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Flunarizine inhibits endothelium-dependent hypoxic facilitation in canine coronary arteries through an action on vascular smooth muscle

Amjad Iqbal & Paul M. Vanhoutte

Department of Physiology and Biophysics, Mayo Clinic and Mayo Foundation, Rochester, MN 55905, U.S.A.

1 Hypoxia augments contractile responses to several vasoactive agents in canine isolated coronary arteries with intact endothelium. Calcium antagonists inhibit the further increases in tension caused by hypoxia. The present experiments were designed to determine whether the calcium-antagonist flunarizine would inhibit hypoxic contractions in isolated blood vessels through an action on the endothelium or on the vascular smooth muscle.

2 Rings of canine coronary arteries, with or without endothelium, were suspended at optimal length for isometric tension recording in organ chambers filled with modified Krebs-Ringer bicarbonate solution.

3 Hypoxia (95% N_2 and 5% CO_2) augmented contractile responses to prostaglandin $F_{2\alpha}$ (2×10^{-6} M); removal of the endothelium abolished this hypoxic facilitation.

4 Flunarizine (5×10^{-5}–5×10^{-7} M) exerted a long-lasting inhibition of the hypoxic facilitation in a concentration-dependent manner. Flunarizine did not inhibit the response to prostaglandin $F_{2\alpha}$.

5 To differentiate between the response of smooth muscle and the endothelium, strips of coronary arteries without endothelium were layered with strips with or without endothelium. Hypoxia augmented contractions only in layered preparations with endothelium. Flunarizine prevented the hypoxic contractions in layered preparations in which only the smooth muscle was treated with flunarizine. In contrast, when only the endothelium was treated, no or minimal inhibition of the hypoxic contraction occurred with flunarizine (10^{-5} and 5×10^{-5} M, respectively).

6 These experiments indicate that the calcium antagonist flunarizine inhibits endothelium-dependent hypoxic facilitation in canine coronary arteries primarily through its action on vascular smooth muscle.

Introduction

Hypoxia augments contractile responses to several vasoactive agents (potassium chloride, 5-hydroxytryptamine, and prostaglandin $F_{2\alpha}$) in canine isolated blood vessels, including the coronary artery (Detar & Bohr, 1972; Vanhoutte, 1976; Van Nueten *et al.*, 1980; De Mey & Vanhoutte, 1982; 1983; Rubanyi & Vanhoutte, 1985; Katusic & Vanhoutte, 1986). Removal of the endothelium greatly reduces or abolishes the hypoxic facilitation of the contraction (De Mey & Vanhoutte, 1982; 1983; Rubanyi & Vanhoutte, 1985; Katusic & Vanhoutte, 1986). Previous studies have shown that endothelial cells release a contracting factor or factors under hypoxic conditions (Rubanyi & Vanhoutte, 1985). Calcium antagonists (diltiazem, flunarizine, lidoflazine) inhibit the further increases in tension caused by hypoxia (Van Nueten *et al.*, 1980; 1983; Katusic *et al.*, 1985). The present experiments were designed to determine whether the inhibition of the hypoxic facilitation by these drugs is due to an action on the endothelium, on the vascular smooth muscle, or on both. To do so we used a layered preparation which allowed treatment of the endothelium and the vascular smooth muscle separately and selected flunarizine, a difluoro derivative of piperazine which inhibits Ca^{2+}-entry (Van Nueten & Janssen, 1973; Holmes *et al.*, 1984; Borgers *et al.*, 1984) and has a longer duration of action than diltiazem, nifedipine, and verapamil (Van Nueten *et al.*, 1978; Godfraind & Dieu, 1981). This allowed us to treat the endothelium and the vascular smooth muscle separately.

Methods

The experiments were peformed using rings or strips of left circumflex coronary arteries taken from

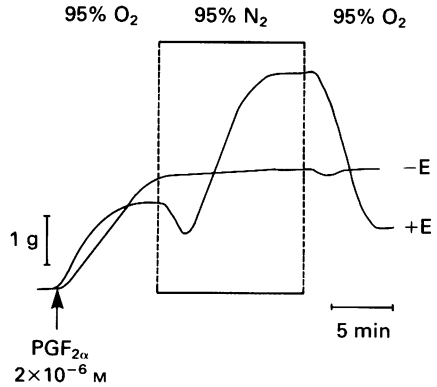

Figure 1 Original recordings of isometric force in paired rings of canine coronary artery with and without endothelium. The rings were contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2×10^{-6} M). After the contractile response had stabilized (10–12 min), hypoxia was induced by switching from a 95% O_2-5% CO_2 to a 95% N_2-5% CO_2 gas mixture. After a transient relaxation (which was endothelium-dependent) hypoxia augmented the contractile response to prostaglandin $F_{2\alpha}$ in the ring with (+E), but not in that without (−E) endothelium. Upon reoxygenation, both rings relaxed transiently, with the response being much greater in the preparation with endothelium.

mongrel dogs of either sex (15–25 kg), anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, intravenously), and subsequently exsanguinated. Immediately after excision, the arteries were placed in cold modified Krebs-Ringer bicarbonate solution (control solution; 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). The blood vessels were cleaned of adherent connective tissue with special care taken not to touch the luminal surface.

Ring preparations

Rings (4–5 mm length) were cut from the left circumflex artery. In some rings the endothelium was removed by gentle rubbing of the intimal surface with a microdissecting forceps. The rings were then suspended in organ chambers filled with 25 ml of control Krebs-Ringer bicarbonate solution (37°C, pH 7.4) bubbled with 95% O_2 and 5% CO_2. They were connected to transducers (Statham Universal UC2) and changes in isometric force recorded. The rings were brought to optimal basal tension by progressive stretch and repeated exposure to potassium chloride (20 mM). The absence of functional endothelium was assessed by lack of relaxation to acetylcholine (10^{-6} M) (Furchgott & Zawadzki, 1980). The vessels were allowed to equilibrate for 45 min following this procedure. Some rings were then incubated with different concentrations of flunarizine (10^{-7} to 10^{-4} M) for 30 min. The drug was removed by repeated washings with control solution, and the rings were allowed to equilibrate for an additional 30 min. Hypoxia was produced by changing the gas mixture to 95% N_2 plus 5% CO_2.

Layered preparation

To assess the differences in the action of flunarizine between the endothelium and the vascular smooth muscle, layered preparations were used (Furchgott & Zawadzki, 1980; Rubanyi & Vanhoutte, 1985). Rings (4 mm length) were cut from the left circumflex coronary artery, and the endothelium was mechanically removed in some rings. The rings were then cut across to obtain circumferential strips. Initially, the circumferential strips were suspended alone in the organ chambers by attaching one end of the strip to the bottom of a stainless-steel plate with two stainless-steel pins. The upper ends of the strips were connected to transducers for the measurement of changes in isometric force. The circumferential strips were allowed to equilibrate for 90 min, and then were brought to their optimal basal tension by repeated exposure to potassium chloride (20 mM). The absence of endothelium was confirmed by lack of relaxation to acetylcholine (10^{-6} M). At this point, some of the circumferential strips were incubated with different concentrations of flunarizine (10^{-5} to 10^{-4} M) for 30 min. The drug was removed by rinsing the organ chambers with control solution. The organ chamber was then lowered and the passive tension of the circumferential strip decreased. Longitudinal strips (with or without endothelium) with the same dimensions as the circumferential strips were prepared from the same coronary artery; in certain experiments, the longitudinal strips were incubated with flunarizine before layering. They were placed (intimal surface against intimal surface) between the circumferential strip and the stainless-steel plate by disconnecting the upper end of the circumferential strip from the force transducer. Reattachment of the circumferential strip to the transducer and stretching it to the original optimal basal tension secured the longitudinal strip. The layered preparation was then allowed to equilibrate for 30 min.

Drugs

The following drugs were used: acetylcholine hydrochloride (Sigma Chemical Company, St. Louis, MO), flunarizine (Janssen Pharmaceutica, Beerse, Belgium), potassium chloride (Sigma), prostaglandin $F_{2\alpha}$ (Sigma). The drugs were prepared daily, diluted

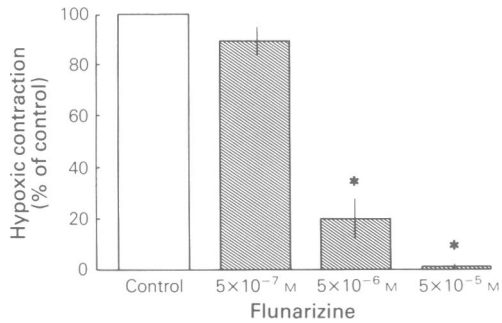

Figure 2 Inhibitory effect of different concentrations of flunarizine on the hypoxic augmentation of prostaglandin $F_{2\alpha}$ (2×10^{-6} M)-induced contractions in canine coronary arterial rings with endothelium ($n = 6$). The rings were incubated with flunarizine for 30 min and repeatedly rinsed with control solution. The rings were then allowed to equilibrate for an additional 30 min, after which time stimulation with prostaglandin $F_{2\alpha}$ was carried out. Hypoxia was introduced after the contractile response to the prostaglandin had stabilized. The data are expressed as % of the hypoxic facilitation occurring in control preparations studied in parallel. Each column shows the mean and vertical lines indicate s.e.mean. The asterisk denotes that the difference from the control response is statistically significant.

using distilled water, and kept on ice. The stock solution of flunarizine was placed in a sonicator for 10 min to ensure complete dissolution of the drug. From the stock solution, 250 μl or less was added to each organ chamber. All drug concentrations are expressed as final organ chamber concentrations (M).

Statistical analysis

Eight preparations were studied in parallel. Results are expressed as means ± s.e.mean. In all experiments, n equals the number of dogs from which blood vessels were taken. Statistical evaluation of the data was by Student's t test for paired and unpaired observations. Differences were considered significant when P was smaller than 0.05.

Results

Ring preparation

Hypoxia caused an augmentation of the contraction induced by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) (hypoxic facilitation; De Mey & Vanhoutte, 1983) only in rings with endothelium (Figure 1). The augmentation was preceded by a transient endothelium-dependent relaxation (Figure 1). Flunarizine caused a concentration-dependent inhibition of the hypoxic facilitation (Figure 2). Flunarizine did not significantly alter basal tension, and no effect on the contraction to prostaglandin $F_{2\alpha}$ was evident (1.3 ± 0.3 g for control vs 1.2 ± 0.2 g for treated rings). The inhibitory effect of flunarizine was present immediately after incubation with the drug, and persisted for up to 120 min after the drug had been washed out (data not shown).

Layered preparations

In circumferential strips (without endothelium) mounted alone, acetylcholine did not cause relaxation (Figure 3A) and hypoxia did not augment prostaglandin $F_{2\alpha}$-induced contractions (Figure 3D,a). In layered preparations containing longitudinal strips without endothelium, the relaxation to acetylcholine (10^{-6} M) was absent (Figure 3B), and no hypoxic facilitation occurred (Figure 3D,b). When circumferential strips were layered with longitudinal strips with endothelium, acetylcholine caused relaxation (Figure 3C), and hypoxia evoked a significant augmentation of prostaglandin $F_{2\alpha}$-induced contractions (Figure 3D,c); as in intact rings, the hypoxic augmentation was preceded by a modest, transient decrease in tension (Figure 3D,c).

Flunarizine caused a marked concentration-dependent inhibition of hypoxic facilitation in layered preparations in which only the circumferential strip without endothelium was treated with the drug (Figure 4). In contrast, in the layered preparations in which only the longitudinal strip with endothelium was exposed to the drug (before being added to the strip without endothelium), no statistically significant inhibition of the hypoxic facilitation occurred with 10^{-5} M flunarizine; at 5×10^{-5} M, there was significant inhibition, but to a significantly lesser degree than in the preparations in which only the smooth muscle had been treated (Figure 4). Flunarizine did not significantly alter either the basal tension of the layered preparations, or their contractile response to prostaglandin $F_{2\alpha}$ (1.4 ± 0.1 g for control and 1.2 ± 0.2 g for treated preparations).

Discussion

The present experiments confirm the endothelium-dependent nature of hypoxic facilitation in the canine coronary artery (Rubanyi & Vanhoutte, 1985). Indeed, no hypoxic augmentation of the contraction to prostaglandin $F_{2\alpha}$ was seen in rings, circumferential strips, or layered preparations without endothelium; whenever the preparations contained endothelium, an augmentation of the contractile response was observed under hypoxic conditions. The experiments with layered preparations also

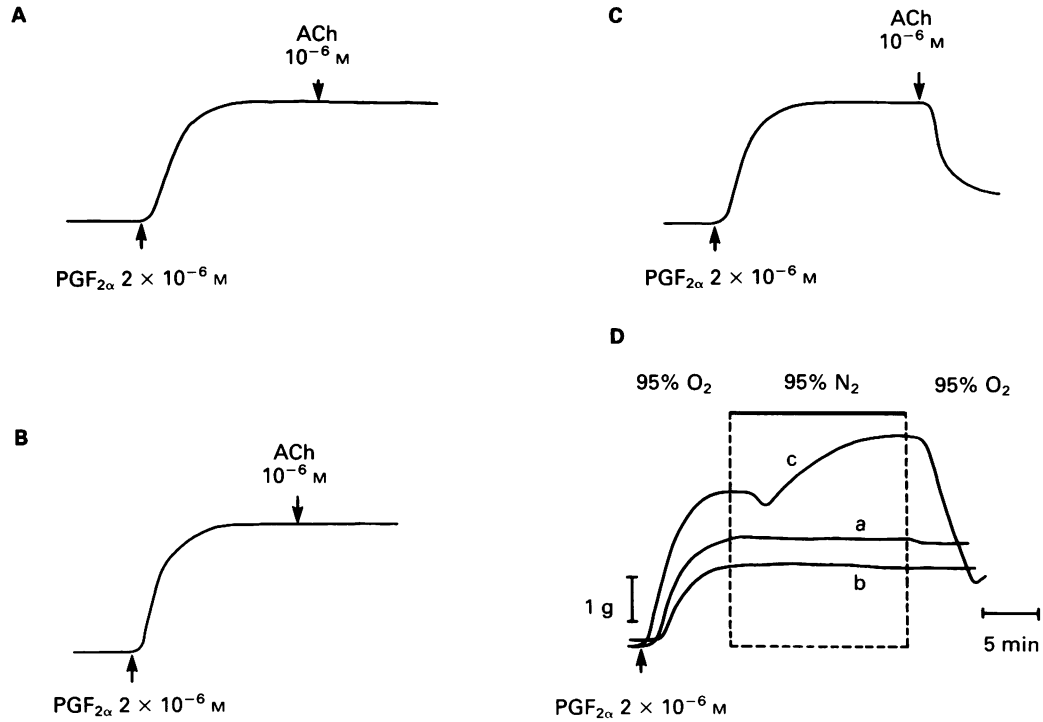

Figure 3 Isometric force recording in circumferential strips of canine coronary arteries without endothelium. Initially, the strips were mounted alone and brought to optimal tension with KCl (20 mM) (not shown). They were then contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 2 × 10^{-6} M) and the absence of endothelium confirmed by the lack of relaxation induced by acetylcholine (ACh, 10^{-6} M; A). The circumferential strips without endothelium were then layered with longitudinal strips without (B) or with (C) endothelium. The layered preparations were contracted with prostaglandin $F_{2\alpha}$ and their response to acetylcholine tested: relaxations were observed in layered preparations which contained endothelium (C) but not in those where the longitudinal strip had been denuded (B). (D) The three types of preparations (circumferential strip without endothelium (a), layered preparations containing a longitudinal strip without endothelium (b) and layered preparations formed of a circumferential strip without plus a longitudinal strip with endothelium (c)) were again contracted with prostaglandin $F_{2\alpha}$ and hypoxia was introduced when the response had stabilized. Hypoxia augmented the contractile response only in the layered preparations containing endothelium (D,c).

confirm that the hypoxic facilitation is due to the release of a vasoconstrictor substance (or substances) from the endothelial cells (Rubanyi & Vanhoutte, 1985). In the rings and the layered preparations containing endothelium, the hypoxic facilitation was preceded by a transient relaxation, which can be attributed to the production of vasodilator prostanoids, as earlier work on the same preparation has shown that endothelium-dependent relaxations to hypoxia can be prevented by indomethacin (Rubanyi & Vanhoutte, 1985).

Flunarizine reduces vasoconstrictor responses induced by different vasoactive agents through inhibition of the entry of Ca^{2+} in vascular smooth muscle (Van Nueten & Janssen, 1973; Van Nueten *et al.*, 1978; Godfraind & Dieu, 1981). The Ca^{2+}-antagonistic properties of flunarizine in vascular smooth muscle have a slower onset and a longer duration of action than those of other Ca^{2+}-antagonists, such as verapamil, diltiazem, or nifedipine (Van Nueten *et al.*, 1978; Godfraind & Dieu, 1981; Godfraind & Miller, 1982; Wadsworth & Moss, 1982). Flunarizine was selected in the present experiments because of its long duration of action, which was evidenced by the experiments where the interval between the incubation with the drug and the exposure to hypoxia was altered. These studies demonstrated that any effect of flunarizine on the hypoxic facilitation was maintained for more than one hour after washing out of the compound. This then allowed flunarizine to be used as a long lasting blocker of Ca^{2+}-entry within the time course

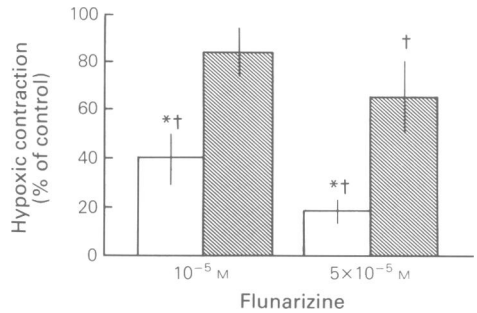

Figure 4 Effect of endothelium on the inhibitory effect of two concentrations of flunarizine in layered preparations of canine coronary arteries (circumferential strips without and longitudinal strips with endothelium). In each experiment, all preparations were from the same coronary artery and were studied in parallel ($n = 6$). In one group, only the circumferential strip without endothelium was exposed to different concentrations of flunarizine before the addition of the strip with endothelium (open columns). In the other group, only the longitudinal strips with endothelium were incubated with flunarizine before being layered with the circumferential strip without endothelium (which was not exposed to flunarizine) (hatched columns). The data are expressed as % of hypoxic contraction occurring in control preparations studied in parallel. Each column represents the mean and vertical lines show s.e.mean. * Denotes that the difference between the two groups is statistically significant ($P < 0.05$); † indicates that the effect of flunarizine is statistically significant ($P < 0.05$).

of the present experiments, enabling us to expose the endothelium and the smooth muscle separately to the Ca^{2+}-antagonistic action of the drug.

Flunarizine did not significantly alter the contractile response to prostaglandin $F_{2\alpha}$ in either the rings or layered preparations. Calcium antagonists have been shown to vary considerably in their ability to inhibit vasoconstrictor responses (Van Nueten & Vanhoutte, 1981). In the canine coronary arteries exposed to calcium-free solution containing 60 mM potassium chloride, addition of prostaglandin $F_{2\alpha}$ produces additional contractions; using ^{45}calcium-labelled coronary arteries exposed to calcium-free solution containing 60 mM potassium, no increased efflux of ^{45}calcium was seen following the addition of prostaglandin $F_{2\alpha}$ (Rooke *et al.*, 1984). These results indicate that prostaglandin $F_{2\alpha}$-induced contractions of the canine coronary arteries may be, at least partially, due to mechanisms independent of an increase in Ca^{2+} entry. This may help to explain why in the present study no significant inhibition of prostaglandin $F_{2\alpha}$-induced contractions was observed with flunarizine.

The major finding of the present study is that flunarizine caused concentration-dependent inhibition of hypoxic facilitation in preparations when only the vascular smooth muscle was exposed to it, but only minimal inhibition when administered to endothelial cells. In the preparations in which only the smooth muscle was exposed to the drug, inhibition of hypoxic facilitation by flunarizine is probably mediated through the inhibition of calcium influx in the vascular smooth muscle cells at the level of the plasma membrane (Godfraind & Miller, 1982; Borgers *et al.*, 1980; 1984).

The observation that incubation of the endothelium only with flunarizine has little or no effect on the hypoxic augmentation, implies that the Ca^{2+}-antagonist has only a minimal effect on the release of endothelium-derived contracting factor(s). The available evidence suggests that Ca^{2+}-antagonists also do not prevent the release of relaxing factor(s) from endothelial cells (Miller *et al.*, 1985; Rubanyi *et al.*, 1988). The modest depression of the hypoxic response, observed after incubation of the strip containing endothelium with high concentrations of flunarizine, probably reflects that some of the compound in the endothelial cells reaches the more intimal smooth muscle after the layering procedure.

The present study thus suggests that the prevention, by Ca^{2+}-antagonists, of endothelium-dependent contractions to hypoxia, is due primarily to the inhibition of Ca^{2+}-entry into the vascular smooth muscle, rather than to interference with the release of constrictor substance(s) from the endothelial cells.

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