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Endothelium-dependent relaxation and hyperpolarization of canine coronary artery smooth muscles in relation to the electrogenic Na-K pump

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1 In the smooth muscle cells of canine coronary artery, acetylcholine (ACh) produced a transient, endothelium-dependent hyperpolarization of the membrane. A similar hyperpolarization was also elicited by exposure to Krebs solution after incubation of the artery in K-free solution for 30 min.

2 A hyperpolarization of reproducible amplitude was generated when ACh was applied at intervals greater than 30 min. Repetitive application of ACh at 15 min intervals caused a successive reduction in the amplitude of hyperpolarization.

3 The reduction in the amplitude of relaxation during five successive applications of ACh at 15 min intervals was less than 10% of the first relaxation.

4 The ACh-induced hyperpolarization was blocked by atropine but not by ouabain, whereas the K-free induced hyperpolarization was blocked by ouabain. In low Na (Li-substituted) solution, ACh still induced a hyperpolarization but the K-free induced hyperpolarization was absent.

5 In coronary artery precontracted by high-K solution, ACh produced an endothelium-dependent relaxation, without membrane hyperpolarization. The associated relaxation was resistant to ouabain but sensitive to atropine.

6 It is concluded that in the canine coronary artery, the electrogenic Na-K pump does not contribute to the endothelium-dependent hyperpolarization or relaxation. The results are consistent with the release of two different inhibitory factors from the vascular endothelium.

Introduction

Arterial smooth muscles are relaxed by acetylcholine (ACh) in an endothelium-dependent manner, and the mediator may be an endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and a hyperpolarizing factor (Chen *et al.*, 1988; Chen & Suzuki, 1989). The EDRF-induced relaxation is accompanied by an increase in the production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), and the actions of this factor are similar to those of the nitrovasodilators (Ignarro & Kadowitz, 1985). Nitric oxide is one of the possible candidates for EDRF (Palmer *et al.*, 1987), and the similarities (Ignarro *et al.*, 1987) and differences (Shikano & Berkowitz, 1987; Shikano *et al.*, 1987) between these two substances are now subject to much investigation.

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The endothelium-derived hyperpolarizing factor (EDHF) acts mainly transiently and its actions are resistant to methylene blue or haemoglobin which are inhibitors of the actions of EDRF (Martin *et al.*, 1985). In the rat aorta, EDHF is responsible for about 20–40% of the ACh-induced endothelium-dependent relaxation (Chen & Suzuki, 1989).

The endothelium-dependent hyperpolarization of smooth muscle membrane produced by ACh is mainly due to an increase in K-conductance in the rabbit saphenous (Komori & Suzuki, 1987a) and ear arteries (Suzuki, 1988) and in rat arteries (Chen *et al.*, 1988; Chen & Suzuki, 1989). However, in the canine coronary artery, Feletou & Vanhoutte (1988) reported that the endothelium-dependent hyperpolarization produced by ACh was sensitive to ouabain whereas the actions of EDRF were resistant to this drug. These data therefore suggested that the hyperpolarization was produced by an activation of the

electrogenic Na-K pump in the smooth muscle membrane rather than an increase in K-conductance. These two contradictory results were obtained from experiments using different methods. In the former group, investigations were carried out in tissues with intact endothelial cells whereas the latter were performed using endothelium-free tissue in a cascade experiment, which makes direct comparison between the two results difficult.

In the present study, we have investigated the possible involvement of an electrogenic Na-K pump in the endothelium-dependent hyperpolarization produced by ACh, using preparation of canine coronary artery with intact endothelial cells. The electrogenic Na-K pump was inhibited by ouabain or Li ions (Kerkut & York, 1971).

Methods

Mongrel dogs of either sex, weighing 10–15 kg, were anaesthetized by i.v. injection of Na pentobarbitone (40 mg kg^{-1}), and exsanguinated from the femoral artery. The heart was excised and kept in Krebs solution at room temperature. The left descending coronary artery with an outer diameter of 0.5–1.0 mm (located mainly in the left ventricular wall) was dissected for a length of 0.5–0.7 cm, and cleaned by removing the connective tissues and heart muscles which surrounded the vessel.

For recording electrical responses of smooth muscle cells, the artery was cut open along its longitudinal axis and mounted in a recording chamber made of lucite plate, with a capacity of about 2 ml. At the bottom of the chamber was a silicon rubber plate (Shin-etsu kagaku, Tokyo, KE-66) on which the isolated vessel, endothelial layer uppermost, was immobilized with tiny pins, and superfused with warmed (35°C) Krebs solution at a flow rate of 2–3 ml min^{-1}.

Glass capillary microelectrodes were made from borosilicate glass tube (outer diameter, 1.2 mm with a core inside, Hilgenberg, West Germany), and filled with 3 M KCl. Their tip resistance ranged from 40–80 MΩ. A microelectrode was inserted into a smooth muscle cell through the endothelial layer, and the electrical responses thus recorded were displayed on a pen-writing recorder (Recticorder, Nihon-kohden RJG-4024). Mechanical responses of smooth muscles of the coronary artery were recorded separately under isometric conditions using a ring segment of the vessel as described previously (Suzuki & Casteels, 1979).

When necessary, the endothelial cells were removed mechanically by the method of Furchgott & Zawadzki (1980) in the case of microelectrode experiments, or by rubbing the internal surface of the vessel with a steel wire for mechanical experiments (Nagao & Suzuki, 1987).

The ionic composition of Krebs solution was as follows (mM): Na^+ 134.7, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 15.5, Cl^- 137 and glucose 11.5. The solution was aerated with O_2 containing 3% CO_2, and the pH of the solution was at 7.2–7.4. The K-free solution was prepared by replacing KCl and KH_2PO_4 with NaCl and NaH_2PO_4, respectively. The high-K solution was prepared by replacing NaCl with KCl. The concentration of Na^+ was reduced to 15.5 mM by substituting NaCl with LiCl (Li-Krebs).

Drugs used were acetylcholine chloride, atropine sulphate (all from Sigma Chemical Co., St. Louis, MO.), and ouabain (g-strophanthin, Merck).

Experimental values are shown as means ± s.d., and statistical significance was determined by Student's t test ($P < 0.05$ was considered significant).

Results

Electrical properties of the ACh-induced hyperpolarization

The resting membrane potential of the canine coronary artery smooth muscles ranged between −50 and −60 mV, and the values recorded from individual cells were stable. Figure 1 shows that application of 10^{-5} M ACh transiently hyperpolarized the membrane and that the membrane potential reverted to the resting membrane potential level within 7–10 min (Figure 1a). When ACh was applied for 2 min, the hyperpolarized membrane was restored to the previous resting potential level within 2–3 min, after a transient depolarization of 3–5 mV (Figure 1b). The ACh-induced hyperpolarization was not seen in the presence of atropine (10^{-6} M) (Table 1) or after removal of the endothelial cells (Figure 1c).

Figure 1d shows the relationship between the amplitude of the ACh-induced peak hyperpolarization and the concentration of ACh. ACh ($> 10^{-7}$ M) produced a concentration-dependent hyperpolarization, with a maximum at 10^{-4} M ACh, only in tissues with intact endothelial cells.

Experiments were also carried out to determine the time required for recovery of the ACh-induced hyperpolarization. ACh (10^{-5} M) was applied twice at various intervals and the amplitude of the hyperpolarization produced by the second exposure to ACh relative to the first was measured. In every trial throughout these experiments, ACh was applied for 1 min, which allowed the peak hyperpolarization to develop. When ACh was applied at 5 min intervals,

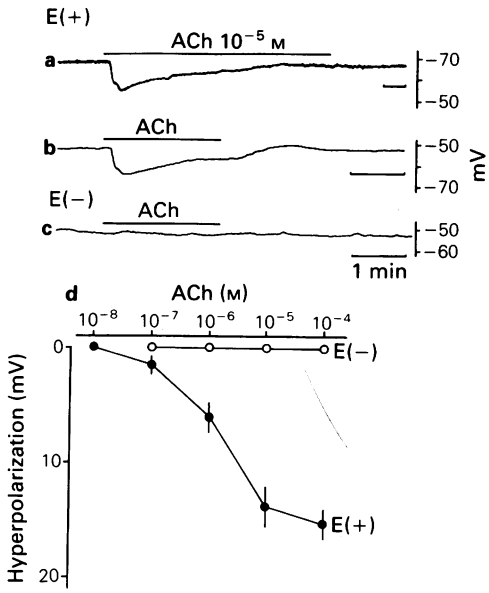

Figure 1 Electrical responses recorded from smooth muscle cells of the canine coronary artery during application of acetylcholine (ACh, 10^{-5} M) in tissues with intact endothelial cells (E (+), a and b) and with endothelial cells removed (E (−), c); (a) and (b) but not (c) were recorded from the same tissue. ACh was applied at the bar above each record. (d) Concentration-dependent hyperpolarizations produced by ACh in smooth muscle cells of canine coronary artery, in tissues with (E (+), ●) and without (E (−), ○) endothelial cells. Means with s.d. shown by vertical lines (n = 5–12).

the hyperpolarization produced by the second ACh challenge was decreased in amplitude and also the rate of potential change slowed (Figure 2a). As shown in Figure 2b, a reproducible amplitude of hyperpolarization was generated by ACh when the intervals between successive exposure were 30 min.

When ACh (10^{-5} M) was applied 5 times successively every 15 min, the amplitude of the resulting hyperpolarization decreased by 8–10% of the previous response, and the 5th response was about 70% of the first (Figure 2c).

Effects of inhibition of the electrogenic Na-K pump on the ACh-induced hyperpolarization

In tissues with intact endothelial cells, the membrane was also hyperpolarized transiently (10–15 min) by administration of Krebs solution after incubation in K-free solution for 30 min (Figure 3a and b). The amplitude of the ACh (10^{-5} M)- and K-free-induced hyperpolarizations obtained from five different tissues were 13.9 ± 1.6 mV ($n = 15$) and 14.2 ± 1.8 mV ($n = 10$), respectively.

Table 1 Membrane potential of smooth muscle cells in the canine coronary artery

Control (resting membrane potential)	-53.5 ± 1.6 mV ($n = 24$)
Endothelial cells removed	-52.9 ± 1.3 mV ($n = 10$)
Atropine 10^{-6} M	-52.8 ± 1.4 mV ($n = 13$)
K-free solution (30–40 min)	-48.4 ± 1.8 mV ($n = 10$)*
Ouabain 10^{-6} M (10–60 min)	-50.7 ± 1.3 mV ($n = 11$)*
Li-Krebs solution	-40.9 ± 1.4 mV ($n = 9$)*
24.9 mM $[K]_o$ solution	-47.6 ± 1.5 mV ($n = 10$)*

Values are means ± s.d.
(n = number of observations). * Significant difference from the control ($P < 0.05$).

Application of ouabain (10^{-6} M) for 30–60 min depolarized the membrane by 1–3 mV (Table 1). In tissues with an intact endothelium and in the presence of ouabain, ACh (10^{-5} M) produced a hyperpolarization of the membrane, the amplitude and configuration of which were identical to those seen in control conditions (Figure 3c). The mean peak amplitude of the hyperpolarization produced by ACh (10^{-5} M) in the presence of ouabain was 14.3 ± 1.7 mV ($n = 8$, $P > 0.05$). The K-free-induced hyperpolarization was, however, completely blocked by ouabain, or on some occasions, converted to a small transient (5–7 min) depolarization (1–3 mV), when Krebs solution was applied after incubation in K-free solution for 30–40 min (Figure 3d).

Substitution of about 88% of Na ion ($[Li]_o$ = 119.2 mM) depolarized the cell membrane by about 12 mV (Table 1). In this Li-Krebs solution, ACh (10^{-5} M) produced a transient hyperpolarization (Figure 3e), but exposure to Krebs solution after incubation in K-free solution did not (Figure 3e). Li-substitution reduced the magnitude of the ACh-induced hyperpolarization (9.5 ± 1.9 mV, $n = 8$, $P < 0.05$) and markedly accelerated its decay (compare Figure 3e with Figure 1a).

ACh-induced hyperpolarization in high $[K]_o$ solution

If the ACh-induced hyperpolarization is produced by an increase in membrane K-conductance, it should be decreased or absent in high $[K]_o$ solution. In rat arteries, the expected $[K]_o$ to block the ACh-induced hyperpolarization was 20–25 mM (Chen & Suzuki, 1989). Experiments were thus carried out to observe the effects of 24.9 mM $[K]_o$ solution on the

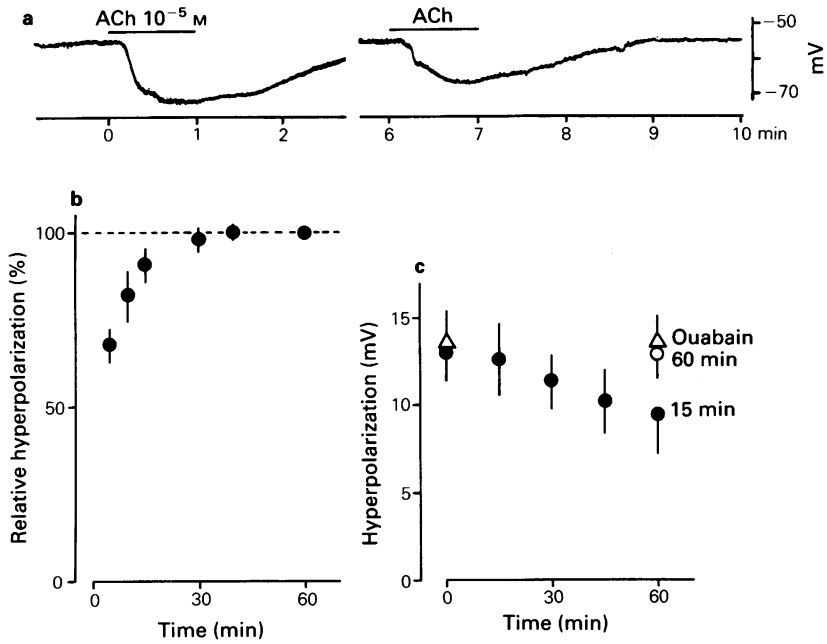

Figure 2 Tachyphylaxis of the acetylcholine (ACh)-induced hyperpolarization in smooth muscle of the canine coronary artery. (a) Hyperpolarizations produced by application of ACh (10^{-5} M, for 1 min) on two occasions separated by a 5 min interval (recordings were interrupted for about 3 min between two ACh applications). (b) Recovery of the ACh response from desensitization. Amplitude of the hyperpolarization produced by the second ACh was measured as a percentage of the first, and plotted against the time between the two applications of ACh. Means with s.d. shown by vertical lines ($n = 5$–7). (c) Tachyphylaxis of the ACh-induced hyperpolarization. Amplitude of the hyperpolarization produced by application of ACh (10^{-5} M) on 5 successive occasions each separated by a 15 min interval (●), or on 2 occasions separated by a 60 min interval in the absence (○) and presence of 10^{-6} M ouabain (△). Each ACh challenge was applied for 1 min, and the peak amplitude of the hyperpolarization was measured.

endothelium-dependent hyperpolarization produced by ACh in the canine coronary artery.

Increasing $[K]_o$ to 24.9 mM depolarized the smooth muscle membrane by about 6 mV (Table 1). Application of ACh (10^{-5} M) in the control condition produced hyperpolarization (Figure 4a, the mean amplitude being 13.8 ± 1.6 mV, $n = 6$). However, this hyperpolarization was decreased in amplitude or disappeared (Figure 4b) in high $[K]_o$ solution (mean amplitude, 0.2 ± 0.7 mV, $n = 5$).

These results strongly suggest that the ACh-induced hyperpolarization generated in the smooth muscle of canine coronary artery is mainly due to an increase in K-conductance of the membrane.

Mechanical properties of the ACh-induced relaxation

Increasing $[K]_o$ to 24.9 mM produced a contraction of smooth muscles in ring segment of the canine coronary artery. The tension elevated by the high-$[K]_o$ solution was 18 ± 4% ($n = 5$) of the maximum value produced by 118 mM $[K]_o$ solution, and a similar level of tension was maintained for up to 2 h by the high-$[K]_o$ solution. In tissues with intact endothelial cells, application of ACh ($> 10^{-8}$ M) in the high-$[K]_o$ solution inhibited the contraction in a concentration-dependent manner and reached a maximum value at 10^{-5} M ACh. This relaxation was not observed in tissues devoid of endothelial cells or in the presence of atropine (10^{-6} M).

Experiments were also carried out to observe the effects of repetitive application of ACh on the amplitude of relaxation. As demonstrated in Figure 5a, ACh (10^{-5} M) inhibited the high-$[K]_o$-induced contraction to about 30%, and the tension was restored to the initial level within 5–7 min of the removal of ACh.

When the amplitude of the ACh-induced relaxation was measured as a percentage of the initial value, successive applications of ACh at 15 min intervals caused some reduction of the inhibitory actions of ACh. The amplitude of relaxation produced by the 5th ACh challenge was about 90% of the first (Figure 5b). Two applications of ACh at 60 min

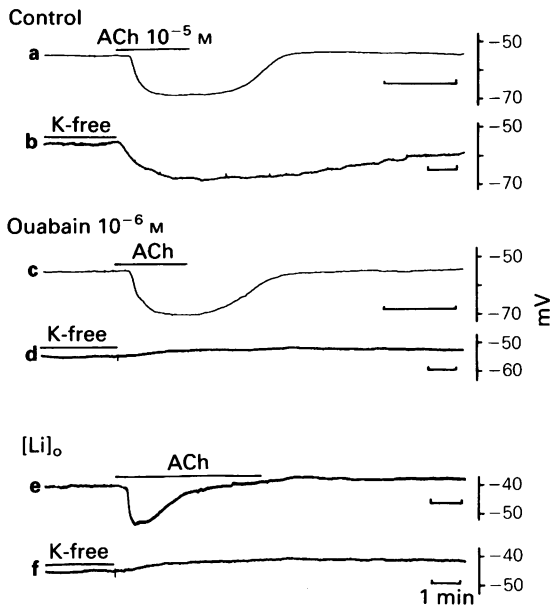

Figure 3 Membrane hyperpolarizations produced by acetylcholine (ACh, 10^{-5} M) (a, c and e) or administration of K ions after incubation in K-free solution for 30 min (b, d and f) in smooth muscle cells of the canine coronary artery. (a) and (b) Control: (c) and (d) in the presence of 10^{-6} M ouabain; (e) and (f) in low $[Na]_o$ solution substituted with Li (Li-Krebs).

intervals relaxed the muscle to the same extent ($P > 0.05$).

In the presence of ouabain (10^{-6} M), ACh still relaxed the muscles which had been precontracted with high-$[K]_o$ solution. The amplitude of the relaxation produced by 10^{-5} M ACh was

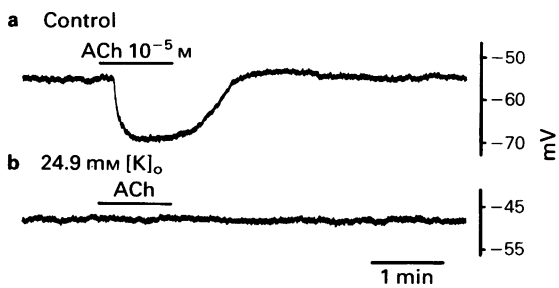

Figure 4 Effects of acetylcholine (ACh, 10^{-5} M) on membrane potential of smooth muscle cells from canine coronary artery, in high $[K]_o$ solution. (a) Control; (b) in 24.9 mM $[K]_o$ solution. ACh was applied for 1 min (bar). (a) and (b) are recordings from the same cell.

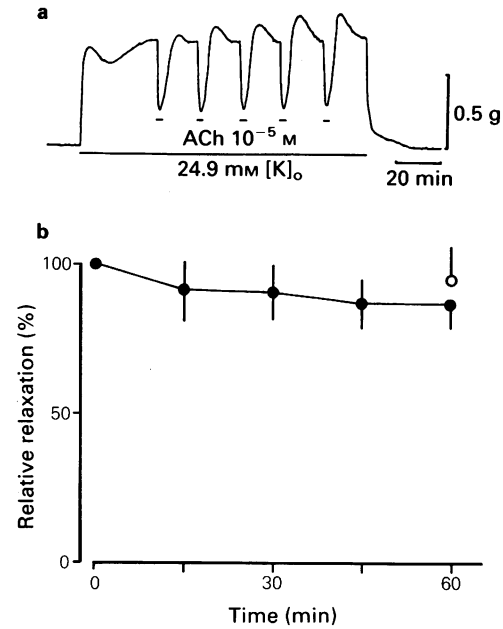

Figure 5 Acetylcholine (ACh)-induced relaxation in the canine coronary artery. (a) A ring segment of the artery was contracted by 24.9 mM $[K]_o$ solution, and exposed to ACh (10^{-5} M) for 3 min on 5 occasions at 15 min intervals. (b) The relaxations produced by application of 10^{-5} M ACh as described in (a) (●) or on 2 occasions separated by a 60 min interval (○) were observed in tissues contracted with 24.9 mM $[K]_o$ solution, and the amplitude of the maximum relaxation relative to the first was measured ($n = 5$).

$63.3 \pm 14.8\%$ ($n = 23$) of the high-$[K]_o$-induced contraction in the control and $54.8 \pm 8.7\%$ ($n = 15$) in the presence of ouabain (10^{-6} M), values which were not significantly different ($0.05 < P < 0.1$).

Discussion

The present experiments have demonstrated that in the smooth muscle cells of canine coronary artery, ACh produced a transient, endothelium-dependent hyperpolarization. These results confirmed observations in other arteries such as the pig coronary artery (Bény *et al.*, 1986), the rabbit saphenous (Komori & Suzuki, 1987a,b), ear (Suzuki, 1988) and aortic vessels (Bèny & Brunet, 1988), the guinea-pig mesenteric (Bolton *et al.*, 1984) and basilar arteries (Nishiye *et al.*, 1989), the rat aorta (Taylor *et al.*, 1988) and main pulmonary arteries (Chen *et al.*, 1988) and the canine mesenteric artery (Komori *et*

al., 1988). Cascade experiments demonstrated that in the canine coronary artery, the endothelium-dependent hyperpolarization by ACh was produced by an unknown substance which is liberated from the endothelial cells (Feletou & Vanhoutte, 1988). The resistance of the ACh-induced hyperpolarization to methylene blue or haemoglobin, an inhibitor of the action of EDRF (Martin *et al.*, 1985) also indicates that the ACh-induced hyperpolarization is not associated with the actions of EDRF (Chen *et al.*, 1988; Chen & Suzuki, 1989).

Tachyphylaxis was observed to the ACh-induced hyperpolarization, and over 30 min between successive exposure to ACh, was required to obtain a reproducible amplitude of hyperpolarization in the canine coronary artery. This period of time was longer than in rat aorta (about 5 min) but was comparable with the characteristics of the histamine-induced hyperpolarization in this artery (Chen & Suzuki, 1989). Although the detailed mechanisms remain to be determined, desensitization of ACh- or histamine-receptors on the endothelial cell membrane seems to contribute to the transient nature of the endothelium-dependent hyperpolarization. The sustained relaxation produced by ACh in the rabbit saphenous artery (Komori & Suzuki, 1987a) and aorta (Bèny & Brunet, 1988) indicates that the release of EDRF is maintained until ACh is removed, and such a property of EDRF is distinct from the transient nature of the hyperpolarization. The transient response of the smooth muscle membrane of the rat aorta to histamine is not due to depletion of EDHF or to desensitization of receptors for EDHF in the smooth muscle membrane (Chen & Suzuki, 1989).

In cascade experiments, the endothelium-dependent hyperpolarization observed in the canine coronary artery was blocked by ouabain (Feletou & Vanhoutte, 1988). However, the present experiments demonstrated that in this artery with intact endothelial cells, ACh produced a hyperpolarization in the presence of ouabain and the results are in agreement with those observed in the rabbit ear artery (Suzuki, 1988). Some arteries require very high concentrations of ouabain to inhibit the electrogenic Na-K pump and in the rat main pulmonary artery, 10^{-3} M ouabain was required to block the K-free-induced hyperpolarization (Suzuki & Twarog, 1982). In the canine coronary artery, 10^{-6} M ouabain was sufficient to block the K-free-induced hyperpolarization but it was insufficient to block the ACh-induced hyperpolarization. Therefore, the ACh-induced hyperpolarization is almost certainly generated by mechanisms other than activation of the electrogenic Na-K pump in this tissue. Reduction or disappearance of the ACh-induced hyperpolarization in high $[K]_o$ solution suggests that this hyperpolarization is accompanied by an increase in K-conductance of the membrane, and the results are thus consistent with the findings in other arteries (Kuriyama & Suzuki, 1978; Komori & Suzuki, 1987a; Chen & Suzuki, 1989). Direct evidence of an increase in K-conductance was obtained in ion-flux experiments (Chen *et al.*, 1988). It remains to be determined why the effects of ouabain on the ACh-induced hyperpolarization differ between the endothelium-intact and endothelium-free (cascade) tissues.

Li ions can permeate the Na-channel, but they cannot be extruded from the cell by the electrogenic Na-K pump (Kerkut & York, 1971). In Li-Krebs solution, ACh produced an endothelium-dependent hyperpolarization, but the K-free-induced hyperpolarization was abolished. These results again indicate that the endothelium-dependent hyperpolarization in the canine coronary artery does not involve activation of the electrogenic Na-K pump. However, the decay of the hyperpolarization was accelerated in the Li-Krebs solution. It is uncertain whether this shortening is due to the effects of Li ions, to a reduction of Na ion concentration, or to other mechanisms.

In the present study, ACh relaxed the high-K-induced contractions, in an endothelium-dependent manner and this relaxation was not altered by ouabain. Therefore, the electrogenic Na-K pump does not seem to contribute to the EDRF-induced relaxation of vascular smooth muscle (Feletou & Vanhoutte, 1988), and as such the mechanism involved may be different from the endothelium-dependent relaxation to arachidonic acid (Rubanyi & Vanhoutte, 1985).

In the canine coronary artery, repetitive application of ACh caused a successive, marked reduction in amplitude of the endothelium-dependent hyperpolarization, with only a slight reduction in the amplitude of the endothelium-dependent relaxation. These results further suggest that the relaxing and hyperpolarizing responses are generated by different factors, and as such are in agreement with our previous observations (Komori & Suzuki, 1987a,b; Chen *et al.*, 1988; Chen & Suzuki, 1989).

It is concluded that in the canine coronary artery, the endothelium-dependent hyperpolarization and relaxation of smooth muscle cells produced by ACh does not seem to involve activation of the electrogenic Na-K pump. Different time courses in the tachyphylaxis occurred between the hyperpolarization and relaxation suggesting that both EDRF and EDHF are involved in the action of ACh in this artery.

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