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Endothelium-dependent hyperpolarization of canine coronary smooth muscle

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1 Experiments were designed to determine whether endothelium-dependent relaxing factor(s) released by acetylcholine from the canine femoral artery influences the membrane potential of coronary arterial smooth muscle.

2 The membrane potential was recorded in small canine coronary arteries (internal diameter $\leqslant 500\,\mu m$; without endothelium) by means of intracellular microelectrodes. The organ bath also contained a strip of left descending coronary artery without endothelium in which isometric force was measured to bioassay relaxing factor(s) as well as segments of femoral artery with endothelium, which served as the source of endothelium-derived relaxing factor(s).

3 Acetylcholine induced endothelium-dependent, transient hyperpolarizations and relaxations that were not affected by indomethacin.

4 Inhibition of the sodium-potassium pump by ouabain or potassium-free solution did not inhibit the relaxation to acetylcholine but prevented the corresponding hyperpolarization.

5 Activation of the sodium-potassium pump of the smooth muscle cells by readmission of potassium ions after incubation in potassium-free solution caused relaxation and marked hyperpolarization.

6 These results suggest that endothelium-derived relaxing factor(s) induces hyperpolarization of vascular smooth muscle of the canine coronary artery, possibly by activation of sodium-potassium pumping, but that this effect on the cell membrane may only partially explain endothelium-dependent relaxations evoked by acetylcholine.

Introduction

The endothelial cells play a major role in mediating the inhibitory effects of acetylcholine in isolated arteries (Furchgott & Zawadzki, 1980; De Mey & Vanhoutte, 1981) by releasing a diffusable substance(s) which can be bioassayed (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985). Interactions between the Na^+, K^+-pump and relaxations induced by acetylcholine have been described (De Mey & Vanhoutte, 1980; Rapoport *et al.*, 1985). Activation of that pump leads to hyperpolarization of vascular smooth muscle (Fleming, 1980; Haddy, 1983; Hermsmeyer, 1983a). The purpose of the present study was to determine whether or not a product(s) released by the endothelium which induces relaxation of bioassay tissues (Rubanyi *et al.*, 1985) influences the membrane potential of the smooth muscle of the canine coronary artery.

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Methods

Experiments were performed on distal left anterior descending arteries, small (internal diameter $\leqslant 500\,\mu m$) coronary arteries taken from the epicardium of the left ventricle and femoral arteries of mongrel dogs of either sex (18 to 28 kg) anaesthetized with sodium pentobarbitone ($30\,mg\,kg^{-1}$, i.v.). The blood vessels were carefully cleaned of adherent connective tissue.

Organ chamber experiments

In preliminary experiments, rings (with and without endothelium) of left anterior descending coronary arteries (approximately 4 mm in length) and small coronary arteries (approximately 7 mm in length) were suspended in 25 ml organ chambers filled with modified Krebs-Ringer bicarbonate solution (bubbled with a 95% O_2 and 5% CO_2 gas mixture; pH 7.4) with the following composition (mM): NaCl

118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium EDTA 0.026 and glucose 11.1 (control solution). Isometric force was measured by means of strain gauges (Gould UC2). Rings were brought to the optimal point of their length-tension relationship by repeated stimulation with KCl (20 mM). The experiments were performed after repeated rinses with control solution, and an equilibrium period of 1 h.

Electrophysiological experiments

Two rings of femoral artery (5 mm in length) were slit along the longitudinal axis and pinned down (endothelium upward) to the bottom of a 3 ml organ bath. In most experiments care was taken to preserve the endothelium as intact as possible, as it served as the source of endothelium-derived relaxing factor(s); however, in some rings the endothelium was mechanically removed by inserting a pair of watchmaker's forceps into the lumen and rolling the ring back and forth on saline-wetted paper (De Mey & Vanhoutte, 1981). Between the two strips of femoral arteries were mounted: (a) a circumferential strip of left anterior descending coronary artery (with the endothelium removed as previously mentioned; Cohen *et al.*, 1983) pinned down at one end and connected at the other to a force transducer (Gould UC2) for the measurement of isometric tension; it was used to bioassay released endothelium-derived factor(s); and (b) a segment (3 mm) of small coronary artery, slit along the longitudinal axis, and pinned down with the adventitial side downward to allow the penetration of microelectrodes from the intimal surface of the blood vessel (Belardinelli *et al.*, 1979; Harder *et al.*, 1979); the dissection and the opening of the small coronary artery resulted in the removal of the endothelium, as demonstrated histologically (Cohen *et al.*, 1983). The arterial strips could be stimulated extracellularly with rectangular pulses (20–100 V, 50–100 ms) by means of platinum plate electrodes placed at either side of the vessels (field stimulation).

Transmembrane potentials were recorded with glass microelectrodes filled with 3 M KCl, with a tip resistance of 30 to 80 megohms. The microelectrode was mounted on a AUS JENA sliding micromanipulator. The potential recorded was amplified by means of a WPI recording preamplifier with capacitance-neutralization. Signals (i.e. electrical and mechanical activity) were monitored on an oscilloscope and continuously recorded on paper (Gould) and on tape (Hewlett Packard); the latter allowed replay for further analysis. Impalements were not accepted as valid unless they were signalled by a sudden change in voltage, and were maintained for at least 3 min; at that point the membrane potential had stabilized. If the membrane potential decreased spontaneously, indicating that cell damage had occurred, the measurement was disregarded. Some impalements were maintained for up to 1 h. Changes in membrane potential caused by acetylcholine were analysed only if the penetration of the electrode was maintained during the infusion of the drug. Tip resistance was monitored before and after impalements; if any major changes due to plugging or breaking had occurred the data were disregarded. The tissues were continuously superfused with control solution (see above) maintained at 37°C and aerated with a gas mixture containing 95% O_2 and 5% CO_2.

Drugs

The following pharmacological agents were used: acetylcholine chloride, indomethacin, prostaglandin $F_{2\alpha}$, ouabain (Sigma Chemical Co., St. Louis, MO) and tetraethylammonium chloride (Eastman Kodak, Rochester, NY). All concentrations are expressed in molar concentrations (M) in the superfusate. Drugs were dissolved in distilled water except indomethacin which was prepared as a stock solution in an equimolar concentration of $NaCO_3$.

Calculations and statistics

Data are shown as means ± standard error of the mean (s.e. mean); N represents the number of cells impaled; in each experiment n indicates the number of tissues from different dogs tested. Statistical evaluation of the data was by Student's t test for paired or unpaired observations. When more than two means were compared, a one-way analysis of variance was used. Scheffe's test for multiple comparison was employed to identify differences among groups. Values were considered to be statistically different when P was smaller than 0.05.

Results

Organ chamber experiments

Acetylcholine-induced relaxation Rings of left anterior descending and small epicardial coronary arteries with endothelium taken from the same dogs were studied in parallel; at optimal length, basal tension averaged 10.4 ± 0.7 g ($n = 12$) for the left anterior descending coronary, and 3.1 ± 0.2 g ($n = 16$) for the small coronary arteries. The rings were contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M) and exposed cumulatively to increasing concentrations of acetylcholine. Acetylcholine induced comparable, concentration-dependent, sustained

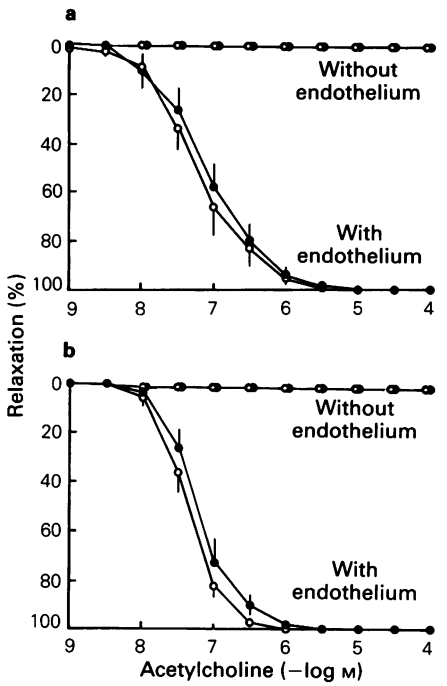

Figure 1 (a) Effect of acetylcholine on rings of left anterior descending (○) and small epicardial (●) coronary arteries with and without endothelium, during contraction to prostaglandin $F_{2\alpha}$ (2×10^{-6} M). The relaxations are expressed as a percentage of the contraction to prostaglandin $F_{2\alpha}$ and shown as means with s.e. mean indicated by vertical lines ($n = 8$ for the left anterior descending coronary artery, $n = 11$ for the small coronary artery). (b) Same experiment in the presence of indomethacin (10^{-5} M; $n = 5$ for the left anterior descending and $n = 6$ for the small coronary artery).

relaxations of both arteries (Figure 1a). The negative logarithms of the EC_{50} to acetylcholine were not statistically different (7.21 ± 0.16 and 7.06 ± 0.13, for the left anterior descending and the small coronary artery, respectively). Indomethacin (10^{-5} M) did not significantly affect the endothelium-dependent response to acetylcholine (Figure 1b): the negative logarithms of the EC_{50} to acetylcholine were not statistically different (7.38 ± 0.08 and 7.23 ± 0.11, respectively). In rings without endothelium, acetylcholine did not induce statistically significant changes in tension (Figure 1).

Potassium induced relaxation Rings of left anterior descending coronary artery without endothelium were equilibrated for 30 min in solution containing a low concentration of potassium (0.6 mM; achieved by removal of KCl and replacing half of the KH_2PO_4 by $NaPO_4$) and then contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M). Increasing the potassium concentration from 0.6 to 8.1 mM (by adding 7.5 mM KCl) induced complete relaxation ($-99.3 \pm 0.75\%$ of the contraction induced by prostaglandin $F_{2\alpha}$; $n = 5$). In order to rule out the involvement of changes in osmolarity in the relaxation induced by the addition of potassium ions, identical experiments were performed with equimolar substitution of NaCl for KCl; K^+ ions induced a relaxation which averaged $99 \pm 7.9\%$ of the contraction induced by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) ($n = 5$; not statistically different from the effect of K^+ without compensation for changes in osmolarity). In further studies, changes in potassium concentration were made without compensation for changes in osmolarity. After incubation in the low potassium solution and ouabain (10^{-6} M), readmission of potassium ions (7.5 mM) induced contraction ($+32.3 \pm 14\%$ of the increase in tension evoked by prostaglandin $F_{2\alpha}$; $n = 5$).

Electrophysiological studies

The smooth muscle cells of the small coronary arteries had stable resting membrane potentials which averaged −55 mV (Table 1). No action potentials were recorded when extracellular electrical pulses (20–100 V, 50–100 ms, 0.1–1 Hz) were applied. The addition of tetraethylammonium (1.5×10^{-2} M) significantly depolarized the cells and evoked the appearance of action potentials which occurred either spontaneously or upon electrical field stimulation (Figure 2, Table 1). Prostaglandin $F_{2\alpha}$ (2×10^{-6} M) induced contraction of the left anterior descending coronary arteries with no significant change in membrane potential in the small coronary arteries (Table 1).

Acetylcholine Strips of unstimulated left anterior descending coronary arteries without endothelium developed spontaneous tone. When femoral arteries with intact endothelium were present in the organ bath, acetylcholine (10^{-5} M) induced complete relaxation of unstimulated left anterior descending arteries and transiently hyperpolarized smooth muscle of the small coronary artery to -61.1 ± 1.6 mV (number of cells: $N = 8$, number of dogs: $n = 6$, statistically different from the membrane potential in control solution; paired t test, $P < 0.05$; Table 2). When the blood vessels were exposed to prostaglandin $F_{2\alpha}$ (2×10^{-6} M), acetylcholine (10^{-5} M) induced complete relaxation of the left anterior descending coronary arteries and hyperpolarized the small arteries to -62.8 ± 2.9 mV

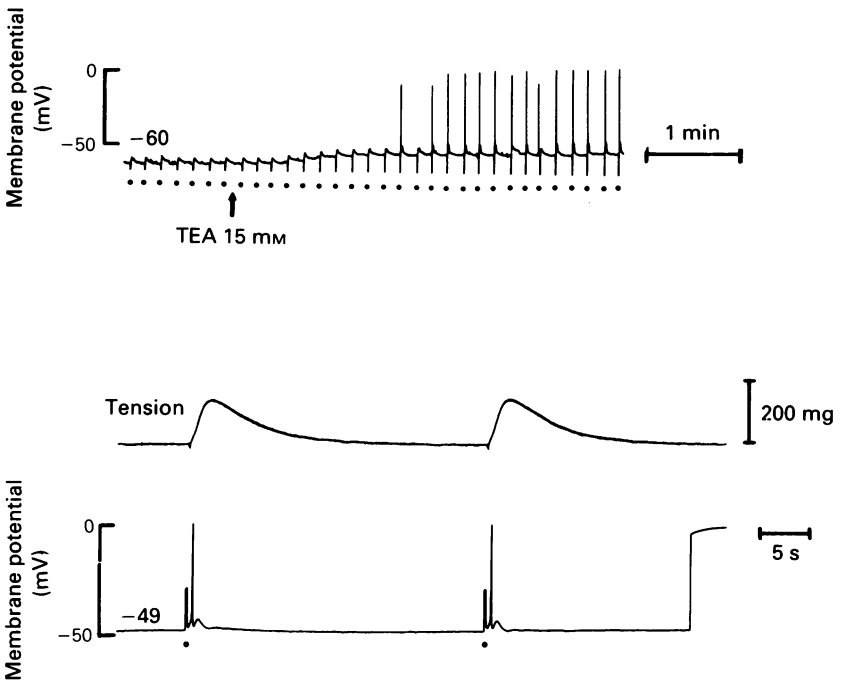

Figure 2 Effect of tetraethylammonium (TEA, 1.5×10^{-2} M) on the membrane potential and the excitability of the smooth cells of the canine coronary artery. Upper trace: Tetraethylammonium induced depolarization and overshooting spikes during electrical stimulation of a small canine coronary artery. ● = electrical stimulation (60 V, 50 ms, 0.1 Hz). Lower trace: Tetraethylammonium induced overshooting spikes during electrical stimulation of small canine coronary artery and contraction of a strip of left anterior coronary artery studied simultaneously. Note the sudden change in potential when the microelectrode is ejected from the vascular smooth muscle. ● = electrical stimulation (60 V, 80 ms, 0.1 Hz).

(N = 5, $n = 4$; statistically different from the membrane potential in solution containing prostaglandin $F_{2\alpha}$; paired t test, $P < 0.05$; Table 2).

Indomethacin (10^{-5} M) induced an increase in tension of the left anterior descending coronary artery. The membrane potential of the small coronary artery was not significantly different from control (Table 1). In the presence of a femoral artery with endothelium, acetylcholine (10^{-5} M) induced relaxation of the left anterior descending coronary artery and hyperpolarized the smooth muscle of the small coronary artery to -64.6 ± 2.3 mV (N = 10, $n = 8$; statistically different from the membrane potential in solution containing indomethacin; paired t test, $P \leqslant 0.05$) (Figure 3; Table 2). The hyperpolarization in the presence of indomethacin was significantly greater than that observed in control solution (Table 2).

The hyperpolarization of the small coronary artery caused by acetylcholine (10^{-5} M) was transient and returned to control values at a time when the relaxation was still maintained (Figure 3).

The removal of endothelium from the femoral artery prevented both the relaxation and the hyperpolarization induced by acetylcholine (10^{-5} M) (Figure 3).

Endothelium-dependent responses and Na^+/K^+ pump

Ouabain After 1 h incubation of the femoral artery (with endothelium) and the two coronary arteries (without endothelium) with indomethacin (10^{-5} M) plus ouabain (5×10^{-6} M), neither the tension nor the membrane potential were significantly different from control (Table 1). The addition of acetylcholine (10^{-5} M) did not significantly affect the tension in the left anterior descending coronary artery or the membrane potential in the small coronary artery (-54.5 ± 1.4 mV; change in membrane potential: -1.0 ± 1.58 mV; N = 4, $n = 4$; Figure 4).

To dissociate the effect of ouabain on the endothelial cells of the femoral artery from that on the smooth muscle cells of the left anterior descending

Table 1 Membrane potential of smooth muscle cells of canine small epicardial coronary artery[a]

	Control solution	*TEA* (1.5×10^{-2} M)	*$PGF_{2\alpha}$* (2×10^{-6} M)	*Indomethacin,* (10^{-5} M)	*Ouabain* (I[b])	*Ouabain* (II[c])	*Potassium-free solution*[d]
Membrane potential (mV)	-55.2 ± 0.4	-45.4 ± 0.7*	-53.8 ± 1.0	-53.4 ± 0.7	-54.4 ± 0.8	-55.7 ± 1.0	-53.8 ± 0.9
Number of cells (N)	108	51	28	24	15	15	14
Number of dogs (*n*)	31	20	13	10	7	6	6

[a] Membrane potential from cells impaled at least 3 min.
[b] Segments of both femoral and coronary arteries were incubated with ouabain (5×10^{-6} M; 60 min) in the presence of indomethacin (10^{-5} M).
[c] Segments of coronary arteries were incubated in ouabain for 1 h. Measurements were made in control solution in the presence of indomethacin (10^{-5} M) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 2×10^{-6} M).
[d] Segments of femoral artery and coronary arteries were incubated in potassium-free solution in the presence of indomethacin (10^{-5} M) and prostaglandin $F_{2\alpha}$ (2×10^{-6} M).
* Denotes value statistically significantly different from that in control solution (one-way analysis of variance; Scheffe's test, $P < 0.05$).
TEA = tetraethylammonium.

Table 2 Hyperpolarization of smooth muscle cells of canine small epicardial coronary artery induced by acetylcholine (10^{-5} M)[a]

	Control solution	*Prostaglandin $F_{2\alpha}$* (2×10^{-6} M)	*Indomethacin* (10^{-5} M)
Nadir of the hyperpolarization (mV)	5.1 ± 1.2	7 ± 1.3	10.8 ± 1.4*
Number of cells (N)	8	5	10
Number of dogs (*n*)	6	4	8

[a] Impalements were performed either in control solution or in the presence of prostaglandin $F_{2\alpha}$ (2×10^{-6} M) or indomethacin (10^{-5} M). After stabilization of the membrane potential, acetylcholine was infused. The values presented are when the impalement was maintained throughout the infusion of acetylcholine until the maximum of hyperpolarization was reached and partial recovery had occurred.
* Denotes value significantly different from that in control solution (one way analysis of variance: Scheffe's test, $P < 0.05$).

coronary artery and the small coronary artery, only the latter were incubated in solution containing indomethacin (10^{-5} M) plus ouabain (5×10^{-6} M), for 1 h. After washout of the ouabain, a femoral artery strip (with or without endothelium; never exposed to ouabain) was introduced into the organ bath. The tension developed by the left anterior descending artery disappeared after the wash out of ouabain; the blood vessels were exposed to prostaglandin $F_{2\alpha}$ (2×10^{-6} M). The membrane potential of the small coronary artery was not significantly different from control (Table 1). Acetylcholine (10^{-5} M) induced relaxation of the strip of the left anterior descending coronary arteries but no change in membrane potential of the small coronary arteries (-54.3 ± 2 mV; change in membrane potential: $+0.58 \pm 0.71$ mV; N = 6, $n = 5$; not significantly different from the membrane potential in the absence of acetylcholine; paired t test; Figure 4). The removal of the endothelium of the femoral artery prevented the relaxation induced by acetylcholine (data not shown).

Potassium-free solution Strips of left anterior descending coronary arteries and small coronary arteries (both without endothelium) were incubated

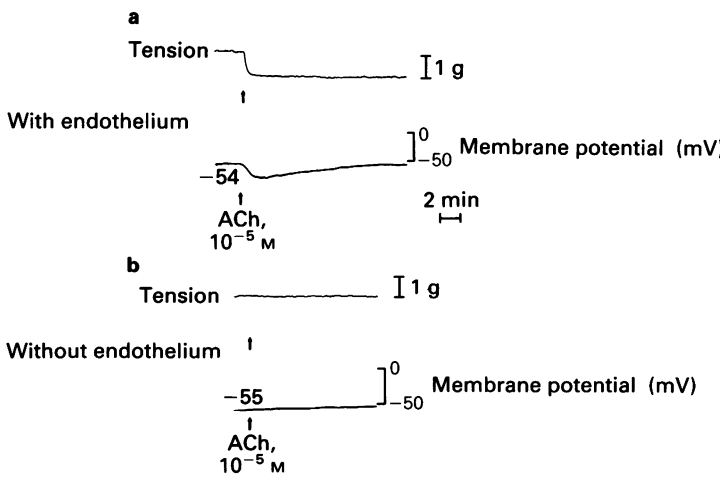

Fig. 3 Endothelium and response of canine coronary artery to acetylcholine (ACh; 10^{-5} M) in the presence of indomethacin (10^{-5} M). (a) Femoral artery with endothelium present in the organ bath. (b) Femoral artery without endothelium present in the organ bath. Tension is recorded from a strip of left anterior descending canine coronary artery without endothelium, and the membrane potential from a small epicardial canine coronary artery without endothelium.

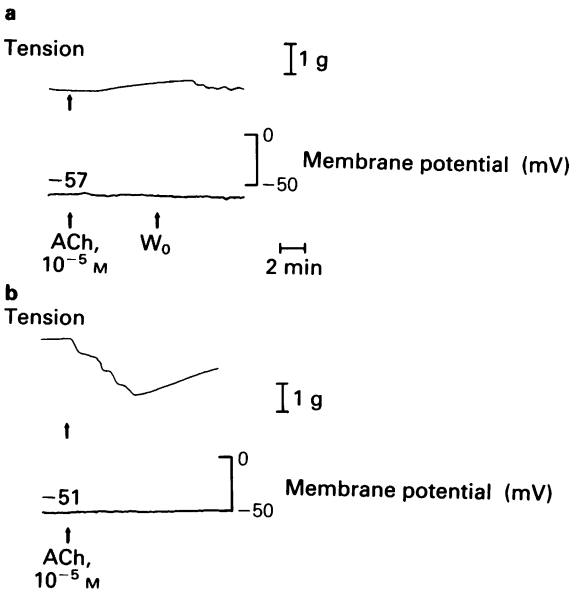

Figure 4 Endothelium-dependent responses in canine coronary arteries and inhibition of the sodium-potassium pump with ouabain (5×10^{-6} M). Experiments performed in the presence of indomethacin (10^{-5} M). Tension is recorded from a strip of left anterior descending canine coronary artery without endothelium, and the membrane potential from a small epicardial canine coronary artery without endothelium. Strips of canine femoral artery with endothelium were the source of endothelium-derived relaxing factor(s) released by acetylcholine (10^{-5} M). (a) Experimental condition: both coronary arteries without endothelium and the femoral artery with endothelium were incubated for 1 h in ouabain (5×10^{-6} M). (b) Experimental condition: both coronary arteries without endothelium were incubated in ouabain for 1 h. The femoral artery with endothelium was introduced in the organ bath after washout of the ouabain. Prostaglandin $F_{2\alpha}$ (2×10^{-6} M) was added to induce tension in the canine coronary artery.

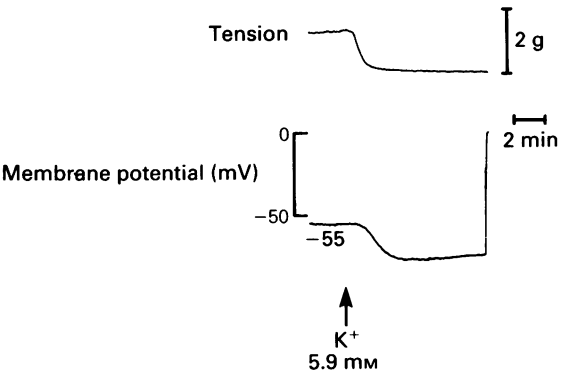

Figure 5 Potassium-induced relaxation and hyperpolarization. The tension is recorded from a strip of canine left anterior descending coronary artery (upper), the membrane potential from a small canine coronary artery (lower); both vessels were without endothelium. There was no femoral artery in the organ bath during these experiments, which were performed in the presence of prostaglandin $F_{2\alpha}$ (2×10^{-6} M) and indomethacin (10^{-5} M). After incubation in potassium-free solution, the addition of potassium ions (5.9 mM) induced relaxation and hyperpolarization. Note the sudden change in potential when the microelectrode is ejected from the vascular smooth muscle cell.

in potassium-free solution in the presence of indomethacin (10^{-5} M) and of prostaglandin $F_{2\alpha}$ (2×10^{-6} M). No femoral artery was present in the organ bath. The addition of potassium ions (5.9 mM or 12 mM) induced transient relaxation of the left anterior descending coronary artery and transient, but statistically significant hyperpolarization of the small coronary artery [to -71.6 ± 2.1 mV (change in potential -17.4 ± 2.7 mV; N = 7, $n = 3$) upon addition of 5.9 mM K^+ and to -69 ± 3 mV (change in potential -16.8 ± 3.5 mV; N = 4; $n = 4$) upon addition of 12 mM K^+] (Figure 5).

Strips of left anterior descending coronary arteries and small coronary arteries (both without endothelium) and segments of femoral arteries (with or without endothelium) were incubated in potassium-free solution for 1 h (in the presence of indomethacin (10^{-5} M) and of prostaglandin $F_{2\alpha}$ (2×10^{-6} M). The membrane potential of the small coronary arteries was not statistically different from control (Table 1). If a femoral artery with endothelium was present in the organ bath, the addition of acetylcholine (10^{-5} M) induced relaxation of the left anterior descending coronary artery strip but did not induce a statistically significant change in the membrane potential of the small coronary artery (membrane potential: -55.0 ± 2.1 mM, N = 5, $n = 4$) (Figure 6). The addition of 5.9 mM K^+ caused further relaxation and transient hyperpolarization [to -75.3 ± 2.3 (change in potential -20.7 ± 2.6 mV) N = 3, $n = 2$] (Figure 6).

The removal of the endothelium of the femoral artery prevented the relaxation induced by acetylcholine (data not shown).

Discussion

The present study confirms that acetylcholine causes endothelium-dependent relaxations of large coronary arteries of the dog (Cohen *et al.*, 1983) and demonstrates that endothelium-derived relaxing factor(s) released by acetylcholine from the femoral endothelium can cause hyperpolarization of the smooth

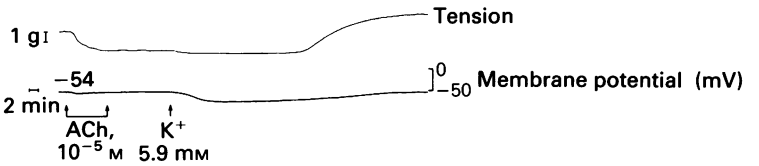

Figure 6 Endothelium-dependent responses in canine coronary arteries and inhibition of the sodium-potassium pump with potassium-free solution. Experiments were performed in the presence of indomethacin (10^{-5} M) and prostaglandin $F_{2\alpha}$ (2×10^{-6} M). Tension is recorded from a strip of left anterior descending canine coronary artery without endothelium (upper), the membrane potential from a small epicardial canine coronary artery without endothelium (lower). Strips of canine femoral artery with endothelium were the source of endothelium-derived relaxing factor(s) released by acetylcholine (10^{-5} M). After 1 h incubation in potassium-free solution acetylcholine (10^{-5} M) induced relaxation but no change in membrane potential. Subsequent readmission of potassium ions (5.9 mM) induced further relaxation and hyperpolarization demonstrating activation of the sodium-potassium pump.

muscle of small coronary arteries. Both the relaxation and the hyperpolarization occur whether the respective smooth muscles develop spontaneous tone or are contracted with indomethacin or prostaglandin $F_{2\alpha}$. Indomethacin, an inhibitor of cyclo-oxygenase (Moncada & Vane, 1979), does not inhibit the relaxation or the hyperpolarization, confirming that the endothelium-derived vasoactive substance(s) released by acetylcholine in the canine femoral artery is not a prostanoid metabolite of arachidonic acid, in particular prostacyclin (Rubanyi *et al.*, 1985).

Under our experimental conditions, it was technically difficult to record tension in the strips of the small coronary arteries. On the other hand, the elastic structures of the left anterior descending coronary artery rendered impalement of its smooth muscle difficult. Preliminary experiments (not shown) indicated that, even if the impalements were successful, the recordings of the membrane potential of the smooth muscle of the larger arteries were not as stable or long lasting as those in that of the small epicardial coronary artery. Hence, we decided to measure the changes in membrane potential in the latter, using, as in earlier work (Rubanyi *et al.*, 1985), the smooth muscle of a larger coronary artery to bioassay the presence of endothelium-derived relaxing factor(s). The stable resting membrane potential of approximately −55 mV recorded in the smooth muscle of the small coronary arteries and the excitability induced by tetraethylammonium (a property shared with other vascular smooth muscle, for review see Hermsmeyer, 1983b), confirms previous work on the same tissue (Belardinelli *et al.*, 1979; Harder *et al.*, 1979). Similar electrical properties of the cell membrane of large and small intramural coronary arteries have been demonstrated by others (Harder *et al.*, 1979). In the present study, the large and small vessels exhibited similar endothelium-dependent relaxations to acetylcholine. The primary purpose of the present study was not to correlate changes in membrane potential and tension. Hence, the experimental approach used, in particular because of the similarity in responsiveness to acetylcholine in the two coronary arteries studied, allows a valid assessment of the electrophysiological effects of endothelium-derived relaxing factor(s).

The direct action of acetylcholine on vascular smooth muscle varies with the species and the vessels studied. It induces contraction without change in membrane potential of the porcine coronary artery (Ito *et al.*, 1979; Itoh *et al.*, 1982); however, this blood vessel does not exhibit endothelium-dependent relaxations to acetylcholine (Kalsner, 1985). In the coronary artery of the guinea-pig, acetylcholine induces contraction despite hyperpolarization (Kitamura & Kuriyama, 1979), but the role of the endothelium in these responses is unknown. The intimal layer plays an obligatory role in the relaxation of isolated blood vessels by acetylcholine (Furchgott & Zawadzki, 1980). In the present study, acetylcholine did not influence membrane potential if endothelial cells were absent. When endothelial cells were present in the organ bath, acetylcholine systematically induced hyperpolarization of the smooth muscle of the small coronary artery. An endothelium-dependent hyperpolarization to carbachol has also been demonstrated in the mesenteric artery of the guinea-pig (Bolton *et al.*, 1984; Bolton & Clapp, 1986). The amplitude of the hyperpolarization caused by acetylcholine is augmented in the presence of indomethacin. The present experiments do not provide an explanation for this augmentation. In the canine coronary artery, endogenous products of cyclo-oxygenase can reduce relaxations induced by β-adrenoceptor agonists (Rubanyi & Vanhoutte, 1985).

Potassium-induced relaxations have been used to assess the degree of activity of Na^+/K^+-ATPase in vascular smooth muscle (Webb & Bohr, 1978). The endothelium-independent relaxations and hyperpolarizations obtained by readmitting potassium ions after incubation in potassium-free solution suggest

the presence of electrogenic sodium pumping in the smooth muscle of the canine coronary artery (Webb & Bohr, 1978; Fleming, 1980; Hermsmeyer, 1983a). De Mey & Vanhoutte (1980) and Rapoport *et al.* (1985) have reported that inhibition of Na^+/K^+ exchanges reduces, but does not prevent relaxations induced by acetylcholine. The organ chamber experiments demonstrated that, both in large and small coronary arteries, the relaxations induced by acetylcholine are sustained. However, the endothelium-dependent hyperpolarization of the smooth muscle of the small coronary artery caused by acetylcholine is a transient phenomenon. The hyperpolarization but not the relaxation is abolished after exposure of the coronary smooth muscle to a concentration of ouabain which must inhibit the Na^+/K^+ pump since it prevented potassium-induced relaxations. Exposure of the femoral endothelium and the coronary smooth muscle to potassium-free solution (conditions known to inhibit the activity of the Na^+/K^+ pump: Fleming, 1980; Webb & Bohr, 1978), prevented the hyperpolarization but not the relaxation induced by acetylcholine, suggesting that one of the actions (hyperpolarization), but not the other (relaxation), of the released endothelium-derived relaxing factor(s) is dependent on Na^+/K^+-ATPase. By contrast, unlike potassium-free solution, ouabain must also affect release of the relaxing factors by the endothelial cells in the femoral artery as neither hyperpolarization nor relaxations to acetylcholine were obtained when all tissues were incubated with the cardiac glycoside. Thus, potassium-free solution and ouabain affect the endothelial cells differently. The present experiments do not provide a satisfactory explanation for this difference; the effect of ouabain on endothelial cells may be due to a pharmacological action not involving Na^+, K^+-ATPase.

Some depolarization of the smooth muscle cells should occur rapidly after treatment with ouabain and potassium-free solution (Fleming, 1980). The present study shows that, after prolonged exposure to either of these treatments, the stabilized change in membrane potential of the smooth muscle cells of the small coronary artery is minimal. This long incubation period was imposed because both ouabain and potassium-free solution cause a transient contraction of the left anterior coronary artery (data not shown). For technical considerations, we decided not to measure acute changes in membrane potential caused by the inhibitors of Na^+, K^+-ATPase, as we were interested only in steady state inhibition of the Na^+, K^+-ATPase pump. Transient contractions and depolarizations to ouabain have been described in the taenia coli of the guinea-pig (Bose, 1974). An alternative explanation for the absence of depolarization by ouabain would be a weak activity of the pump under our control experimental conditions. The fact that relaxations and hyperpolarizations induced by acetylcholine did not evolve identically in response to ouabain and potassium-free solution may indicate that the two phenomena are due to different endothelial-derived factor(s) (Hoeffner & Vanhoutte, 1987), the release of one of which is not prevented by ouabain. In any event, the abolition by the inhibitors of Na^+, K^+-ATPase of the hyperpolarization suggests that endothelium-derived factor(s) released by acetylcholine may activate the Na^+, K^+ pump in coronary smooth muscle. However, changes in membrane conductance, i.e. increasing conductance of potassium or chloride ions, could be an alternative or complementary explanation for the hyperpolarization induced by acetylcholine (Kuriyama & Suzuki *et al.*, 1978; Bolton, 1984). Provided that the changes in membrane potential were the same in the bioassay tissues where tension was measured, the persistence of a relaxation of the smooth muscle in potassium-free solution and in the presence of ouabain at a time when the hyperpolarization has disappeared, suggests that hyperpolarization cannot fully explain the sustained endothelium-dependent relaxations caused by acetylcholine. These most probably can be attributed to activation of the production of cyclic GMP (Holzmann, 1982; Rapoport & Murad, 1983; Furchgott *et al.*, 1984).

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