{2+} was necessary for the prolonged phase of hyperpolarization, the depolarization and oscillation phases of the ACh response, but not the initial part of the hyperpolarization, which appeared to depend on the release of Ca^{2+} from intracellular stores.

Relationship between membrane potential and cytosolic calcium

The data presented here for endothelium in intact aorta support a relationship between membrane potential and $[Ca^{2+}]_i$. The ACh-evoked electrical response mirrors the typical pattern of agonist-evoked changes in $[Ca^{2+}]_i$ reported in various endothelial cultures (Colden-Stanfield *et al.* 1988; Hallam *et al.* 1989; Sage *et al.* 1989; Lückhoff & Busse, 1990; Neylon & Irvine, 1990; Gillespie *et al.* 1991). The initial

peak and the plateau phase of hyperpolarization could be accounted for by the biphasic elevation in $[Ca^{2+}]_i$ reported to be evoked by endothelial agonists, assuming activation of Ca^{2+}-activated K^+ channels over the typical range of $[Ca^{2+}]_i$ of 100–500 nM, as indicated for such channels in various cell types (Rudy, 1988). Furthermore, the oscillations in membrane potential reported here could reflect oscillations in $[Ca^{2+}]_i$, as reported to be evoked by various agonists in cultured endothelial cells (Jacob *et al.* 1988; Sage *et al.* 1989; Neylon & Irvine, 1990; Gillespie *et al.* 1991; Laskey *et al.* 1992).

However, not all our observations of the effects of ACh on membrane potential can be explained by simply supposing that the changes in membrane potential reflect changes in a Ca^{2+}-activated K^+ conductance in turn following changes in $[Ca^{2+}]_i$. The depolarization phase of the ACh response, which in some preparations reached 0 mV, has no correlate with known measurements of changes in $[Ca^{2+}]_i$ and suggests some as yet unknown mechanism of ionic channel regulation may be present. This mechanism appears to be activated by prolonged entry of Ca^{2+} through Ni^{2+} sensitive channels, and so presumably a prolonged elevation in $[Ca^{2+}]_i$. One possibility is that elevated $[Ca^{2+}]_i$ might inactivate voltage-dependent K^+ channels as reported in neutrophils (Bregestovski, Redkozubov & Alexeev, 1986). Such a mechanism, if present in endothelial cells, might also contribute to the generation of oscillations in $[Ca^{2+}]_i$ and membrane potential, and merits further investigation.

Electrical coupling between endothelium and vascular smooth muscle

Morphological studies indicate that intercellular contacts occur not only within endothelium and within vascular smooth muscle but also between endothelial and vascular smooth muscle cells. These myoendothelial gap junctions are well documented in arterioles and precapillary sphincters (Rhodin, 1967) and to a lesser extent in larger vessels (Spagnoli, Villaschi, Neri & Palmieri, 1982). Although most physiological data argue for chemical communication between endothelium and vascular smooth muscle, the possibility of some role for myoendothelial electrical coupling cannot be excluded, particularly in the microcirculation. However, our data provide no support for this hypothesis in rat aorta. The voltage-operated Ca^{2+} channel blockers nifedipine and verapamil had no effect on ACh-evoked responses and depolarization with KCl or by current injection did not evoke any response which could be attributed to coupling to excitable cells.

In conclusion, results presented here indicate that the endothelium of an intact vessel shows some properties which differ from those observed in cultured cells and allows the study of the ACh response which is not possible in cultured cells. This preparation will therefore prove useful for the investigation of the electrophysiology of endothelium under conditions which are perhaps as close as possible to the physiological.

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