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EFFECTS OF VASOACTIVE AGONISTS ON THE MEMBRANE POTENTIAL OF CULTURED BOVINE AORTIC AND GUINEA-PIG CORONARY ENDOTHELIUM

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SUMMARY

1. The effects of bradykinin, ATP, adenosine, histamine and thrombin on the membrane potential of confluent monolayers of cultured bovine aortic endothelial cells (BAECs) and guinea-pig coronary endothelial cells (GCECs) were studied at 37 °C using the whole-cell mode of the patch-clamp technique.

2. The amplitude histogram of the resting potentials of BAEC monolayers showed a bimodal distribution with one peak around −25 mV and another peak around −85 mV. Transitions from one potential level to the other were observed. The bistable membrane potential can be explained by an N-shaped current–voltage relation of the endothelial cell membrane.

3. When BAECs with a low resting potential (−10 to −30 mV) were superfused with maximally effective concentrations of ATP (2–10 μM) an initial hyperpolarization of −80 to −90 mV was observed which decayed to a plateau of about −60 mV within 1 min. When ATP was removed after 2–3 min the membrane potential returned to control level within 1 min. This was followed by a second hyperpolarization of 10–20 mV, which decayed within 15 min.

4. In the absence of extracellular calcium, ATP produced only a brief transient hyperpolarization in aortic endothelium. The plateau and the secondary hyperpolarization were abolished. These findings are consistent with the idea that the changes in membrane potential reflect changes in intracellular free Ca^{2+} and that the initial peak is due to release of Ca^{2+} from intracellular stores, whereas the plateau and the secondary hyperpolarization depend on transmembrane Ca^{2+} influx.

5. Bradykinin evoked potential changes similar to ATP in BAECs, except that the secondary hyperpolarization during wash-out was absent. When the membrane potential was more negative than −80 mV, ATP and bradykinin induced only a small initial hyperpolarization followed by a depolarization of up to 20 mV.

6. In aortic endothelium, ADP (10 μM) evoked a much smaller response than ATP.

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Adenosine (10 μM), thrombin (2 units/ml), acetylcholine (10 μM) and histamine (10 μM) had only a very small effect on the membrane potential, if any.

7. The amplitude histogram of the membrane potential of GCECs showed only one peak around −35 mV. In coronary endothelium, application of bradykinin, ATP, histamine, thrombin, acetylcholine and adenosine all evoked a transient hyperpolarization of 10–40 mV lasting 1 min or less, which then turned into a depolarization.

8. The K^+ channel openers cromakalim (BRL 34915) and lemakalim (BRL 38227) did not affect the membrane potential of GCECs or BAECs. This suggests that ATP-sensitive K^+ channels are absent in endothelial cells.

9. Reducing the oxygen tension (P_{O_2}) of the superfusate from 150 mmHg to 12–32 mmHg also had virtually no effect on the resting potential of BAECs and GCECs. However, in sixteen out of twenty-six experiments 15 min hypoxia produced an increase in input resistance; the median of the increase was 9%. This may be due to partial uncoupling of the cells in the monolayer caused by closure of intercellular gap junctions.

10. It is concluded that in spite of many common properties there are some marked differences between coronary (mainly microvascular) and aortic (macrovascular) endothelial cells in their electrical response to vasoactive substances. The implications of the present findings for the mechanisms of hypoxic vasodilatation in the heart are discussed.

INTRODUCTION

Blood vessels respond to physical stimuli such as transmural pressure and shear stress at the vessel wall and to chemical stimuli such as neurotransmitters, circulating hormones or locally released vasoactive substances, with changes in (i) wall tension, (ii) diameter, (iii) permeability to various solutes and (iv) transmural hydraulic conductivity (Johnson, 1980; Michel, 1984; Olesen, 1989). Most of these adaptive responses are mediated or modulated by the endothelium, and each organ and each segment of their vascular beds has its own ultrastructurally and functionally distinct type of endothelium (Simionescu & Simionescu, 1984; Shepro & D'Amore, 1984). The molecular mechanisms mediating the response to physical and chemical stimuli are only partly understood. One of the most important functions of the endothelium is the release of autacoids such as endothelium-derived relaxing factor (EDRF) or prostaglandins, which act on vascular smooth muscle cells, various blood cells, and also on the endothelial cells themselves. EDRF is generally assumed to be identical to nitric oxide (NO) (Moncada, Radomski & Palmer, 1988; Kelm & Schrader, 1990) and is synthesized from the amino acid *l*-arginine, probably via a Ca^{2+}- and β-nicotinamide–adenine dinucleotide phosphate (NADPH)-dependent mono-oxygenase (Moncada, Palmer & Higgs, 1989; Mülsch, Bassenge & Busse, 1989).

The release of EDRF by vasoactive agonists such as acetylcholine, bradykinin or ATP is associated with changes in the free cytosolic calcium concentration ($[Ca^{2+}]_i$) (Lückhoff & Busse, 1986; Hallam, Jacob & Merritt, 1988; Schilling, Ritchie, Navarro & Eskin, 1988) and with a hyperpolarization of the endothelial cell membrane (Busse, Fichtner, Lückhoff & Kohlhardt, 1988; Olesen, Davies & Clapham, 1988;

Mehrke & Daut, 1990). This hyperpolarization may be due to an increased open probability of Ca^{2+}-activated K^+ channels caused by a rise in $[Ca^{2+}]_i$ (Sauvé, Parent, Simoneau & Roy, 1988; Colden-Stanfield, Schilling, Possani & Kunze, 1990). On the other hand, the rise in $[Ca^{2+}]_i$ induced by bradykinin or ATP was found to be increased by hyperpolarization of the endothelium (Schilling, 1989; Laskey, Adams, Johns, Rubanyi & van Breemen, 1990; Lückhoff & Busse, 1990). This may be due to an agonist-induced Ca^{2+} influx through plasmalemmal Ca^{2+} channels.

The interdependence of membrane potential (E_m) and intracellular Ca^{2+} is likely to be important for the regulation of EDRF synthesis. However, it is not yet clear, how closely the agonist-induced changes in membrane potential are correlated with changes in intracellular Ca^{2+} and which feedback loops are involved in determining $[Ca^{2+}]_i$ and membrane potential in the steady state. In order to increase our understanding of these processes we have studied the changes in membrane potential induced by various vasoactive agonists in cultured endothelial cells from bovine aorta.

The electrical behaviour of bovine aortic endothelial cells (BAECs), i.e. of typical macrovascular endothelial cells, was then compared with the electrical behaviour of cultured endothelial cells isolated from guinea-pig heart, which are primarily of microvascular origin. The main reason for making this comparison was to investigate possible differences between micro- and macrovascular endothelium in general. Large arteries and veins are separate anatomical entities, and their response to different physical and chemical stimuli varies according to their position in the vascular tree. In contrast, the microvasculature is structurally and functionally part of the tissue it supplies, and its response varies according to the nature and activity of the surrounding tissue. Thus it is conceivable that the mechanisms controlling release of autacoids may differ between large vessels and the microvasculature. Our results show that there are indeed considerable differences between aortic and coronary endothelial cells in their electrical response to vasoactive compounds.

The second reason for carrying out this comparative electrophysiological study was to clarify the mechanisms underlying hypoxic vasodilation in the heart. In trying to understand these mechanisms it is important to know to what extent the wealth of experimental data available from macrovascular endothelium can be used to make inferences on the events occurring in the coronary microvasculature. In addition, it is necessary to discriminate between direct effects of hypoxia on endothelial cells or vascular smooth muscle cells and indirect effects of hypoxia due to locally released vasoactive substances. Therefore we measured the effects of hypoxia on the membrane potential of cultured coronary and aortic endothelial cells.

From experiments on isolated perfused hearts it was concluded that hypoxic dilatation of coronary arteries is mediated by ATP-sensitive K^+ channels in coronary smooth muscle cells (Daut, Maier-Rudolph, von Beckerath, Mehrke, Günther & Goedel-Meinen, 1990). In order to test whether such channels are also present in the endothelium we measured the effects of the K^+ channel opener cromakalim, which activates these channels, on the membrane potential of cultured coronary and aortic endothelial cells. These experiments may contribute to our understanding of the complex cascade of events occurring in the coronary microvasculature during hypoxia and ischaemia.

METHODS

Bovine aortic endothelial cells (BAECs) were isolated and cultured as described previously (Lückhoff, Busse, Winter & Bassenge, 1987). In brief, aortae were opened longitudinally and incubated at 37 °C with collagenase (CLS I, Biochrom; 120 units/ml) for 20 min. Detached cells were harvested by flushing the intima with a syringe. After washing by gentle centrifugation the cells were seeded in standard 35 mm Petri dishes (Falcon). In a few experiments ($n = 5$) the cells were resuspended by treatment with trypsin (0·05 mg/ml) and subcultured with a split ratio of 1:3.

The isolation and culture of guinea-pig coronary endothelial cells (GCECs) has been described in detail recently (Mehrke & Daut, 1990). In brief, isolated guinea-pig hearts were perfused with nominally Ca^{2+}-free solution (Joklik modified minimal essential medium; 4 min), followed by perfusion with digesting solution containing 0·5 mg/ml collagenase, 1 mg/ml dispase, 0·01 mg/ml trypsin and 0·5 mg/ml bovine serum albumin, essentially fatty acid free (all from Sigma). After 10–12 min perfusion was stopped and the heart was cut into small pieces and agitated in digesting solution. The dispersed cells were separated by density gradient centrifugation, washed, and seeded in 35 mm Petri dishes (Nunc or Falcon). Both BAECs and GCECs were incubated at 37 °C in medium 199 (GIBCO) to which 20% fetal calf serum (Sigma) was added. The incubator contained water-saturated atmosphere containing 95% air and 5% CO_2.

The electrical recordings were performed with primary cultures of BAECs and GCECs, except in a few experiments (BAECs, $n = 5$; GCECs, $n = 3$) where first subcultures were used. No systematic differences were noted in these cells. The Petri dishes were mounted on an inverse microscope (Zeiss IM 35) and an area of 2×10 mm was separated off by means of a frame (height 1 mm) made of polyvinyl chloride. The rest of the cells were discarded. The cells in this recording chamber were superfused with physiological salt solution (PSS) containing (mM): 138 NaCl, 3 KCl, 0·8 $MgCl_2$, 1·8 $CaCl_2$, 1 Na H_2PO_4, 2 *l*-glutamine, 10 glucose, 5 HEPES. The pH was adjusted to 7·4 (at 37 °C) with Tris. The chamber was perfused by means of motor-driven syringes. The solution could be changed within 5 s by switching a four-port valve connected to two motor-driven syringes running at precisely the same speed (usually 0·2 ml/min). All solutions were warmed to 37 °C using a heat exchanger made of glass, located immediately before the entry to the recording chamber.

Hypoxia could be introduced by switching to a superfusing solution equilibrated with 100% N_2. In these experiments glass syringes and Teflon tubing tightly fitted into a stainless steel tube were used to minimize uptake of oxygen. The P_{O_2} in the recording chamber was determined before and after the electrical recordings by sucking the superfusate into a stainless steel tube positioned immediately above the cells (touching the bottom of the Petri dish) and measuring the P_{O_2} of the effluent with a Clark type oxygen electrode (Eschweiler, Kiel, Germany) calibrated before and after the experiment. With a given hypoxic solution the final P_{O_2} in the bath could be varied by changing the flow rate of both the control and the hypoxic solution.

Standard patch-clamp techniques were used for electrical recording. The patch pipettes were made of thin-walled glass (Clark, Reading) using an automatic pipette puller (Zeitz Instruments, Verdistr. 2, D-8900 Augsburg, Germany). The pipette solution contained (mM): 150 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 Na_2ATP, 1 NaH_2PO_4, 5 HEPES. The pH was adjusted to 7·2 with Tris. In some experiments the EGTA concentration was reduced to 1 mM (Fig. 4). Further details about the apparatus and about the identification of coronary endothelial cells are given in Daut, Mehrke, Nees & Newman (1988) and in Mehrke & Daut (1990). The results reported below are usually given as mean ± standard deviation (S.D.), n denotes the number of successful whole-cell recordings from confluent monolayers of endothelial cells. Individual recordings with one patch pipette lasted between 30 min and 4 h.

RESULTS

Input resistance and resting potential of confluent monolayers

Confluent monolayers of cultured endothelial cells were superfused with physiological salt solution pre-warmed to 37 °C. Gigaohm seals between the patch pipette and a cell in the monolayer were established by approaching the glass pipette carefully and applying gentle negative pressure (10–30 cmH_2O) and the patch was ruptured by repetitive negative current pulses. The resting potential of monolayers

of BAECs ranged between −10 and −98 mV (see below). The input resistance of the monolayers was usually only a few megaohms, as illustrated in Fig. 1. On the left a typical 'whole-cell' clamp record of a confluent monolayer of BAECs is shown. A 7 mV depolarizing voltage step from the resting potential of −91 mV required a

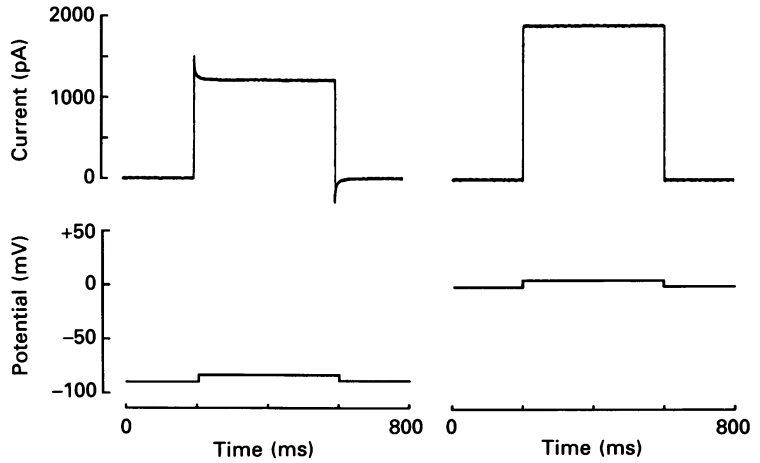

Fig. 1. Typical measurement of the input resistance of a confluent monolayer of BAECs (left) and of the resistance of the patch electrode (right). Voltage steps of +7 mV amplitude were applied in the whole-cell mode of the patch-clamp technique. The current required to displace the pipette potential by 7 mV is shown in the upper traces. The monolayer had a resting potential of −91 mV (lower trace, left). After withdrawal of the pipette (and cleaning of the tip by application of positive pressure) the bath potential was 0 mV (lower trace, right).

steady-state current of about 1·9 nA. The input resistance of the monolayer plus the resistance of the patch electrode was 5·8 MΩ. The slow current tails after the voltage steps are due to the large capacitance of the monolayer which is charged through the distributed resistance of the gap junctions between individual endothelial cells (Daut *et al.* 1988).

After withdrawal of the patch electrode the same voltage step was repeated without attachment of a monolayer, as illustrated on the right-hand side of Fig. 1. It can be seen that the capacitive transient had disappeared and the series resistance of the pipette was only about 60% lower (3·7 MΩ) than the combined resistance of pipette and monolayer. Thus the input resistance of the monolayer itself was only about 2 MΩ. Not all of the monolayers had input resistances as low as this. In the present study the input resistances of BAECs were in the range 2–30 MΩ, the median was 12 MΩ ($n = 33$). This may be compared to the input resistance of single endothelial cells, which is 1–2 GΩ. (Daut *et al.* 1988). Thus our present data on BAECs confirm the idea that the endothelium, at least when cultured in monolayers, forms an electrical syncytium in which the individual cells are coupled by numerous gap junctions.

Figure 2*A* shows an amplitude histogram of the resting potential of all monolayers

from BAECs. All resting potentials were stable within ± 2 mV for at least 30 min ($n = 67$). It is obvious that there was a bimodal distribution of resting potentials with peaks around -25 and -85 mV. There was no systematic difference in input resistance between cells that had low or high resting potentials. The bimodal

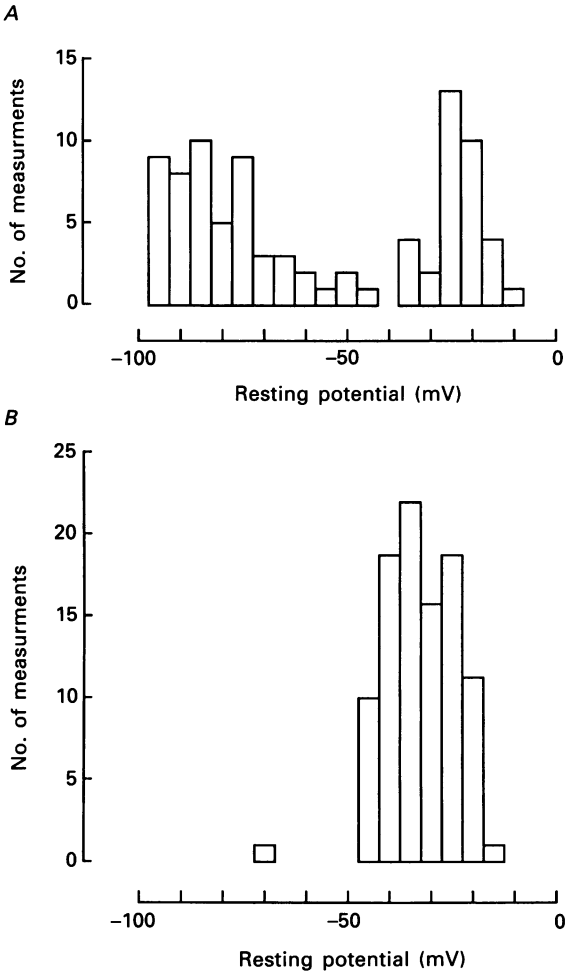

Fig. 2. The distribution of the resting potential of confluent monolayers of cultured endothelial cells. *A*, amplitude histogram of the resting potential of BAECs. *B*, amplitude histogram of the resting potential of GCECs. Only potential recordings that were stable for at least 30 min were evaluated.

distribution may be due to the fact that the current–voltage relation of endothelial cells is very flat and N-shaped, as has been reported by Cannell & Sage (1989) for bovine pulmonary artery endothelial cells. Thus the opening of very few channels or a small variation in the current produced by the electrogenic sodium pump (see Daut *et al.* 1988) could shift the membrane potential from one stable point to the other.

In a second series of experiments we used coronary endothelial cells isolated from

guinea-pig heart. These cells were obtained by collagenase digestion of isolated, perfused guinea-pig hearts. Since the lumped luminal surface area of the capillaries is much larger than that of the larger blood vessels most of our cultured cells are probably of microvascular origin. The resting potential of GCECs was on average -32 ± 8 mV ($n = 100$). The input resistance, corrected for electrode resistance, was in the range 3–30 MΩ (median 11 MΩ). The amplitude histogram depicted in Fig. 2*B* shows that the measured resting potentials could be fitted reasonably well by a single Gaussian distribution, which is in sharp contrast to the results obtained in BAECs cultured in the same medium and superfused with the same solution.

Effects of ATP, ADP and adenosine on bovine aortic endothelium

A large number of compounds have been shown to induce the release of EDRF or prostaglandins in isolated large blood vessels. Some of these vasoactive agonists, for example ATP, induced characteristic potential changes in the monolayers of BAECs. A typical experiment is illustrated in Fig. 3*A*. When the monolayer was superfused with 2 μM-ATP a rapid hyperpolarization to −80 mV was observed, which turned into a plateau of about −60 mV within 1–2 min. After removal of ATP from the bath the cells depolarized to their control resting potential within 30 s. This depolarization was followed by a secondary hyperpolarization to −33 mV, which decayed within 10–15 min. Figure 3*A* also shows that repeated application of the same compound to the same monolayer produced very similar potential changes; 2 μM-ATP was enough to elicit a maximal response in the primary cultures of aortic endothelium. Application of 10 μM-ATP in different monolayers (see Fig. 3*B*) produced a hyperpolarization similar to that shown in Fig. 2*A*.

Spectrofluorometric studies on various types of endothelial cells have shown that endothelium-dependent vasodilators evoke an initial peak in intracellular calcium, which is due to Ca^{2+} release from intracellular stores, followed by a sustained plateau of intracellular Ca^{2+}, which is caused by Ca^{2+} influx from the extracellular space (reviewed by Adams, Barakeh, Laskey & van Breemen, 1989). In a previous electrophysiological study on cultured coronary endothelial cells we have suggested, that the hyperpolarization evoked by vasodilatatory agonists is caused by the opening of Ca^{2+}-activated K^+ channels and that the hyperpolarization mirrors the change in submembrane free Ca^{2+} (Mehrke & Daut, 1990). Thus the removal of external Ca^{2+} should abolish the plateau of the hyperpolarization. Figure 3*B* shows that this is indeed the case. First 10 μM-ATP was added under control conditions, and 3 min after removal of external Ca^{2+} (nominally Ca^{2+}-free bathing solution) the same concentration of ATP was added again. The initial peak of the hyperpolarization, attributable to release of Ca^{2+} from internal stores, was still present, although its amplitude was somewhat reduced (from −78 to −66 mV). However, the plateau of the hyperpolarization and the secondary hyperpolarization after removal of ATP were completely abolished. Similar results were obtained in four further BAEC monolayers. These findings are consistent with the idea that the changes in membrane potential reported here reflect changes in intracellular Ca^{2+} and that there are two different Ca^{2+} sources contributing to the overall response of the cells. Figure 3*B* also shows that removal of external Ca^{2+} evoked a transient hyperpolarization. The underlying mechanism is still unclear.

In order to get some information on the type of purinoceptor involved we applied both ATP and ADP to the same monolayers. The amplitude of the hyperpolarization elicited by 10 μM-ADP was less than 30% of that elicited by 10 μM-ATP ($n = 4$). 10 μM-adenosine had no effect on the membrane potential ($n = 5$). These findings

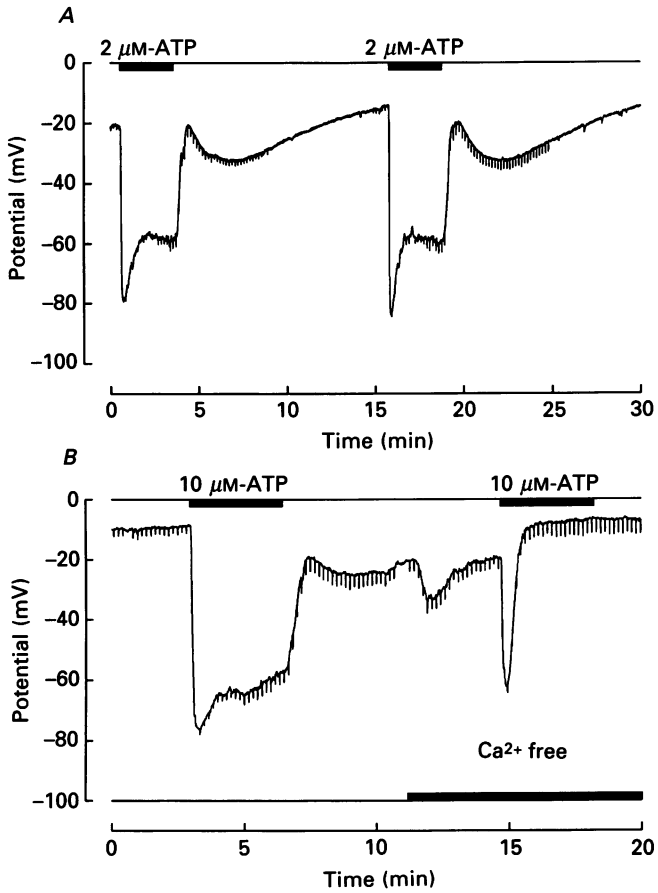

Fig. 3. Effects of ATP on the membrane potential of confluent monolayers of BAECs. *A*, superfusion of a monolayer with 2 μM-ATP for 3 min, repeated after interval of 16 min. The brief deflections in the potential trace on the left are due to rectangular current pulses (amplitude, −200 pA; duration, 250 ms) applied through the patch pipette (see text). *B*, superfusion of a different monolayer with 10 μM-ATP in the presence (left) and absence (right) of external Ca^{2+} ions.

suggest that the effects of ATP were mediated by the P_2 purinoceptor, which has a rank order of potency ATP > ADP > adenosine (Burnstock & Kennedy, 1986). This interpretation is in line with the results of Sauvé *et al.* (1988) and with our previous observation that in coronary endothelial cells the hyperpolarization evoked by ATP could not be inhibited by theophylline, an antagonist of P_1 purinoceptors (Mehrke & Daut, 1990). Thrombin at a concentration of 2 units/ml had only a small effect (< 10 mV) on the membrane potential of bovine aortic endothelium ($n = 4$).

Effects of bradykinin and ATP and at different resting potentials

Application of the vasodilatory peptide bradykinin produced a maximal hyperpolarization similar to that observed with ATP, as can be seen from Fig. 4*A*. However, there were some characteristic differences between the effects of bradykinin and ATP. During application of the maximally effective concentration of 20 nM-bradykinin the plateau of the hyperpolarization decayed more rapidly than with ATP. Furthermore, the secondary hyperpolarization after removal of the drug, which was always observed with ATP, was much smaller (< 5 mV) or absent with bradykinin. The brief potential deflections in the negative direction in Figs 3 and 4 are caused by constant current pulses of 250 ms duration applied through the patch pipette to monitor the input resistance of the monolayers. As explained above, the input resistance reflects mainly the resistance of the gap junctions connecting the different cells in the monolayer. During the initial phase of the hyperpolarization the input resistance was somewhat decreased, probably as a result of a change in membrane slope conductance. During the plateau phase the input resistance was usually unchanged. This suggests that during application of ATP the cells in the monolayer were still electrically coupled and that the patch pipette still measured the potential of a whole monolayer.

In some experiments, however, the input resistance increased during and after application of ATP or bradykinin (see Fig. 4*A*). This may be due to an increase in the lumped resistance of the gap junctions caused by the rise in intracellular Ca^{2+}. In order to test this hypothesis the EGTA concentration in the patch pipette was reduced from 10 to 1 mM in some experiments. This caused an increase in the free Ca^{2+} concentration of the pipette solution to about 1 μM and a reduction of the Ca^{2+} buffering power. In the absence of vasoactive agonists the input resistance of the monolayers was unchanged with this patch solution ($n = 10$). This is illustrated in Fig. 4*B* (left). Before addition of bradykinin our standard test pulses of -200 pA produced only a very small potential deflection that can hardly be seen at this amplification. However, during and after application of bradykinin the measured input resistance of the monolayer increased dramatically. When the standard pipette solution (containing 10 mM-EGTA, see Methods) was used in another cell of the same monolayer the input resistance remained very low after application of 20 nM-bradykinin, as can be seen on the right-hand side of Fig. 4*B*.

These experiments suggest that with 1 mM-EGTA in the pipette the Ca^{2+}-buffering and -extrusion mechanisms of the patched cell were able to keep the free intracellular Ca^{2+} concentration well below 1 μM. However, under these conditions application of bradykinin apparently increased the free cytosolic Ca^{2+} concentration enough to partially close the gap junctions between individual cells in the monolayer. With our standard pipette solution such effects could not be detected because the patched cell, and possibly the immediately surrounding cells, were dialysed with the pipette solution, which buffers the intracellular Ca^{2+} concentration to very low values. The gap junctions of the patched cell and of the immediately surrounding cells are the most relevant ones in determining the input resistance, whereas all of the cells in the monolayer contribute about equally to the measured membrane potential (Daut *et al.* 1988).

Most of the BAEC monolayers studied had resting potentials more negative than −50 mV (see Fig. 2*A*). When the membrane potential was between −80 and −90 mV ATP and bradykinin evoked only a small hyperpolarization, which was followed by a depolarization, as illustrated in Fig. 4*C*. The small amplitude of the hyper-

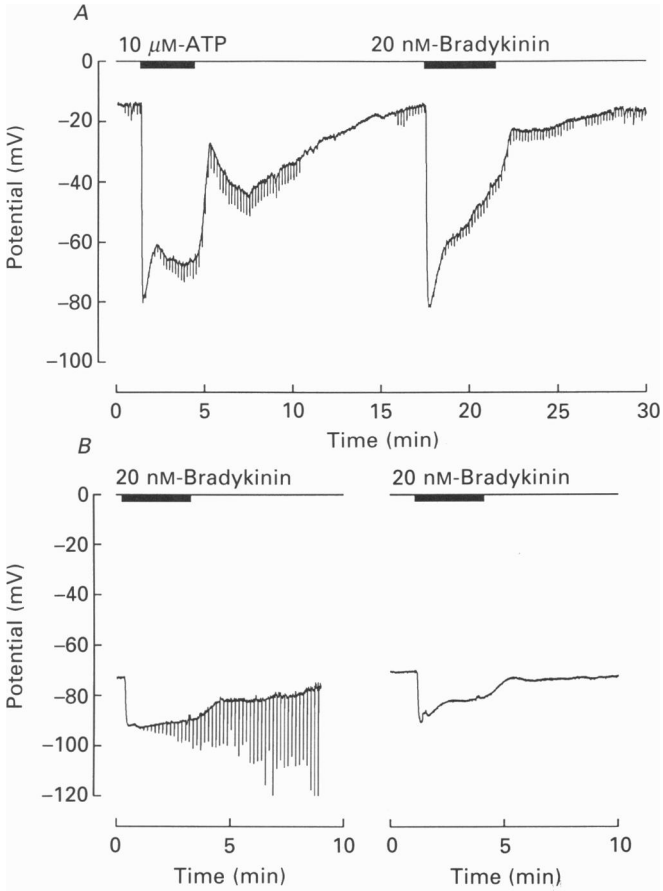

Fig. 4. For legend see facing page.

polarization can be explained by the fact that the resting potential was close to the potassium equilibrium potential (E_K). When the resting potential of a monolayer was more negative than −90 mV the initial hyperpolarization was almost completely absent and a depolarization of up to 20 mV could be induced by bradykinin and ATP, as illustrated in Fig. 4*D* ($n = 5$). Comparison of the four panels of Fig. 4 shows that the different phases of the electrical response to 20 nM,-bradykinin appear to have a different potential dependence and thus may reflect opening of different ion channels, or of a different mixture of ion channels. The initial phase lasting 10–30 s was always in the hyperpolarizing direction, even when the resting potential was negative to −80 mV. In contrast, the second phase lasting 2–3 min appears to have a reversal potential of about −80 mV (see Discussion).

As mentioned above, the bimodal distribution of resting potential may be the

result of a very flat or even negative region of the current–voltage relation. This hypothesis is supported by the observation that in several monolayers of BAECs the resting potential changed from one level to the other in the course of an experiment

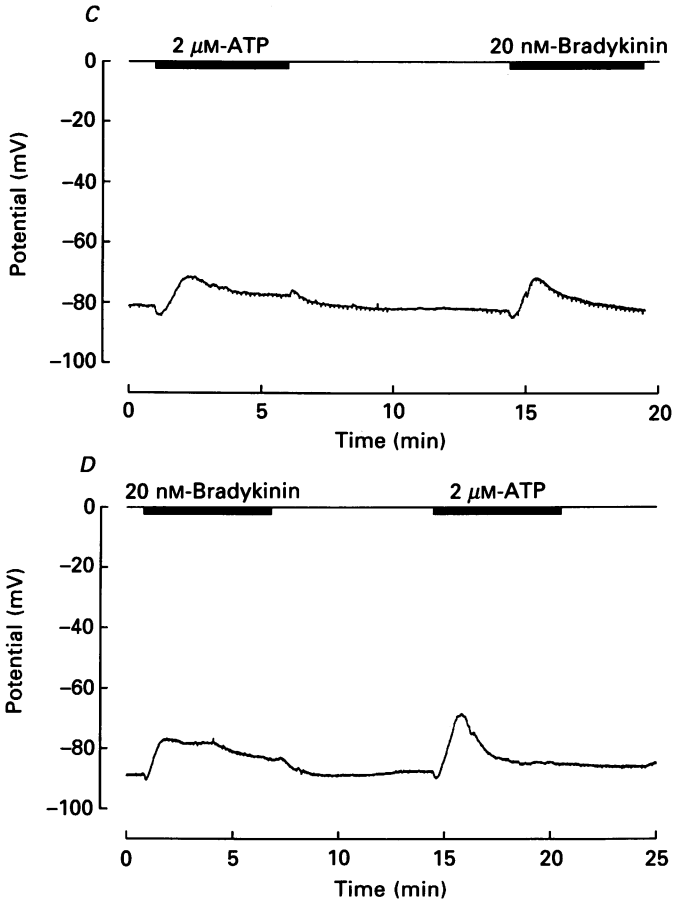

Fig. 4. The response to ATP and bradykinin of four monolayers of BAECs with different resting potentials. *A*, comparison of the effects of 10 μM-ATP and 20 nM-bradykinin in a monolayer with a resting potential of −14 mV; before application of bradykinin the amplitude of the current pulses was reduced from −200 to −100 pA. *B*, the response to 20 nM-bradykinin of a monolayer with a resting potential of −73 mV (−100 pA current pulses). The left-hand record was obtained with a pipette containing 1 mM-Ca^{2+} and 1 mM-EGTA, the right-hand record was from the same monolayer with a pipette containing 1 mM-Ca^{2+} and 10 mM-EGTA. *C* and *D*, the response to 2 μM-ATP and to 20 nM-bradykinin of a monolayer with a resting potential of −80 mV (*C*) and of a monolayer with a resting potential of −89 mV (*D*).

($n = 6$), as illustrated in Figure 5*A*. For the first 2 h the monolayer had a resting potential more negative than −80 mV and 2 μM-ATP produced only a small potential change. Then the resting potential changed to −28 mV within 15 min. A second application of 2 μM-ATP evoked a much larger change in membrane potential. However, after washing out the ATP the potential again returned to the more negative potential. The new level was usually maintained for more than an

hour and was apparently homogeneous throughout the monolayer, because the same potential was measured when different cells of the monolayer were patched. These experiments show that the electrical behaviour of aortic endothelium resembles the electrical behaviour of cardiac Purkinje fibres, which also have two levels of resting

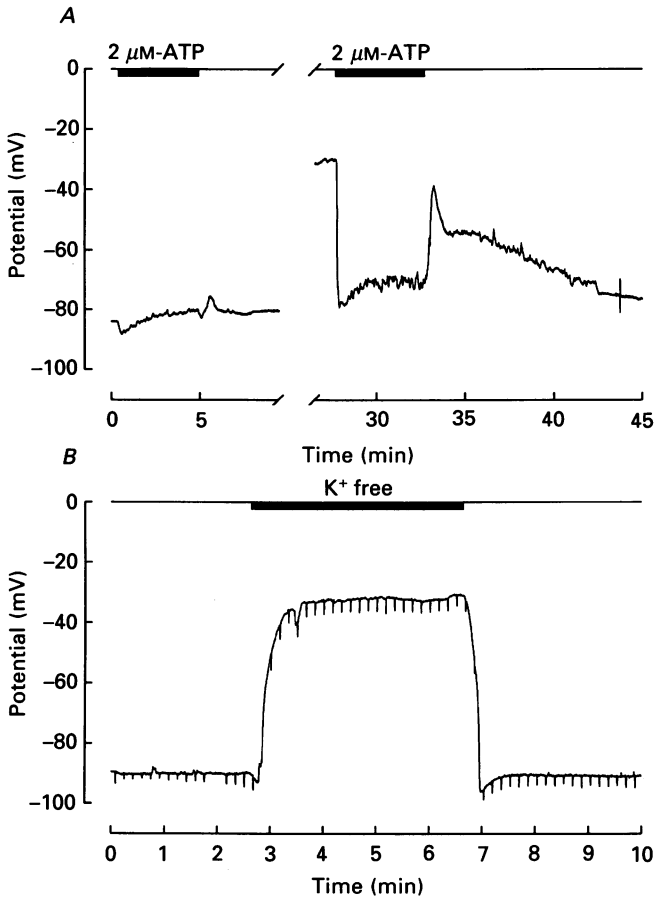

Fig. 5. *A*, spontaneous variation of the membrane potential of a BAEC monolayer and its effect on the response to 2 μM-ATP. After the first application of ATP the monolayer depolarized. Note the time interval between the two parts of the record. After the second application of ATP the membrane potential returned to its original level for more than 30 min. *B*, the effect of removal of external potassium on the membrane potential of a monolayer of BAECs.

potential when exposed to reduced external potassium (Gadsby & Cranefield, 1977). There is yet another electrical property of the endothelium that is reminiscent of cardiac Purkinje fibres. When exposed to K^+-free solution monolayers of bovine aortic endothelium depolarized to about -30 mV, as can be seen from Fig. 5*B* ($n = 3$). This depolarization was most likely due to the potassium dependence of the conductance of the inward rectifier K^+ channels and to inhibition of the electrogenic sodium pump (Daut *et al.* 1988).

Application of 2 μM-acetylcholine ($n = 4$) had virtually no effect on the membrane potential of BAECs (not shown). The effects of histamine were more variable. Application of 10 μM-histamine to monolayers with a resting potential < 40 mV produced a hyperpolarization of < 5 mV ($n = 4$). However, during superfusion with

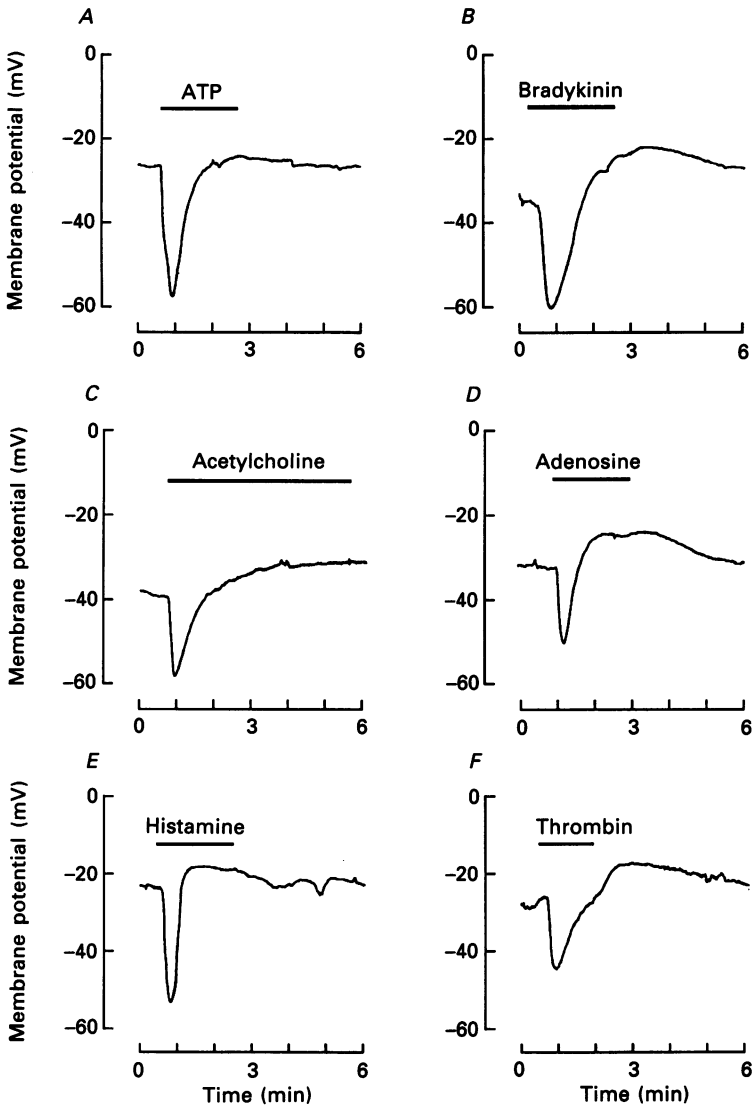

Fig. 6. The effects of various endothelium-dependent vasodilators on the membrane potential of confluent monolayers of GCECs. The different monolayers were superfused with 1 μM-ATP (*A*); 10 nM-bradykinin (*B*); 2 μM-acetylcholine (*C*); 2 μM-adenosine (*D*); 10 μM-histamine (*E*) and 2 units/ml thrombin (*F*).

very high concentrations of histamine (100 μM) we observed a brief hyperpolarization of up to 25 mV in two out of five experiments (not shown). This effect was still very much smaller than the response to 2 μM-ATP in the same monolayer. Thrombin and adenosine had only very small effects on the resting potential of BAECs. All of these substances are known to cause release of EDRF in various vascular beds.

Effects of vasoactive compounds on guinea-pig coronary endothelium

The electrical response of confluent monolayers of GCECs to various agonists was quite different from that observed in BAECs. In coronary endothelium ATP and bradykinin usually evoked a transient hyperpolarization of 10–40 mV, which then turned into a depolarization. Two typical records are shown in Fig. 6*A* and *B*. The duration of the hyperpolarization, measured half-way between resting potential and peak negativity, was shorter than 1 min in GCECs, and it was followed by a sustained depolarization. A similar response could be induced in GCECs by application of acetylcholine (2 μM), thrombin (2 units/ml), histamine (10 μM) and adenosine (1 μM), as can be seen from panels *C–F* of Fig. 6. This is in marked contrast to the results obtained in BAECs. Thus the endothelial cells isolated from guinea-pig heart appear to express receptors not present in cultured macrovascular endothelial cells isolated from bovine aorta.

Acetylcholine elicited a hyperpolarization in all of the GCEC monolayers studied ($n = 11$). The lack of effect ACh in BAECs is probably due to rapid loss of receptors in primary culture (Lückhoff & Busse, 1990). It is not yet known why in cultured BAECs the ACh receptors are lost much more rapidly than in GCECs. The response to adenosine was seen only in about 60% of the monolayers of GCECs (see Mehrke & Daut, 1990). Thrombin evoked an electrical response in 85% of the experiments carried out on monolayers of GCECs ($n = 47$); histamine elicited a hyperpolarization in 87·5% of the experiments ($n = 16$). The much larger effect of thrombin and histamine in GCECs as compared to BAECs may reflect a greater abundance of thrombin and histamine receptors in the microvasculature. The main point of Fig. 7 is to illustrate the qualitative similarity of the response to GCECs to the six endothelium-dependent vasodilators tested. These findings suggest that all of these substances cause an increase in intracellular Ca^{2+}. Since an increase in intracellular, Ca^{2+} can stimulate the release of EDRF our results are consistent with the idea that acetylcholine, thrombin, histamine, and adenosine can induce release of EDRF in guinea-pig coronary arteries.

The difference between GCECs and BAECs in the time course of the electrical response to bradykinin is illustrated in Fig. 7. Panel *A* is from a confluent monolayer of BAECs and panel *B* is from a confluent monolayer of GCECs. Both records show the typical response to maximally effective concentrations of bradykinin (20 nM). In GCECs the peak of the hyperpolarization was not as negative as in BAECs, it was usually in the range -45 to -75 mV. Furthermore, the hyperpolarization decayed much more rapidly and in most experiments turned into a depolarization within 90 s after application of bradykinin. In Ca^{2+}-free solution the hyperpolarization elicited by bradykinin in GCECs was only reduced to 72% (Mehrke & Daut, 1990), which contrasts with the drastic effect of Ca^{2+}-free solution in BAECs shown above (Fig. 3*B*) (see Discussion). A more detailed description of the effects of bradykinin and

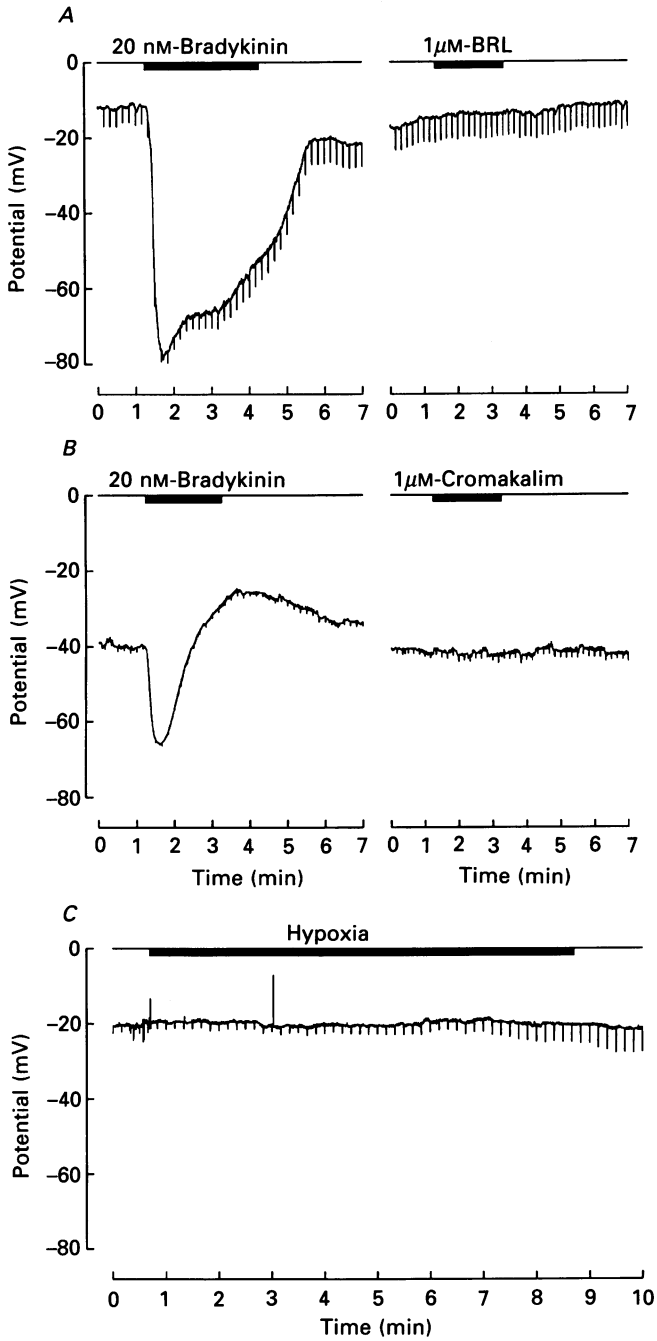

Fig. 7. *A*, the effects of 20 nM-bradykinin on a monolayer of BAECs (left) and the lack of effect of the K^+ channel opener lemakalim (1 μM) on the same monolayer (right). *B*, the effects of 20 nM-bradykinin on a monolayer of GCECs (left) and the lack of effect of the K^+ channel opener cromakalim (1 μM) on the same monolayer (right). *C*, the effect of hypoxia (P_{O_2} = 18 mmHg) on the membrane potential of a monolayer of BAECs. Current pulses of −200 pA were applied. Note the increase in the resulting potential deflection.

ATP on GCECs observed under similar conditions has been given previously (Mehrke & Daut, 1990).

Effects of K^+ channel openers and hypoxia

It has recently been shown that openers of ATP-sensitive K^+ channels (K^+_{ATP} channels) such as cromakalim can cause a marked dilatation of coronary arteries in isolated, perfused guinea-pig hearts and that hypoxic vasodilatation may also be related to the opening K^+_{ATP} channels (Daut *et al.* 1990). In order to find out whether such K^+ channels exist in the endothelium we superfused cultured BAECs and GCECs with the K^+ channel openers cromakalim (BRL 34915) and lemakalim (BRL 38227), which is the active enantiomer of cromakalim. Figure 7*A* shows that 1 μM-lemakalim had no effect in BAECs that responded with a typical hyperpolarization to bradykinin. Figure 7*B* illustrates that 1 μM-cromakalim had no effect on the membrane potential of a monolayer of GCECs. The same result was obtained in eight further experiments with BAECs and GCECs.

Since the release of EDRF from endothelial cells may be stimulated by moderate hypoxia (Pohl & Busse, 1989) we also looked at the effects of hypoxia on the resting potential. By equilibrating the superfusate with 100% N_2 the P_{O_2} in the recording chamber was reduced to 12–32 mmHg (see Methods). The mean P_{O_2} of the hypoxic solution was 22 ± 6 mmHg. Figure 7*C* shows a typical experiment in which a reduction of the P_{O_2} from 150 to 18 mmHg caused only a minor change in the membrane potential of GCECs. In GCECs the change in resting potential measured during 15 min hypoxia in the range 12–32 mmHg was $+0{\cdot}5 \pm 1{\cdot}4$ mV ($n = 13$); in BAECs it was $+0{\cdot}5 \pm 1{\cdot}3$ mV ($n = 19$).

However, the superfusion of the cells with hypoxic solution was not entirely without effect. In twenty-six experiments (ten on GCECs, sixteen on BAECs) we measured the change in input resistance after 15 min of hypoxia. In nine experiments the input resistance was unchanged, but in the other seventeen experiments the input resistance increased by up to 300%, as illustrated in Fig. 7*C*. The median of the change in input resistance was + 9% ($n = 26$). In most of the experiments the increase in input resistance was reversed within 20 min during reoxygenation. Since the measured input resistance of the monolayer is primarily determined by the gap junctions of the patched cell we believe that there was a tendency for the cells to uncouple during hypoxia, perhaps as a consequence of changes in intracellular Ca^{2+} and/or pH (see Discussion).

DISCUSSION

Similarities and differences between aortic and coronary endothelial cells

In BAECs with a low resting potential, application of ATP of bradykinin induced a rapid initial hyperpolarization followed by the sustained plateau (Figs 3*A* and 4*A*). The time course of these potential changes was remarkably similar to the time course of the changes in intracellular Ca^{2+} measured in other macrovascular endothelial cells (Hallam, Jacob & Merritt, 1988, 1989; Schilling *et al.* 1988) using spectrofluorometric techniques. Like the plateau in $[Ca^{2+}]_i$, the plateau of the hyperpolarization was abolished in the absence of external Ca^{2+} (Fig. 2*B*). This result

is consistent with our previous findings in GCECs (Mehrke & Daut, 1990) suggesting that the agonist-induced changes in the membrane potential of endothelial cells are mainly due to changes in sub-membrane intracellular calcium and the subsequent increase in the open probability of Ca^{2+}-activated K^+ channels. Single-channel recordings suggest that the 'threshold' for Ca^{2+} activation of these channels may be below 400 nM (Sauvé *et al.* 1988), possibly even below 100 nM (Colden-Stanfield *et al.* 1990).

The time course of the changes in intracellular Ca^{2+} is important because $[Ca^{2+}]_i$ probably controls the release of EDRF and prostacyclin (Lückhoff, Pohl, Mülsch & Busse, 1988; Moncada *et al.* 1989). The initial phase of the hyperpolarization and the initial rapid rise in intracellular Ca^{2+} are independent of extracellular Ca^{2+} and are due to release of Ca^{2+} from intracellular stores. The second phase of the hyperpolarization and the sustained plateau of $[Ca^{2+}]_i$ depend on external Ca^{2+} and are due to Ca^{2+} influx from the extracellular space (for review, see Adams *et al.* 1989). The removal of external Ca^{2+} had a much more drastic effect in aortic than in coronary endothelium. In BAECs the hyperpolarization evoked by bradykinin or ATP lasted as long as the vasodilators were present, i.e. at least 3–4 min. Removal of external Ca^{2+} shortened the hyperpolarization evoked by ATP by an order of magnitude (Fig. 2). In GCECs the transient hyperpolarization induced by bradykinin or ATP in normal extracellular Ca^{2+} lasted less than 1 min (Figs 6 and 7), and it was reduced by only 28%, on average, in Ca^{2+}-free solution (Mehrke & Daut, 1990). These findings suggest that the agonist-induced increase in transmembrane Ca^{2+} influx was much larger in aortic than in coronary endothelial cells. This conclusion is supported by spectrofluorometric (Indo-1) measurements showing that the Ca^{2+} transient induced by bradykinin decayed much more rapidly in GCECs than in BAECs (A. Lückhoff, R. Busse, G. Mehrke, U. Pohl & J. Daut, unpublished results). Whether this represents a general difference between microvascular and macrovascular endothelium remains to be investigated.

Another difference between aortic and coronary endothelial cells can be inferred from the distribution of the measured resting potentials. In BAECs the resting potential histogram showed a bimodal distribution with peaks around −25 and −85 mV, whereas in GCECs the resting potentials measured in different monolayers showed a roughly Gaussian distribution around −35 mV (Fig. 2). The bimodal distribution in BAECs is consistent with voltage-clamp measurements in BAECs and in single bovine pulmonary artery endothelial cells showing a very flat current–voltage relation (Takeda, Schini & Stoeckel, 1987) with a negative slope between −50 and −25 mV (Cannell & Sage, 1989). An interesting consequence of the negative slope of the current–voltage relation of the endothelial cell membrane is that the current–voltage relation could cross the zero-current axis three times, which causes the membrane potential to become bistable. This means that a very small increase in outward current would be sufficient to change the resting potential from a relatively positive value (near −25 mV) to a more negative value near the potassium equilibrium potential, which is in agreement with our experimental results (Fig. 5). The results of Takeda *et al.* (1987) suggest that cultured BAECs show a bimodal distribution of resting potentials also at room temperature. It will be interesting to see whether the membrane potential of macrovascular endothelium *in*

vivo can also switch between two different states. The fact that two levels of resting potential have not been observed in GCECs suggests that the current–voltage relation in GCECs has no negative slope. This conclusion is in agreement with our previous voltage-clamp experiments in single GCECs (Daut *et al.* 1988).

The molecular mechanism underlying the negative slope of the current–voltage relation in BAECs may be a decrease in the open probability of the inwardly rectifying K^+ channel with depolarization, as has been found in cardiac ventricular muscle (Kurachi, 1985). A similar inward rectifier K^+ channel has been observed in BAECs (Takeda *et al.* 1987). It should be noted, however, that the shape of the current–voltage relation is not a constant property of macrovascular endothelial cells. When intracellular Ca^{2+} rises the current–voltage relation will probably become more linear because the open probability of Ca^{2+}-activated K^+ channels increases with depolarization.

Various groups have recently shown that hyperpolarization increases Ca^{2+} influx (Schilling, 1989; Laskey *et al.* 1990; Lückhoff & Busse, 1990). This leads to a further hyperpolarization through the opening of more Ca^{2+}-activated K^+ channels. The potential dependence of Ca^{2+} influx and of the open probability of the inward rectifier K^+ channels represent positive feedback mechanisms. These mechanisms may allow for a regenerative hyperpolarizing response, i.e. a hyperpolarization evoked locally in the depolarized state could be propagated along the wall of a blood vessel. This might be an important amplification factor for the release of EDRF.

In coronary endothelium there are clearly two opposing events activated by vasodilatatory agonists: the rise in intracellular Ca^{2+} leading to a hyperpolarization, and the subsequent depolarization (Figs 6 and 7), which has been attributed to closing of K^+ channels by protein kinase C (Mehrke & Daut, 1990). At very negative resting potentials a diphasic membrane potential change was also observed in BAECs (Fig. 4). The initial phase lasting 10–30 s was always in the hyperpolarizing direction. Since the potassium equilibrium potential at 37 °C was calculated to be about −104 mV (assuming an intracellular potassium concentration of 150 mM) the initial phase may represent the opening of K^+ selective channels. The subsequent phase lasting several minutes appeared to reverse polarity at about −80 mV. This phase might be related to the fact that additional, less K^+ selective channels are opened during the plateau, or to the fact that some of the K^+ channels may be closed by second messengers, as postulated for GCECs. Thus the chemical events responsible for the depolarization in GCECs may also be present in BAECs. On the other hand, a depolarization was never observed in aortic endothelial cells with low resting potential. This can be explained by the fact that the depolarizing effect may be overridden by the hyperpolarization evoked by the Ca^{2+} influx from the extracellular space.

The antagonism between a depolarizing and a hyperpolarizing force may be the cause of the oscillations in cytosolic Ca^{2+} (Sage, Adams & van Breemen, 1989; Laskey *et al.* 1990) and in membrane potential (Mehrke & Daut, 1991) which have been observed in endothelial cells under some conditions. The secondary hyperpolarization observed after removal of ATP from the bathing solution (Figs 3 and 4) may be related to the simultaneous presence of agonist-induced hyperpolarizing and depolarizing mechanisms in BAECs, which are activated and removed with different

kinetics. The secondary hyperpolarization after removal of ATP might reflect the fact that the dissociation of ATP from the receptor is faster than the return of intracellular Ca^{2+} to its resting value. The absence of the secondary hyperpolarization after removal of bradykinin could then be explained by the much higher affinity of bradykinin, which might be associated with a lower rate of dissociation from the receptor.

Implications for the regulation of coronary blood flow

We have found that cultured coronary endothelial cells, but not aortic endothelial cells, hyperpolarize upon application of low concentrations of adenosine, thrombin, histamine and acetylcholine (Fig. 6). High concentrations of adenosine (Lückhoff & Busse, 1986), histamine (this paper) and acetylcholine (Olesen *et al.* 1988) sometimes elicit a small response in cultured BAECs, and freshly isolated BAECs usually respond to acetylcholine (Busse *et al.* 1988). Since all of our cells were cultured for about the same length of time we believe that this difference in sensitivity to various transmitters may reflect a genuine difference in the density of the respective receptors in the two types of endothelium.

Our observation that adenosine elicited the same electrical response as the other endothelium-dependent vasodilators suggests that extracellular adenosine can also cause a rise in intracellular Ca^{2+} and thus a release of EDRF and prostacyclin from coronary endothelium in culture. *In vivo*, adenosine is present both in the blood (Sollevi, Torssell, Öwall, Edlund & Lagerkranser, 1987) and in the interstitial space (Decking, Jüngling & Kammermeier, 1988) at vasoactive concentrations. The endothelium continuously releases and takes up adenosine and forms a metabolic barrier that allows only a slow exchange between intravascular and perivascular adenosine (Nees, Herzog, Becker, Böck, Des Rosiers & Gerlach, 1985; Gerlach, Becker & Nees, 1987). Furthermore, the endothelium degrades extracellular adenine nucleotides to adenosine via membrane-bound ectonucleotidases (Gordon, 1986). Thus release of EDRF by intravascular and perivascular adenosine or ATP may play a role in the regulation of platelet aggregation (Alheid, Reichwehr & Förstermann, 1989) and in the regulation of coronary flow. It should be noted, however, that in a recent study on isolated perfused guinea-pig hearts no additional release of endothelium-derived nitric oxide could be detected after intra-arterial application of adenosine (Kelm & Schrader, 1990). One way to reconcile these contradictory findings would be to assume that the adenosine receptors are primarily located in the arteriolar segment of the coronary vessels and that the amount of nitric oxide release is too small to be detected in the effluent.

In the terminal arterioles numerous gap junctions between endothelial cells and the underlying one or two layers of vascular smooth muscle cells have been found (Rhodin, 1980; Simionescu & Simionescu, 1984). In a recent electron microscopical study on primate hearts myoendothelial gap junctions were found to be more abundant in coronary resistance vessels than in large coronary arteries (K.-F. Bürrig, personal communication). Therefore it appears possible that in the small arterioles, which to a large extent determine the rate of blood flow, smooth muscle cells and endothelial cells form a functional unit that has the same resting potential. This would imply that the electrical behaviour of the endothelium cells could

influence coronary blood flow not only by releasing EDRF and prostaglandins but also by modulating the membrane potential of smooth muscle cells of the coronary resistance vessels. The principal mechanism by which small changes in membrane potential are transduced into large changes in smooth muscle tone is the steep potential dependence of the steady-state open probability of Ca^{2+} channels in vascular smooth muscle cells (see Nelson, Patlak, Worley & Standen, 1990).

It has recently been shown that hypoxic vasodilatation in isolated perfused guinea-pig heart can be mimicked by openers of ATP-sensitive K^+ channels (K^+_{ATP} channels) like cromakalim, and can be completely prevented by the antidiabetic sulphonylurea glibenclamide, which blocks K^+_{ATP} channels (Daut *et al.* 1990). However, if K^+_{ATP} channels were present in the endothelium as well, the vasodilatory effect of K^+ channel openers could be partly due to a hyperpolarization of the endothelium, which would give rise to an increase Ca^{2+} influx, and thus to an increased release of EDRF. In our present experiments cromakalim was found to have no effect on the membrane potential of BAECs and GCECs (Fig. 7). Since the specific membrane resistance of coronary endothelial cells is very high (Daut *et al.* 1988) the whole-cell clamp of confluent monolayers is a very sensitive method for detecting agonist-induced transmembrane currents. Thus our present results suggest that neither BAECs nor GCECs possess K^+_{ATP} channels or any other channels sensitive to K^+ channel openers like cromakalim. These findings support the idea that hypoxic dilatation of coronary arteries is mainly due to the opening of K^+_{ATP} channels in coronary smooth muscle cells (Daut *et al.* 1990).

During ischaemia the P_{O_2} in the heart falls to values close to zero, but during moderate to severe hypoxia the intravascular P_{O_2} in arteries which are not occluded is probably between 10–30 mmHg. Our observation that in some of the experiments the input resistance of the monolayers increased during hypoxia in the range 12–32 mmHg (Fig. 7) was unexpected. This might represent a pathophysiologically important protective mechanism that prevents the propagation of electrical signals between hypoxic and normoxic endothelial cells. However, our method does not give us a reliable estimate of the magnitude of such changes in gap-junction resistance, because $[Ca^{2+}]_i$ and pH in the patched cell, and possibly in the neighbouring cells, are buffered by the patch solution (see Fig. 4). Thus the effects of hypoxia on the resistance of the gap junctions would be underestimated in experiments in which the cytosol of the impaled cell was well dialysed with the patch solution, and the degree of electrical uncoupling would depend on the rate of diffusional exchange between patch pipette and cytosol. A different experimental approach is needed to obtain a quantitative description of the possible effects of P_{O_2} on cell-to-cell coupling in the endothelium.

In contrast, the measurement of the membrane potential with the whole-cell clamp of confluent monolayers represents the average of hundreds of cells (Daut *et al.* 1988) and is unlikely to be affected by the composition of the patch solution as long as the cells are not completely uncoupled (see Fig. 4*B*). We have found that the membrane potential of cultured GCECs does not change significantly during a decrease of P_{O_2} down to 12 mmHg (Fig. 7). This suggests that endothelial cells do not contribute to hypoxic vasodilatation in the intact guinea-pig heart by means of a hyperpolarizing mechanism. It has been found, however, that cultured endothelial

cells can release prostaglandins and EDRF in increased amounts when exposed to low P_{O_2} values (Pohl & Busse, 1989) and that intracellular Ca^{2+} increases moderately during hypoxia (Lückhoff, Pohl & Busse, 1986). Thus we cannot exclude that in our experiments hypoxia induced a small increase in $[Ca^{2+}]_i$ that was not sufficient to affect the endothelial membrane potential. Nevertheless, our results suggest that hypoxia *per se* (in the range 12–32 mmHg) may be much less effective in eliciting endothelium-dependent dilatation of coronary arteries than endogeneous vasoactive compounds that may be released from cardiac muscle cells, blood cells and autonomic nerves during cardiac hypoxia and ischaemia.

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