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Absence of effect of calcium antagonists on endothelium-dependent relaxation in rabbit aorta

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1 The effect of chronic feeding of New Zealand White rabbits with nicardipine (60 mg kg^{-1} daily for 5 weeks) on the endothelium-dependent relaxation (EDR) to acetylcholine (ACh) was examined *in vitro*. The effect of acute exposure to nicardipine and diltiazem (10 μmol l^{-1}) in the tissue bath was also examined.

2 A bioassay system for endothelium-dependent relaxation factor (EDRF) in which a rabbit aortic ring with endothelium removed was used as recipient and a segment of rabbit aorta with endothelium as donor (producing EDRF in response to ACh) was developed. This system enabled the effect of nicardipine on the synthesis/release and on the relaxation to EDRF to be studied separately.

3 The maximum relaxations to ACh in control and nicardipine-fed animals were 43.6 ± 5.5 and 53.8 ± 6.7% (mean ± s.e.mean) of the contractile response to noradrenaline (NA, 1 μmol l^{-1}) ($n = 6$, $P > 0.05$). Similarly the EDR to ACh was not significantly altered by acute exposure (30 min) to nicardipine or diltiazem. The maximum relaxations without and with nicardipine were 32.4 ± 4.2% and 28.0 ± 3.1% of the contraction to NA (1 μmol l^{-1}) ($n = 11$, $P > 0.05$).The corresponding data for diltiazem were 42.1 ± 5.7 and 36.4 ± 7.3% respectively ($n = 11$, $P > 0.05$).

4 Both calcium antagonists inhibited the contraction induced by potassium (100 mmol l^{-1}). Nicardipine and diltiazem in concentrations of 100 μmol l^{-1} reduced the potassium-induced contraction to 33.0 ± 9.0% and 53.8 ± 6.7% of control respectively ($n = 6$, $P < 0.05$).

5 In the bioassay experiments the infusion of nicardipine on (a) the recipient tissue only and (b) the donor and the recipient tissue had no significant effect on the relaxant response observed in the recipient tissue when superfused with Krebs-bicarbonate buffer containing ACh via the donor tissue ($n = 6$, $P > 0.05$).

6 These results indicate that nicardipine and diltiazem had no significant effect on synthesis/release and the relaxant response to EDRF in the rabbit aorta. Thus the translocation of Ca^{2+} accompanying the EDR to ACh in the rabbit aorta is likely to utilize Ca^{2+} channels not blocked by these calcium antagonists.

Introduction

The studies of Furchgott and his colleagues (Furchgott & Zawadzki, 1980) have demonstrated that acetylcholine (ACh) produces a relaxation of the rabbit aorta which is mediated by the endothelial cells. This endothelium-dependent relaxation (EDR) has been demonstrated in a variety of blood vessels taken from many different species (Vanhoutte & Rimele, 1982–1983). Subsequent studies have confirmed that several pharmacological agents release this 'endothelium-dependent relaxation factor' (EDRF) (Furchgott, 1983).

The mechanisms governing the release of EDRF have been investigated extensively, in particular, the possible role of calcium ions. For instance, it has been shown that the calcium ionophore A23187 produces a relaxant response dependent on endothelium (Zawadzki *et al.*, 1980) and that actylcholine (ACh) activates the muscarinic receptors resulting in the influx of Ca^{2+} into the endothelial cells. Additional information linking calcium ions to the release of EDRF has been sought by investigating the effect of calcium channel blockers upon EDR and by using buffers containing

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low calcium concentrations (Singer & Peach, 1982; Long & Stone, 1985; Winquist *et al.*, 1985). As the above studies produced conflicting results the present investigation was undertaken to examine the effect of the calcium channel blocker nicardipine which is a dihydropyridine derivative on EDR in the rabbit aorta. Three separate investigations were undertaken: (i) the effect of chronic oral administration of nicardipine on EDR; (ii) the effect on EDR of incubating the rabbit aorta *in vitro* with nicardipine; and (iii) the effect of nicardipine on the synthesis/release of EDRF by the rabbit aorta and on the response to EDRF in the preparation.

As a subsidiary study to (ii) above, the effect of diltiazem (a benzothiazepine derivative) on EDR was also examined. In addition, the effect of nicardipine and diltiazem on a contraction induced by potassium was assessed to confirm their calcium antagonistic properties under the experimental conditions of this study.

Methods

The experiments were performed on male New Zealand White rabbits purchased from a single vendor. The details of the methods used in the three related studies are given below.

(i) *Effect of chronic oral administration of nicardipine on EDR*

Animals, 12 weeks of age, were randomized into control and experimental groups. The animals were numbered and housed individually under similar conditions. Both groups were given an ordinary rabbit diet (Baby Rabbit Pellets, Masterfeeds Division, Maple Leaf Mills, London, Ontario, Canada) and water *ad libitum*. Nicardipine (60 mg kg^{-1} daily, in 2 divided doses) was administered to the experimental group. The pure compound was made into a suspension in water and administered by the oral infusion technique (Moreland, 1965). In this technique a wooden speculum (15 × 2 × 0.6 cm) with an adequate sized opening was placed transversely in the mouth behind the incisors. The suspension of nicardipine in water was administered via a rubber tube inserted into the oesophagus.

A group of 6 animals from both control and experimental groups were killed after 5 weeks of administration of nicardipine. Each animal was anaesthetized by injecting pentobarbitone sodium (25 mg kg^{-1}) into the marginal ear vein. A midline thoracotomy was performed and the aorta removed for further experimentation. Before removal of the aorta, a sample of blood was obtained via a cardiac puncture for estimation of the serum concentration of nicardipine. This blood was centrifuged at 2000 r.p.m. for 10 min and serum stored at − 70°C for subsequent analysis.

Tissue bath studies After removal of excess connective tissue the thoracic aorta was cut into rings approximately 5 mm long. Special care was taken to avoid contact with the luminal surface of the rings in order to preserve the endothelium. The endothelium was removed deliberately in some rings by inserting the tip of a small forceps into the luminal surface of the ring and turning back and forth for 20 s on a filter paper wetted with Krebs-bicarbonate buffer (Senaratne & Kappagoda, 1984). The rings were suspended in tissue baths of 22 ml capacity containing Krebs-bicarbonate buffer solution at a pH of 7.4. The solution was maintained at 37°C with the aid of a heater/circulator (Model No E15, Haake Mess-Technik, Karlsruhe, Federal Republic of Germany) and continuously aerated with a gas mixture containing 95% O_2: 5% CO_2. The rings were mounted on two stainless steel triangular clips, the lower clip being attached to a movable support and the upper clip to a force displacement transducer (Model No FT 03C, Grass Instrument Co., Quincy, Ma., U.S.A.). Before the experiments the rings were stretched to an optimal basal tension of 8.0 g. (This optimal basal tension of 8.0 g was established on the basis of length-active tension curves obtained by use of a fixed concentration of noradrenaline, 0.1 μmol l^{-1}, in preliminary experiments.) The preparations were left in the tissue bath for a period of 90 min for equilibration before the experimental protocol was begun. The fluid in the tissue bath was changed every 30 min during this period.

The responses to ACh were examined in aortic rings from each animal. The endothelium was removed from one of these rings. All preparations were contracted after the 90 min equilibration period by adding noradrenaline (NA) 1 μmol l^{-1} to the tissue bath. After the contraction had reached a plateau, a concentration-effect curve to ACh was obtained by adding ACh to the tissue bath in a cumulative manner (1 nmol l^{-1} to 10 μmol l^{-1}). This protocol was followed on the aortae obtained from experimental and control animals.

(ii) *Effect on EDR of incubating the rabbit aorta* in vitro *with nicardipine and diltiazem*

Rabbits, 12 weeks of age, were killed and the descending thoracic aorta removed as described above. Rings were prepared from each aorta for the tissue bath studies. Endothelium was removed from one of these rings as described above. Each ring was contracted with NA (1 μmol l^{-1}) and concentration-effect curves to ACh (1 nmol l^{-1} to 10 μmol l^{-1}) were obtained as before.

After the concentration-effect curves to ACh were obtained the fluid in the tissue baths was replaced with fresh Krebs-bicarbonate buffer and the tension allowed to return to the baseline value with frequent replacement of the buffer. At this stage the calcium antagonists nicardipine or diltiazem (10 μmol l^{-1}) were added to the tissue baths containing an aortic ring with intact endothelium. Another ring with intact endothelium served as a control with no calcium antagonist added to the bath fluid. After 30 min of incubation with the calcium antagonists, all the rings were contracted with NA (1 μmol l^{-1}) and the concentration-effect curve to ACh repeated as above. The calcium antagonists were present in the respective tissue baths throughout the experiment. Only one calcium antagonist was tested on one ring. The concentration-effect curve to ACh in the presence of the calcium antagonist was compared with the parallel (second) control concentration-effect curve.

At the end of each experiment ((i) and (ii) above) rings with and without endothelium were prepared for scanning electron microscopy (see below).

The effect of nicardipine and diltiazem on a contraction induced by potassium was also studied in a separate series of animals. Aortic rings with intact endothelium were mounted and left to equilibrate for 90 min as described above. At the end of this period the Krebs-bicarbonate buffer was replaced with buffer containing 100 mmol l^{-1} potassium chloride (see below for composition of latter buffer). The induced contraction by potassium was allowed to reach a plateau. At this stage the buffer containing 100 mmol l^{-1} potassium was removed and replaced with Krebs-bicarbonate buffer. Nicardipine (10 or 100 μmol l^{-1}) and diltiazem (100 μmol l^{-1}) were added to the tissue baths at this point (only a single concentration of the calcium antagonist was tested in one aortic ring). An aortic ring with no calcium antagonist served as a control. After 30 min of incubation with the calcium antagonists the buffer containing 100 mmol l^{-1} potassium was reintroduced and the response determined in the presence of the calcium antagonist.

(iii) *Effect of nicardipine on the synthesis/release of EDRF by the rabbit aorta and on the response to EDRF*

In these experiments, a bioassay was developed to examine the effect of nicardipine upon the synthesis/release of the EDRF from the rabbit aorta.

The assay is based on transferring the factor from a donor tissue with endothelium to a ring of recipient tissue with endothelium removed in which its biological activity could be monitored. Rabbits (12 weeks of age) were killed as before and 4–5 cm lengths of thoracic aorta were removed for use as donor tissues. Care was taken to avoid damage to the endothelial cells in the donor tissues. A separate 5 mm long ring of aorta without endothelium was used as the recipient.

The bioassay apparatus is shown in Figure 1. The donor aorta (d) was placed in a plastic chamber (c) which was enclosed in a water-jacket and filled with Krebs-bicarbonate buffer. The opposite walls of this chamber were pierced by two stainless steel cannulae (o.d. 3.2 mm, i.d. 2.2 mm) whose positions could be adjusted according to the length of donor aorta. The donor aorta was mounted between the ends of the two cannulae inside the chamber. The donor aorta was perfused intra-luminally with oxygenated Krebs-bicarbonate buffer (95% O_2:5% CO_2; pH 7.4) from a reservoir (R) by means of a positive displacement roller pump (P) (Miniplus 2, Gilson Medical Electronics, Villiers, France). A multiple channel infusion pump (Ac) (Model 2212, Harvard Apparatus Co. Inc, Newport Beach, Ca., U.S.A.) was used to introduce Krebs-bicarbonate buffer or ACh into the perfusate at a point proximal to the donor. The perfusate emerging from the chamber was dripped over a recipient ring of aorta (r) which was connected to a strain gauge for recording isometric tension (Model FT 03C, Grass Instruments Co., Quincy, Ma., U.S.A.). A second infusion pump (At) (Model 341A, Sage Instruments, Cambridge, Ma., U.S.A.) was used to add atropine to the superfusate directly as shown in Figure 1. (Thus the recipient tissue alone was treated with atropine). With the aid of a three way stop-cock placed between the reservoir (R) and the tissue chamber, it was possible to introduce a second reservoir as an alternate source of perfusate. The reservoirs and the tissue chamber were maintained at 37°C with the aid of a heater circulator (Model E15, Haake Mess Technik Gmbt. U. Co., Karlsruhe, Federal Republic of Germany). During the experiments the chamber C was drained through an outlet at the bottom and refilled every 45 min with fresh Krebs-bicarbonate buffer.

The flow rates in the three pumps were set as follows. The positive displacement roller pump P was set to deliver 2.3 ml min^{-1} of Krebs-bicarbonate buffer from the reservoir. The multiple channel infusion pump (Ac) delivered 0.45 ml min^{-1} of either Krebs-bicarbonate buffer or increasing concentrations of ACh into the perfusate. The second pump (At) delivered atropine at a rate of 0.25 ml min^{-1}. Thus the total flow of superfusate onto the recipient ring was maintained constant at 3.0 ml min^{-1}. All the connections in the apparatus were made with polyethylene tubing.

Tissues were mounted as described above and 60 min were allowed for stabilization of the preparation. During the first 30 min of this stabilization period a basal tension of 8.0 g was applied to the recipient in a stepwise manner.

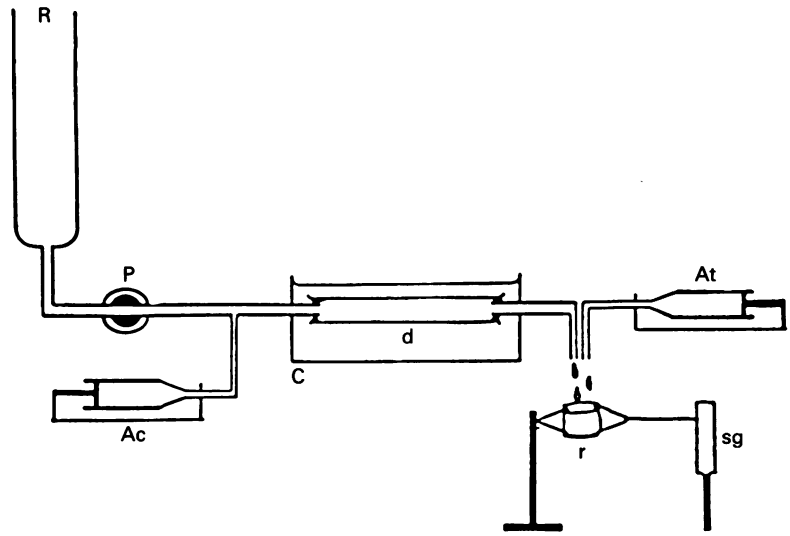

Figure 1 The bioassay apparatus: a 4–5 cm length of donor rabbit aorta (d) was mounted inside the chamber (C) filled with Krebs buffer. Krebs buffer in reservoir (R) was used to perfuse the donor intraluminally with the aid of a positive displacement roller pump (P). Effluent through the donor was used to superfuse the recipient (r) from which the endothelium had been removed. This recipient was connected to a strain gauge (sg) for isometric tension recording. Acetylcholine (ACh) was infused proximal to the donor in four concentrations by a multiple channel infusion pump (Ac) and atropine was infused onto the recipient by a single channel infusion pump (At). The atropine mixed with the superfusate on the surface of the recipient tissue (r).

At the end of 60 min, prostaglandin $F_{2\alpha}$ (10 μmol l^{-1}) was added to the Krebs-bicarbonate buffer (perfusate from pump P) to produce a contraction in the recipient. When this contraction in the recipient had reached a steady state, the perfusate was supplemented with Krebs-bicarbonate buffer from Ac. The pump At (Figure 1) was started at the same time and Krebs-bicarbonate buffer containing atropine (10 μmol l^{-1}) was added directly onto the recipient. With these two manipulations (i.e. the infusion of buffer proximal to the donor and the infusion of atropine on recipient) a slight fall in the contraction to prostaglandin $F_{2\alpha}$ was seen due to a dilution effect. When the contraction had stabilized, ACh was infused proximally at four different concentrations (3.16, 10, 31.6 and 100 μmol l^{-1}). Each concentration was infused for 5 min and Krebs-bicarbonate buffer was infused for 5 min between each concentration of ACh. In this way the responses in the recipient to the four concentrations of ACh were obtained.

Over the next 30 min a mixture of atropine sulphate (10 μmol l^{-1}) and nicardipine (1 mmol l^{-1}) was infused onto the recipient from At. Meanwhile prostaglandin $F_{2\alpha}$ continued to be infused through the donor and directed onto the recipient. At the end of this 30 min period, ACh was added again in the above four concentrations from pump Ac proximal to the donor and the response in the recipient determined. During the infusion of ACh, the supplement from At containing atropine sulphate (10 μmol l^{-1}) and nicardipine (1 mmol l^{-1}) was continued. Next, nicardipine (100 μmol l^{-1}) dissolved in Krebs-bicarbonate buffer (containing prostaglandin $F_{2\alpha}$) was infused through the donor via pump P for 30 min. At the end of this period, ACh was infused as above and the response in the recipient determined while maintaining the infusion of nicardipine.

In this part of the study, the following sequence of concentration-effect curves relating EDR to ACh were studied: (1) Control curve with atropine alone on recipient (introduced by pump At). (2) Curve with atropine and nicardipine on recipient (introduced by pump At). (3) Curve with atropine alone on recipient (introduced by pump At) and nicardipine in perfusate from reservoir R. Thus both the donor and recipient were exposed to nicardipine.

The bioassay technique used in the current investigation allows the synthesis/release and actions of EDRF to be studied separately. Preliminary experiments demonstrated that infusion of ACh on donor aorta with endothelium and superfusing the recipient aorta (without endothelium) with this perfusate, produced a relaxant response. Infusion of atropine on the recipient had no inhibitory effect on the relaxation. However infusion of atropine on the donor or the removal of endothelium from the donor

abolished the relaxation observed in the present study in the recipient ring. Infusion of ACh on the recipient without endothelium produced no relaxation. These observations confirmed that the relaxation is mediated by EDRF and is similar to that described by Griffith *et al.* (1984), Rubanyi *et al.* (1985) and Long & Stone (1985).

Electron microscopy

At the end of this protocol, pieces from the donor aorta and the recipient ring were prepared for scanning electron microscopy (Jayakody *et al.*, 1985). The tissues were fixed initially with 2.5% gluteraldehyde in Millonig's buffer (48 h) followed by post-fixation with 1% osmium tetroxide (45 min). The specimens were dehydrated with ethanol and then put in a critical point dryer (Model No. LPD 100, see VAC, Pittsburg, Pa., U.S.A.) for 4 min at 41°C and 8200 kPa with CO_2. The tissues were then mounted on aluminium stubs with silver glue, sputter coated with gold, and examined in a scanning electron microscope (Model 505, Philips, Eindhoven, The Netherlands). These methods are modifications of those described by Glauert (1975) and Dawes (1981).

Estimation of nicardipine in serum

The nicardipine in serum was estimated by use of a capillary column gas chromatography method. The sensitivity of the assay was 0.002 μmol l^{-1}. The assay was carried out by Syntex Research, Palo Alto, Ca., U.S.A.

Drugs

The pharmacological agents used were: acetylcholine chloride, atropine sulphate, calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$), diltiazem hydrochloride, noradrenaline bitartrate, verapamil hydrochloride (Sigma Chemical Co., St Louis, Mo., U.S.A.); prostaglandin $F_{2\alpha}$ (Upjohn Company of Canada, Don Mills, Canada); nicardipine hydrochloride was obtained as a gift from Syntex Research (Palo Alto, Ca., U.S.A.). The Krebs-bicarbonate buffer solution used was of the following composition (mmol l^{-1}): NaCl 116.0, KCl 5.4, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 22.0, $NaH_2PO_4.H_2O$ 1.2, glucose 10.1, $MgCl_2.6H_2O$ 1.2, $CaNa_2EDTA$ 0.023 and ascorbic acid 1.1.

In experiments in which the potassium-induced contractions were studied, the buffer was of the following composition (mmol l^{-1}): NaCl 21.0, KCl 100.0, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 22.0, $NaH_2PO_4.H_2O$ 1.2, glucose 10.1, $MgCl_2.6H_2O$ 1.2, $CaNa_2EDTA$ 0.023 and ascorbic acid 1.1.

Stock solutions of the drugs used in tissue bath experiments were prepared in distilled water and added to the 22 ml tissue baths in volumes of 146 or 100 μl. All concentrations are expressed as the final concentration in the tissue bath fluid. For bioassay experiments the solutions were prepared in Krebs-bicarbonate buffer.

The concentration of prostaglandin $F_{2\alpha}$ in the reservoir R was 10 μmol l^{-1}. The fluid in the reservoir was diluted 1.304 times by the fluid entering from the infusion pumps (Ac and At), resulting in a final prostaglandin $F_{2\alpha}$ concentration of 7.7 μmol l^{-1} at the recipient. The ACh infused (0.45 ml min^{-1}) into the perfusate (2.3 ml min^{-1}) was diluted approximately 6 times. The concentrations of ACh in the infusion pump were 3.16, 10.0, 31.6 and 100 μmol l^{-1}, and after dilution the concentrations in the fluid perfusing the donor were 0.5, 1.6, 5.3 and 16.6 μmol l^{-1}. Atropine infused onto the recipient was diluted 12 times. The concentration of atropine in the pump At was 10 μmol l^{-1} and after dilution the final concentration on the recipient was 0.83 μmol l^{-1}. When nicardipine 1.0 mmol l^{-1} was infused on the recipient after dilution the final concentration on the recipient was 83.3 μmol l^{-1}. Similarly when nicardipine (100 μmol l^{-1}) was infused on donor after dilution, the final concentration on the donor was 76.7 μmol l^{-1}. The flow rates from the infusion pumps were kept constant throughout the study.

Statistical analysis

In each type of experiment the number of rings studied was also the number of rabbits used. The data are expressed as mean ± standard errors of the mean (s.e.mean). A P value less than 0.05 was considered as significant for all statistical analyses. The concentration-effect curves to acetylcholine in control and experimental animals were compared by regression analysis followed by analysis of covariance (Snedecor & Cochran, 1980).

Results

(i) *Effect of chronic oral administration of nicardipine on EDR*

At the start of the study, the rabbits weighed 2.1 ± 0.2 kg ($n = 12$). Both control animals and animals fed nicardipine gained weight on the standard diet over the period of study and weighed 3.2 ± 0.2 kg ($n = 6$) and 3.1 ± 0.6 kg ($n = 6$) respectively at the time they were killed. The weight gains in the control and nicardipine-fed animals were not significantly different from each other ($P > 0.05$).

The aortic rings from both control and nicardipine-fed groups did not exhibit any spontaneous contrac-

tions in the basal state. Preparations from both groups of animals contracted with the addition of NA (1 μmol l^{-1}) reaching a plateau within 10–15 min. The mean tensions attained in control and nicardipine-fed animals were 7.7 ± 0.9 g and 7.0 ± 0.5 g respectively ($n = 6$, $P > 0.05$). When ACh was added, rings from both animal groups demonstrated a concentration-dependent relaxation. This relaxation commenced at an ACh concentration of approximately 10 nmol l^{-1} and reached a maximum at 1.0 to 3.16 μmol l^{-1}. The maximum relaxations to ACh in control and nicardipine-fed animals were 43.6 ± 5.5 and 53.8 ± 6.7% (mean ± s.e.mean) of the contractile response to NA ($n = 6$, $P > 0.05$). The concentration-effect curves to ACh in the two groups of animals are shown in Figure 2 ($n = 6$, $P > 0.05$). The serum nicardipine levels in the two groups of animals are shown in Table 1. No relaxation to ACh was seen in rings in which the endothelium was removed deliberately (maximum relaxation: 0.0 ± 0.0%, $n = 12$).

(ii) *Effect on EDR of incubating the rabbit aorta* in vitro *with nicardipine hydrochloride and diltiazem*

The aortic rings contracted on exposure to NA (1 μmol l^{-1}); the active tension generated was 7.6 ± 0.6 g ($n = 11$). In the presence of nicardipine (10 μmol l^{-1}) the active tension generated by the same concentration of NA was 7.3 ± 0.6 g. This was not significantly different from that without nicardipine ($P > 0.05$). The relaxant response to ACh was not significantly altered by nicardipine (Figure 3, $P > 0.05$). The maximum relaxations without and with nicardipine were 32.4 ± 4.2% and 28.0 ± 3.1% of the contractile response to NA ($n = 11$, $P > 0.05$). Similarly, concentration-effect curves to ACh were obtained before and after incubation with diltiazem (10 μmol l^{-1}). The active tensions generated to NA before and after incubation with diltiazem were 5.7 ± 0.4 g and 7.6 ± 0.4 g respectively ($n = 11$, $P < 0.05$). The maximum relaxations to ACh before and after diltiazem were 42.1 ± 5.7 and 36.4 ± 7.3% of the contractile response to NA (Figure 4, $P > 0.05$). These findings are summarized in Figures 3 and 4.

The aortic rings contracted on exposure to potassium (100 mmol l^{-1}). The active tension generated was 11.4 ± 1.3 g ($n = 6$). In the presence of nicardipine (10 μmol l^{-1}) the active tension generated by the same concentration of potassium was 6.4 ± 1.0 g. This tension was significantly different from that without nicardipine ($P < 0.05$). Similarly the active tensions generated by potassium (100 mmol l^{-1}) before incubation with nicardipine (100 μmol l^{-1}) and diltiazem (100 μmol l^{-1}) were 10.0 ± 0.9 g and 14.5 ± 1.7 g respectively. The corresponding values after incubation with the calcium antagonists were 3.3 ± 0.9 g and 7.8 ± 1.0 g ($n = 6$, $P < 0.05$ for each).

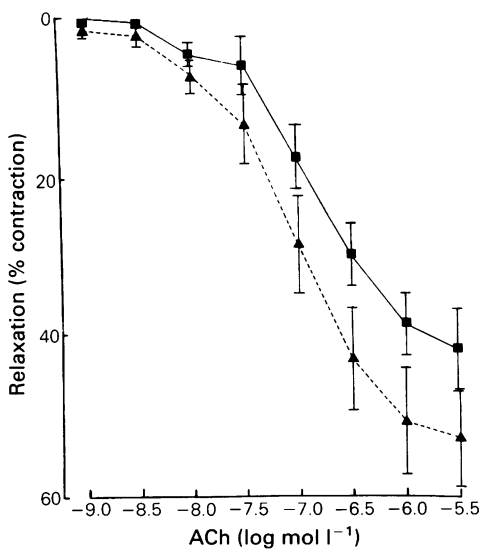

Figure 2 Cumulative concentration-effect curves to acetylcholine (ACh) in control animals (■) and animals fed nicardipine 60 mg kg^{-1} daily for five weeks (▲). The abscissa scale shows the concentration of ACh and the ordinate scale shows the relaxation expressed as a percentage of the contraction to noradrenaline (1 μmol l^{-1}). There was no significant difference between the two curves ($n = 6$, $P > 0.05$).

Table 1 Concentrations of nicardipine in serum

Rabbit No.	*Serum nicardipine* (μmol l^{-1})
CN1	0.02
CN2	0.06
CN3	0.02
CN4	0.02
CN5	0.01
CN6	0.09
C2	0.00
C4	0.00

The concentration of nicardipine (μmol l^{-1}) in serum from rabbits fed nicardipine 60 mg kg^{-1} day^{-1} (as 2 divided doses) for 5 weeks (Rabbit Nos CN1-CN6). The last dose of nicardipine was given at 20 h 00 min and the blood collected at 08 h 00 min the next day at the time the rabbit was killed. No nicardipine was detected in the serum of control animals (Rabbit Nos C2, C4) which were not given nicardipine.

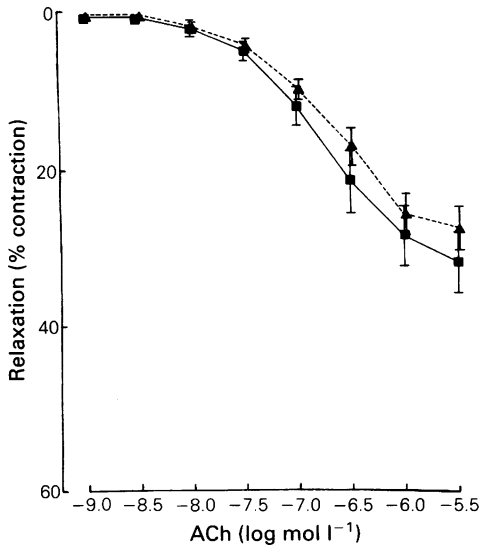

Figure 3 Cumulative concentration-effect curves to acetylcholine (ACh) in control rings (■) and rings exposed to nicardipine (10 μmol l^{-1}) for 30 min (▲). The abscissa scale shows the concentration of ACh and the ordinate scale shows the relaxation expressed as a percentage of the contraction to noradrenaline (1 μmol l^{-1}). There was no significant difference between the two curves ($n = 11$, $P > 0.05$).

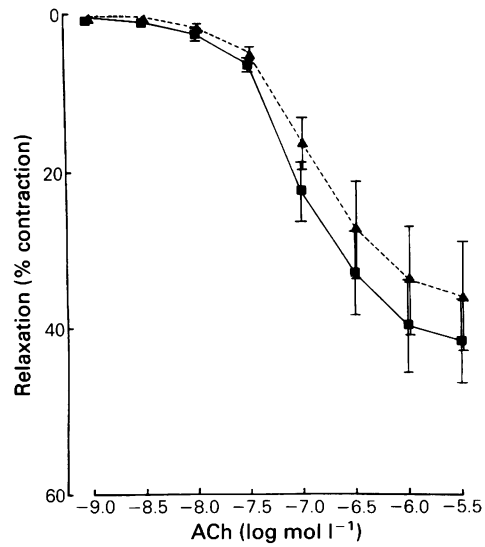

Figure 4 Cumulative concentration-effect curves to acetylcholine (ACh) in control rings (■) and rings exposed to diltiazem (10 μmol l^{-1}) for 30 min (▲). The abscissa scale shows the concentration of ACh and the ordinate scale shows the relaxation expressed as a percentage of the contraction to noradrenaline (1 μmol l^{-1}). There was no significant difference between the two curves ($n = 11$, $P > 0.05$).

(iii) *The effect of nicardipine hydrochloride on the synthesis/release of EDRF and on the response to EDRF*

The protocol was completed in six preparations. Thus, six concentration-effect curves were generated in the control state, six curves were generated with the recipient alone exposed to nicardipine and six curves with nicardipine on both the donor and recipient. Therefore, three sets of concentration-effect curves were compared in the final statistical analyses. The active tensions produced by prostaglandin $F_{2\alpha}$ in the three curves were 4.52 ± 0.43 g, 4.45 ± 0.42 g and 4.43 ± 0.44 g respectively. The maximum relaxations to ACh were 23.5 ± 4.7%, 26.8 ± 5.0% and 27.2 ± 4.2% of the contractile response to prostaglandin $F_{2\alpha}$ respectively (Table 2). These values were not different significantly ($P > 0.05$). The concentration-effect curves are shown in Figure 5.

Scanning electron microscopy showed that in the rings where the endothelium was not mechanically removed, over 80% of the endothelial surface was still intact at the end of the experiments. The endothelial surface appeared smooth with discernible cell margins.

Table 2 Relaxant responses to acetylcholine (ACh) in recipient ring of rabbit aorta expressed as a percentage of the contraction to prostaglandin $F_{2\alpha}$: effects of nicardipine on recipient and an donor plus recipient

	Concentrations of ACh (μmol l^{-1})			
	0.5	*1.6*	*5.3*	*16.6*
Control (%)	1.4 ± 0.9	10.2 ± 2.6	13.1 ± 1.7	22.3 ± 5.2
Nicardipine on recipient (%)	1.7 ± 0.7	13.6 ± 3.5	14.8 ± 2.8	26.7 ± 5.5
Nicardipine on donor (+ recipient) (%)	1.2 ± 0.6	7.8 ± 3.4	12.7 ± 2.7	27.2 ± 4.7

Responses are mean ± s.e.mean, $n = 6$. Prostaglandin $F_{2\alpha}$ as a concentration of 7.7 μmol l^{-1} was used. The control responses, the responses with nicardipine (83.3 μmol l^{-1}) on recipient and the responses with nicardipine (71.3 μmol l^{-1}) on donor (+ recipient) are shown.

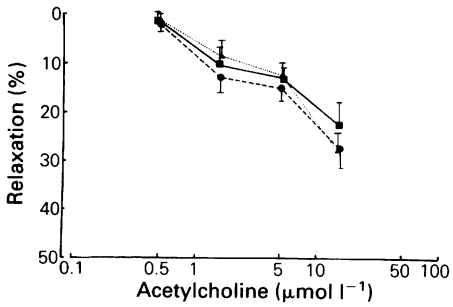

Figure 5 Concentration-effect curves obtained from the bioassay protocol. The abscissa scale shows the concentration of acetylcholine (ACh) and the ordinate scale shows the relaxation expressed as a percentage of the contraction to prostaglandin $F_{2\alpha}$ (7.7 μmol l^{-1}). The concentration-effect curves to acetylcholine when nicardipine was infused on the recipient only (▲) and on the donor (●) were not significantly different from control (■) ($n = 6$, $P > 0.05$).

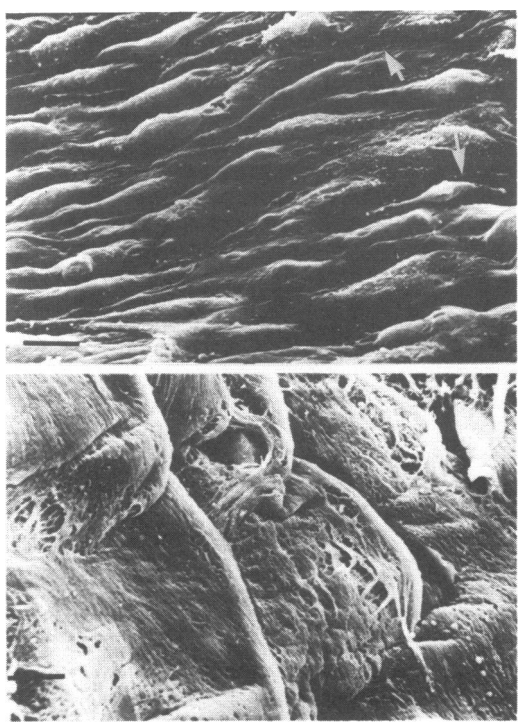

Figure 6 Scanning electron micrographs; top: endothelial cells on intimal surface of a rabbit aortic ring fixed and processed at end of an experiment. Arrows show the cell margins [× 1,310]. Bottom: intimal surface of a ring mechanically denuded of endothelium, showing fibres of the sub-endothelial layer [× 1,250]. The black bars represent 10 μm.

An occasional endothelial cell was found to be absent (Figure 6). The appearance of the endothelium of the animals that were fed nicardipine was similar to those of controls. In the rings where the endothelium was mechanically removed, no endothelial cells were present. The fibres of the sub-endothelial layer were seen (Figure 6).

Discussion

Since the original description of the phenomenon of endothelium-dependent relaxation by Furchgott & Zawadzki (1980), considerable interest has arisen in the elucidation of the mechanism of the production of EDRF. There is evidence that the relaxant factor could be a metabolite of arachidonic acid (Furchgott, 1983; Peach *et al.*, 1985), being generated by the action of phospholipase A_2 on membrane-bound phospholipids. Calcium ions facilitate the action of this enzyme. Further, the calcium ionophore A23187 has been shown to mediate EDR in blood vessels.

In order to 'consolidate' this link between Ca^{2+} and EDR, several investigators have examined the influence of zero calcium buffers and calcium antagonists upon this phenomenon. For instance, Singer & Peach (1982) showed that (1) extracellular calcium depletion inhibited the methacholine-induced relaxation and also that (2) the calcium channel blockers nifedipine and verapamil inhibited methacholine- and A23187-induced relaxations. Similarly, Long & Stone (1985) using a bioassay system have shown that the ACh-evoked release and probably also the basal release of EDRF, is dependent upon the presence of extracellular Ca^{2+}. Other investigators have failed to abolish the EDR to ACh with the calcium antagonists verapamil and nifedipine (Winquist *et al.*, 1985).

However, in the tissue bath experiments presented in this paper the calcium antagonists nicardipine and diltiazem had no inhibitory effect on the endothelium-dependent relaxation to ACh. This could be due to one of several factors: (1) Nicardipine and diltiazem may not act as calcium antagonists in the rabbit aorta. This explanation is unlikely as both nicardipine and diltiazem inhibited the potassium-induced contraction under the present experimental conditions. Also, nicardipine has been previously shown to inhibit $^{45}Ca^{2+}$ uptake and the contractile response induced by high extracellular potassium concentrations in the rabbit aorta (Terai *et al.*, 1981). Similar studies with diltiazem support its action as a calcium antagonist in the rabbit aorta (Ito *et al.*, 1978; Van Breemen *et al.*, 1981). Although the above studies refer to calcium channels in vascular smooth muscle it is assumed that these compounds may exert a similar calcium antagonistic action at the cell membrane of the

endothelial cells. (2) Calcium ions are not involved in the EDR to ACh in the rabbit aorta. This appears unlikely as the EDR to ACh has been shown to be inhibited by exposing the tissue to zero Ca^{2+} buffer *in vitro* (Singer & Peach, 1982). Further, the induction of EDR by the calcium ionophore, A23187 and the calcium agonist Bay K 8644 (Rubanyi *et al.*, 1986), makes this explanation unlikely. (3) Calcium translocation during release of EDRF is not blocked by nicardipine and diltiazem. Although both nicardipine and diltiazem have been shown to inhibit Ca^{2+} influx induced by high extracellular potassium in the rabbit aorta (see above), these two drugs (as well as other calcium antagonists) do not block all calcium channels. It is generally believed that there are at least 3 types of calcium channels in the sarcolemma (Weiss, 1981): (a) voltage-operated channels which allow influx of Ca^{2+} when the cell membrane is depolarized, (b) receptor-operated channels which mediate Ca^{2+} influx following occupation of receptors by agonists without a necessary change in the membrane potential and (c) channels mediating Ca^{2+} influx at rest. Of these channels, depending on the vascular bed and animal species, the calcium antagonists could act predominantly on the voltage-operated channels or receptor-operated channels (Cauvin *et al.*, 1983). The Ca^{2+} influx induced by depolarization of most cell membranes with high extracellular potassium is inhibited by these drugs (Vanhoutte, 1981). However, the contractile responses produced by exogenous noradrenaline are often resistant to the action of calcium antagonists (Vanhoutte & Rimele, 1981; Jayakody *et al.*, 1986). Thus it is possible that any Ca^{2+} influx associated with EDR to ACh in the rabbit aorta may utilize calcium channels not blocked by these drugs.

Findings similar to the present study have been reported by Winquist *et al.* (1985), in rat and rabbit aortae where endothelium-dependent relaxations were not altered appreciably by verapamil (10 μmol l^{-1}) and only affected modestly by a single concentration of nifedipine (0.5 μmol l^{-1}). Winquist *et al.* (1985) concluded that, although the presence of extracellular calcium is required critically for the expression of endothelium-dependent relaxation, the associated calcium translocation is not blocked by organic calcium entry blockers.

Infusion of nicardipine on the recipient alone to reach a tissue concentration of 83.3 μmol l^{-1} for 30 min had no significant effect on the relaxation to EDRF. Hence, calcium channels blocked by nicardipine appear to be not involved in the relaxation mediated by EDRF in the rabbit aorta. Similarly, the infusion of nicardipine (76.7 μmol l^{-1}) on the donor for 30 min had no significant effect on the sythesis/release of EDRF evoked by ACh. Thus, the calcium channels blocked by nicardipine appear not to be involved in synthesis/release of EDRF by ACh in rabbit aorta.

The findings of the present study suggest that the calcium channels which are blocked by nicardipine (which is a dihydropyridine compound) do not appear to be involved in (1) synthesis/release of EDRF by ACh in rabbit aorta or (2) the relaxant response mediated by EDRF in rabbit aorta. In addition, diltiazem (which is a benzothiazepine compound) is also without effect. Finally, verapamil (which is a phenylalkylamine compound) has been shown to have no effect on the release of EDRF in the bioassay experiments (unpublished data).

As drugs from three structurally different classes of calcium antagonists have had no significant inhibitory effect on endothelium-dependent relaxation of rabbit aorta to ACh, it is concluded that the calcium translocation which is believed to occur during (1) synthesis/release of EDRF, and (2) relaxation produced by EDRF is not blocked by calcium antagonists.

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